PROCEEDINGS OF THE SECOND SYMPOSIUM OF THE INTERNATIONAL WORKING GROUP ON PLANT VIRUSES WITH FUNGAL VECTORS

Editor: Chuji Hiruki

McGill University, Montreal, Canada
July 25-27, 1993

ISBN 0-9639572-0-1

90 Madison Street, Suite 208, Denver, CO 80206
Printed in U.S.A.

PREFACE

The International Working Group on Plant Viruses with Fungal Vectors (IWGPVFV) was founded in 1988 during the 5th International Congress of Plant Pathology in Kyoto, Japan, under the chairmanship of Professor C. Hiruki to bring together scientists actively involved in research on plant viruses transmitted by soil-borne fungi, soil-borne fungi that transmit viruses and their life-cycles and ecology, and the vector-virus relationships and epidemiology of the diseases. Information and materials resulting from their research are exchanged among group members and are distributed to others not directly involved in such research upon request.

Workshops and symposia of the group are organized either separately or in conjunction with plant pathology or virology conferences at intervals of no more than three years. The first symposium of IWGPVFV was held at Braunschweig in 1990, in conjunction with the 8th International Congress of Virology in Berlin, with more than 100 international participants.

The second symposium was held at McGill University in Montreal, Canada, on 25-27 July, 1993. The scientific program consisted of both oral and poster sessions. Their contributions encompassed a broad range of research activities, as seen in the Proceedings, which contains 37 papers in five sections and provides up-to-date information on 1) molecular biology of both viruses and fungi, 2) virology and virus pathology, 3) mycology, 4) disease resistance, and 5) epidemiology and disease management. The strength of the group is based on the fact that IWGPVFV is multidisciplinary in nature and close collaboration exists among scientists of different backgrounds. The success of such collaboration in projects of mutual interest is clearly reflected in their contributions.

The organization of the symposium was greatly facilitated by a conference grant from the Natural Sciences and Engineering Research Council of Canada and by financial support from the firms listed on the following page. We are also greatly indebted to Dr. C.M. Rush for his extremely effective fund raising work and his assistance in arranging for the publication of the Proceedings.

The third symposium of IWGPVFV will be held at Scottish Crops Research Institute, United Kingdom, in 1996.

Chuji Hiruki
Editor
Past Chairman, IWGPVFV

Programme Committee

C. Hiruki, Chair
University of Alberta, Edmonton, Alberta, Canada

M.J.C. Asher
Broom's Barn Experimental Station, Bury St. Edmunds, Suffolk, U.K.

S.A. Lommel
North Carolina State University, Raleigh, NC, U.S.A.

Local Organizing Committee

J.F. Peterson, Chair
McGill University-Mcdonald Campus, Ste-Anne-de-Bellevue, Quebec, Canada

J.S. Barr
Biosystematics Research Centre, Agriculture Canada, Ottawa, Canada

S. Haber
Research Station, Agriculture Canada, Winnipeg, Manitoba, Canada

C. Hiruki
University of Alberta, Edmonton, Alberta, Canada

ACKNOWLEDGEMENTS

We are indebted to the following organizations and companies for financial support. This support has greatly facilitated the organization of the symposium and the publication of the Proceedings.

Natural Sciences and Engineering Research Council of Canada

American Crystal Sugar Company

Dow Elanco

Gustafson

Holly Hybrids

Holly Sugar Plant Pathology Department

Spreckels Sugar Company

CONTENTS

	Page
Preface	3
Acknowledgements	5
Contents	7
Keynote Address	
Similarities between *Hypoviruses* of the chestnut blight fungus, plant potyviruses and barley yellow mosaic virus D.L. Nuss	11
Section 1	
Molecular Biology	
Primary structure and genetic organization of peanut clump virus RNA2 S.K. Manohar, C. Schmitt, E.Herzog, M. Dollet, H. Guilley, K. Richards and G. Jonard	15
Sequence studies of Indian peanut clump furovirus RNA J.S. Miller, V. Wesley, M.A. Mayo, D.V.R. Reddy, R.A. Naidu, M.K. Jana and V.K. Parniak	19
Potato mop-top virus: A third type of furovirus genome organisation B. Reavy, S. Kashiwazaki, K.P. Scott and B.D. Harrison	23
Nucleotide sequences and genome organizations of soil-borne wheat mosaic and sorghum chlorotic spot viruses Y. Shirako, T.M.A. Wilson, R.C. French and J.H. Strauss	27
Identification of sequences involved in beet necrotic yellow vein virus encapsidation and diffusion A. Hehn, C. Schmitt, A.-M. Haeberle, D. Gilmer, C. Stussi-Garaud, H. Guilley, K. Richards and G. Jonard	31
Detection and sequence analysis of deletion mutants of beet necrotic yellow vein virus by RT-PCR M. Saito, T. Kiguchi and T. Tamada	35
Biological and molecular biological studies on barley mild mosaic virus strains S. Kashiwazaki, K. Nomura, S. Okuyama and H. Hibino	39

Section 2
Virology

Mechanical inoculation of sugar beet roots with beet soil-borne virus in the absence of *Polymyxa betae* 43
P.J. Hutchinson, C.M. Henry and R.H.A. Coutts

Detection of beet necrotic yellow vein and beet soil-borne viruses in sugarbeet by means of tissue print-immunoblotting 47
A. Kaufmann and R. Koenig

Histological studies of the infection process of beet necrotic yellow vein virus in roots of beet plants 51
O.E. Scholten, H. Paul, D. Peters, J.W.M. van Lent and R.W. Goldbach

Beet soilborne virus: Preparation of monoclonal antibodies and influence of the 'AHLUM' isolate on mechanically inoculated sugarbeet 55
R. Koenig, L. Barbarossa, A. Kaufmann, J. Vetten and D.-E. Lesemann

Texas 7 a possible strain of beet necrotic yellow vein virus 59
C.M. Rush, R.C. French and G.B. Heidel

Variations among furoviruses associated with sugarbeet 63
G.C. Wisler, J.E. Duffus and H.-Y. Liu

Rhizomania - are both beet necrotic yellow vein virus (BNYVV) and beet soil-borne virus (BSBV) involved? 67
K. Lindsten

Interactions between beet soil-borne virus (BSBV-2) and beet necrotic yellow vein virus (BNYVV) 71
H. Prillwitz and E. Schlösser

Nordic isolates of potato mop-top virus, comparison of reactions with monoclonal antibodies and observations of spraing symptoms in potato cultivars 75
M. Sandgren

Temporal aspects of winter wheat infection by wheat spindle streak mosaic virus (WSSMV) in New York, USA 79
J.E. Carroll, G.C. Bergstrom and S.M. Gray

Effects of some factors on symptom expression of lettuce big-vein virus in lettuce (*Lactuca sativa*) 83
J.A. Walsh

Section 3
Mycology

Development of molecular methods for the identification of *Polymyxa* species 87

E. Ward, E.S. Mutasa, M.J. Adams, C.R. Collier and M.J.C. Asher

RAPD analysis of *Polymyxa betae* 91

A.L. Pilgeram and J.E. Duffus

Zoospore encystment of *Olpidium brassicae* is triggered by fusion between the body and flagellum membrane 95

C. Hiruki

Incidence of infection with *Spongospora subterranea*, the vector of potato moptop virus, in four wild potato species 99

E. Mäkäräinen, H. Rita, E. Teperi and J.P.T. Valkonen

Epidemiological aspects of powdery scab of potatoes caused by *Spongospora subterranea* 103

U. Merz

Section 4
Virus-Fungus Interaction

Virus-vector interactions in the Rhizomania syndrome 107

H. Prillwitz and E. Schlösser

A summary of research on watercress yellow spot virus and fungal vector *Spongospora subterranea* f. sp. *naturtii* 111

J.A. Walsh and C.M. Clay

Flame chlorosis, a novel, soil-transmitted, viruslike disease of spring cereals and wild grasses is linked to *Pythium* spp. 115

S. Haber, D.J.S. Barr, J. Chong, A. Tekauz and W. Kim

Immunogold cytochemistry demonstrates the presence of maize dwarf mosaic virus inside *Puccinia sorghi* urediospores confirming the vector ability of this fungus 119

M.B. von Wechmar and M.A. Jaffer

Mucor circinelloides Van Tieghem F. Lusitanicus (Bruderleing) Schipper hosts and vectors tobacco necrosis virus 123

M.B. von Wechmar, M.A. Jaffer and M. Purves

Transmission of seven cucurbit viruses by host-specific strains of *Olpidium radicale* 127

R.N. Campbell and S.T. Sim

Section 5
Resistance

Mechanisms of resistance to *Polymyxa betae* in wild *Beta* species 129
M.J.C. Asher and K.J. Barr

Variation in the level of infection with *Polymyxa betae* and its effect on infection with beet necrotic yellow vein virus in beet accessions of the sections *Beta* and *Corollinae* 133
H. Paul, B. Henken, O.E. Scholten, Th.S.M. De Bock and W. Lange

Comparison of serology and a reverse transcription-polymerase chain reaction assay for evaluating hard red winter wheat (*Triticum aestivum* L.) cultivars for reaction to wheat soil-borne mosaic 137
R.E. Pennington, J.L. Sherwood and R.M. Hunger

Section 6
Epidemiology and Management

Occurrence and recurrence of symptoms of augusta disease caused by tobacco necrosis virus in tulip 141
C.J. Asjes

Detection and quantification of viruses and their vectors in soil 145
M.J. Adams

Distribution of peanut clump virus (PCV), a virus with high symptom variability 149
M. Dollet, J. Dubern, F. Waliyar and S.K. Manohar

Effect of fluazinam on infection of sugar beets by *Polymyxa betae*, vector of beet necrotic yellow vein virus 153
H. Uchino, K. Kanzawa and T. Tamada

SIMILARITIES BETWEEN *HYPOVIRUSES* OF THE CHESTNUT BLIGHT FUNGUS, PLANT POTYVIRUSES AND BARLEY YELLOW MOSAIC VIRUS

Donald L. Nuss

Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

Summary

Members of the virus family *Hypoviridae* cause a variety of phenotypic changes, including virulence attenuation, in the chestnut blight fungus, *Cryphonectria parasitica* . Recent progress in the molecular characterization of several *hypoviruses* have revealed surprising amino acid sequence similarities with the plant potyviruses and with the related fungus-vectored barley yellow mosaic virus. The apparent conservation of sequence domains between a group of viruses that replicate in a plant pathogenic fungus and a group of insect- and fungus-transmitted plant viruses have interesting evolutionary implications.

Introduction

Interactions between viruses and fungi can take several forms. Certain soil-inhabiting fungi can serve as effective vectors of plant-infecting viruses during the course of interactions with plant root systems. The viruses involved in such interactions appear not to replicate in their fungal vectors and, thus, must be considered plant viruses rather than fungal viruses (mycoviruses). In the more common form of virus-fungus interaction, fungi serve as hosts for virus multiplication. However, it is important to note that mycoviruses differ from conventional animal and plant viruses in that they do not exhibit an infection cycle with an extracellular phase. Rather, the relationship between mycoviruses and their fungal hosts can best be characterized as a persistent infection.

Mycoviruses can be further distinguished by particle morphology, genome composition and the presence or absence of symptom expression. Most mycoviruses have genomes composed of double-stranded (ds) RNA encapsidated in an icosahedral particle (Buck, 1986). The majority of icosahedrial mycovirus cause no obvious phenotypic alterations of the fungal host. Notable exceptions include the viral elements associated with the killer phenotype of *Saccharomyces cerevisiae* (Wickner, 1992) and La France disease of *Agaricus bisporus* (Hicks and Haughton, 1986).

Un-encapsidated virus-like dsRNA genetic elements have also been found associated with membrane or mitochondrial fractions of several fungal species. The presence of a number of these unconventional viral elements is correlated with attenuation of fungal virulence. These include the dsRNAs associated with debilitation 'decline' of *Rhizoctonia solani* (Castanho and Butler, 1978), the d-factors found in 'diseased' strains of the Dutch elm disease fungus, *Ophiostoma ulmi* (Brasier, 1983) and the large dsRNAs responsible for virulence attenuation (transmissible hypovirulence) of the chestnut blight fungus, *Cryphonectria parasitica* (Anagnostakis, 1982). Of the un-encapsidated dsRNA, only the *C. parasitica* hypovirulence agents have been characterized at the molecular level. Surprisingly, these fungal dsRNAs were found to exhibit a number of structural and amino acid sequence similarities with the single-stranded (ss) RNA plant potyviruses and the related fungus-vectored barley yellows mosaic virus (BaYMV). A discussion of the evolutionary implications posed by these similarities follows, preceded by a brief review of the molecular biology of the *C. parasitica* viral dsRNAs.

Molecular Biology of the Virus Family Hypoviridae

Several viral-like dsRNAs isolated from hypovirulent *C. parasitica* strains have now been characterized to the point that the Sixth Report of International Committee on Taxonomy of Viruses (in Press) approved the formation of a new virus family, the *Hypoviridae* . The current view of the basic genetic organization and expression strategy for the *hypoviruses* is typified by that derived for the prototypic member, L-dsRNA present in hypovirulent *C. parasitica* strain EP713 (now designated CHV1-713), based on the complete nucleotide sequence and partial expression analysis (Fig. 1) (Choi, et al., 1991a; 1991b; Shapira et al., 1991a; Shapira and Nuss, 1991). Although this genetic element is found predominantly in a dsRNA form in cell-free extracts of infected mycelia, the basic structural and genetic properties are reminiscent of a replicative form of a conventional ssRNA virus. One strand contains a stretch of approximately 40 adenosine residues (poly A) at the 3'-end that is paired with a stretch of uridine residues (poly U) found at the 5'-terminus of the complimentary strand. The Poly A-containing strand contains all of the protein coding domains in the form of two long open reading frames, ORF A (622 codons) and ORF B (3,165 codons). ORF A encodes a 69 kDa polyprotein that is autocatalytically processed to produce two proteins, p29 from the amino-terminal domain and p40 from the carboxy-terminus. A papain-like catalytic domain was mapped to p29 and the cleavage dipeptide was identified as Gly 248/Gly-249. The junction between ORF A and ORF B consist of the pentanucleotide 5'-UAAUG-3' in which the UAA portion serves as the termination codon of ORF A and the AUG portion is the 5'-proximal initiation codon in ORF B. A second papain-like protease activity was shown to be autocatalytically released from the amino-terminus of the ORF B-encoded polyprotein in the form of a 48 kDa protein designated p48. Similar genetic organizations, with minor variations, have now been reported for two other members of this virus family (Hillman et al., 1992).

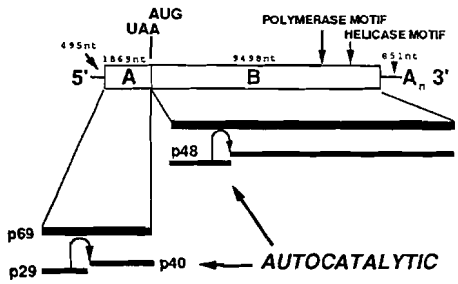


Fig. 1:
Current view of the genetic organization and basic expression strategy for CHV1-713 L-dsRNA. Details are described in the text and in Shapira et al., (1991a) and Nuss, (1992). Adapted with permission from Shapira et al., (1991a).

From a practical perspective, the cloning and sequence analysis described above has led to the construction of a full-length infectious cDNA clone of CHV1-713 L-dsRNA (Choi and Nuss, 1992). This development has allowed the engineering of hypovirulent *C. parasitica* strains that exhibit specific phenotypic traits (Craven et al., 1993) and has significant implication for current efforts to use hypovirulent fungal strains as effective biocontrol agents (Chen et al., 1993).

Possible Common Ancestry for Members of the Hypoviridae , the Plant Potyviruses and the Fungus-Vectored Barley Yellows Mosaic Virus

The first similarity between the *hypoviruses* and the plant potyviruses was uncovered during characterization of the CHV1-713-encoded papain-like protease, p29. This protease resembled the potyvirus-encoded protease HC-Pro in terms of the autocatalytic nature of the cleavage reactions, conserved amino acid sequences adjacent to the essential cysteine and

histidine residues identified for the two proteases, the composition of the cleavage dipeptide and the distances between the essential residues and the cleavage sites (Choi et al., 1991a). A similar pattern was subsequently found for the ORF B-encoded papain-like protease, p48 (Shapira and Nuss, 1991). An additional conserved motif located within the carboxy-terminal portion of ORF B corresponded to an RNA helicase domain found within the potyvirus cylindrical inclusion protein (Shapira et al., 1991a). These observations prompted Koonin et al. (1991) to conduct a computer-assisted analysis of the CHV1-713 L-dsRNA. As a result, five distinct domains with significant sequence similarity to previously described conserved domains within the potyvirus polyprotein were identified, including a RNA dependent RNA polymerase (RDRP) motif (Fig 2). Four of these domains showed similarity to sequence domains that are conserved between the potyviruses and the related fungus-vectored BaYMV (Fig 2). Moreover, phylogenetic analysis of alignments of the CHV1-713 RDRP sequence with all known viral RDRP sequences indicated a much closer similarity to polymerases encoded by ssRNA viruses than to polymerases encoded by dsRNA viruses and suggested a common ancestry for CHV1-713, the plant potyviruses and BaYMV.

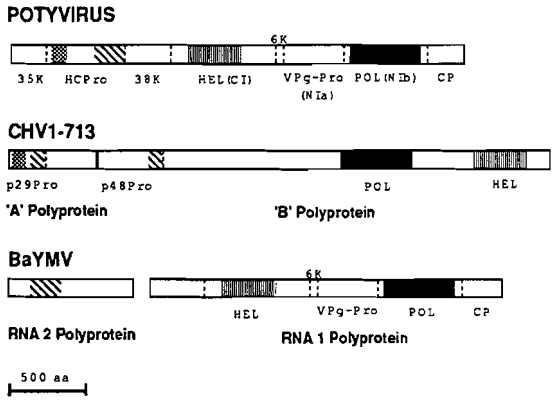


Fig. 2:
Schematic comparison of the organization of the polyproteins of potyviruses, BaYMV and the *hypovirus* CHV1-713. Related domains are highlighted. The termination codon for CHV1-713 ORFA is indicated by a solid vertical line. Abbreviations: POL, RNA polymerase; HEL, helicase; Pro, proteinase; CP, capsid protein; aa, amino acid. Adapted from Koonin et al., (1991).

As can be appreciated by inspection of the organization of conserved domains encoded by CHV1-713, potyviruses and BaYMV (Fig 2), an evolutionary pathway from a common ancestor would necessarily involve extensive modular re-arrangements. In this regard, Koonin et al., (1991) envisioned the following scenario for the evolution of CHV1-713 from a potylike ancestral RNA: a) gene duplication to form the p29 and p40 protease domains, b) deletion of the ancestral capsid gene, c) transposition of the helicase and polymerase genes and d) emergence of a termination codon to separate ORF A and ORF B. Although purely speculative, this scenario has several intriguing components. Since fungal hyphae contain no physical barriers to prevent movement of viral RNA and compatible fungal strains readily undergo anastomosis, one could easily imagine that an extracellular route of infection and the requisite packaging functions would become dispensable once the ancestral virus encountered the environment of the perspective fungal host. Interestingly, one could also draw a functional parallel between the separation of ORF A and ORF B within CHV1-713 L-dsRNA by the introduction of the pentanucleotide 5'-UAAUG-3' and the apparent separation of similar coding domains into individual RNA segments in the case of BaYMV.

The fact that nearly all mycoviruses have genomes composed of dsRNA (Buck, 1986), while the large majority of plant viruses have genomes composed of ssRNA, also raises several larger issues such as whether the host of the postulated ancestral virus was a plant or a fungus and whether its genome was in the form of ssRNA or dsRNA. Since fungi are known to interact with plants at many different levels in nature, one could imagine that an ancestral dsRNA mycovirus could have been transmitted from a fungal host to a plant followed by evolution to a

ssRNA virus along the lines recently suggested by Bruenn (1991). Alternatively, the loss of a requirement for packaging of genomic RNA by an ancestral ssRNA plant virus, once introduced into a fungal host, could have resulted in an altered ratio of genomic ssRNA to replicative form dsRNA, leading to a situation in which a dsRNA form became predominant. Given the propensity with which CHV1-713 dsRNA undergoes internal deletion events and the recent evidence suggesting recombination events involving L-dsRNA and cellular RNA (Shapira et al., 1991b), one could also imagine subsequent pathways leading to the generation of segmented dsRNA viral genomes. The availability of infectious cDNA clones of both CHV1-713 and several plant potyviruses suggests the possibility that some of these potential interactions and evolutionary pathways could be tested experimentally in the near future.

Acknowledgement

This review was adapted in part from the following publications: Koonin et al.(1991); Nuss (1992).

References

Anagnostakis, S.L. (1982) Biological control of chestnut blight. Science 215:446-471.

Brasier, C.M. (1983) A cytoplasmically transmitted disease of *Ceratocystis ulmi*, Nature 305:220-222.

Bruenn, J.A. (1991) Relationship among the positive strand and double-stranded RNA viruses as viewed through their RNA dependent RNA polymerases. Nucleic Acids Res. 19:217-226.

Buck, K.W. (1986) Fungal virology- an overview, p. 2-84. In K.W. Buck (ed.), Fungal Virology. CRC Press, Boca Raton, FL.

Castanho, B. and Butler, E.E. (1978) *Rhizoctonia* decline: Studies on hypovirulence and potential use in biological control. *Phytopathology* 68:1511-1514.

Chen, B., Choi, G.H. and Nuss, D.L. (1993) Mitotic stability and nuclear inheritance of integrated viral cDNA in engineered hypovirulent strains of the chestnut blight fungus. EMBO J. 12:2991-2998.

Choi, G.H., Shapira, R., and Nuss, D.L. (1991a) Co-translational autoproteolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. Proc. Natl. Acad. Sci. USA, 88:1167-1171.

Choi, G.H., Pawlyk, D.M., and Nuss, D.L. (1991b) The autocatalytic protease p29 encoded by a hypovirulence-associated virus of the chestnut blight fungus resembles the potyvirus-encoded protease HC-Pro. Virology, 183:747-752.

Choi, G.H. and Nuss, D.L. (1992) Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257:800-803.

Craven, M.G., Pawlyk, D.M., Choi, G.H. and Nuss, D.L. (1993) Papin-like protease p29 as a symptom determinant encoded by a hypovirulence-associated virus of the chestnut blight fungus. J. Virol. 67:in Press.

Hicks, R.G.T. and Haughton, K.L. (1986) Detection of double-stranded RNA in shake culture of *Agaricus bisporus* affected by 'La France' disease. Trans. Brit. Mycol. Soc. 86:579-584.

Hillman, B.I., Tian, Y., Bedker, P.J., and Brown, M.P. (1992) A North American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA. J. Gen. Virol. 73:681-686.

Koonin, E.V., Choi, G.H., Nuss, D.L., Shapira, R. and Carrington, J.C. (1991) Evidence for common ancestry of a chestnut blight hypovirulence associated double-stranded RNA and a group of positive-strand RNA plant viruses. Proc. Natl. Acad. Sci. USA 88:10647-10651.

Nuss, D.L. (1992) Biological control of chestnut blight: An example of virus mediated attenuation of fungal pathogenesis. Microbiol. Rev. 56:561-571.

Shapira, R., Choi, G.H., and Nuss, D.L. (1991a) Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. EMBO J. 10:731-739.

Shapira, R., Choi, G.H., Hillman, B.I., and Nuss, D.L. (1991b) The contribution of defective RNAs to the complexity of viral-encoded double-stranded RNA populations present in hypovirulent strains of the chestnut blight fungus, *Cryphonectria parasitica*. EMBO J. 10:741-746.

Shapira, R., and Nuss, D.L. (1991) Gene expression by a hypovirulence-associated virus of the chestnut blight fungus involves two papin-like protease activities. J. Biol. Chem. 226:19419-19425.

Wickner, R.B. (1992) Double-stranded and single-stranded RNA viruses of *Saccharomyces cerevisiae*. Annu. Rev. Microbiol. 46:347-375.

PRIMARY STRUCTURE AND GENETIC ORGANIZATION OF PEANUT CLUMP VIRUS RNA 2

S. K. Manohar[1], C. Schmitt[2], E. Herzog[2], M. Dollet[1], H. Guilley[2], K. Richards[2] and G. Jonard[2]

[1] IRHO, Laboratoire de Phytovirologie des Regions Chaudes, CIRAD-ORSTOM
BP 5035, 34032 Montpellier (Cedex 01), France

[2] Institut de Biologie Moléculaire des Plantes du CNRS et de l'Université Louis Pasteur
12 Rue du Général Zimmer, Strasbourg 67084 Cedex France

Summary

RNA 2 from an African isolate of the probable furovirus peanut clump virus (PCV) has been cloned and sequenced. The RNA is 4503 nucleotides and contains six long open reading frames (ORFs). The 5'-proximal ORF encodes the coat protein which is followed immediately in another reading frame by an ORF for a 39 kDa protein. Both the coat protein and the 39 kDa protein are expressed from full-length RNA during *in vitro* translation. Downstream of the 39 kDa ORF is a 'Triple Gene Block' which displays closest homology to the Triple Gene Block of barley stripe mosaic virus. The 3'-proximal ORF encodes a putative 7 kDa protein. Analysis of other PCV isolates has revealed that the 39 kDa ORF can undergo deletion without interfering with infectivity of the virus upon mechanical inoculation to leaves, suggesting that the 39 kDa protein is involved in the natural infection cycle, e.g. fungus transmission. Minor isolate-related sequence changes (nucleotide substitutions) have also been detected in the coat protein cistron.

Introduction

Peanut clump virus (PCV), a soil-borne virus believed to be vectored by *Polymyxa graminis*, is responsible for a serious desease of groundnut in Africa (see Manohar *et al.*, (1993) for references). PCV can be transmitted mechanically to leaves of *Chenopodium amaranticolor*, *Nicotiana benthamiana* and several other hosts. The virions are rod-shaped and contain single-stranded RNA of approximately 4.2 kb and 6 kb as measured by gel electrophoresis under nondenaturing conditons (Fig. 1); the capsid protein is ~23 kDa (Thouvenel and Fauquet, 1981).

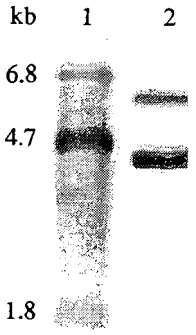


Fig. 1 Agarose gel electrophoresis of RNA of BNYVV isolate St123 (lane 1)and PCV isolate PCV2 (lane 2). Ethidium Br staining.

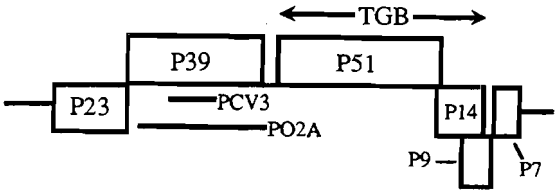


Fig.2 Genetic map of PCV2 RNA 2. The nature of the mapped deletions in isolates PCV3 and PO2A are indicated beneath the map.

Sequence and Genome Organization of PCV RNA 2

To facilitate comparisons of PCV with other fungus-borne viruses such as beet necrotic yellow vein virus (BNYVV), cDNA corresponding to RNA 2 of PCV (isolate PCV2) has been cloned and sequenced (Manohar *et al.*, 1993). The complete RNA is 4503 nucleotides in length and contains six long ORFs (Fig. 2). ORF 1 was identified as the viral coat protein (P23), based upon the size of the predicted translation product, its amino acid composition and *in vitro* translation experiments (see below). It is noteworthy that, other than the initiation codon, P23 contains no methionine residues.

The P23 cistron is followed immediately by an ORF potentially encoding a 39 kDa protein (P39), which is in another reading frame. The initiation codon of the 39 ORF overlaps the termination codon of the coat protein cistron (...CC*AUGA*G...; the P39 initiation codon is underlined and the P23 termination codon is in italics).

The 39 kDa ORF is separated from the following ORF, encoding a 51 kDa protein (P51), by a short intergenic region. P51 contains consensus sequences for a purine nucleotide binding pocket and associated motifs thought to function as an RNA helicase. P51 and the putative products of the two following ORFs, P14 and P9, display extensive sequence homology with counterparts encoded by a 'Triple Gene Block' (TGB) in barley stripe mosaic virus (BSMV) RNAß , BNYVV RNA 2, potexviruses and carlaviruses (see Morozov *et al.*, 1989 for a review). The TGB proteins of PCV are most closely related to those of BSMV. It has been shown for BSMV, BNYVV and white clover mosaic potexvirus (Petty *et al.*, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992) that the TGB proteins are involved in cell-to-cell movement of the virus during infection and it is probable that the corresponding species in PCV RNA 2 have a similar role. The role, if any, of the 7 kDa protein encoded by the short 3'-proximal ORF is unknown.

Translation products of viral RNA

Fig. 3 presents the results of PAGE fractionation of ^{3}H-leucine-labelled *in vitro* translation products of total PCV RNA. Two abundant translation products of about 25 kDa and 40 kDa are present as well as a pair of bands of >100 kDa which presumably arise from translation of RNA 1. The 25 kDa and 40 kDa species were also produced when gel-purified RNA 2 or RNA 2 transcripts were used in translation (data not shown). Of the various translation products, only the 25 kDa band was immunoprecipitated by antiserum raised against PCV, demonstrating that this species is the viral coat protein. The 25 kDa species was not detected when ^{35}S-methionine was substituted for ^{3}H-leucine in the translation mix (data not shown), consistent with the absence of internal met residues in P23 (Presumably the N-terminal Met of P23 is eliminated post-translationally in the *in vitro* translation experiments).

The approx. 40 kDa translation product has been shown to correspond to P39 (data not shown). The mechanism of expression of P39, whether it is produced by reinitiation of translation by ribosomes after termination of the coat protein cistron, internal translation initiation or leaky scanning is currently under investigation.

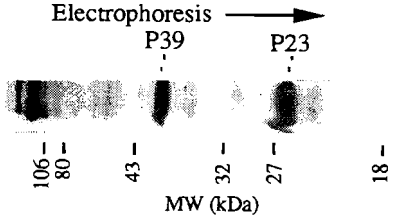


Fig. 3 SDS-PAGE (12% gel) of ^{3}H-leucine labelled translation products obtained upon translation of PCV RNA (isolate PCV2) in rabbit reticulocyte lysate.

```
PCV5                A C           T C                 T   C
PO2A          T       G                                   A
PCV2  ATGTCTAACATTGCTGAGGTTAGTCGCGGAGGAGGACATTACGGTGTCGATCCGTGGCGCCAGCATATTA
PCV2  M S N I A E V S R G G G H Y G V D P W R Q H I I
PCV5

PCV5   C    C        A               C G    T    G AT    G       TA
PO2A                                 G                          A
PCV2  TTAAGAATAGGATTAACGCTGATTGGTGGATTCGCTTAGATCACTGGGAAACCTTGTTAGCGGATCTGCG
PCV2    K N R I N A D W W I R L D H W E T L L A D L R
PCV5                T                       N

PCV5        A    C          C       G A       T       T    A    A T
PO2A          C    G              G
PCV2  AGGGGTGAGTTTTGAAGTCAACAGTTCGCGTTCTCAGGTTGCTGACTTCATTAACAGGGTTCCTAAGGAC
PCV2   G V S F E V N S S R S Q V A D F I N R V P K D
PCV5

PCV5     G C G C A T          C    T T             C A C C
PO2A                       C                          C
PCV2  TTACCTGCTGGTGTGTCCGTGAGGTTTCCAGGCCCCCGGGGTAATCTCGGCAGTACGAATTATACCGAAG
PCV2  L P A G V S V R F P G P R G N L G S T N Y T E V
PCV5

PCV5   G    T     G G       A C    A            A A    T C
PO2A   T                              A                        A
PCV2  TCTATTTCGTTAGAATAAAATCTGAGCTTAAACAGAAGTTATTAAGTTTGATTGCTGCCGCAGATCAGGG
PCV2    Y F V R I K S E L K Q K L L S L I A A A D Q G
PCV5             V

PCV5  T G C T       G T G C G    T    A A C       A T C
PO2A                G                          C    T
PCV2  AAAAAATCGCGACGTGGAAATCGGTCGTCCAAACGCCCCAGTTGTGAGTACTGGTGCGGGCGGTAACCAA
PCV2   K N R D V E I G R P N A P V V S T G A G G N Q
PCV5                                       T

PCV5       T A          A T          A T    A  C G G       G
PO2A                A    A
PCV2  GCCATCGTTGCTCAGAGAGGTGTCAATACTGTGAGGGACCAACAGCCTTTAAGAGACGGTTCCTTGCACT
PCV2  A I V A Q R G V N T V R D Q Q P L R D G S L H Y
PCV5

PCV5   C    T C A       T     C C T    C          C    T    C    AGT
PO2A
PCV2  ATCGGTATTTGGTGCAAGACATTGAGTTGGCGGGTGCTGAACAGTTTGATCGTGCGTTATTTGAAGAGAC
PCV2    R Y L V Q D I E L A G A E Q F D R A L F E E T
PCV5       F                                                    V

PCV5     T       T          C    CG      C    A G C
PO2A     T    T                                        G
PCV2  TTTCAGTCTCAACTGGACTGTCGTTGCTCCACCTGCTGGTGGTGGTGGCGGTGGTGCACCA
PCV2   F S L N W T V V A P P A G G G G G G A P
PCV5                          A
```

Fig. 4 Nucleotide sequence of the coat protein cistron of the type isolate of PCV (PCV2) and the deduced amino acid sequence. Nucleotide changes in isolates PO2A and PCV5 (above) and changed amino acids in isolate PCV5 (below). The nucleotide changes in PO2A did not provoke changes in the protein sequence.

Deletions and sequence heterogeneity in RNA 2 of different PCV isolates

Examination of RNA 2 of different PCV isolates has revealed two types of sequence variation. As shown previously (Manohar *et al.*, 1993), several isolates of PCV (PCV 3 and PO2A) were found to contain shortened forms of PCV RNA 2. Sequence analysis has revealed that the RNA 2 of these isolates contain deletions in the P39 ORF (Fig. 2). In BNYVV RNA 2, the 54 kDa ORF separating the 5'-proximal coat protein from the first gene of the TGB has been shown to contain a determinant which is not required for infection of plants by mechanical inoculation but is required for transmission by the fungal vector *Polymyxa betae* (Tamada and Kusume, 1991). Our results indicate that P39 of PCV RNA2 is likewise nonessential for infection of leaves by mechanical inoculation since isolates PCV3 and PO2A, which lack sizable segments of the P39 gene, are still infectious to mechanically inoculated plants. It will be interesting to determine if such deleted isolates are competent for transmission by *P. graminis*.

RNA 2 was also subject to point mutations. The region corresponding to the coat protein of isolates PCV5 and PO2A was converted to cDNA by reverse transcription, amplified by the polymerase chain reaction (PCR) and cloned. Sequence analysis (Fig. 4) revealed that the coat protein cistron of PO2A differed at 21 positions from that of the type isolate (PCV2) but that none of these changes altered the sequence of the coat protein. The coat protein cistron of isolate PCV5, on the other hand, differed at 103 positions with seven amino acid substitutions. We conclude that PCV2 and PO2A are more closely related to one another than to PCV5, at least for the portion of the genome containing the coat protein cistron.

References

Beck, D. L., Guilford, P. J., Voot, D. M., Anderson, M. T., and Forster, R. L. S. (1991). Triple gene block proteins of white clover mosaic potexvirus are required for transport. *Virology* **183**: 695-702.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA-2. *Virology* **189**: 40-47.

Manohar, S. K., Guilley, H., Dollet, M., Richards, K., and Jonard, G. (1993). Nucleotide sequence and genetic organization of peanut clump virus RNA 2 and partial characterization of deleted forms.*Virology* **194**: in press.

Morozov, S. Y., Dolja, V. V., and Atabekov, J. G. (1989). Probable reassortment of genomic elements among elongated RNA-containing plant viruses. *J. Mol. Evol.* **29**: 52-62.

Petty, I. T. D., French, R., Jones, R. W., and Jackson, A. O. (1990). Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. *EMBO J.* **9**: 3453-3457.

Tamada, T., and Kusume, T. (1991). Evidence that the 75 K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus *polymyxabetae. J. Gen. Virol.* **82**: 1497-1504.

Thouvenel, J. C. and Fauquet, C. (1981). Peanut clump virus. *CMI/AAB Description of plant viruses.* n° 235, 4 pp.

SEQUENCE STUDIES OF INDIAN PEANUT CLUMP FUROVIRUS RNA

J.S. Miller[1], V. Wesley[2], M.A. Mayo[1], D.V.R. Reddy[2], R.A. Naidu[2], M.K. Jana[3] & V.K. Parniak[4].

(1) Scottish Crop Research Institute, Invergowrie, Dundee, UK, (2) ICRISAT, Patancheru, Hyderabad, A.P., India, (3) IIT, Kharagpur 721 302, West Bengal, India and (4) CCMB, Hyderabad 500 007, A.P. India.

Summary

Indian peanut clump virus (IPCV) has a bi-partite genome and is transmitted by *Polymyxa graminis*: it has been classified in the furovirus genus. Cloning and sequencing of cDNA made to RNA from IPCV particles has revealed that the 5'-proximal ORF on RNA-2 codes for the coat protein and that RNA-1 codes for a putative methyl transferase gene. The 3'-halves of each RNA have yet to be sequenced. IPCV coat protein is 61% identical to the coat protein of peanut clump virus from West Africa (PCV). This value, together with the lack of serological relatedness between them, confirms that IPCV and PCV should be considered as separate viruses. Comparisons among the coat proteins of a range of rod-shaped viruses showed that IPCV and PCV coat proteins resembled that of barley stripe mosaic hordeivirus more than those of other furoviruses.

Introduction

Indian peanut clump virus (IPCV) causes a serious disease in groundnut crops (Reddy *et al.*, 1988). It is soil-borne and is transmitted by the soil-inhabiting fungus *Polymyxa graminis* to groundnut and graminaceous hosts (Reddy *et al.*, 1988). IPCV can persist in soil for several years and is difficult to control; no resistance to IPCV has yet been found in groundnut germplasm (Reddy *et al.*, 1988).

The virus has rod-shaped particles of two lengths comprising a 24K coat protein and RNA molecules of either c.6kb (RNA-1) or c.4kb (RNA-2) (Reddy *et al.*, 1985). RNA-2 is the mRNA for the coat protein (Mayo and Reddy, 1985).

Although classified in the furovirus group because of its fungus transmission and rod-shaped particles (Brunt, 1991), IPCV is serologically unrelated to West African peanut clump virus (PCV) and other furoviruses. Isolates of IPCV from different places in India differ appreciably serologically (Reddy *et al.*, 1985; Nolt *et al.*, 1988).

In projects aimed at developing cloned probes to detect infection by IPCV and at isolating the coat protein genes for transformation work, we have sequenced about half of each RNA of IPCV. The results obtained so far have been used in sequence comparisons to infer taxonomic relationships of IPCV with other furoviruses.

Materials and Methods

cDNA synthesis and cloning

RNA was extracted from purified virus particles as described by Mayo and Reddy

(1985). cDNA was synthesised as described by Gubler and Hoffman (1983) using a commercial kit (Boehringer) and cloned in *Sma* I-cut pUC19. Clones specific to each IPCV RNA were identified by Northern blotting.

Nucleotide sequencing

Nucleotide sequences were determined by dideoxy chain termination (Sanger *et al.*, 1977). Template DNA was either single-stranded M13 DNA or double-stranded DNA from cDNA clones. Sequences were assembled using STADEN software (Staden, 1982) and analysed using the GCG package (Devereux *et al.*, 1984). Sequences were compared with those for PCV, barley stripe mosaic (BSMV), soil-borne wheat mosaic (SBWMV), beet necrotic yellow vein (BNYVV) and Nicotiana velutina mosaic viruses (NVMV).

Results

Nucleotide sequences

Two large contiguous sequences have been obtained. One corresponds to the 5'-half of RNA-2 which includes the coat protein gene and the other corresponds to the 5'-end of RNA-1 which includes part of what appears to be a methyl transferase gene.

In RNA-2 (Fig. 1), the 5'-most open reading frame (ORF) encodes a 24K protein which resembles the coat protein of PCV. When this gene was cloned into pET-15b, transformed *E. coli* expressed protein which reacted with antiserum to IPCV particles. The next ORF downstream is in the (-1) frame with respect to the coat protein gene and encodes a c. 39K protein (p39).

The sequence assembled for RNA-1 contains one ORF which extends 3' of the sequenced region. The encoded protein resembles a methyl transferase.

Sequence comparisons

Sequence comparisons among coat proteins of viruses with rod-shaped particles made using GAP revealed values greater than the non-specific value (c. 20%) only when IPCV was compared with PCV (61% match) and BSMV (37% match). Sequences were compared by multiple sequence alignments using CLUSTALV. The deduced relationships among the coat proteins (Fig. 2) show that BSMV is the virus most like IPCV and PCV and that coat proteins of other furoviruses are relatively distant from each other.

Fig. 3 shows the similarity between IPCV and BSMV coat proteins in a DOTPLOT. Two large areas of homology are clearly visible.

Discussion

Although IPCV and PCV are similar biologically, the value of a 61% match between the coat protein sequences confirms that the viruses are distinct and not one virus. In their coat protein sequences, IPCV and PCV more resemble BSMV than other furoviruses. Indeed there is little coat protein sequence similarity among furoviruses. This is the first report of strong coat protein sequence similarities between furoviruses and BSMV although some similarities have been found between non-structural proteins of BSMV and those of PCV (Manohar *et al.*, 1993), BNYVV (Bouzoubaa *et al.*, 1987) and SBWMV (Shirako and Wilson, 1993). It is possible that BSMV (which is not fungus-transmitted) and furoviruses had a common origin or have evolved by sharing genes by recombination in a common host.

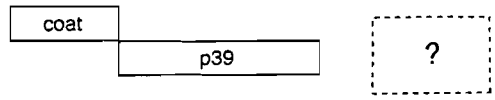


Fig. 1 Diagram of the deduced location of the coat protein and p39 genes in IPCV RNA-2. ? indicates the unsequenced region

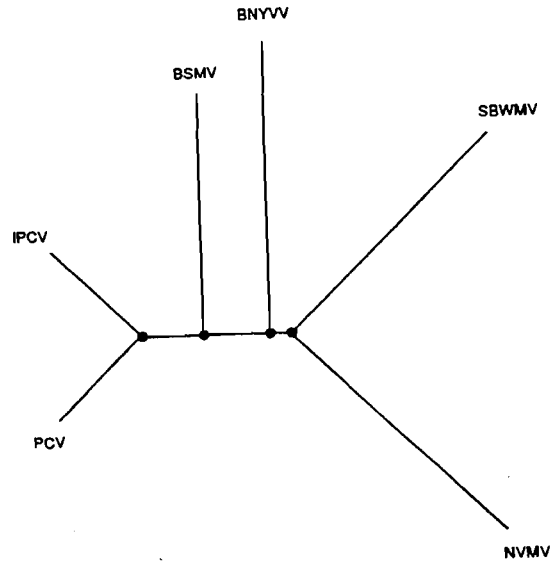


Fig. 2 Diagram of similarity relationships between furovirus coat proteins deduced from a CLUSTALV multiple alignment analysis

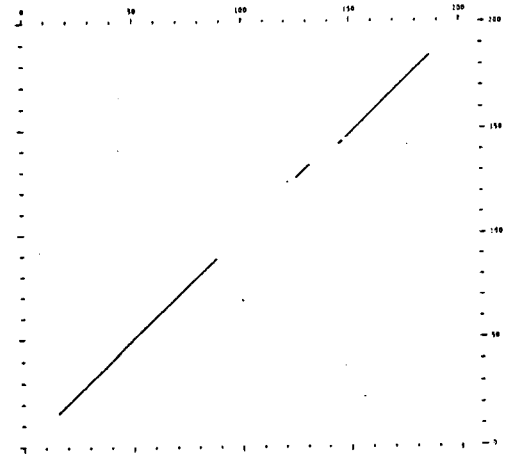


Fig. 3 DOTPLOT showing simirity between coat protein sequences of BSMV (vertical sequence) and IPCV (horizontal sequence)

References

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G. & Richards, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *J. Gen. Virol.* **68**:615-626.

Brunt, A.A. (1991). Furovirus group. In *Classisfication and Nomenclature of Viruses. 5th Report of the International Committee on Taxonomy of Viruses.* Edited by R.I.B. Francki, C.M. Fauquet, D.L.Knudson & F. Brown, p. 377-379. *Arch. Virol.* (Suppl. 2).

Devereux, J., Haeberli, P., & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**:387-395.

Gubler, U. & Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**:263-269.

Manohar, S.K., Guilley, H., Dollet, M., Richards, K. & Jonard, G. (1993). Nucleotide sequence and genetic organisation of peanut clump virus RNA-2 and partial characterisation of deleted forms. *Virology* **195**:33-41.

Mayo, M.A. & Reddy , D.V.R. (1985). Translation products of RNA from Indian peanut clump virus. *J. Gen. Virol.* **66**:1347-1351.

Nolt, B.L., Rajeshwari, R., Reddy, D.V.R., Bharathan, N. & Manohar, S.K. (1988). Indian peanut clump virus isolates: Host range, symptomatology, serological relationships, and some physical properties. *Phytopathology* **78**:310-313.

Reddy, D.V.R., Robinson, D.J., Roberts, I.M. & Harrison, B.D. (1985). Genome properties and relationships of Indian peanut clump virus. *J. Gen. Virol.* **66**:2011-2016.

Reddy, D.V.R., Nolt, B.L., Hobbs, H.A., Reddy, A.S., Rajeshwari, R., Rao, A.S., Reddy, D.D.R. & McDonald, D. (1988). Clump disease in India: isolates, host range, transmission and management. In *Viruses with Fungal Vectors.* Edited by J.I. Cooper and M.J.C. Asher, pp. 239-246.

Sanger, F., Nicklen, S. & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.(USA)* **74**:5463-5467.

Shirako, Y. & Wilson, T.M.A. (1993). Complete nucleotide sequence and organisation of h bipartite genome of soil-borne wheat mosaic virus. *Virology* (In Press).

Staden, R. (1982). Automation of the computer handling of gel reading data produced by h shotgun method of DNA sequencing. *Nucl.Acids Res.* **10**:4731-4751.

Acknowledgements

This work was supported by the Overseas Development Administration Plant Science Programme administered by the Centre for Arid Zone Studies, Bangor.

POTATO MOP-TOP VIRUS: A THIRD TYPE OF FUROVIRUS GENOME ORGANISATION.

B. Reavy (1), S. Kashiwazaki (2), K.P. Scott (1), and B.D. Harrison (3)

(1) Scottish Crop Research Institute, Invergowrie, Dundee, UK, (2) National Agriculture Research Centre, Tsukuba, Ibaraki 305 JAPAN, (3) Dept. of Biological Sciences, University of Dundee, Dundee, UK

Summary

Potato mop-top virus (PMTV) is a furovirus transmitted by *Spongospora subterranea*. The genome of isolate T of PMTV consists of 3 single-stranded RNA species of 6.5kb (RNA1), 3kb (RNA2) and 2.5kb (RNA3), which are distinct. RNA 2 contains 4 large open reading frames (ORFs) which encode proteins of 51K, 13K, 21K and 8K. The three largest resemble the triple gene block proteins of some other plant viruses. The 8K protein is rich in cysteine. RNA 3 contains the virus coat protein gene, which ends in an amber termination codon, readthrough of which would result in synthesis of a 67K read-through protein. PMTV resembles soil-borne wheat mosaic (SBWMV) and beet necrotic yellow vein (BNYVV) furoviruses in this feature and the respective proteins of PMTV are more similar to those of SBWMV than to those of BNYVV. However the RNA3 of PMTV is not directly equivalent to RNA2 of SBWMV as it lacks a 3'-terminal cysteine-rich protein. PMTV resembles BNYVV and differs from SBWMV in having a triple gene block. PMTV RNA 2 and 3 together are functionally equivalent to BNYVV RNA 2 . However, the putative triple gene block proteins of PMTV resemble the corresponding proteins of barley stripe mosaic hordeivirus more closely than those of BNYVV.

Introduction

Potato mop-top virus (PMTV) occurs world-wide and infects potato crops and wild *solanum* species. In addition to yield losses it causes sporadic mopping and yellow blotching of the upper leaves of plants and brown arcs in the flesh of tubers of sensitive species. The virus was first identified by Calvert & Harrison in 1966 and the virus particles are fragile and rod-shaped with two predominant lengths of 100-150 nm and 250-300 nm (Harrison & Jones, 1970). Serological relationships are reported between PMTV and both tobacco mosaic virus (TMV) (Kassanis *et al.*, 1972) and soil borne wheat mosaic furovirus (SBWMV), but not with beet necrotic yellow vein furovirus (BNYVV) (Randles *et al*, 1976). PMTV is transmitted by the plasmodiophoromycete fungus *Spongospora subterranea* (Jones & Harrison, 1969) and has been placed in the furovirus group on the basis of its fungal vector and particle morphology (Francki *et al*, 1991).

Sequence analysis of SBWMV and BNYVV indicate that these two furoviruses have considerably different genome organisations (Shirako & Wilson, 1993). The type strain of PMTV is the Todd-O isolate (PMTV-T). The genome of PMTV-T consists of three RNA

species of 6.5kb, 3.2kb and 2.5 kb (Kallender *et al.*, 1990; Scott *et al.*, 1990) and is thus unlike both SBWMV, which has two RNA species, and BNYVV, which has at least four. We have begun sequence analysis of PMTV-T to determine its relationships with members of the furoviruses and to examine diversity of genome organisation within the furovirus group.

Materials and Methods

cDNA synthesis and cloning:
RNA was purified from virus particles by proteinase K digestion followed by phenol extraction and ethanol precipitation. cDNA was synthesised essentially as described by Gubler and Hoffman (1983) using a commercial kit (Pharmacia). Two cDNA libraries were made. For the first library, cDNA synthesis was primed with random hexamer oligonucleotides and the product was methylated using *Eco*RI methylase, ligated to *Eco*RI linkers, digested with *Eco*RI and ligated into λ ZAPII arms with complementary *Eco*RI ends (Stratagene). For the second library, total PMTV RNA was polyadenylated with polyA-transferase and cDNA synthesis was performed using oligo(dT) as a primer. The products of this synthesis were cloned into pBluescript previously digested with *Sma*I and dephosphorylated using alkaline phosphatase.
Nucleotide sequencing:
Nucleotide sequences were determined by dideoxy chain termination (Sanger *et al.*, 1977). Nested deletions of some clones were generated by exonuclease III digestion (Henikoff, 1984) and sequenced using double-stranded templates (Stratagene). Specific smaller fragments of other clones were obtained by restriction enzyme digestion and isolated from low melting-point agarose gels for subcloning into M13 vectors and sequencing using single-stranded templates (Sambrook *et al.*, 1989). The sequence of nucleotides at the 5' end of the RNA was determined by primer extension using specific primers on PMTV RNA templates. Sequence analysis was performed using the GCG package (Devereaux, *et al.*, 1984).

Results

Attempts to synthesise cDNA from PMTV RNA using oligo(dT) as a primer were unsuccessful unless the RNA was first polyadenylated indicating that the PMTV RNA species are not contain polyadenylated. The complete sequences of RNAs 2 and 3 were determined and are discussed separately.
PMTV RNA2
The second largest RNA species of PMTV consists of 2962 nucleotides and encodes four open reading frames (ORFs), of which the three largest have homologies with the triple-gene-block proteins of a number of other plant viruses. In all three proteins this homology was strongest with the triple-gene-block proteins of barley stripe mosaic hordeivirus (BSMV). The first ORF starts at nucleotide 369 and ends at 1760 encoding a 51K protein. This protein contains an NTP-binding motif (CVPGSGKT) and, by sequence homologies, appeared to be equivalent to the 58K and 42K triple-gene-block proteins of BSMV and

BNYVV, respectively. The sequence identity was greatest (32%) with the BSMV protein. The second ORF was between nucleotides 1747 and 2106 and encodes a 13K protein which was equivalent to the 14K and 13K triple gene block proteins of BSMV and BNYVV respectively. Again the sequence similarity of this protein was greatest (52%) with the corresponding BSMV protein. The third ORF was between nucleotides 1961 and 2533 and codes for a 21K protein. The only significant homology this protein has is a 29% identity with the 17K triple gene block protein of BSMV. A fourth, small ORF between nucleotides 2461 and 2667 codes for an 8K cysteine-rich protein. No significant homologies were found between this protein and any othersin the databases. The 3' untranslated region is 295 nucleotides.

PMTV RNA3

The smallest RNA species of PMTV contains 2,315 nucleotides. An ORF between nucleotides 289 and 817 encodes a 19.7K protein. In-frame fusions of the carboxy-terminal 77 amino acids of this ORF with the β-galactosidase gene in pBluescript plasmids produced a protein which reacted in western blots with monoclonal antibodies to PMTV indicating that this ORF was the coat protein gene. The 19.7K protein has 29.9% identity with the coat protein of SBWMV and 19.8% identity with that of BNYVV. The PMTV coat protein gene terminates in a UAG (amber) termination codon and is followed by an in frame ORF which terminates at a UAA codon, nucleotides 2098-2100. Readthrough of the amber termination codon could therefore result in synthesis of coat protein/readthrough protein with a total molecular weight of 67K. PMTV would thus be similar to SBWMV and BNYVV, both of which have potential coat protein readthrough genes. Some sequence similarity was found between the readthrough proteins of PMTV and SBWMV, but there was no significant homology between the PMTV readthrough protein and the BNYVV readthrough protein. The 3' untranslated region of RNA3 is 218 nucleotides long.

Discussion

The genome organisation of PMTV is the third to be determined for a furovirus. Comparisions indicate that all three viruses differ and suggest that PMTV, BNYVV and SBWMV represent three distinct subgroupings within the furovirus group. SBWMV is the type member of the furovirus group and contains two RNA species. The larger (RNA1) of these two RNAs is similar in organisation to the RNAs of tobamoviruses and tobraviruses, and resembles TMV RNA with the exception that the coat protein gene is absent from the 3' region of the RNA. The coat protein of SBWMV is encoded on the smaller RNA molecule (RNA2) where it is followed immediately by a readthrough protein gene and an ORF coding for a 19K cysteine-rich protein (Shirako and Wilson, 1993). PMTV RNA 3 is similar in organisation to SBWMV RNA2, but lacks an ORF coding for a cysteine-rich protein 3' to the readthrough protein. Sequence similarities between the encoded proteins also suggest that PMTV RNA3 is most closely related to SBWMV RNA2. The genome of BNYVV consists of at least four RNA species. The largest of these (RNA1) is quite distinct from the RNA1 of SBWMV and contains a single ORF coding for a 237K protein which presumably functions as a replicase (Bouzoubaba *et al.*, 1987). BNYVV RNA2 is similar to that of SBWMV and PMTV RNA3 in containing the virus coat protein gene

followed by an in-frame readthrough protein gene but the similarities end there. The BNYVV readthrough protein gene is followed by a triple-gene-block and a 15K cysteine-rich protein. PMTV RNAs 2 and 3 together would thus appear to be functionally equivalent to BNYVV RNA2, but the coat protein and readthrough protein encoded by PMTV RNA3 are most similar to those of SBWMV, and the triple-gene-block proteins encoded by PMTV RNA2 are most similar to BSMV. It is therefore unlikely that PMTV RNAs 2 and 3 arose as a splitting of a BNYVV-like RNA2 or conversely that BNYVV RNA2 arose as a joining together of PMTV-like RNAs 2 and 3. The evolutionary relationships between members of the furovirus group, and between the furovirus group and other plant viruses are complex and would appear to involve exchange of gene modules such as the triple-gene-block. There appear to be independent exchanges of genetic information within individual RNA species and hence independent evolutionary relationships of different parts of the same RNA molecule.

References

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., and Richards, K. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. J. Gen. Virol. **67**: 1689-1700

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G., and Richards, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. J. Gen. Virol. **68**: 615-626

Calvert, E.L. and Harrison, B.D. (1966). Potato mop-top, a soil-borne virus. Plant Pathol. **15**: 134-139

Devereux,J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. 12,386-395

Francki, R.I.B., Fauquet, C.M., Knudson, D.L., and Brown, F. (1991) Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. Arch. Virol. Suppl. 2

Gubler, U. and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene **25**: 263-269

Harrison, B.D. and Jones, R.A.C. (1970). Host range and some properties of potato mop-top virus. Ann. Appl. Biol. **65**: 393-402

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**: 351-359

Jones, R.A.C. and Harrison, B.D. (1969). The behaviour of potato mop-top in soil and evidence for its transmission by *Spongospora subterranea*. Ann Appl. Biol. **63**: 1-17

Kallender, L.H., Buck, K.W., and Brunt, A.A. (1990). Association of three RNA molecules with potato mop-top virus. Neth. J. Plant Pathol. **96**: 47-50

Kassanis, B., Woods, R.D., and White, R.F. (1972). Some properties of potato mop-top virus and its serological relationship to tobacco mosaic virus. J. Gen. Virol. **14**: 123-132

Randles, J.W., Harrison, B.D., and Roberts, I.M. (1976). Nicotiana velutina mosaic virus: purification, properties and affinities with other rod-shaped viruses. Ann. Appl. Biol. **63**: 1-17

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Eds N. Ford, C. Nolan and M. Ferguson. Cold Spring Harbour Press

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. (USA) **89**: 5715-5719

Scott,K.P., Reavy, B., and Harrison, B.D. (1990). Preliminary analysis of the RNA of potato moptop virus. Schrift. der Deutsch. Phytomed. Gesell.1: 95-97

Shirako, Y. and Wilson, T.M.A. (1993). Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. Virol. (In Press)

NUCLEOTIDE SEQUENCES AND GENOME ORGANIZATIONS OF SOIL-BORNE WHEAT MOSAIC AND SORGHUM CHLOROTIC SPOT VIRUSES

Y. Shirako[1], T. M. A. Wilson[2], R. C. French[3], and J. H. Strauss[1]

[1]Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA; [2]Department of Virology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK; [3]USDA, ARS, University of Nebraska, Lincoln, NE 68583, USA

Summary

Nucleotide sequences of RNA 1 and RNA 2 of soil-borne wheat mosaic virus (SBWMV; RNA 1 from 1981 Nebraska isolate; RNA 2 from 1981 and 1988 Nebraska isolates and 1990 Illinois isolate) and sorghum chlorotic spot virus (SCSV; RNA 1 and RNA 2 from 1986 Kansas isolate) were determined. SBWMV RNA 1 (7.1 kb) encodes, from 5' to 3', overlapping 150-kDa and 209-kDa putative replicase proteins and a 37-kDa putative transport protein. SBWMV RNA 2 (3.6 kb) encodes, from 5' to 3', the 19-kDa capsid protein, an in-frame 84-kDa readthrough protein, and a 19-kDa cysteine-rich protein. In both RNAs, the 3' terminal regions may form a tRNA-like structure of the tymovirus-type. The nucleotide sequences of the 1981 and the 1988 Nebraskan SBWMV RNA 2 were identical. RNA 2 of the 1990 Illinois isolate differed from the Nebraskan RNA 2 by 9.7% at the nucleotide level and by 3.0% at the amino acid level; the amino acid sequences of the capsid proteins were identical. SCSV has the same genome organization and 3' end tRNA-like structure as SBWMV. SCSV RNA 1 (6.9 kb) codes for overlapping 145-kDa and 204-kDa proteins and a 37-kDa protein. SCSV RNA 2 (3.5 kb) codes for the 19-kDa capsid protein, an 81-kDa readthrough protein, and an 18-kDa cysteine-rich protein. The RNA polymerase domain was the most highly conserved protein between SBWMV and SCSV with 72% amino acid identity and the small cysteine-rich protein was the most divergent with 34% identity.

Introduction

Soil-borne wheat mosaic virus (SBWMV) and sorghum chlorotic spot virus (SCSV) are members of the furovirus group infecting cereal plants (Brunt, 1991). The two viruses are similar in particle morphology with stiff, rod-shaped particles 20 nm in diameter and 260-300 nm and 140-160 nm in length, and are distantly related in serological studies. However, SBWMV and SCSV differ significantly in biological properties (Kendall *et al.*, 1988). SBWMV systemically infects wheat and barley but not maize, whereas SCSV infects maize but neither wheat nor barley. The optimum temperature for SBWMV replication is around 17°C and that for SCSV is 25° or higher. In addition, SBWMV has been reported in most winter wheat growing areas in the US as well as in France, Italy, Egypt, Japan, China, and Brazil. SCSV has been isolated from only a few fields in Nebraska and Kansas (Brakke and Langenberg, 1988).

In this study, nucleotide sequences of SBWMV and SCSV RNAs were determined to elucidate the relationships between the two cereal furoviruses as well as their relationships to other rod-shaped plant RNA viruses. In addition, the nucleotide sequences of RNA 2 of SBWMV isolated in different years or in different locations were determined to ascertain the extent of heterogeneity among SBWMV isolates.

Materials and Methods

Virus isolates

Wheat leaves infected with SBWMV were collected in a nursery at the University of Nebraska, Lincoln in 1981 or 1988. SBWMV-infected wheat leaves were also collected in a test field of the University of Illinois at Urbana in 1990 and kindly provided by A. D. Hewings. The 1981 Nebraskan isolate was propagated in wheat plants after mechanical transfer and virus was purified from systemically infected leaves. Viruses of 1988 Nebraskan and 1990 Illinois isolates were directly purified from field materials. Corn leaves infected with SCSV were kindly provided by S. A. Lommel. Virus was propagated in corn plants after mechanical inoculation and was purified from systemically infected leaves.

cDNA cloning

SBWMV RNA was extracted from purified virus by an SDS/phenol method and RNA 1 and RNA 2 were fractionated by sucrose density gradient centrifugation. The 3' end was polyadenylated with *E. coli* poly(A) polymerase and first strand cDNA synthesis was primed with dT(17). Second strand synthesis by *E. coli* DNA polymerase I was primed with RNase H-generated RNA fragments. The ds cDNA was cloned into a plasmid vector either directly or after restriction endonuclease digestion. SCSV RNA was SDS/phenol-extracted from purified virus, electrophoresed in an agarose gel, and purified from the sliced gel cDNA was synthesized using a random deoxyhexanucleotide and the second strand was synthesized by the RNase H/*E. coli* DNA polymerase I method. The ds cDNA was treated with *Eco*RI methylase, ligated with *Eco*RI linker and cloned into an *Eco*RI-digested plasmid vector. cDNA to the 3' terminal region of SCSV RNA was prepared with a d(T)17 primer to *in vitro* polyadenylated RNA or with a primer complimentary to the terminal 17 nucleotides (nt) and converted to ds DNA, followed by cloning into a plasmid vector.

Nucleotide sequencing

cDNA clones to SBWMV RNA were subcloned utilizing convenient restriction endonuclease sites in the inserts. Fifty random cDNA clones to SCSV RNA were directly used for sequence analysis. Nucleotide sequence was determined by the dideoxy method using primers annealing to the vector sequence flanking the insert or primers specifically annealing to the internal sequence. The 5' terminal sequence was determined directly from viral RNA by a dideoxy method.

Results and Discussion

Nucleotide sequence and genome organization of SBWMV

The complete nucleotide sequences of SBWMV RNA 1 (1981 Nebraska isolate) and RNA 2 (1988 Nebraska isolate) have been published (Shirako and Wilson, 1993). A schematic diagram illustrating the genome organization of the virus is shown in Fig. 1. RNA 1 is 7099 nt and codes for 150-kDa and 209-kDa, putative replicase proteins in the 5' terminal region, and a 37-kDa putative transport protein of the dianthovirus-type (Xiong *et al.*, 1993; Mushegian, personal communication) in the 3' terminal region. The 209-kDa protein is translated by a partial readthrough of the opal termination codon following the 150-kDa protein. RNA 2 is 3593 nt and encodes the 19-kDa capsid protein in the 5' terminal region. The opal termination codon of the capsid protein gene can be partially suppressed to produce an in-frame 84-kDa readthrough protein. In the 3' terminal region, there is another open reading frame for a 19-kDa cysteine-rich protein. A 28K protein, which was originally considered to be another capsid readthrough protein (Hsu and Brakke, 1985; Shirako and Ehara, 1986), appears to be initiated at a non-AUG codon upstream of the capsid protein gene. In both RNAs, the 5' end is capped and the 3' terminal region may be folded into a tRNA-like structure identical to that formed by tymoviral RNA (Mans *et al.*, 1991). An amino acid sequence comparison of RNA replicase genes indicated that SBWMV is

closely related to barley stripe mosaic, tobacco rattle and tobacco mosaic viruses but only distantly related to beet necrotic yellow vein virus (BNYVV). Taken together with the differences which exist in the 3' end structures (Putz *et al.*, 1983) and in the cell-to-cell movement proteins (Gilmer *et al.*, 1992), this result suggests that SBWMV and BNYVV should be placed in separate virus groups.

Divergence in SBWMV RNA 2 sequences between US isolates

Nucleotide sequences of RNA 2 of Nebraskan SBWMV isolated in 1981 and Illinois SBWMV isolated in 1990 were also determined and compared with that of the 1988 Nebraskan SBWMV RNA 2. The 1981 Nebraskan SBWMV RNA 2 was 3593 nt and the nucleotide sequence was identical to that of the 1988 Nebraskan SBWMV RNA 2. The two Nebraskan isolates were obtained from the same field that was infested with SBWMV for years, and the evolution of SBWMV RNA 2 genome in nature appears to be very slow. On the other hand, RNA 2 of Illinois SBWMV isolated in 1990 differed in sequence (Fig. 2). This RNA 2 was the same size as the Nebraskan RNA 2 and the sizes of the 5', 3' and internal untranslated regions and of the coding regions were identical to those of the Nebraskan RNA 2. The capsid protein was completely conserved as to amino acid sequence but there was 12.6% divergence at the nucleotide level. The three other proteins encoded had amino acid sequence differences. The overall difference between the Nebraskan and Illinois SBWMV RNA 2 sequences was 9.7% at the nucleotide level and 3.0% at the amino acid level, suggesting that the Nebraskan and Illinois SBWMV might have diverged several hundreds of years ago.

Genome organization of SCSV and its relationship to SBWMV

The nucleotide sequences of all coding regions as well as that of the 3' terminal untranslated regions of SCSV RNA 1 and RNA 2 were determined from cloned cDNA (Fig. 1). The 5' terminal regions of RNA 1 and RNA2 have not yet been sequenced. SCSV RNA 1 is approximately 6900 nt and encodes, from 5' to 3', a 145-kDa protein whose opal termination codon can be partially suppressed to produce a 204-kDa readthrough protein, and a 37-kDa protein, followed by 3' untranslated region of 368 nt. SCSV RNA 2 is approximately 3500 nt and encodes, from 5' to 3', the 19-kDa capsid protein whose opal termination codon can be partially read through to produce an 81-kDa protein, and a cysteine-rich 18-kDa protein, followed by the 539 nt of the 3' untranslated region. An initiation codon for a 25K protein (Kendall *et al.*, 1988) equivalent to the SBWMV 28K protein has not been identified, but the 25K protein is probably initiated at a non-AUG codon upstream from the capsid protein gene similar to the case for the SBWMV 28K protein. The nucleotide sequence of the 3' untranslated region is highly conserved between RNA 1 and RNA 2, and in both RNAs the terminal 90 nt region possibly forms a tRNA-like structure identical to that formed by tymoviral RNA. A poly(A) stretch of 27 nt at the middle portion of the 3' untranslated region was a feature unique to SCSV RNA 2. Thus SCSV has exactly the same genome organization and structure as SBWMV. Percent identities at the amino acid level in each protein domain of the two viruses are shown in Fig. 1. The RNA polymerase domain in the readthrough regions of SBWMV 209-kDa and SCSV 204-kDa proteins was the most conserved, whereas the small cysteine-rich protein encoded at the 3' proximity of RNA 2 was the most diverged. These results indicate that SCSV is not a host range variant or a high temperature strain of SBWMV but a distinct member of the furovirus group.

References

Brakke, M. K., and Langenberg, W. G. (1988). Experiences with soil-borne wheat mosaic virus in North America and elsewhere. *In* "Viruses with Fungal Vectors" (J. I. Cooper and M. J. C. Asher, Eds.), pp. 183-202. Association of Applied Biologist, Warwick, England.

Brunt, A. A. (1991). Soil-borne wheat mosaic virus group. *In* "Classification and nomenclature of Viruses, Fifth Report of the International Committee on Taxonomy of Viruses" (R. I. B. Francki, C. M. Fauquet, D. L. Knudson, and F. Brown, Eds.), pp. 377-379. Springer-Verlag, New York.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. *Virology* 189: 40-47.

Hsu, Y. H., and Brakke, M. K. (1985). Cell-free translation of soil-borne wheat mosaic virus RNAs. *Virology* 143: 272-279.

Kendall, T. L., Langenberg, W. G., and Lommel, S. A. (1988). Molecular characterization of sorghum chlorotic spot virus, a proposed furovirus. *J. Gen. Virol.* 69: 2335-2345.

Mans, R. M. W., Pleij, C. W. A., and Bosch, L. (1991). tRNA-like structures: structure, function and evolutionary significance. *Eur. J. Biochem.* 201:303-324.

Putz, C., Pinck, M., Fritsch, C., and Pinck, L. (1983). Identification of the 3'- and 5'-ends of beet necrotic yellow vein virus RNAs. *FEBS Lett.* 154: 41-46.

Shirako, Y., and Ehara, Y. (1986). Comparison of the in vitro translation products of wild-type and a deletion mutant of soil-borne wheat mosaic virus. *J. Gen. Virol.* 67:1237-1245.

Shirako, Y., and Wilson, T. A. M. (1993). Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* 195: 16-32.

Xiong, Z., Kim., K. H., Giesman-Cookmeyer, D., and Lommel, S. A. (1993). The role of the red clover necrotic mosaic virus capsid and cell-to-cell movement proteins in systemic infection. *Virology* 192: 27-32.

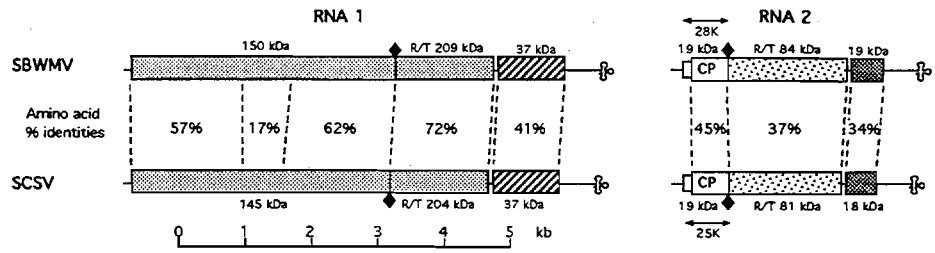


Fig. 1 Genome organizations of RNA 1 and RNA 2 of SBWMV and SCSV.

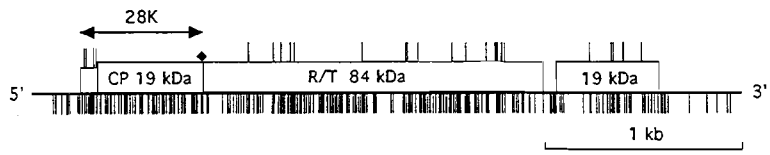


Fig. 2 Comparison of SBWMV RNA 2 sequence between Nebraskan and Illinois isolates. Ticks above the boxes indicate differences in amino acids and ticks below the horizontal line indicate differences in nucleotides.

IDENTIFICATION OF SEQUENCES INVOLVED IN BEET NECROTIC YELLOW VEIN VIRUS ENCAPSIDATION AND DIFFUSION

A. Hehn[1], Corinne Schmitt[1], Anne-Marie Haeberlé[2], D. Gilmer[1], Christiane Stussi-Garaud[1], H. Guilley[1], K. Richards[1] and G. Jonard[1]

[1]Institut de Biologie Moléculaire des Plantes du CNRS et de l'Université Louis Pasteur
12 rue du Général Zimmer, Strasbourg 67084 Cedex, France

[2]INRA, Station Grandes Cultures, Laobratoire de Pathologie Végétale
68021 Colmar Cedex, France

Summary

Functions associated with the six proteins encoded by beet necrotic yellow vein virus RNA 2 have been investigated by reverse genetics, made possible by the availablity of biologically active RNA transcripts. The proteins of the Triple Gene Block, P42, P13 and P15, were necessary for cell-to-cell movement of the virus in the course of infection of leaves but were not required for replication in single cells (protoplasts). Mutations in P14, encoded by the 3'-proximal gene of RNA 2, partially inhibited virus multiplication in both leaves and protoplasts. Mutations in the C-terminal portion of the P75 readthrough domain are known to interfere with vector transmission. Mutations in the N-terminal portion of the readthrough domain and the coat protein interfered with formation of virions. These findings suggest that P75 is involved in virus assembly and may remain associated with virions as a minor structural component.

Introduction

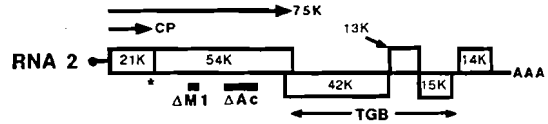
Beet necrotic yellow vein virus (BNYVV) is a multicomponent plus-strand RNA virus vectored by *Polymyxa betae* Keskin and responsible for rhizomania disease of sugar beet (for review see Richards and Tamada, 1992). Of the four genome components detected in European isolates, RNA 1 is necessary and sufficient for RNA replication (Bouzoubaa *et al.*, 1990; Gilmer *et al.*, 1992) while RNAs 3 and 4 influence virus proliferation in root tissue (Tamada *et al.*, 1990; Koenig *et al.*, 1991) and vector transmission (Tamada and Abe, 1989), respectively.

The availability of biologically active synthetic transcripts of each of the BNYVV RNAs (Quillet *et al.*, 1989) has permitted investigation of gene function by 'reverse genetics', in which mutations are introduced into the genome at the cDNA level. Then RNA is transcribed from the cDNA and the biological properties of the mutated transcripts are examined. Here we describe recent findings using this approach to study functions of the genes carried by BNYVV RNA 2.

Genetic organization of RNA 2 and subcellular localization of RNA 2-encoded proteins.

BNVV RNA 2 carries six ORFs (Fig. 1). The 5'-proximal ORF encodes the viral coat protein (CP). This ORF terminates in an amber termination codon which undergoes translational readthrough into the following 54 kDa ORF (the 'readthrough domain') about 10% of the time to produce a longer 'readthrough protein', P75, which has the coat protein sequence at its N-terminus (Bouzoubaa *et al.*, 1986). The readthrough domain is followed by a Triple Gene Block (TGB) which displays sequence homologies with similar gene clusters in barley stripe mosaic virus (BSMV), potexviruses and carlaviruses (reviewed by Morozov *et al.*, 1989). The 3'-proximal ORF encodes a cysteine-rich 14 kDa protein.

Using serological techniques, all the RNA 2-encoded proteins have been detected in BNYVV-infected *Chenopodium quinoa* leaves except P15 (Niesbach-Klösgen *et al.*, 1990). CP and P75 were present in all subcellular fractions (Fig. 1), suggesting that these species may have



ORF	CELL COMPARTMENT			
	CELL WALL	1000g PELLET (NUC+CHLORO)	30,000g PELLET (MEMBRANES)	30,000g SUPER (CYTOSOL)
CP	←	+		→
P75	←	+		→
P42	±	−	+	−
P13	±	−	+	−
P15	?			
P14	−	−	−	+

Fig. 1 Genetic organization of BNYVV RNA 2 and subcellular localization of its gene products (see Niesbach-Klösgen *et al.*, 1990). The asterisk denotes the suppressible termination codon separating the CP and 54 k ORFs. Black rectangles indicate the deletions in Mutants ΔM1 and ΔAc (see below). TGB = Triple Gene Block.

a tendency to aggregate or 'stick' nonspecifically to cell structures. P42 and P13 were predominantly associated with the membrane-enriched fraction (Fig. 1), a location which may be functionally significant in view of the role of these species in cell-to-cell diffusion (see below). P14 was present almost exclusively in the cytosol.

The Triple Gene Block proteins are required for cell-to-cell movement in leaves.

Mutants RNA 2 transcripts were prepared in which each ORF was disabled by deletion or frameshift mutation. The mutants were then inoculated, along with wild-type RNA 1 transcript, to leaves and protoplasts of *C. quinoa*. Such experiments (Fig. 2; also see Gilmer *et al.*, 1992) revealed that none of the TGB proteins are required for RNA replication because mutation of these genes did not interfere with infection of protoplasts. No infection of whole leaves, on the other hand, was detected (Fig. 2), indicating that the TGB proteins intervene in cell-to-cell spread of the infection. A similar conclusion has been reached for the TGB proteins of barley stripe mosaic virus (Petty *et al.*, 1990) and white clover mosaic potexvirus (Beck *et al.*, 1991).

Mutations in P14 produced a more complex phenotype. Such mutations significantly diminished both protein and RNA production (particularly of RNA 2) in protoplasts and plants but did not completely knock out RNA replication (Fig. 2). Several different types of mutation produced this inhibitory effect, including frameshift mutations (Mutants P and L; Fig. 2), elimination of the initiation codon (not shown) and introduction of termination codons by point mutation (Mutants M and N; Fig. 2). The capacity of different types of mutation in P14 to produce the same phenotype makes it unlikely that the effect on replication and protein synthesis is due to interference with a *cis*-acting signal on RNA 2.

All the P14 mutations also altered the symptoms on leaves, producing pinpoint necrotic local lesions rather than large chlorotic lesions characteristic of the wild type (Gilmer *et al.*, 1992). The mechanism by which P14 intervenes in the infection process is currently under investigation.

P75 readthrough protein and BNYVV assembly.

Mutations disabling the coat protein and P75 were not lethal in whole leaf infections (Schmitt *et al.*, 1992). As expected, the coat protein mutants did not package the viral RNA, as shown by

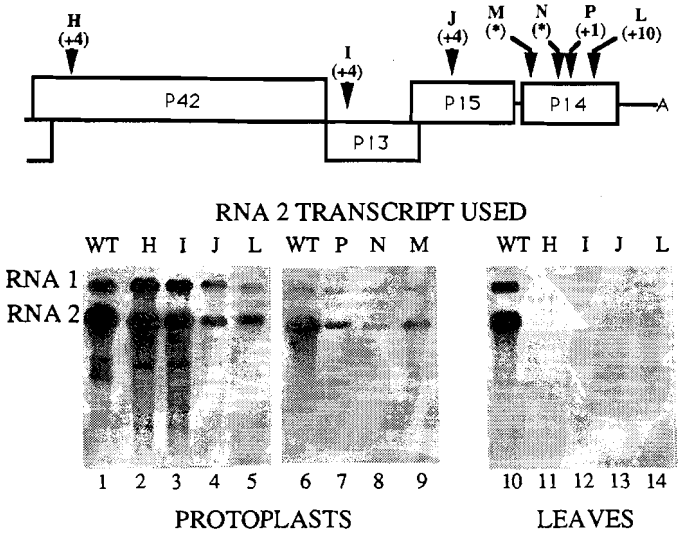


Fig. 2 Effect of mutations in the 3'-proximal genes of RNA 2 on virus multiplication in *C. quinoa* protoplasts and leaves. Each mutation site is indicated by an arrow and the number of nts added to produce a frameshift is given in parentheses. Point mutations that introduce a termination codon are indicated by asterisks. The results of Northern hybridization on RNA extracted from protoplasts or leaves inoculated with RNA 1 plus each mutant is shown below. In lanes 7-9, 20 times more RNA was loaded than in lane 6. WT = wild type.

the sensitivity of viral RNA in crude extracts to nucleolytic degradation (Schmitt *et al.*, 1992). Mutations in the C-terminal portion of the P75 readthrough domain, such as the deletion mutant ΔAc (Fig. 1), did not interfere with virion formation (Schmitt *et al.*, 1992) but blocked transmission by *P. betae* (authors' unpublished observations). This finding confirms an earlier study of the properties of naturally occuring RNA 2 mutants carrying deletions near the P75 C-terminus (Tamada and Kusume, 1991; T. Tamada, personal communication). Finally, deletions in the N-terminal protion of the P75 readthrough domain, such as ΔM1 (Fig. 1) interfered with virion assembly (Schmitt *et al.*, 1992). The effect on assembly is not due to deletion of a sequence on the viral RNA acting in assembly (e.g., an assembly initiation sequence) because the encapsidation of RNA 1was blocked as well (Schmitt *et al.*, 1992).

The effect of deletions in the P75 readthrough domain on both virion assembly and vector transmission suggests that P75 is associated with virions, perhaps in a transient fashion or as a minor structural component. Direct evidence for an association between P75 and BNYVV particles has recently been provided by immunogold electron microscopy using a gold-labelled antiserum specific for the P75 readthrough domain (Haeberlé *et al.*, submitted). BNYVV has rod-shaped virions and the electron microscopy observations suggest that P75 is associated with an extremity of the virus particle. A possible model accounting for the electron microscopy observations and the mutagenesis results is shown in Fig. 3. In the model, it is assumed that one or a few P75 molecules specifically initiate virion assembly. The interaction with the viral RNA would occur *via* the coat protein moiety of P75 with the readthough domain conferring specificity to and possibly accelerating the process. Once assembly nucleation has occured, rod elongation could proceed by stepwise addition of CP subunits. In infected tissue, physical association between P75 and growing or completed particles would evidently have implications for the mechanism by which P75 intervenes in vector transmission of the virus.

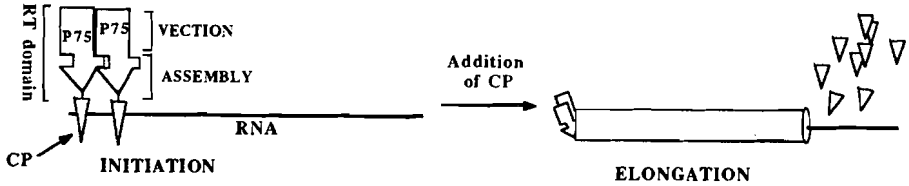


Fig. 4 Two-step model for BNYVV assembly (not to scale). The number of P75 molecules involved in assembly initiation is not known. RT domain = readthrough domain of P75.

References

Beck, D. L., Guilford, P. J., Voot, D. M., Andersen, M. T., and Forster, R. L. S. (1991) Triple gene block proteins of white clover mosaic potexvirus are required for transport. *Virol.* **183**:695-702.

Bouzoubaa, S. and Scheidecker, D. (1990) Infection of *Chenopodium quinoa* protoplasts with synthetic transcripts of beet necrotic yellow vein virus RNAs. in Proceedings of the First International Symposium of the Working Group on Plant Viruses with Fungal Vectors. pp 21-23. Koenig, R.(ed) , Verlag Eugen Ulmer, Stuttgart.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K., and Jonard, G. (1986) Nucleotide Sequence of beet necrotic yellow vein virus RNA-2. *J. Gen. Virol.* **67**:1689-1700.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. (1992) Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3'-proximal genes located on RNA 2. *Virol.* **189**:40-47.

Koenig, R., Jarausch, R., Li, Y., Commandeur, U., Burgermeister, W., Gehrke, M., and Lüddecke, P. (1991) Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanical inoculation of sugarbeets. *J. Gen. Virol.* **72**:2243-2246.

Morozov, S. Y., Dolja, V. V., and Atabekov, J. G. (1989) Probable reassortment of genomic elements among elongated RNA-containing plant viruses. *J. Mol. Evol.* **29**:52-62.

Niesbach-Klösgen, U., Guilley, H., Jonard, G., and Richards, K. (1990) Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. *Virol.* **178**:52-61.

Petty, I. T. D., French, R., Jones, R. W., and Jackson, A. O. (1990) Identification of barley stripe mosaic virus genes involved in virus replication and systemic movement. *EMBO J.* **9**:3453-3457.

Quillet, L., Guilley, H., Jonard, G., and Richards, K. (1989) In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. *Virol.* **172**:293-301.

Richards, K. and Tamada, T. (1992) Mapping functions on the multicomponent genome of beet necrotic yellow vein virus. *Annu. Rev. Phytopath.* **30**:291-313.

Schmitt, C., Balmori, E., Jonard, G., Richards, K. E., and Guilley, H. (1992) *In vitro* mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 54-kDa readthrough protein is important for efficient virus assembly. *Proc. Natl. Acad. Sci. USA* **89**:5715-5719.

Tamada, T. and Abe, H. (1989) Evidence that beet necrotic yellow vein virus RNA 4 is essential for efficient transmission by the fungus *Polymyxa betae*. *J. Gen. Virol.* **70**:3391-3398.

Tamada, T. and Kusume, T. (1991) Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus *Polymyxa betae*. *J. Gen. Virol.* **72**:1497-1504.

Tamada, T. Saito, M. Kiguchi, T., and Kusume, T. (1990) Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. in Proceedings of the First International Symposium of the Working Group on Plant Viruses with Fungal Vectors. pp 41-44. Koenig, R.(ed), Verlag Eugen Ulmer, Stuttgart.

DETECTION AND SEQUENCE ANALYSIS OF DELETION MUTANTS OF BEET NECROTIC YELLOW VEIN VIRUS BY RT-PCR

M. Saito, T. Kiguchi and T. Tamada

Hokkaido Central Agricultural Experiment Station, Naganuma, Hokkaido, 069-13 Japan

Summary

The reverse transcription and polymerase chain reaction (RT-PCR) was applied to detect beet necrotic yellow vein virus (BNYVV) isolates containing full-length RNA-3 and mutant isolates containing RNA-3 deleted to various extents between nucleotides 380 and 1266. Five oligonucleotide primers were tested in different combinations. The results showed that at least two different combinations of 5' and 3' primers could be used to detect the deletion mutants, depending on the size of the deletion. The detection limit of RT-PCR was about 1 pg of RNA. The test sensitivity was affected by several factors, such as primer combination, number of reaction cycles, and relative quantities of template RNA in the sample. The RT-PCR was shown to be useful for the detection of deletion mutants of BNYVV RNA-3.

Introduction

Beet necrotic yellow vein virus (BNYVV), responsible for rhizomania disease of sugar beet (*Beta vulgaris* L.), is transmitted by the soil-borne fungus *Polymyxa betae*. Field isolates of BNYVV usually contain four or five RNA species. All four BNYVV species RNA-1, -2, -3 and -4 (French isolate) have been sequenced (reviewed by Richards and Tamada, 1992), and, in addition, we have determined the complete nucleotide sequences of RNA-2, -3, -4 and -5 of Japanese isolates. RNA-1 and RNA-2 are needed for virus infection, whereas the other smaller RNA species (RNA-3, -4 and -5) behave like satellite RNA molecules in mechanically inoculated leaves of host plants (Tamada *et al.*, 1989; Richards and Tamada, 1992). Isolates of BNYVV maintained in the laboratory are customarily propagated by mechanical inoculation onto leaves of test plants. In that case, however, it is known that smaller RNAs contained in BNYVV isolates sometimes undergo deletion during serial propagation in leaves (Bouzoubaa *et al.*, 1985; 1991). For example, manual inoculation of field isolates of BNYVV produces mutant isolates which elicit different types of local lesions on the leaves of *Tetragonia expansa*. These mutant isolates contain RNA-3 which has internal deletions in the open reading frame (ORF) that encodes a 25K polypeptide (Bouzoubaa *et al.*, 1991).

Recently, polymerase chain reaction (PCR) has been developed to detect very small amounts of nucleic acids (Saiki *et al.*, 1988). This technique has been used to diagnose plant RNA viruses by doing a reverse transcription reaction before the polymerase chain reaction (Vunsh *et al.*, 1990; Wetzel *et al.*, 1991;Kohnen *et al.*, 1992; Robinson, 1992; Lim *et al.*, 1993). We applied RT-PCR using several oligonucleotide primers to detect and analyze BNYVV deletion mutants. In this paper, we describe the effect of several factors on the detection of isolates of full-length and deletion mutants of BNYVV RNA-3 by RT-PCR.

Materials and methods

Virus isolates: The laboratory isolates, S-3 (RNA-1+2+3) containing normal size RNA-3 and S-3c (RNA-1+2+3c) containing deleted RNA-3 (named RNA-3c), whose deleted region is from nt 725 to 1087 of RNA-3, were used. These isolates were obtained from a field isolate S by single lesion transfers in *Tetragonia expansa* leaves. In addition, another four isolates, 5YS-4, K-52-3, 45 and 21, which were thought to contain deleted forms of RNA-3, were used. They were obtained from several field isolates by mechanical inoculation to *T. expansa*. Laboratory isolates S-4 (RNA-1+2+4) containing RNA-4, and D-5 (RNA-1+2+5) containing RNA-5, were used as controls.

Sample preparation: RNA samples were prepared from virus particles purified partially from inoculated *T. expansa* leaves as described by Tamada *et al.* (1989).

RT-PCR: Five kinds of 15- to 22-mer oligonucleotides used for detection of BNYVV RNA-3 were synthesized on an Applied Biosystems DNA synthesizer Model 392: upstream primers, 3A, 5'-TCTCTTTATTTATCACCAAC-3' (nucleotides (nt) 211-230), 3B, 5'-CGAATTCATGGGTGATATATTA-3' (Eco RI + nt 442-458, mismatched nucleotides are underlined) and 3C, 5'-TCTTAGCACAAATGG-3' (nt 527-541), and downstream primers, 3D, 5'-GTGATAACTCTAATC-3' (nt 1098-1112) and 3E, 5'-TTTCACACCC AGTCAGTACA-3' (nt 1715-1734). The genetic map of RNA-3 (1773b) and primer positions are shown in Fig. 1. First strand cDNA was synthesized using a commercial kit (Amersham, First strand cDNA synthesis kit) according to the manufacturers instructions. A quarter of the total volume was used for polymerase chain reaction (Promega, *Taq* DNA polymerase). Thirty-five reaction cycles were done, each comprising denaturation for 1 min at 94°C, annealing for 1 min at 50°C and primer extension for 2 min at 72°C. Plasmids pMSC8 containing cloned RNA-3 cDNA was used as a control for the PCR.

Sequencing: PCR products were ligated into pUC119 in *Sma* I or *Hinc* II sites and cloned into *E. coli* NM522 cells. The double-stranded recombinant DNAs were sequenced using an automated fluorescent DNA sequencer (373A, Applied Biosystems).

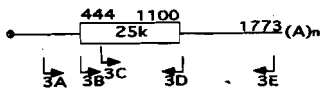


Fig.1 Genetic map of BNYVV RNA-3.

Results

Factors affecting the detection of RNA-3 and RNA-3c by RT-PCR. As shown in Fig. 2, RT-PCR tests using different combinations of primers, 3B/3D, 3C/3D and 3A/3E, were made to detect a normal form (S-3) and a deleted form of RNA-3 (S-3c). The result showed that DNAs of each expected size corresponding to RNA-3 (671bp, 586bp and 1524bp, respectively) and RNA-3c (308bp, 223bp and 1161bp, respectively) were amplified in each of the primer combinations. Single specific bands were detected in each of isolate S-3 and S-3c by the use of 3B/3D primer. However, a primer combination of 3C/3D or 3A/3E gave rise to additional weak bands besides the expected bands in isolate S-3, indicating that primer combination of 3B/3D was more suitable than that of 3C/3D to detect this deletion mutant. The other smaller RNA species, RNA-4 and -5 were not amplified using any of these three primer combinations.

Fig.2 RT-PCR products using different combinations of primers. M, λDNA/[HindIII+EcoRI]; 1, pMSC8; 2, S-3; 3, S-3c; 4, S-4; 5, D-5; 6, water control. The arrowheads indicate the position of PCR products corresponding to full-length RNA-3.

To examine the sensitivity of RT-PCR, 35 and 45 reaction cycles were compared on serially diluted RNA of isolate S-3 using the combination of 3B/3D. Fig. 3 shows that the detection limit was about 1 pg RNA. Increasing the number of PCR cycles from 35 to 45 increased the intensity of the specific band of RNA-3, but non-specific bands did not appear. Another experiment on the effect of primer combination on sensitivity indicates that about ten times greater PCR product was obtained by using 3B/3D than 3A/3D (data not shown). Thus, primer combinations are thought to be an important factor affecting sensitivity of the detection.

A further experiment was done to determine the effect of mixtures of various concentrations of normal-sized RNA-3 and deleted RNA-3c. The result shows that both the normal form and the deleted form were detected easily by RT-PCR using primers 3B/3D (Fig. 4). In this experiment, 0.05-0.1ng of each RNA was detected when mixtures of RNA species were used as samples. However, this value was much higher than that shown in Fig. 3. These differences were thought to be due to the great excess of the total amount of template RNA used in this experiment.

Detection of different deletion mutants of RNA-3 by RT-PCR. Several different deletion mutants of RNA-3 were found to be detected by RT-PCR using different primer combinations (Fig. 5). Deleted RNA-3 contained in 5YS-4 was detected by use of either primer combination of 3A/3D or 3B/3D. Both normal forms and deleted forms of RNA-3 contained in isolates 45 and 21 were also similarly detected by either primer combination 3A/3D or 3B/3D. In the case of K52-3, however, a small PCR product (about 0.6kbp) was amplified by primer combination of 3A/3E, but not by the combination of 3A/3D or 3B/3D, suggesting that nucleotides between these primer positions may be lost as described below.

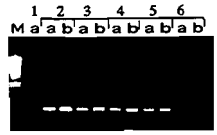


Fig.3 Sensitivity of RT-PCR.
M, λDNA/HindIII; 1, control(pMSC8); 2-6, S-3 RNA. 2, 1000pg; 3, 100pg; 4, 10pg; 5, 1pg; 6, 0.1pg. a, 35 cycles; b, 45 cycles. Primer combination, 3B/3D.

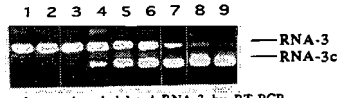


Fig.4 Detection of normal and deleted RNA-3 by RT-PCR.
1, RNA-3 5ng + RNA-3c 0.025ng; 2, RNA-3 5ng + RNA3-c 0.05ng; 3, RNA-3 5ng + RNA-3c 0.1ng; 4, RNA-3 5ng + RNA3-c 0.5ng; 5, RNA-3 5ng + RNA-3c 2.5ng; 6, RNA-3 1ng + RNA3-c 2.5ng; 7, RNA-3 0.2ng + RNA-3c 2.5ng; 8, RNA-3 0.1ng + RNA3-c 2.5ng; 9, RNA-3 0.05ng + RNA-3c 2.5ng. Primer combination, 3B/3D.

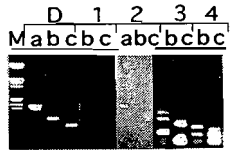


Fig. 5 RT-PCR products of deletion mutants.
M, λDNA/[HindIII+EcoRI]; D, pMSC8; 1, 5YS-4; 2, K52-3; 3, 45; 4, 21; Primer combinations; a, 3A/3E; b, 3A/3D; c, 3B/3D.

Sequence analysis of deletion mutants of RNA-3. Deleted forms of RNA-3 contained in isolates 5YS-4, K52-3, 45 and 21 were sequenced. The deleted region of 5YS-4 was from nt 731 to 799. The deleted regions of isolates 45 and 21 were from nt 462 to 1034 and from nt 469 to 1062, respectively. These mutants retained the 25K ORF in frame. Isolate K52-3 lost 887nt from nt 380 to 1266, indicating that this mutant lost completely the 25K ORF. All of the mutants sequenced were internal deletions, and the deleted regions varied with the isolates.

Discussion

Our results showed that BNYVV RNA-3 and its deletion mutants were detected easily by RT-PCR using different primer combinations. Sequence analysis revealed that all the five mutants tested contained deletions. Deleted regions of four mutants (S-3c, 5YS-4, 45 and 21) were within 25K ORF, but the ORF of one mutant (K52-3) was lost completely. In the case of the former, the primer combination of 3B/3D is suitable for detection, whereas with the latter, (deletions larger than about 1.0kb), the primer combination 3A/3E must be used. Thus, as least two different combinations of primers were needed to detect deletion mutants in laboratory or field isolates of BNYVV.

The detection limit of BNYVV RNA-3 was about 1pg RNA. The sensitivity of RT-PCR obtained in our experiments was higher than that of bean yellow mosaic virus RNA (60pg) reported by Vunsh *et al.* (1990). However, it was reported that it is possible to detect 1-10fg RNA in several plant viruses (Wetzel *et al.*, 1991; Kohnen *et al.*, 1992; Lim *et al.*, 1993). We found that it is important to select a primer combination suitable for detection, as reported by Kohnen *et al.* (1992). We have also shown that the sensitivity is affected by other factors, such as amount of RNA in the samples in the first strand synthesis, or amount of cDNA in the PCR mixture. RT-PCR assays using primer combinations specific to the other smaller RNA species RNA-4 and -5 are in progress.

Acknowledgement

We are grateful to Dr. L. Torrance for helpful comments on the text.

References

Bouzoubaa, S., Guilley, H., Jonard, G., Richards, K. and Putz, C. (1985). Nucleotide sequence analysis of RNA-3 and RNA-4 of beet necrotic yellow vein virus, isolates F2 and G1. J. Gen. Virol. 66:1553-1564.

Bouzoubaa, S., Niesbach-Kloesgen, U., Jupin, I., Guilley, H., Richards, K. and Jonard, G. (1991). Shortened forms of beet necrotic yellow vein virus RNA-3 and -4: internal deletions and a subgenomic RNA. J. Gen.l Virol. 72:259-266.

Kohnen, P.D., Dougherty, W.G. and Hampton, R.O. (1992). Detection of pea seedborne mosaic potyvirus by sequence specific enzymatic amplification. J. Virol. Meth. 37:253-258.

Lim, S.T., Wong, S.M., Yeong, C.Y., Lee, S.C. and Goh, C.J. (1993). Rapid detection of cymbidium mosaic virus by the polymerase chain reaction (RT-PCR). J. Virol. Meth. 41:37-46.

Richards, K.E. and Tamada, T. (1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Annu. Rev. Plant Pathol. 30:291-313.

Robinson, D.J. (1992). Detection of tobacco rattle virus by reverse transcription and polymerase chain reaction. J. Virol. Meth. 40:57-66.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Sharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Tamada, T., Shirako, Y., Abe, H., Saito, M., Kiguchi, T. and Harada, T. (1989). Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. J. Gen. Virol. 70:3399-3409.

Vunsh, R., Rosner, A. and Stein, A. (1990). The use of polymerase chain reaction (PCR) for the detection of bean yellow mosaic virus in gladiolus. Anna. Appl. Biol. 117:561-569.

Wetzel, T., Candresse, T., Ravelonandro and Dunez, J. (1991). A polymerase chain reaction assay adapted to plum pox potyvirus detection. J. Virol. Meth. 33:355-365.

BIOLOGICAL AND MOLECULAR BIOLOGICAL STUDIES ON BARLEY MILD MOSAIC VIRUS STRAINS

Satoshi Kashiwazaki[1], Ken Nomura[2], Satoshi Okuyama[2] and Hiroyuki Hibino[1]

[1]National Agriculture Research Center, Tsukuba, Ibaraki 305, Japan and
[2]Faculty of Agriculture, Ibaraki University, Ami, Ibaraki 300-03, Japan

Summary

Three strains of barley mild mosaic virus, BaMMV-Ka1 and BaMMV-Na1 from Japan and BaMMV-M from Germany, were differentiated by the range of barley cultivars infected by mechanical inoculation. BaMMV-Ka1 and BaMMV-M were similar in symptomatology, but BaMMV-Na1 differed from them. BaMMV-Ka1 and BaMMV-M were serologically indistinguishable, and were related but distantly to BaMMV-Na1. Cross-absorption of antisera to BaMMV-Ka1 and BaMMV-Na1 with virus particles eliminated cross reaction between BaMMV-Ka1 or BaMMV-M and BaMMV-Na1 in ELISA. Sequence analysis indicates that the capsid proteins of BaMMV-Ka1 and BaMMV-Na1 each contain 251 amino acids and have 94% identity. Most of the sequence differences between the two capsid proteins are found in the N-terminal regions, and might explain their serological differences.

Introduction

Yellow mosaic disease causes serious damage to barley production in Japan, China, Korea and several European countries. Two different viruses, barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV), are known to be associated with this disease. BaYMV and BaMMV are similar in particle morphology and symptomatology, and are soil-borne with a common vector fungus *Polymyxa graminis*. However, they are serologically unrelated and are different in some biological and physical properties (Huth & Adams, 1990). Although use of resistant barley cultivars is the only practical measures to avoid damage caused by yellow mosaic, some BaYMV strains have been found to be pathogenic to cultivars having the resistance genes *Ym*, *Ym2* or *ym4* (Kashiwazaki *et al.*, 1989b; Friedt *et al.*, 1990). In 1989-1990, we obtained two BaMMV isolates from yellow mosaic-resistant cultivars at two different sites in Japan, BaMMV-Ka1 isolated from cv. Ishukushirazu with the resistance gene *ym3* at Kagawa and BaMMV-Na1 from cv. Kinuyutaka with *Ym* at Yamaguchi. We compared their properties with those of a BaMMV isolate from Germany (designated as BaMMV-M) and two BaYMV strains II-1 and III from Japan (Kashiwazaki *et al.* 1989b).

Material and Methods

The virus isolates were maintained and propagated on barley cvs. New Golden or Ishukushirazu by mechanical inoculation. Reactions of barley cultivars to virus isolates were examined by mechanical inoculation in growth chambers controlled at 13-15°C. The virus isolates were purified from infected barley leaves as described by Usugi and Saito (1976). Antisera to BaMMV-Ka1 and BaMMV-Na1 were produced in rabbits by three intramuscular and a subsequent intravenous injections. Antisera to BaMMV-M and BaYMV-III

(Kashiwazaki *et al.* 1989b) and antiserum to BaYMV-II-1 (Usugi and Saito, 1976) were also used. To absorb antisera with virus particles, antisera were mixed with purified virus particles, incubated at 4℃ over night, and then the virus particles were removed by ultracentrifugation. Procedures of double immunodiffusion tests, ELISA, SDS-PAGE of capsid proteins and acrylamide-agarose gel electrophoresis of RNAs were described by Kashiwazaki *et al.* (1989b).

Results

Reactions of twenty-six barley cultivars to the three BaMMV isolates and the two BaYMV strains after the mechanical inoculation are summarized in Table 1. Systemic mosaic symptoms were observed in all plants of the test cultivars infected with BaMMV or BaYMV. All the three BaMMV isolates infected cvs. Ishukushirazu, Chikurin Ibaraki 1 Ea52 and Haganemugi, all of which have the resistance gene *ym3*. BaMMV-Na1 and BaMMV-M infected cvs. Misato Golden, Mikamo Golden and Kinuyutaka, all of which have the resistance gene *Ym* derived from cv. Mokusekko 3, whereas BaMMV-Ka1 did not infect them. However, none of the BaMMV isolates or the BaYMV strains infected Mokusekko 3. BaMMV-Ka1 and BaMMV-M infected cvs. Tosan Kawa 73 and Shiromugi 6, which were not infected with BaMMV-Na1.

Table 1. Reactions of barley cultivars to BaMMV isolates Ka1, Na1 and M, and BaYMV strains II-1 and III by mechanical inoculation

	Virus isolate				
	BaMMV			BaYMV	
Barley cultivar	Ka1	Na1	M	II-1	III
Two-rowed barley					
New Golden	+	+	+	+	+
Akagi Nijo	+	+	+	+	+
Haruna Nijo	+	+	+	-	+
Amagi Nijo	+	+	+	-	+
Misato Golden (*Ym*)	-	+	+	-	+
Mikamo Golden (*Ym*)	-	+	+	-	+
Kinuyutaka (*Ym*)	-	+	+	NT	NT
Ishukushirazu (*ym3*)	+	+	+	-	-
Diana (*ym4*)	-	-	-	NT	NT
Six-rowed barley					
Joshushiro Hadaka	+	+	+	-	+
Taishomugi	+	+	+	+	-
Senbon Hadaka	+	+	+	-	-
Asamamugi	+	+	+	-	-
Haganemugi (*ym3*)	+	+	+	-	-
Chikurin Ibaraki 1 Ea52 (*ym3*)	+	+	+	NT	NT
Tosan Kawa 73	+	-	+	+	+
Shiromugi 6	+	-	+	+	+
Kashimamugi	-	-	-	-	+
Mokusekko 3 (*Ym*)	-	-	-	-	-
Mihori Hadaka 3 (*Ym2*)	-	-	-	-	-
Chikurin Ibaraki 1	-	-	-	NT	NT
Masakadomugi (*ym3*)	-	-	-	NT	NT
Chosen (*ym3*)	-	-	-	-	-
Kikai Hadaka	-	-	-	-	-
Shikei 8362	-	-	-	-	-
Franka (*ym4*)	-	-	-	NT	NT

Letters in the parenthesis indicate resistance genes to BaYMV or BaMMV (*see* Friedt *et al.*, 1990).
+=infected ; -=not infected ; NT=not tested.

The symptoms on the barley cultivars caused by the three BaMMV isolates were generally milder than those caused by the two BaYMV strains. BaMMV-Kal and BaMMV-M were very similar in the symptoms, but BaMMV-Nal were different from them. For example, on New Golden, BaMMV-Nal induced less necrosis and yellowing than did BaMMV-Kal or BaMMV-M. However, on cv. Senbon Hadaka, BaMMV-Nal incited severer mosaic and necrosis than did BaMMV-Kal or BaMMV-M.

In immunodiffusion tests, antisera to BaMMV-Kal, BaMMV-Nal and BaMMV-M formed precipitin lines against all the three BaMMV isolates, but none of the antisera reacted with BaYMV-II-1 or BaYMV-III (Fig. 1). Neither antisera to BaYMV-II-1 or BaYMV-III reacted with the three BaMMV isolates. When tested with antisera to BaMMV-Kal or BaMMV-M, the precipitin lines formed against BaMMV-Kal and BaMMV-M were fused, but they spurred over the precipitin lines against BaMMV-Nal. When tested with antiserum to BaMMV-Nal, the precipitin lines formed against the three BaMMV isolates were fused each other.

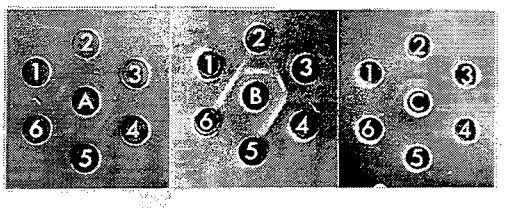


Fig. 1. Serological reactions between BaMMV isolates in double immunodiffusion tests. Center wells were filled with undiluted antisera to BaMMV-M (A), BaMMV-Kal (B) and BaMMV-Nal (C). Peripheral wells were filled with purified preparations of BaMMV-Kal (1,4), BaMMV-Nal (2), BaMMV-M (3), BaYMV-III (5) and BaYMV-II-1 (6).

Table 2. Serological relationships between BaMMV isolates Kal, Nal and M as shown by absorbance values (A_{410}) in ELISA

		Antigen		
Antiserum	Absorption	BaMMV-Kal	BaMMV-Nal	BaMMV-M
BaMMV-Kal	No	>2	0.59	1.70
BaMMV-Kal	BaMMV-Nal	0.72	0.00	0.76
BaMMV-Nal	No	1.68	1.92	1.26
BaMMV-Nal	BaMMV-Kal	0.01	0.33	0.02
BaMMV-M	No	1.69	0.54	1.33

In ELISA, antisera to BaMMV-Kal and BaMMV-M reacted strongly with BaMMV-Kal and BaMMV-M, but weakly with BaMMV-Nal (Table 2). Also, antiserum to BaMMV-Nal reacted strongly with BaMMV-Nal but weakly with BaMMV-Kal and BaMMV-M. However, absorption of antisera to BaMMV-Kal and BaMMV-Nal with particles of BaMMV-Nal and BaMMV-Kal, respectively, eliminated cross reaction between BaMMV-Kal or BaMMV-M and BaMMV-Nal

SDS-PAGE of purified preparation of the three BaMMV isolates each gave one major band of capsid protein and some minor bands of degradation products of the capsid protein.

The M_r of the capsid protein was estimated as 31.5 x 10^3 (K) for BaMMV-Na1 and 31 K for BaMMV-Ka1 and BaMMV-M. The samples of BaYMV-II-1 and BaYMV-III contained the capsid protein with M_r of 33 K (BaYMV-II-1) or 32 K (BaYMV-III), and the degradation products in much more quantity than those of BaMMV.

Electrophoresis of RNAs released from purified preparations of the three BaMMV isolates each gave two bands with M_r of 2.57 x 10^6 (RNA-1) and 1.35 x 10^6 (RNA-2). The RNA-1 bands of the three BaMMV isolates comigrated with those of BaYMV-II-1 and BaYMV-III, but the RNA-2 bands migrated slightly faster than did those of BaYMV (M_r 1.40-1.45 x 10^6).

Sequence analysis has indicated that 3'-terminal halves of RNA-1 of BaMMV-Ka1 and BaMMV-Na1 both encode a part of a long polyprotein, containing a capsid protein at its end, followed by a 3' non-coding region (Kashiwazaki *et al.*, 1992). The two strains share 88% and 92% nucleotide identity in the coding and non-coding regions, respectively. The capsid proteins of the two strains each contain 251 amino acids and have 94% identity. There are 15 amino acid differences between the two proteins and 12 of them are found in the N-terminal one-thirds.

Discussion

The present study demonstrates the variability in pathogenicity of BaMMV, as has been reported for BaYMV (Kashiwazaki *et al.*, 1989b). Based on the pathogenicity towards barley cultivars, three BaMMV strains are differentiated. Although yellow mosaic-resistant barley cultivars developed in Japan have either *Ym* or *ym3*, both BaMMV-Ka1 and BaMMV-Na1 are pathogenic to cultivars with *ym3* and BaMMV-Na1 is also pathogenic to cultivars with *Ym*. Thus, other resistance gene(s) are required to develop new cultivars resistant to both strains.

Our results indicate that the three BaMMV strains are grouped into two serotypes. BaMMV-Ka1 and BaMMV-Na1 can easily be differentiated by ELISA using cross-absorbed antisera. Their serological differences could be caused by the amino acid differences in the N-terminal regions of their capsid proteins, which are probably located on the surface of virus particles (Kashiwazaki *et al.*, 1989a). However, the relation between sequence differences between the two strains and their differences in biological properties, such as pathogenicity, remains to be studied.

References

Friedt, W., Ordon, F. and Götz, R. (1990). Genetics of resistance to the 'barley yellow mosaic virus complex' and status of breeding. Proc. 1st Symp. Int. Work. Group on Plant Viruses with Fungal Vectors, Braunschweig. pp.117-120.

Huth, W. and Adams, M. J. (1990). Barley yellow mosaic virus (BaYMV) and BaYMV-M: two different viruses. Intervirology 31:38-42.

Kashiwazaki, S., Hayano, Y., Minobe, Y., Omura, T., Hibino, H. and Tsuchizaki. T. (1989a). Nucleotide sequence of the capsid protein gene of barley yellow mosaic virus. J. Gen. Virol. 70:3015-3023.

Kashiwazaki, S., Ogawa, K., Usugi, T., Omura, T and Tsuchizaki, T. (1989b). Characterization of several strains of barley yellow mosaic virus. Ann. Phytopath. Soc. Japan 55:16-25.

Kashiwazaki, S., Nomura, K., Kuroda, H., Ito, K. and Hibino, H. (1992). Sequence analysis of the 3'-terminal halves of RNA 1 of two strains of barley mild mosaic virus. J. Gen. Virol. 73:2173-3281.

Usugi, T. and Saito, Y. (1976). Purification and serological properties of barley yellow mosaic virus and wheat yellow mosaic virus. Ann. Phytopath. Soc. Japan 42:12-20.

MECHANICAL INOCULATION OF SUGAR BEET ROOTS WITH BEET SOIL-BORNE VIRUS IN THE ABSENCE OF *POLYMYXA BETAE*.

P.J. Hutchinson[1,2], C.M. Henry[2] and R.H.A. Coutts[1]

[1]Department of Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, U.K.
[2]MAFF, Central Science Laboratory, Hatching Green, Harpenden, Hertfordshire AL5 2BD, U.K.

Summary

Using a mechanical inoculation procedure, sugar beet seedlings were successfully inoculated at low frequency with two isolates of beet soil-borne virus (BSBV 86-109 and 452N) in the absence of the fungus vector *Polymyxa betae*. Successful infection was assessed by immunoblot analysis of plant extracts. The 86-109 isolate caused a significant decrease in the size and weight of mechanically inoculated beet plants as compared to controls while the 452N isolate caused little effect.

Introduction

Beet soil-borne virus (BSBV) is a poorly characterized virus found in sugar beet roots and was originally described by Ivanoviç and Macfarlane (1982). BSBV has furovirus-like particles and like the serologically unrelated beet necrotic yellow vein virus (BNYVV) (Torrance *et al.*, 1988, Lesemann *et al.*, 1989), is transmitted by the plasmodiophorid fungus *Polymyxa betae* (Keskin) (Henry *et al.*, 1986). Several similar poorly characterized tubular viruses have been isolated from sugar beet in Europe and the USA (reviewed in Hutchinson *et al.*, 1990, 1992). Lesemann *et al.* (1989) identified two different serotypes of BSBV termed 'Ahlum' and 'Wierthe' which are distantly related to one another (Barbarossa *et al.*, 1992). Double-stranded (ds) RNA analysis and reciprocal hybridisation revealed similarities between the original isolate from Norfolk, UK, another UK isolate, 452N, (Hill and Torrance, 1989) a Swedish isolate, 86-109 and a Belgian isolate, 1530 but none between these and BNYVV at least at the levels of stringency investigated. These studies suggested that the genome of BSBV N has three genomic RNA components (RNA 1, 2 and 3) with sizes of 6.2, 2.9 and 2.5 kb respectively following repeated mechanical passaging of the virus (Hutchinson *et al.*, 1992) and the Ahlum isolate appears to be similar (Kaufmann *et al.*, 1992a). Using end-labelled probes specific for each dsRNA, we found that RNA 2 shares little if any sequence homology with RNA 1 but might have some limited homology with RNA 3 (unpublished results). The effects of BSBV on the growth of sugar beet and on beet yield are unknown but should not be discounted (Henry *et al.*, 1986). The vector of BSBV, *Polymyxa betae*, can itself restrict seedling root growth (Blunt *et al.*, 1991) and can cause severe stunting of sugar beet under conditions leading to heavy fungal infection (Keskin, 1964, Ivanoviç *et al.*, 1983). Infection with *P. betae* in combination with BSBV causes slightly greater damage to sugar beet plants than *P. betae* alone (Ivanoviç *et al.*, 1983) and the 96-109 isolate has been associated with yellowing of sugar beet leaves in the field (K. Lindsten pers comm.). Under natural conditions in Britain the incidence and severity of *P. betae* on beet plants during spring and early summer is low and soil temperature is thought to be the major factor limiting its early development in sugar beet (Blunt *et al.*, 1991) but this does not discount its effects during later beet sowing in Britain or its effects in warmer countries.

Mechanical inoculation of sugar beet roots with BNYVV has shown that isolates containing a complete genome, similar to that found in natural infections, can cause heavy damage to sugar beet roots in the absence of *P. betae* and confirmed that BNYVV is the cause of rhizomania and that *P. betae* mainly acts as the virus vector (Koenig and Burgermeister, 1989, Koenig *et al.*, 1991). With these results in mind, we have attempted to mechanically inoculate sugar beet seedlings with two BSBV isolates, 86-109 and 452N to ascertain their effects on the growth of the plants under the same conditions to those where the virus multiplies following inoculation with viruliferous *P. betae*.

Materials and methods

Plant propagation

All plants were maintained in insect-proof glasshouses as previously (Henry *et al.*, 1986). *Beta vulgaris* cv Monoire seeds were surface sterilized, pre-germinated in darkness and then germinated in the laboratory at *c.* 20°C for two to three days until the radicles emerged before transfer to 10 cm pots containing sterile Micafil (Blunt *et al.*, 1991) and then watered with quarter strength modified Hoagland nutrient solution.

Inoculation of B. vulgaris seedlings with P. betae resting spores

Air-dried *B. vulgaris* roots containing *P. betae* cystosori were derived from BSBV N-infected or healthy sugar beet plants. For inoculation, the exposed roots of five beet seedlings in single pots were sprinkled with 0.1g of viruliferous or non-viruliferous root powder, re-covered with Micafil and placed in the glasshouse in blackened perspex reservoirs with the lower 1.5 cm of the pots submerged in the liquid nutrient and then fed on alternate days with the same nutrient. Seedlings inoculated with viruliferous or non-viruliferous *P. betae* were cultured in separate reservoirs.

Mechanical inoculation of B. vulgaris seedlings with BSBV

Beet seedlings were mechanically inoculated with either the 86-109 or the 452N isolate using a method adapted from Koenig and Stein (1990). Six to nine day old hydroponically-grown seedlings (twenty-five per treatment) were placed in a plastic 50 ml tube together with 3 ml of inoculum containing 0.09g carborundum (500 mesh) and the tube vortexed for 1 min on a Genie TM Vortex mixer at a speed setting of 6 to 7. The inoculum consisted of 1 volume of freshly expressed sap from BSBV-infected or healthy *Chenopodium quinoa* leaves produced according to Henry *et al.*, (1986) and 3 volumes of 50 mM sodium phosphate buffer (pH 7.2). After vortexing, the plantlets were left for 5 min in the inoculum. Following inoculation the seedlings were washed in sterile distilled water and each planted in sterilised Micafil in disposable plastic cups and arranged in a randomized design across internal benchs within the glasshouse (Blunt *et al.*, 1991). Holes were not cut in the cup bases, to prevent cross-contamination. Sufficient nutrient solution was applied sparingly to the plants on alternate days. The liquid nutrient and roots were monitored periodically by microscopic analysis for the presence of fungus.

Sampling and analysis

Six weeks after mechanical inoculation and culture plant heights for each treatment were measured. One week later, randomly selected plants from each treatment were depotted and root samples removed at several places. The plants were then repotted, and replaced into the greenhouse. Simultaneously roots inoculated with viruliferous and non-viruliferous *P. betae* were also sampled All root tissue was tested for BSBV by immunoblotting (Hutchinson *et al*, 1990). Both the antisera and conjugate to 86-109 (which cross-reacts with isolates, 452N and N) were used at a dilution of 1 in 200 in these assays. Eight weeks after mechanical inoculation ten plants from each of the treatments were depotted, washed free of Micafil and the weights of leaf, tap and lateral roots were measured. Root sap samples were prepared from five randomly selected plants from each treatment and tested by ELISA and immunobloting (Hutchinson *et al.*, 1990). The dry weights of all samples from each treatment were calculated by drying the samples at 80°C for at least 24 h until no further weight change occurred. The fresh and dry weights were compared by analysis of variance.

Results

Plant roots inoculated with viruliferous or non-viruliferous *P. betae* all contained large numbers of characteristic cystosori six to seven weeks after inoculation. Root and leaf extracts from plants inoculated with *P. betae* carrying BSBV N and non-viruliferous *P. betae* were compared by immunoblot analysis. Extracts from the infected plants contained a virus-specific protein of Mr. *ca.* 20kDa which was absent from control plants. This protein, which was restricted to the roots of virus-infected plants, is the coat protein (CP) of isolate N and is of a similar Mr to the CP of the Ahlum serotype (17 kDa; Barbarossa *et al.*, 1992). The distribution of the BSBV N CP in the roots was similar to that recorded for field-grown, BSBV-infected sugar beet (Kaufmann *et al.*, 1992b).

Over the eight week experimental period all beet plants that were mechanically inoculated with *C. quinoa* extracts were free from contamination with *P. betae.* Six weeks after inoculation the height of the plants

inoculated with isolate 452N isolate were not significantly different from mock-inoculated controls. However the height of plants inoculated with the isolate 86-109 were significantly reduced as compared to the controls, with a mean reduction of 11.5%. At both seven and eight weeks after mechanical root inoculation none of the extracts, from a randomised selection of root samples, gave positive results in ELISA. However immunoblotting of the same extracts revealed than on average 1 in 5 root samples inoculated with either the 452N or the 86-109 isolate contained the virus CP eight weeks after inoculation which was absent from extracts of mock-inoculated beet plants. The virus CP was not detectable in leaves or shoots and was at a higher concentration in tap roots than in lateral roots. The fresh and dry weights of the leaves, tap and lateral roots of all plants were compared eight weeks after inoculation.

Mechanical root inoculation with isolate 86-109 caused a significant reduction in fresh and dry weight of leaves, tap and lateral roots as compared to mock-inoculated controls (Fig. 1). Reductions in leaf and tap root fresh weight were highly significant. Total fresh and dry weights of plants inoculated with isolate 86-109 were reduced by 47% and 34% respectively as compared to the mock-inoculated controls. By contrast, inoculation with isolate 452N caused no significant effects to the fresh or dry weights of the sugar beet organs (Fig. 1).

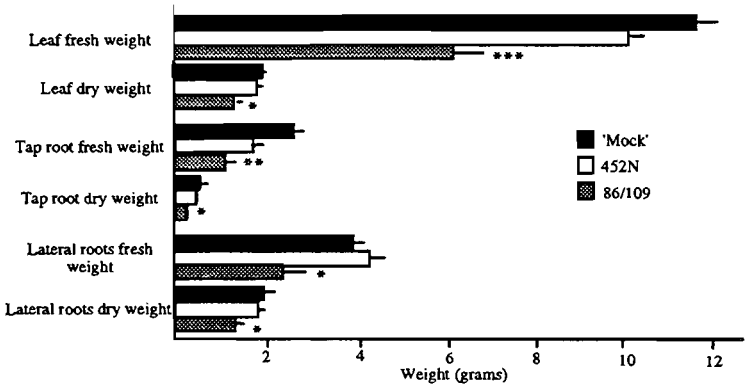


Fig. 1 Mean fresh and dry weight measurements of the leaves, tap and lateral roots of sugar beet plants mechanically inoculated with either the BSBV 452N or 86-109 isolate or 'mock' inoculated. Bars represent the standard error of the means: * significant at P = 0.005, ** significant at P = 0.01, *** significant at P = 0.001, n = 10. These results are representative of a number of different experiments.

Discussion

The results presented suggest that replication of at least isolate 86-109 (following mechanical inoculation to sugar beet cv Monoire in the absence of *P. betae*) can significantly reduce growth under glasshouse conditions where the virus proliferates in infected roots. The isolate 452N however had little effect on growth under similar conditions.

Immunoblotting detected BSBV infection more readily than did ELISA. However the virus undoubtedly escaped detection in some plants due to its low concentration, particularly as BSBV was detected by immunoblotting in one out of five beet extracts tested eight weeks after mechanical inoculation irrespective of which BSBV isolate was inoculated. While both isolates could infect and replicate in sugar beet in the absence of the fungus vector only 86-109 damaged the plants. This was not a dosage effect of the inoculum, since the titre of both isolates in crude extracts of infected *C. quinoa* leaves used for inoculation were similar. Both isolates have a similar pathogenicity in *C. quinoa* and the dsRNA profile of inoculated leaf extracts were also similar (Hutchinson *et al.*, 1992). Differences in the RNA composition of BNYVV inoculum used for the mechanical inoculation of sugar beet roots have been associated with variable pathogenicity in sugar beet (Koenig *et al.*, 1991) but whether this is also the case for BSBV is not known.

It remains to be seen whether BSBV is an important pathogen in the field but both the virus (Henry *et al.*, 1986) and its vector are widespread in the UK and may share alternate hosts (Barr and Asher, 1992). BSBV has

been associated with BNYVV in at least one outbreak of rhizomania in the UK (Hill and Torrance, 1989) and Germany (Kaufmann *et al.*, 1992b) but any synergistic effects are undocumented. Thus caution should be exercised in discounting BSBV as a benign sugar beet pathogen until more is known about its replication and molecular organisation.

Acknowledgements

We are grateful to Dr. K. Lindsten for supplying the Swedish BSBV 86-109 isolate and antisera to the virus. This work was funded by the Science and Engineering Research Council and the Sugar Beet Research and Education Committee.

References

Barbarossa, L., Vetten, H.J., Kaufmann, A., Lesemann, D-E., and Koenig, R. (1992). Monoclonal antibodies to beet soil-borne virus. *Ann. appl. Biol* **121**, 143-150.

Barr, J. and Asher M.J.C. (1992). The host range of *Polymyxa betae* in Britain. *Plant Path.* **41**, 64-68.

Blunt, S.J., Asher, M.J.C. and Gilligan, C.A. (1991). Infection of sugar beet by *Polymyxa betae* in relation to soil temperature. *Plant Path.* **40**, 257-267.

Henry, C.M., Jones R.A.C. and Coutts, R.H.A. (1986) Occurrence of a soil-borne virus of sugar beet in England. *Plant Path.* **38**, 585-591.

Hill, S.A. and Torrance, L. (1989). Rhizomania disease of sugar beet in England. *Plant Path.* **38**, 114-122.

Hutchinson, P.J., Henry, C.M. and Coutts, R.H.A. (1990). A comparison of three European isolates of beet soil-borne virus with beet necrotic yellow vein virus. *Proc. of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors* pp. 121-123 Eugen Ulmer GmbH & Co, Stuttgart.

Hutchinson P.J., Henry C.M. and Coutts, R.H.A. (1992). A comparison using dsRNA analysis between beet soil-borne virus and some other tubular viruses isolated from sugar beet. *J. Gen.* **73**, 1317-1320.

Ivanoviç M. and Macfarlane, I. (1982). A tubular virus associated with infection of sugar beet by *Polymyxa betae*. *Rep. Rothamsted Experimental Station for 1981*, pp. 190-191.

Ivanoviç M., Marfarlane , I. and Woods, R.D. (1983). Viruses of sugar beet associated with *Polymyxa betae*. *Rep. Rothamsted Experimental Station for 1982*, pp. 189-190.

Kaufmann, A., Li, Y., Koenig, R., Breyel, E., Maiss, E., Lüddecke, P. and Commandeur, U. (1992a). Single and double-stranded RNAs associated with an isolate of beet soil-borne virus. *Intervirol.* **33**, 97-102.

Kaufmann, A., Koenig, R. and Lesemann, D-E. (1992b). Tissue print immunoblotting reveals an uneven distribution of beet necrotic yellow vein and beet soil-borne viruses in sugar beets. *Arch. Virol.* **126**, 329-335.

Keskin, B. (1964). *Polymyxa betae* n: sp. ein parasite in den Wurzein van *Beta vulgaris* Tournefort besonders während der Jugendentwicklung der Zuckerrübe. *Archiv für Mikrobiologie* **49**, 348-374.

Koenig, R. and Burgermeister, W. (1989). Mechanical inoculation of sugar beet roots with isolates of beet necrotic yellow vein virus having different RNA compositions. *J. Phytopath.* **124**, 249-255.

Koenig, R. and Stein, B. (1990). Distribution of beet necrotic yellow vein virus in mechanically inoculated sugar beet plantlets of cultivars with different degrees of rhizomania resistance. *Proc. of the First Symposium of the International Working Group of Plant Viruses with Fungal Vectors*, pp 87-90. Eugen Ulmer GmBH & Co. Stuttgart.

Koenig, R., Jarausch, W., Li, Y., Commandeur, U., Burgermeister, W., Gehrke, M. and Lüddecke, P. (1991). Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanically inoculated sugar beets. *J. Gen. Virol.* **72**, 2243-2246.

Lesemann, D-E., Koenig, R., Lindsten, K. and Henry, C. (1989). Serotypes of beet soil-borne virus from FRG and Sweden. *EPPO Bull.* **19**, 539-540.

Torrance, L., Pead, M.T. and Buxton, G. (1988). Production and some characteristics of monoclonal antibodies against beet necrotic yellow vein virus. *Ann. appl. Biol.* **113**, 519-530.

DETECTION OF BEET NECROTIC YELLOW VEIN AND BEET SOIL-BORNE VIRUSES IN SUGARBEET BY MEANS OF TISSUE PRINT-IMMUNOBLOTTING

A. Kaufmann, and R. Koenig

Biologische Bundesanstalt, Institut für Biochemie und Pflanzenvirologie,
Messeweg 11, D 38104 Braunschweig, Germany.

Summary

Tissue print-immunoblotting (TiPIB) was found to be an easy method for studying the distribution of beet necrotic yellow vein (BNYVV) and beet soil-borne (BSBV) viruses in sugarbeet. Viral antigens were detected by means of alkaline-phosphatase-labelled virus-specific antibodies either in a color or a chemiluminescence reaction using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate or AMPPD [3-(2'-spiroadamantan)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetan], respectively, as enzyme substrates. Both, BNYVV and BSBV, were found to be unevenly distributed in tap roots of field grown sugarbeet. BNYVV antigen-containing areas in the tap roots were usually found underneath a root beard. BNYVV- and BSBV-infected areas did not coincide. In some samples BNYVV antigen was detected predominantly in the xylem vessels, but in many others it occurred also in the parenchyma and other tissues. The limited spread of BNYVV in tap roots and the rare infections of the upper parts of naturally infected sugarbeet suggest that the translocation of viable virus through the xylem and a reentry into living cells, if they occur at all, are not very efficient. In partially resistant sugarbeet varieties the BNYVV concentration - judged from the intensity of the color reaction - tended to be lower than in highly susceptible ones.

Introduction

In 1990 Lin et al. have described a new serological method - now usually designated as tissue print immunoblotting (TiPIB) - for the localization of plant viruses in infected tissues. We have adopted this method to the detection of beet necrotic yellow vein (BNYVV) and beet soil-borne viruses (BSBV) in sugarbeet (Kaufmann et al., 1992). In the following we give a detailed description of the method and report on some of the results which were obtained.

Method

- sugarbeet tap roots are cut horizontally or longitudinally with a sharp knife

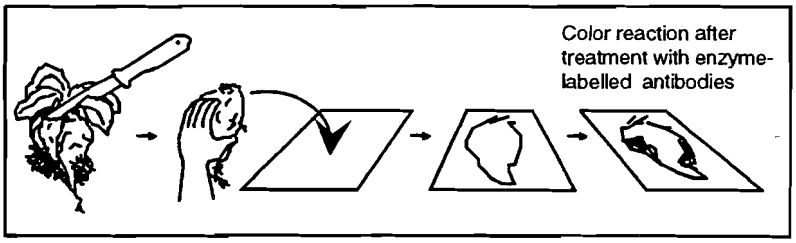


Fig. 1 Tissue print-immunoblotting with sugarbeet tap roots

- the freshly exposed cutting surface is immediately pressed firmly against a nylon membrane (Boehringer, Nylon-Membran, positivily charged, order No. 1209299)

- this tissue print can be stored in a refrigerator and, if needed, further prints can be made on unused parts of the membrane at a later time
- the free protein binding sites are blocked by incubating the membrane with gentle rocking for at least two hours (or over night) in a solution containing 2% bovine serum albumin or skim milk in **PBS** (8 g NaCl/0.2 g KH_2PO_4/0.2 g KCl/1.44 g Na_2HPO_4 x $2H_2O$/0.2g NaN_3 per liter, pH 7.4)
- residual plant parts sticking to the membranes are removed by three brief washes in **PBS-Tween** (PBS as above + 0.05% Tween 20, Merck)
- the blots are incubated for 2 hours at room temperature with alkaline phosphatase-labelled antibodies to BNYVV or BSBV (diluted c. 1:1000 in PBS) - after use the antibody solution can be stored at 4°C, it can be reused for further blots at a later time
- the blots are washed 3 times with PBS-Tween (see above)
- for detecting the binding of the antibodies the blots are incubated in a substrate solution prepared by adding
  66 µl *NBT stock solution (75 mg NBT/ml 70% dimethyl formamide) and
  50 µl *BCIP stock solution (50 mg BCIP/ml 100% dimethyl formamide) to
  15 ml substrate buffer (0.1 M Tris/HCl/0.1 M NaCl/50 mM $MgCl_2$, pH 9.5)
  (*NBT - nitroblue tetrazolium, Sigma, order No. B-8503,
  *BCIP - 5-bromo-4-chloro-3-indolylphosphate, Sigma, order No. N-6876)
  Virus antigen-containing regions stain purple after 15-20 min, the reaction is stopped when the background starts to become purple by washing the blots in 0.02 M Tris/HCl pH 7.5/5 mM EDTA
- the blots should be stored in a dark place for color preservation

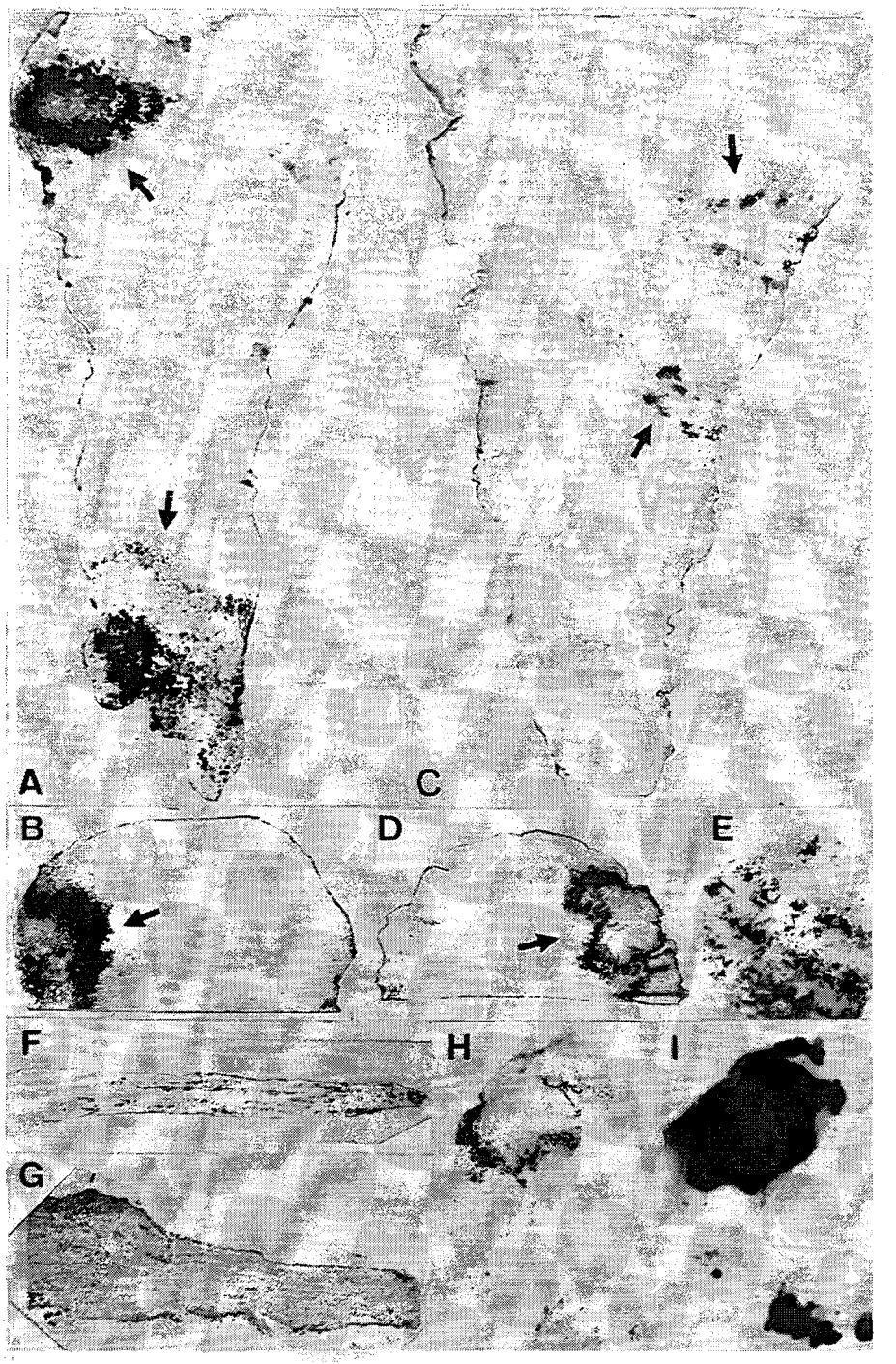
A considerably higher sensitivity can be reached in a chemiluminescent assay, e.g. with lumigen PPD [4-methoxy-4-(phosphatphenyl)spiro(1,2-dioxetan-3,2'-adamantan; Boehringer order No. 1357 328] or AMPPD [3-(2'-spiroadamantan)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetan] as substrates for alkaline phosphatase (Fig. 2 I).

Results and Discussion

TiPIB revealed a patchy distribution of BNYVV in tap roots of field grown sugarbeet which was more pronounced in some years (Fig. **2A** to **D**, **H**, **I**) than in others (Fig. **2F** and **G**). More generalized infections were mainly seen in the lower parts of the tap roots (Fig. **2A**). The distribution of virus-containing areas was similar in highly susceptible (e.g. Fig. **2A**) and in partially resistant cultivars (e.g. Fig. **2C**), but in the latter the staining was often less heavy and the affected areas tended to be smaller. Virus-containing areas were usually found beneath the root beards suggesting that the latter provide the way of entrance for the virus into the tap roots. Beneath some root beards we did not find a virus-containing area which indicates that the virus had not yet proceeded from the rootlets into the tap root. The limited size of the affected areas suggests that the spread of the virus in the tap roots is slow.

Usually BNYVV antigen was found throughout an affected area, but occasionally its concentration in the xylem vessels was especially high (Fig. **2E**). This partly confirms a report by Giunchedi & Poggi Pollini (1988) who found the viral antigen exclusively in the xylem, whereas we did so only occasionally. Translocation through the xylem has previously been postulated for a few other viruses (Schneider & Whorley, 1959; Chambers & Francki, 1966; Jones, 1975). The limited spread of BNYVV in tap roots and the rare infections of the upper parts of naturally infected sugarbeet suggest that the translocation of viable virus through the xylem and a reentry into living cells, if they occur at all, are not very efficient.

BSBV also showed a patchy distribution in the tap roots of field grown sugarbeet. Experiments in which blots of the two halves of doubly infected sugarbeet were developped separately either with with BNYVV- or BSBV-specific antibodies, revealed that the BSBV- and the BNYVV-containing areas did not coincide. In two plants in which we had failed to


A
C
B
D
E
F
H
I
G

detect BSBV in the rootlets by means of ISEM, we could detect it by means of TiPIB in the tap roots. Its presence in the affected areas could then be confirmed by means of ISEM. This indicates that TiPIB may be more sensitive than other techniques in which viruses in localized infections may be overlooked due to dilution.

A detailed account of this work has been given by Kaufmann et al. (1992). A somewhat different procedure of tissue print-immunoblotting has recently also been described for sugarbeet by Resca *et al.* (1992).

Acknowledgement

We are greatly indebted to the Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung (grant Br 28/91 FWW) and to the Deutsche Forschungsgemeinschaft (grant Ko 518/11-1) for financially supporting different aspects of this work.

References

Chambers, T.C., and Francki, R.I.B. (1966). Localization and recovery of lettuce necrotic yellows virus from xylem tissues of Nicotiana glutinosa. *Virology* **29**:673-676.

Giunchedi, L., and Poggi Pollini, C. (1988). Immunogold-silver localization of beet necrotic yellow vein virus antigen in susceptible and moderately resistant sugar-beets. *Phytopath. Medi.t* **27**:1-6.

Jones, R.A.C. (1975). Systemic movement of potato mop-top virus in tobacco may occur through the xylem. *Phytopath. Z.* **82**:352-355.

Kaufmann, A., Koenig, R., and Lesemann, D.-E. (1992). Tissue print-immunoblotting reveals an uneven distribution of beet necrotic yellow vein and beet soil-borne viruses in sugarbeets. *Arch.Virol.* **126**:329-335.

Lin, N.S., Hsu, Y.H., and Hsu, H.T. (1990). Immunological detection of plant viruses and a mycoplasmalike organism by direct tissue blotting on nitrocellulose membranes. *Phytopathology* **80**:824-828.

Resca, R., Giunchedi, L., and Poggi Pollini, C.(1992). Localizzazione immunologica del virus agente della rizomania nell'impronta su membrana di radici di barbabietola. Sementi Elette **XXXVII**: 21-24.

Schneider, I.R., and Worley, J.F. (1959). Rapid entry of infectious particles of southern bean mosaic virus into living cells following transport of the particles in the water stream. *Virology* **8**:243-249.

Fig. 2 Tissue print-immunoblots of longitudinal and cross sections of tap roots of BNYVV-infected highly susceptible (**A**, **B**, **E**, **F** and **G**) and partially resistant (**C**, **D**, **H** and **I**) sugarbeet varieties. On the original immunoblots the purple bluish color of the immunostaining (marked by arrows in **A**, **B**, **C** and **D**) was readily distinguished from a brownish color which was not due to the immunostaining, e.g. on the outlines of roots. The outlines of the roots in **A**, **B**, **C**, **D**, **F** and **G** have been enhanced by pencilling. The enlargement was about 10 fold in **E**, whereas **A** to **D**, and **F** to **I** show the natural sizes. **H** and **I** show the same tissue print-immunoblot, in **H** - as in **A** to **G** - the alkaline-phosphatase-labelled virus-specific antibodies were detected in a color reaction with NBT/BCIP, in **I** in a chemiluminescence reaction with AMPPD. **E** shows areas with virus in especially high concentrations in xylem vessels, the xylem was identified on the original section by means of staining with phloroglucinol and HCl.

HISTOLOGICAL STUDIES ON THE INFECTION PROCESS OF BEET NECROTIC YELLOW VEIN VIRUS IN ROOTS OF BEET PLANTS.

O.E. Scholten[1], H. Paul[1], D. Peters[2], J.W.M. van Lent[2] and R.W. Goldbach[2]

[1]DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, 6700 AA Wageningen, The Netherlands & [2]Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

Summary

Histological studies, using immunogold-silver labelling, were applied to get a better understanding of the distribution of beet necrotic yellow vein virus (BNYVV) in roots of beet plants. In rootlets of the susceptible *Beta vulgaris* cultivar 'Regina', virus was detected in the epidermis, endodermis and parenchyma cells, including the interstitial parenchyma. In general, virus could not be found in the vascular tissue of these rootlets. In the resistant *B. vulgaris* subsp. *maritima* WB42, only very little virus could be detected in the epidermal and cortex parenchyma cells. Resistance to BNYVV seemed to be based on a restricted virus replication.

Introduction

Beet necrotic yellow vein virus (BNYVV), classified as a furovirus, causes rhizomania in beet (*Beta vulgaris* L.). The virus is transmitted by the soil-borne fungus *Polymyxa betae* Keskin (Fujisawa and Sugimoto, 1976; Abe and Tamada, 1986). Zoospores, released from resting spores of the fungus, may contain virus and infect root hairs or epidermal cells. After penetration of a zoospore into the roots, zoosporangia or resting spores are produced, from which new zoospores can be released.

Previous histologogical studies did not always lead to similar conclusions. Tamada (1975) described the absence of virus particles in many cells. Putz and Vuittenez (1980) detected aggregates of virus particles by electron microscopy in root parenchyma cells of beets. In vascular tissues the virus could not be observed. Giunchedi and Poggi Pollini (1988) however, demonstrated specific immunogold-silver labelling of the coat protein in xylem vessels of tap roots and rootlets. Using tissue print immunoblotting, Kaufmann *et al.* (1992) partly confirmed these results by detecting occasionally some virus in xylem vessels. Several resistant accessions are available (Lewellen *et al.*, 1987; Whitney, 1989). Information about mechanisms of resistance is necessary for optimal use in plant breeding programmes. The aim of this study was to compare the localisation of the virus in susceptible and resistant beet plants in order to get a better understanding of the underlying mechanisms.

Materials and Methods

Plant material and inoculation of test plants
The plant material consisted of the susceptible *Beta vulgaris* cultivar 'Regina' and the resistant accession *B. vulgaris* subsp. *maritima* (L.) Arcang. WB42. Individual seedlings were inoculated by 2000 or 10,000 zoospores of a viruliferous zoospore suspension. Plants were grown in the greenhouse at 22/17 °C (day/night).

Immunogold-silver labelling
Pieces of rootlets, containing resting spores of *P. betae*, were embedded in LR White. About 1-2 µm sections were used for immunogold-silver labelling (Van Lent and Verduin, 1987). Antiserum against the coat protein of BNYVV was kindly supplied by A.M. Haeberlé (INRA, Colmar, France). Up to 100 mg of the rootlets was analysed by ELISA to determine the virus concentrations.

Results

Two weeks after inoculating seedlings with 2000 zoospores, high virus concentrations were found in roots of the susceptible cultivar 'Regina', while low virus concentrations were detected in roots of the resistant accession WB42.

Using immunogold-silver labelling, aggregates of the virus could be detected in rootlets of 'Regina' in the epidermis, cortex parenchyma, endodermis and intersitial parenchyma, but in general not in the vascular tissues (Figure 1A). Even after inoculating with 10,000 zoospores only very little labelling appeared in a few xylem vessels. In rootlets of the resistant plants of WB42 small amounts of virus were detected in a few epidermis and parenchyma cells (Figure 1B).

Discussion

Although Giunchedi and Poggi Pollini (1988) detected beet necrotic yellow vein virus in xylem vessels, the present study confirmed the observations of Kaufmann *et al.* (1992), that label only occasionally can be found in xylem vessels. The mechanism of resistance in WB42 seemed to be based on restricted virus replication, as the amount of label per cell in WB42 was less than in 'Regina'. Whether the cell-to-cell transport in WB42 was also restricted was not clear, because of the low virus concentration in these rootlets. Immunogold-silver labelling proved to be a suitable technique for the histological comparison of the infection process in susceptible and resistant plants.

Acknowledgments
The authors thank Betty Henken for technical assistance.

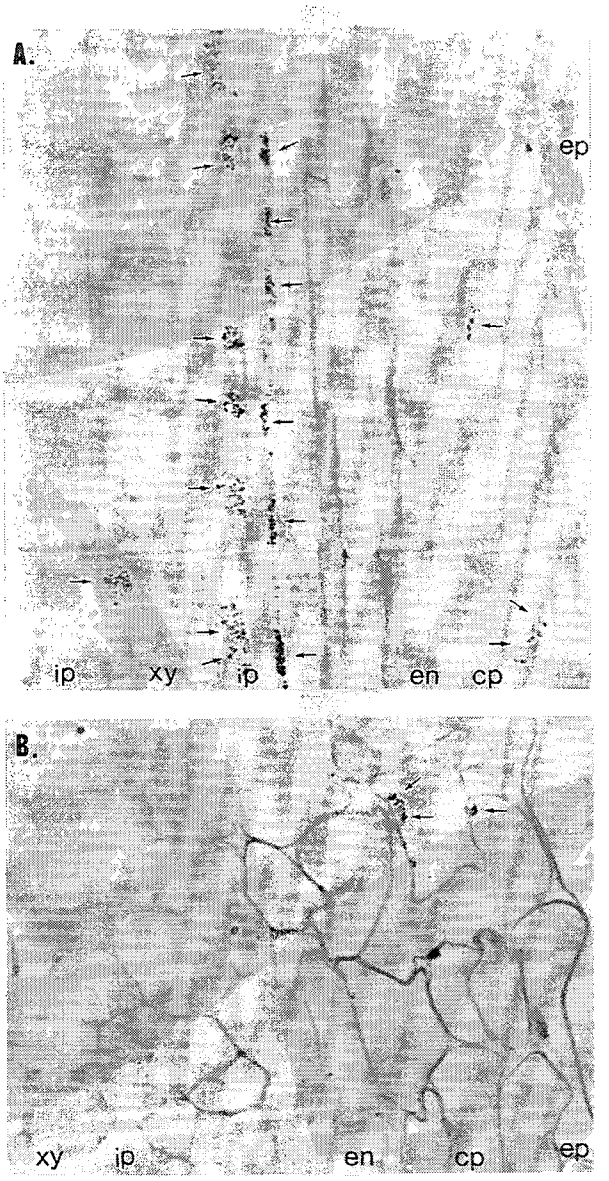


Figure 1. Immunogold-silver labelling of BNYVV (←) in sections of rootlets, inoculated with zoospores, **A.** *Beta vulgaris* cultivar 'Regina' and **B.** *B. vulgaris* subsp. *maritima* WB42, (ep= epidermis, cp= cortex parenchyma, en= endodermis, ip= interstitial parenchyma, xy = xylem).

References

Abe, H., and Tamada, T. (1986). Association of beet necrotic yellow vein virus with isolates of *Polymyxa betae* Keskin. *Ann. Phytopath. Soc. Japan* **52**:235-247.

Fujisawa, I., and Sugimoto, T. (1976). Transmission of beet necrotic yellow vein virus by *Polymyxa betae*. *Ann. Phytopath. Soc. Japan* **43**:583-586.

Giunchedi, L., and Poggi Pollini, C. (1988). Immunogold-silver localization of beet necrotic yellow vein virus antigen in susceptible and moderately resistant sugar-beets. *Phytopath. Medit.* **27**:1-6.

Kaufmann, A., Koenig, R., and Lesemann, D.-E. (1992). Tissue print-immunoblotting reveals an uneven distribution of beet necrotic yellow vein virus and beet soil-borne viruses in sugarbeets. *Arch. Virol.* **126**:329-335.

Lewellen, R.T., Skoyen, I.O., and Erichsen, A.W. (1987). Breeding sugarbeet for resistance to rhizomania: evaluation of host-plant reactions and selection for and inheritance of resistance. Proceedings 50th Wintercongress IIRB, Brussels, p. 139-156.

Putz, C., and Vuittenez, A. (1980). The intracellular location of beet necrotic yellow vein virus. *J. Gen. Vir.* **50**:201-204.

Tamada, T. (1975). Beet necrotic yellow vein virus. CMI/AAB Descript. Plant Viruses, no 144, 4 pp.

Van Lent, J.W.M., and Verduin, B.J.M. (1987). Detection of viral antigen in semi-thin sections of plant tissue by immunogold-silver staining and light microscopy. *Neth. J. Plant Path.* **93**:261-272.

Whitney, E.D. (1989). Identification, distribution and testing for resistance to Rhizomania in *Beta maritima*. *Plant Disease* **73**:287-290.

BEET SOILBORNE VIRUS: PREPARATION OF MONOCLONAL ANTIBODIES AND INFLUENCE OF THE 'AHLUM' ISOLATE ON MECHANICALLY INOCULATED SUGARBEET

R. Koenig, L. Barbarossa, A. Kaufmann, J. Vetten, and D.-E. Lesemann

Biologische Bundesanstalt, Institut für Biochemie und Pflanzenvirologie,
Messeweg 11, D 38104 Braunschweig, Germany.

Summary

Rod-shaped viruses with no serological relationship to beet necrotic yellow vein virus have been isolated repeatedly from sugarbeet. Most of the isolates which we have obtained in Germany were lost after one or two passages on *Ch. quinoa*. Two isolates, however, which were named 'Ahlum' and 'Wierthe' after the places where they were first isolated proved to be stable during repeated passages on *Ch. quinoa*. With polyclonal antisera they were found to be only distantly related serologically. Four monoclonal antibodies (MAb) were obtained for the 'Ahlum' serotype. Three of them were specific for this serotype, but a fourth one had a broader specificity and detected also the 'Wierthe' serotype. One MAb was bound to the particles along the entire surface, whereas two others including the broad-specificity one were bound only to one particle extremity. The fourth MAb is apparently specific for a cryptotope, its binding could not be detected by means of immunogold electron microscopy. The 'Ahlum' serotype caused up to 20% yield losses on mechanically inoculated sugarbeet.

Introduction

Beet soil-borne virus (BSBV) (Henry *et al.*, 1986) is a Polymyxa-transmitted rod-shaped virus with no serological relationship to beet necrotic yellow vein virus (BNYVV). In its genome properties, i.e. the size of its RNAs and their lack of a poly(A) tail, it is more similar to the true furoviruses than to BNYVV (Kaufmann *et al.*, 1992b). Lesemann *et al.* (1989) have identified two serotypes of BSBV in Germany. The 'Ahlum' serotype is serologically closely related to the original English isolate and the Swedish isolate 86-109, whereas the 'Wierthe' serotype is only rather distantly related to these viruses; in immunoelectron microscopical decoration tests the homologous and heterologous titres of antisera to the two serotypes are separated by three to six two-fold dilution steps. Since it is difficult to purify the various isolates of BSBV, antisera usually contain antibodies to normal plant constituents in addition to antibodies to virus particles. We have therefore prepared monoclonal antibodies (MAb) against the 'Ahlum' serotype of BSBV which allow a more sensitive detection of this serotype than polyclonal antibodies (PAb). One of these MAb also detects the 'Wierthe' serotype (Barbarossa *et al.*, 1992).

Since little is known about the effect of BSBV isolates on sugarbeet, we have mechanically inoculated sugarbeet seedlings with the 'Ahlum' and 'Wierthe' serotypes of BSBV. On the varieties Rizor and Carla yield losses of about 20% were observed with the 'Ahlum' serotype (Kaufmann *et al.*, 1993).

Materials and Methods

Materials and methods have been described in detail by Barbarossa *et al.* (1992) and Kaufmann *et al.* (1993).

Results and Discussion

A. MAb from mice immunized with the 'Ahlum' serotype of BSBV

From three fusion experiments we obtained four stable hybridoma lines secreting MAb to the 'Ahlum' serotype of BSBV. Each of the four MAb showed a unique reaction behaviour (Table 1). Three of the MAb, i.e.1, 2 and 4, were specific for the 'Ahlum' serotype of BSBV, they failed to react with the 'Wierthe' serotype. The fourth MAb (MAb 3), however, detected, also this serotype. Its reactivity in ELISA could be demonstrated only on plates which had been precoated with PAb to the 'Wierthe' serotype, because PAb to the 'Ahlum' serotype fail to trap sufficient amounts of virus particles for reactions to be seen.

Table 1. Reactivity of monoclonal (MAb) and polyclonal antibodies (PAb) to the 'Ahlum' serotype of beet soil-borne virus in various serological tests

Antibody	Reactivity in ELISA with the		Immunogold labelling of particles	Reactivity in Western blotting
	Ahlum serotype[1]	Wierthe serotype[2]		
MAb 1	+	-	entire length of particles	-
MAb 2	+	-	one extremity	-
MAb 3	+	+	one extremity	-
MAb 4	+	-	none	-
PAb	+	(+)	entire particle	+

[1] Coating of plates with PAb to the 'Ahlum' serotype, [2] coating of plates with PAb to the 'Wierthe' serotype

The electron microscopical immunogold technique revealed that only one of the four MAb (MAb 1) was bound along the entire length of the particles. MAb 2 and 3 were bound only to one extremity of the particles (Table 1; Fig. 1). Since with a mixture of MAb 2 and 3 the gold labelling was also seen only on one particle extremity, the two MAb seem to be specific for epitopes on the same particle extremity. These two epitopes, however, are apparently not identical, because one was detected only on the particles of the 'Ahlum' serotype, whereas the other one was found also on those of the 'Wierthe' serotype.

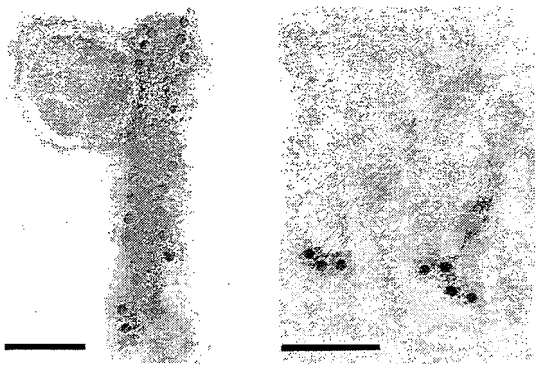


Fig. 1. Binding of MAb 1 along the entire length and of MAb 2 on one extremity of the particles detected by means of goat anti-mouse antibodies conjugated with 10 nm colloidal gold. Bars equal 100 nm.

MAb 4 is apparently specific for a cryptotope, because no binding of this MAb to the particles was detected by means of electron microscopy. The reactivity of crude sap preparations from BSBV-infected plants with MAb 4 suggests that this cryptotope is presumably exposed on some degradation product or precursor of the viral coat protein occurring in such preparations.

All four BSBV MAb are apparently specific for discontinuous epitopes, because none of them reacted with denatured viral coat protein on Western blots (Table 1).

We found that in ELISA all four MAb allowed a more sensitive detection of the 'Ahlum' serotype of BSBV than a polyclonal antiserum (Barbarossa et al., 1992). Interestingly, MAb 2 which is bound only to one particle extremity allowed a somewhat more sensitive detection of the virus than MAb 1 which is bound along the entire length of the particles. Possibly the avidity of MAb 2 is higher than that of MAb 1.

B. Influence of the' Ahlum' and 'Wierthe' serotypes of BSBV on sugarbeet yield after mechanical inoculation

By means of mechanical seedling inoculation (Koenig and Burgermeister, 1989; Koenig and Stein, 1990) we have attempted to assess the amount of damage done to sugarbeet by the 'Ahlum' and 'Wierthe' serotypes of BSBV. The experiments were done with the varieties Desirée and Rizor which are partially resistant to rizomania and Carla which is highly susceptible.

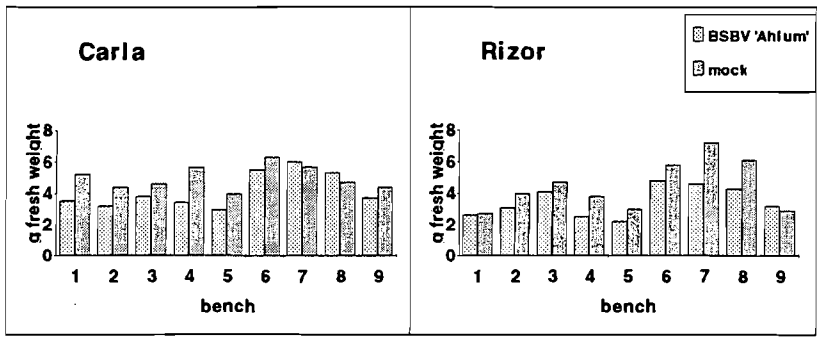


Fig. 2. The yield of tap roots from the sugarbeet varieties Carla and Rizor on individual greenhouse benches 11 weeks after mechanical inoculation with sap from BSBV 'Ahlum'-infected *Ch. quinoa* leaves or mock-inoculation with sap from healthy *Ch. quinoa* leaves. Each bench had contained in a randomized arrangement 6 or 7 plants of each variety and treatment.

Of each variety 60 plants were inoculated either with the 'Ahlum' or the 'Wierthe' serotype of BSBV or were mock-inoculated with sap from healthy *Ch. quinoa*. The 540 pots with BSBV- and mock-inoculated plants were placed in a randomized order in diagonal rows on nine greenhouse benches. Although there were no obvious differences between these benches, the average yield of tap roots was much higher on some benches (e.g. 6 and 7) than on others (e.g. 5 and 9), regardless of the variety and treatment (shown for mock-inoculated plants and plants inoculated with the 'Ahlum' serotype in Fig. 2). If we had placed the virus-inoculated plants on bench 5 and the controls on bench 6 or 7 we would have overestimated the virus-induced damage. If the opposite placement had been chosen we would have underestimated the amount of virus-induced damage. When the plants of all varieties and treatments were placed in a randomized arrangement on the benches, an average yield loss of about 20% in the BSBV-inoculated plants of the varieties Carla and Rizor was observed (Fig. 2). No yield reduction was seen with the variety Desirée after inoculation with the 'Ahlum' serotype or with

all three varieties after inoculation with the 'Wierthe' serotype. Characteristic leaf symptoms were not seen in any of our experiments.

Prillwitz and Schlösser (1992) have introduced BSBV by means of *P. betae* into sugarbeet in order to assess the BSBV-induced losses. Both, mechanical inoculation and inoculation by means of *P. betae* have their advantages and disadvantages. When *P. betae* is used as a vector, it may be difficult to distinguish between virus and vector effects with the result that the virus-induced yield losses are overestimated. Mechanical inoculation has the disadvantage that BSBV which spreads only slowly in tap roots (Kaufmann *et al.*, 1992a; Kaufmann and Koenig, 1993) is introduced only once into the plants, whereas with *P. betae* continuous reinfections are possible. Also, isolates which have been kept for years on *Ch. quinoa* may have lost some of their pathogenicity for sugarbeet. So the estimated yield losses may be too low. In our experiments the 'Wierthe' serotype had a lower pathogenicity for sugarbeet than the 'Ahlum' serotype. This might be a genuine property of this serotype or it may have been acquired during prolonged cultivation on the 'unnatural' local lesion host *C. quinoa*. - Our own observations and those of Prillwitz and Schlösser (1992) who found yield losses of 40% suggest that at least some strains of BSBV are damaging to sugarbeet and that the search for BSBV resistance should be included in breeding programms.

Acknowledgement
We are greatly indebted to the Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung for financial support.

References

Barbarossa, L., Vetten, H.J., Kaufmann, A., Lesemann, D.-E. and Koenig, R. (1992). Monoclonal antibodies to beet soil-borne virus. *Ann. Appl. Biol* **121**:143-150.

Henry, C.M., Jones, R.A.C., and Coutts, R.H.A. (1986). Occurence of a soil-borne virus of sugar beet in England. *Plant Pathology* **35**:585-591.

Kaufmann, A., and Koenig, R. (1993). Detection of beet necrotic yellow vein and beet soil-borne viruses in sugarbeet by means of tissue print-immunoblotting. This volume, p.....

Kaufmann, A., Koenig, R., and Rohloff, H. (1993). Influence of beet soil-borne virus on mechanically inoculated sugarbeet. Plant Pathology **42**:413-417.

Kaufmann, A., Koenig, R. and Lesemann, D.-E. (1992a). Tissue print-immunoblotting reveals an uneven distribution of beet necrotic yellow vein and beet soil-borne viruses in sugar beets. *Arch. Virol.* **126**:329-335.

Kaufmann, A., Li, Y., Koenig, R., Breyel, E., Maiß, E., Lüddecke, P. & Commandeur, U. (1992b). Single- and double-stranded RNAs associated with an isolate of beet soil-borne virus. *Intervirology* **33**:97-102.

Koenig, R. and Burgermeister, W. (1989). Mechanical inoculation of sugar beet roots with isolates of beet necrotic yellow vein virus having different RNA compositions. *J. Phytopathol.* **124**:249-255.

Koenig, R., and Stein, B. (1990). Distribution of beet necrotic yellow vein virus in mechanically inoculated sugar beet plantlets of cultivars with different degrees of rizomania resistance. *Schriftenreihe der Deutschen Phytomedizinischen Gesellschaft* **1**:87-89.

Lesemann, D.-E., Koenig, R., Lindsten, K., and Henry, C. (1989). Serotypes of beet soil-borne furovirus from FRG and Sweden. *Bulletin OEPP/EPPO Bulletin* **19**: 539-540.

Prillwitz, H., und Schlösser, E. (1992). Beet soil-borne virus an Zuckerrüben: Vorkommen, Symptome und Einfluß auf die Pflanzenentwicklung. *Mitteilg. Biol. Bundesanst. (48. Deutsche Pflanzenschutztagung)* **283**:223.

TEXAS 7 A POSSIBLE STRAIN OF BEET NECROTIC YELLOW VEIN VIRUS.

C. M. Rush[1], R. C. French[2], and G. B. Heidel[1]

[1]Texas Agricultural Experiment Station, P.O. Drawer 10, Bushland, TX 79012 and [2]USDA-ARS, University of Nebraska, 406 Plant Science, Lincoln, NE 68583, respectively

Summary

Restriction enzyme analysis, sequencing of PCR products, and Northern hybridization studies were used to evaluate the relationship between beet necrotic yellow vein virus (BNYVV) and an unnamed furovirus from sugar beet designated Texas 7 (Tx7). Using published sequence data from a European isolate of BNYVV, two pairs of primers specific for each RNA species were synthesized. All primer pairs produced PCR products of the expected size with BNYVV samples. With Tx7 samples, a primer pair specific for the 3' end of BNYVV RNA1 also produced a PCR product slightly smaller than that expected for a BNYVV sample. Restriction analysis indicated the Tx7 PCR product was similar to the BNYVV product but contained a deletion of approximately 30 bases near the 3' end. Sequence analysis indicated the Tx7 PCR product had approximately 75% nucleotide and 96% amino acid homology with the BNYVV PCR product. Northern blots, using labeled BNYVV PCR products specific for each RNA as probes, also indicated similarities between Tx7 and BNYVV. Probes from the 3' end of BNYVV RNA1, 2 and 4 all hybridized with individual Tx7 RNAs but not probes for BNYVV 5' ends or probes for RNA3. However, when labeled BNYVV cDNA was used as a probe, it hybridized with Tx7 RNA3. Likewise, labeled Tx7 cDNA hybridized with BNYVV RNAs. From these results, we conclude Tx7 is very closely related to BNYVV and is possibly a mild strain with a major deletion in RNA3.

Introduction

In 1988, a virus similar in particle morphology to BNYVV was isolated from Texas sugar beets and designated Tx7 (Liu and Duffus, 1988). Subsequent investigation revealed Tx7 has four distinct polyadenylated RNA molecules of approximately 6.5, 4.2, 1.2 and 1.0 kb (Heidel et al., 1993). Tx7 has a host range similar to that of BNYVV, is vectored by *Polymyxa betae* and frequently is found in the same fields as BNYVV (Heidel and Rush, 1993). Although Tx7 has much in common with BNYVV, it produces symptoms different from those caused by BNYVV on several hosts. Foliar symptoms of Tx7 on sugar beet include a pale yellow discoloration which primarily follows the major leaf veins, mottling, and a slight puckering or distortion. Foliar symptoms can fade or completely disappear from field beets transplanted into the greenhouse, only to reappear later. In such plants, Tx7 can often be detected by ELISA in nonsymptomatic foliage. Tx7 has no obvious adverse effects on root development. On *Chenopodium quinoa*, Tx7 causes diffuse, pale yellow local lesions which may eventually spread along leaf veins. On *Beta macrocarpa* and *B. maritima*, Tx7 causes necrotic local lesions which ultimately develop into systemic infections on *B. maritima* but not *B. macrocarpa*. Because the symptoms produced by Tx7 are similar to those described for RNA3 deletion mutants of BNYVV (Bouzoubaa et al.,

1988), studies were conducted to further evaluate differences and similarities between Tx7 and BNYVV. Studies were also initiated to evaluate how certain host plants reacted to dual inoculations with these two viruses.

Materials and Methods

Virus maintenance and purification: Tx7 and BNYVV isolates were maintained in the greenhouse on *C. quinoa* by mechanical inoculation. Procedures for viral purification were similar to those described for purifying sorghum chlorotic spot virus (Kendall et al., 1988). Tx7 isolates used in PCR and hybridization studies were passed through *C. quinoa* four times, while isolates of BNYVV were mechanically transmitted 5-9 times before use.

PCR studies: Two primer pairs were made for each specific BNYVV RNA using published sequence data from European isolates (Bouzoubaa et al., 1985; Bouzoubaa et al., 1986; Bouzoubaa et al., 1987). For each RNA, one primer pair matched the 3' end and one the 5' end. Purified BNYVV and Tx7 RNA were used as templates for first strand cDNA synthesis in reverse transcriptase reactions. cDNA amplification was carried out in a 50 μl reaction using 5 μl cDNA, 10 pmol of each primer, 0.2 mM of each dNTP and 5 U *Taq* DNA polymerase in reaction buffer provided with the enzyme (Robertson et al., 1991). PCR products were visualized after electrophoresis in a 1% agarose gel by staining with ethidium bromide. Products were cut with various restriction enzymes, and observed fragment sizes were compared with predicted values. One product from RNA1, near the 3' end, was sequenced after gel purification and cloning into pGEM3z (Promega).

Northern blots: After PCR product identity was verified, radioactive probes were made. PCR products were gel purified and used as templates in second round PCR reactions which included ^{32}P labeled dCTP. Radioactive cDNA probes were also produced from unfractionated BNYVV and Tx7 RNA. Northern blots were hybridized with probes as previously described (Church and Gilbert, 1984).

Dual inoculation studies: Local lesions of Tx7 and BNYVV on *C. quinoa* were macerated in 0.1 M KPB pH 7.5 plus 0.02 M Na_2SO_3 and used to inoculate *C. quinoa*, *B. macrocarpa*, and *B. maritima*. Plants were inoculated with each virus independently or with mixed inoculum. Symptom expression was recorded after approximately two weeks, and ELISA tests were conducted to verify the presence of pathogens.

Results

PCR studies: All primer pairs produced expected products in PCR reactions using BNYVV cDNA except the pair specific for the 5' end of RNA3. When Tx7 cDNA was used, only the primer pair specific for the 3' end of RNA1 gave a product close to that expected for BNYVV. Restriction analysis of the Tx7 product, which was slightly smaller than that expected for BNYVV (approximately 1000 vs. 1056 kb, respectively), indicated a high degree of sequence homology. Failure of ThaI to cut the BNYVV product indicated the Texas isolate of BNYVV differed slightly from the European isolate from which the sequence data was derived. ThaI did cut Tx7 and gave products of the size expected for BNYVV as did SpeI, NheI, and DraI.

Sequence analysis of the Tx7 product indicated 75% nucleotide and 96% amino acid sequence homology with the BNYVV product. The deletion in the Tx7 product first

observed after electrophoresis of PCR products was determined to be 30 bases in the noncoding region of the 3' terminus.

Hybridization studies: Radioactive probes specific for sequences near the 3' end of BNYVV RNA1, 2 and 4 hybridized strongly with BNYVV RNA and, to a lesser degree, with Tx7 RNA. Neither the specific probe for BNYVV RNA3 nor any probes specific for sequence near the 5' termini hybridized with Tx7, although all hybridized strongly with BNYVV RNA. However, cDNA probes made with an oligo dT primer and nonfractionated BNYVV and Tx7 RNA hybridized with homologous and heterologous RNA, indicating some sequence homology between the four Tx7 and BNYVV RNAs.

Dual infection studies: All hosts inoculated with BNYVV developed bright yellow local lesions which eventually went systemic in *B. macrocarpa* and *B. maritima*. *Chenopodium quinoa* inoculated with Tx7 developed diffuse, pale yellow local lesions. *Beta macrocarpa* and *B. maritima* inoculated with Tx7 developed necrotic spots surrounded by purple halos. The virus eventually went systemic in *B. maritima* but not *B. macrocarpa*. *Chenopodium quinoa* inoculated simultaneously with BNYVV and Tx7 developed a mottled appearance very different from symptoms on plants inoculated with BNYVV or Tx7 alone. When *B. macrocarpa* and *B. maritima* were inoculated with both viruses, the Tx7 symptom phenotype was dominant. Mixed infections did not change systemic reactions of either virus.

Discussion

Numerous similarities between BNYVV and Tx7 indicate that Tx7 is very closely related to, if not a strain of, BNYVV. They have similar host ranges, exhibit some serological cross reactivity and have coat proteins of similar size. Both are vectored by *P. betae*, and both possess 3' polyadenylated quadripartite genomes (Heidel et al., 1993). To our knowledge, Tx7 is the only recognized furovirus, other than BNYVV, with a quadripartite genome.

Tx7 and BNYVV also have nucleotide sequence homology as shown by PCR and hybridization experiments in this study. Homology exists between all four RNAs, and the degree of homology is greatest near the 3' termini. The greatest molecular variation between Tx7 and BNYVV appears to occur in RNA3. RNA3 of Tx7 is approximately 1.2 kb compared with 1.7 kb with BNYVV. BNYVV PCR probes hybridized with Tx7 RNA1, 2, and 4, but not with Tx7 RNA3. Since BNYVV RNA3 is primarily responsible for symptom phenotype, it is interesting that Tx7, which differs from BNYVV in symptom expression, also differs from BNYVV on a molecular level at RNA3.

There have been numerous reports that wild type isolates of BNYVV possess four full-length RNA species, and that isolates propagated on leaves often possess deleted forms of RNA3 and 4 (Hamilton et al., 1981; Tamada et al., 1990). BNYVV isolates, with deleted forms of RNA3, produce symptoms similar to those produced by Tx7, including diffuse chlorotic spots, necrotic lesions, and an absence of root symptoms (Hamilton et al., 1981; Tamada et al., 1990). Additionally, when "wild type" isolates and RNA3 deletion mutants of BNYVV are inoculated together to *C. quinoa*, the mutant symptom phenotype is dominant (Jupin et al., 1992). In our study, Tx7 symptoms were dominant when Tx7 and BNYVV were both inoculated to *B. maritima* and *B. macrocarpa*.

There is no question Tx7 is very closely related to BNYVV and exhibits numerous characteristics of BNYVV RNA3 deletion mutants. Furthermore, if one uses the criteria

suggested by Hamilton et al. (1981) to differentiate viral strains from new viruses, Tx7 should be classified as a strain of BNYVV. If Tx7 is a strain of BNYVV, it is, to our knowledge, the first reported "wild type" isolate with a deleted form of RNA3. However, because of the potential confusion a "mild strain" of BNYVV might create among regulatory agencies, we are withholding our opinion concerning strain designation until further data is gathered.

References

Bouzoubaa, S., Guilley, H., Jonard, G., Jupin, I., Quillet, L., Richards, K., Scheidecker, D., and Ziegler-Graff, V. (1988). Genome organization and function of beet necrotic yellow vein virus. Develop. Appl. Biol. **2**:99-110.

Bouzoubaa, S., Guilley, H., Jonard, G., Richards, K., and Putz, C. (1985). Nucleotide sequence analysis of RNA-3 and RNA-4 of beet necrotic yellow vein virus isolates F2 and G1. J. Gen. Virol. **66**:1553-1564.

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G., and Richards, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. J. Gen. Virol. **68**:615-626.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K., and Jonard, G. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. J. Gen. Virol. **67**:1689-1700.

Church, G. M., and Gilbert, W. (1984). Hybridization protocols for DNA probes. *in:* Genomic Sequencing. Proc. Nat. Acad. Sci. **81**:1991-1995.

Hamilton, R. I., Edwardson, J. R., Francki, R. I. B., Hsu, H. T., Hull, R., Koenig, R., and Milne, R. G. (1981). Guidelines for the identification and characterization of plant viruses. J. Gen. Virol. **54**:223-241.

Heidel, G. B., and Rush, C. M. (1993). Distribution of beet necrotic yellow vein virus, beet distortion mosaic virus and an unnamed soilborne sugar beet virus in Texas and New Mexico. Plant Dis. (accepted)

Heidel, G. B., Rush, C. M., Kendall, T. L., and Lommel, S. A. (1993). Partial characterization of a soilborne sugar beet virus in Texas. Page. 159 *in:* Proc. 27th Biennial Meeting American Soc. Sugar Beet Technologists., Anaheim, CA.

Jupin, I., Guilley, H., Richards, K. E., and Jonard, G. (1992). Two proteins encoded by beet necrotic yellow vein virus RNA 3 influence symptom phenotype on leaves. The EMBO J. **11**:479-488.

Kendall, T. L., Langenberg, W. G., and Lommel, S. A. (1988). Molecular characterization of sorghum chlorotic spot virus, a proposed furovirus. J. Gen. Virol. **69**:2335-2345.

Liu, H. Y., and Duffus, J. E. (1988). The occurrence of a complex of viruses associated with rhizomania of sugarbeet. (Abstr.) Phytopathology **78**:1583.

Robertson, N. L., French, R., and Gray, S. M. (1991). Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses. J. Gen. Virol. **72**:1473-1477.

Tamada, T., Saito, M., Kiguchi, T., and Kusume, T. (1990). Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. Pages 41-44 *in:* Proc. Symp. Int. Work. Group on Plant Viruses with Fungal Vectors, 1st, Braunschweig, Germany.

VARIATIONS AMONG FUROVIRUSES ASSOCIATED WITH SUGARBEET.

G.C. Wisler, J.E. Duffus, and H.-Y. Liu.

USDA-ARS, 1636 E. Alisal St., Salinas, CA 93905.

Summary

The nature of relatedness between five beet necrotic yellow vein virus (BNYVV) isolates, (three from California, one from Nebraska, and one from Idaho) and eight other rigid, rod-shaped virus isolates of sugarbeet (two from Texas, five from Nebraska, and one from Idaho) was evaluated in this study using western blot analyses, host range studies, and RT-PCR. Antisera to the BNYVV virion showed strong reactions in western blot analyses at 22-kDa for the five BNYVV isolates, and weak reactions at 24-kDa for the eight other rod-shaped virus isolates. Reciprocal tests using antisera to the whole virion of a rod-shaped virus from Texas showed strong bands at 24-kDa for all eight rod-shaped isolates and weak bands at 22-kDa for the five BNVYY isolates. Antisera to the C-terminus of the BNYVV capsid protein, and the 75-kDa and 14-kDa nonstructural proteins of BNYVV reacted only with the five BNYVV isolates. Antisera to the 25-kDa nonstructural protein reacted to three of five BNYVV isolates. Antisera to the 42-kDa nonstructural protein reacted with all five BNYVV isolates at ca. 42-kDa, and with the eight other isolates at ca. 44-kDa. Seven BNYVV monoclonal antibodies reacted only with the five BNYVV isolates (ca. 22-kDa).

The MW of three BNYVV isolates (two from California, one from Idaho) was identical in RT-PCR for each BNYVV primer pair tested. A rod-shaped virus isolate from Texas and one from Nebraska did not react in preliminary RT-PCR analyses. Host range analyses also indicate BNYVV isolates to be distinct from the other rod-shaped virus isolates of sugarbeet.

Introduction

BNYVV was first detected in the U.S. in 1983 (Duffus et al., 1984). It is a member of the furovirus group, with rigid rod-shaped particle morphology, and is transmitted by *Polymyxa betae* Keskin. Symptoms include bearding of the roots and a loss both in yield and in sugar production. Disease control of BNYVV includes the use of resistant varieties and selective planting based on soil tests for the presence of *P. betae* contaminated with BNYVV.

Several other viruses with similar particle morphology to BNYVV have been isolated from sugarbeet roots from Texas (Liu and Duffus, 1987), Nebraska, and Idaho. Some of these isolates have been shown to cross react with antisera to the BNYVV virion in ELISA and in western blot analyses. Thus, the possibility exists for cross-reactivity in diagnostic tests which are based on serology of the capsid protein. This study addresses (1) the nature of relatedness among BNYVV isolates from California, Nebraska, and Idaho, and (2) the relationship between the BNYVV isolates and eight other rigid, rod-shaped viruses of sugarbeet from Texas, Nebraska, and Idaho. Results from western blot analyses indicate that the five BNYVV isolates are distinct from the other rod-shaped viruses based on: (1) the MW of the coat protein, (2) the specific reactivity to antisera made to the C-terminal capsid protein of BNYVV (courtesy K. Richards), (3) reactivity with antisera to the 75-kDa, 25-kDa, and 14-kDa nonstructural proteins (courtesy of K. Richards), (4) reactivity with seven monoclonal antibodies to BNYVV (courtesy of G. Grassi and L. Torrance), and (5) characteristic symptoms on *Chenopodium quinoa*, *Beta macrocarpa*, and *B. vulgaris*. Preliminary data from RT-PCR analyses also indicate these BNYVV isolates to be distinct from two other rod-shaped virus isolates (one from Texas and one from Nebraska), but are identical to one another. Differences seen between BNYVV isolates are in the extent or presence of systemic leaf symptoms in *B.vulgaris*.

Materials and Methods

Western blot analyses:

The western blot procedure was conducted essentially as described by Towbin et al. (1979) using a Bio-Rad Mini-Protean II Electrophoresis Cell and Trans-Blot Electrophoretic

Transfer Cell according to manufacturer's instructions.. Twelve and 15% SDS-polyacrylamide gels were used primarily. Plant tissue selected for assay were triturated in an extraction buffer (1:2, w:v) consisting of 75 mM Tris-HCl, pH 6.1, 9 M Urea, 7.5% 2-mercaptoethanol, and 4.5% SDS (Rodriguez-Cerezo and Shaw, 1991). Samples were squeezed through dampened miracloth, heated at 95 C for two min, and centrifuged at 5,000 *g* for two min. Samples were stored at -20 C. Each isolate was tested in both *B. vulgaris* and *C. quinoa*.

Generally, polyclonal antisera and monoclonal ascites fluids were diluted 1/1000, and monoclonal tissue culture supernate at 1/10. Antisera to the C-terminal 60 amino acids of the BNYVV capsid protein, and the 14-, 25-, 42-, and 75-kDa nonstructural proteins (Niesbach-Klosgen et al., 1990) were kindly supplied by K. Richards. Monoclonal antibodies (MAb) 41 and 47 were supplied by G. Grassi, and MAb 6,7,8,9, and 10 were supplied by L. Torrance.

Host plant inoculations:

Test plants used for inoculations were held in the dark for 16-24 hr prior to inoculations. Mechanical inoculations were made by triturating tissues in a mortar and pestle with 0.1 M potassium phosphate buffer, pH 7.2, containing 0.1% Na_2SO_3, with the addition of 600 mesh carborundum. The slurry was rubbed onto leaves, and plants were rinsed gently with water after inoculation.

Reverse transcriptase-polymerase chain reaction (RT-PCR):

Aliquots of crude virus preparations were subjected to a phenol:chloroform extraction, followed by an ethanol precipitation. Viral RNA was used as a template for production of cDNA using reverse transcriptase (Superscript, Gibco BRL), followed by amplification by PCR as described by Robertson et al. (1991). Primers used were made according to the published sequence of BNYVV (Bouzoubaa et al., 1985, 1986, 1987; Rush, 1993). PCR products were analyzed on a 1.0% agarose gel, stained with 0.5µg/ml ethidium bromide, and viewed with ultraviolet light.

Results

Antisera to the whole virion of BNYVV reacted strongly in western blots with the five BNYVV isolates (three from California, one from Nebraska, and one from Idaho), with a MW of ca. 22kDa. This same antiserum reacted weakly with eight other rigid, rod-shaped isolates of sugarbeet, with a MW of ca. 24 kDa. In contrast, antisera to the C-terminal 60 amino acids of the BNYVV capsid protein was specific to BNYVV and reacted only with the five BNYVV isolates at ca. 22 kDa. Antisera to the nonstructural 14-kDa and 75-kDa proteins likewise reacted only with the five BNYVV isolates, at 14-kDa and 75-kDa, respectively . Antisera to the 25-kDa nonstructural protein reacted with two isolates from California, and with one from Nebraska, but not with an isolate of BNYVV from Idaho, or with an isolate from California which had been maintained in the greenhouse by mechanical inoculation for several years. All seven MAbs reacted specifically to BNYVV at ca. 22-kD (Fig.1). All but one isolate (NE-KW) reacted with the 42-kDa antiserum: BNYVV isolates were ca. 42-kDa, whereas the others were ca. 44-kDa. Reciprocal tests in western blots were made using antisera to the virions of the two rod-shaped virus isolates from Texas; beet soil-borne mosaic virus (BSBMV-1 and -2). These antisera reacted strongly with the eight other rod-shaped virus isolates at ca. 24-kDa and weakly with the five BNYVV isolates at ca. 22-kDa. Western blot results are summarized in Table 1.

Host range studies of the BNYVV isolates showed similar reactions on the indicator plants tested. All five isolates produced characteristic chlorotic to bright yellow local lesions which spread into the veins in *C. quinoa*. All five also produced yellow local lesions and systemic infection on *B. macrocarpa* . Host range studies of the other virus isolates from sugarbeet gave more variable reactions on the indicator plants tested. For example, symptoms on *C. quinoa* ranged from diffuse, chlorotic local lesions for BSBMV-1 and -2, to necrotic lesions for NE-10 and NE-KW. Symptoms on *B. macrocarpa* ranged from necrotic local lesions for NE-8-1 to a systemic necrosis of the midrib veins for ID-31051 and NE-8-3.

Preliminary tests from RT-PCR analyses show identical size products for the three BNYVV isolates tested to date (Table 2). Primer pairs which represent regions of each of the four RNAs were used. The products were similar to the expected sizes in each case except for primers 1 and 6. In this case, the expected size was 195 bp and the products obtained for all three BNYVV isolates were ca. 300 bp. Primer pair 1 and 8, which corresponds to RNA-3, gave a

very faint reaction for the greenhouse isolate of BNYVV (CA-GH). This agrees with western blot data where CA-GH did not react with antisera to the 25-kDa protein encoded by RNA-3.

Discussion

Results from this study suggest that there is a possible serological relationship between the BNYVV isolates and the eight other rigid, rod-shaped viruses of sugarbeet. This is based on reciprocal cross-reactivity in western blots as shown in this report. The reciprocal crossreactivity was seen when antisera to the whole virions of BNYVV, BSBMV-1 and -2 were used. Antisera to the C-terminus of the BNYVV coat protein, however, was specific to BNYVV, and did not cross-react with the other isolates. Shukla has reported similar results for potyviruses (Shukla et al., 1991), where antisera to the N-or C- termini of the coat protein is highly specific, whereas antisera to the core is cross-reactive. The 42-kDa antisera was also cross-reactive among all isolates used in this study. This is not surprising, since this protein appears to be conserved among several plant virus groups, including potex-, carla-, furo-, and hordeiviruses (Beck et al., 1991). This 42-kDa protein has been implicated in cell-to-cell transport.

The eight non-BNYVV isolates addressed in this report have not been shown to cause the Rhizomania disease symptomatology, and show different symptoms in a host range study from those reported for BNYVV. However, the effect of these virus isolates on sugarbeet production is not known. Typical furo-like virus particle morphology has been noted among these isolates. These data suggest that these other isolates may belong to the furovirus group. However, fungal transmission has not been determined in all cases.

The results of this study indicate that it is possible, in ELISA tests using antisera to the whole virion of BNYVV, that another, related isolate could be mistaken for BNYVV. Although western blot analysis is not suited to large scale diagnostic tests, with the proper controls, it can distinguish between BNYVV and the other rigid, rod-shaped isolates addressed in this study. To date, no isolate similar to these eight other types has been detected from California. Future studies will focus on the development of a more specific antiserum to BNYVV, which can be used on a commercial basis for Rhizomania testing services.

References

Beck, D.L., Guilford, P.J, Voot, D.M., Anderson, M.T., and Forster, R.L.S. 1991. Triple gene block proteins of white clover mosaic potexvirus are required for transport. Virology 183:695-702.

Duffus, J.E., Whitney, E.D., Larsen, R.C., Liu, H.-Y., and Lewellen, R.T. 1984. First report of rhizomania of sugarbeet caused by beet necrotic yellow vein virus in the western hemisphere. Plant Disease 68:251.

Liu, H.-Y., and Duffus, J.E. 1988. The occurrence of a complex of viruses associated with rhizomania of sugarbeet. Phytopathology 78:1582 (Abstr.).

Niesbach-Klosgen, U., Guilley, H., Jonard, G., and Richards, K. 1990. Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins., Virology 178:52-61.

Rush, C.M., Heidel, G.B., French, R.C., and Lazar, M.D. 1993. Relationship between BNYVV and an unnamed soilborne sugar beet virus from Texas. J. Sugar Beet Res. (Abstr.) In Press.

Ward, C.W., and Shukla, D.D. 1991. Taxonomy of potyviruses: current problems and some solutions. Intervirology 32:269-296.

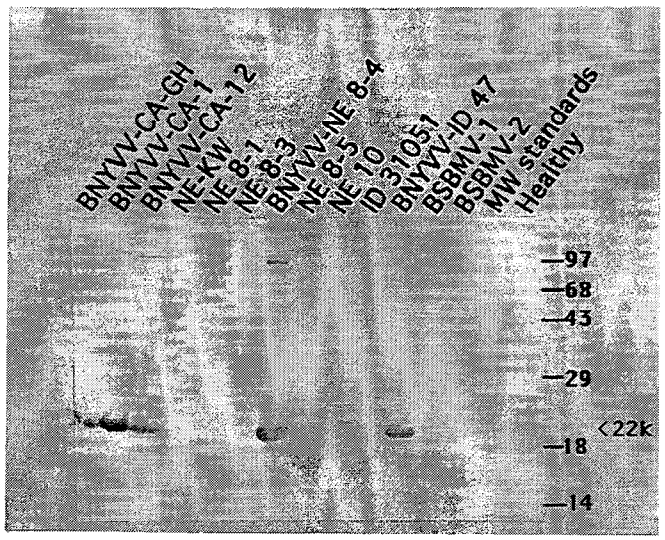


Fig. 1. Western blot analysis of several rigid, rod-shaped viruses of sugarbeet. Blot was probed with monoclonal antiserum to the BNYVV coat protein (Mab 41, courtesy G. Grassi).

RHIZOMANIA - ARE BOTH BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) AND BEET SOIL-BORNE VIRUS (BSBV) INVOLVED?

Klas Lindsten

Department of Plant- and Forest Protection, Swedish University of Agricultural Sciences,
S-750 07 Uppsala, SWEDEN

Summary

Beet soil-borne virus (BSBV) is difficult to detect and has earlier escaped detection.It can be detected in infested soils, with the method used, already after one week of baiting but BNYVV usually requires a longer time except when the amount of inoculum is very high.BSBV seems to infect bait plants faster than BNYVV.Slower infections take place in nature but a preinfection with BSBV is likely to occur in rhizomania fields. The damage caused by BSBV is less than that by BNYVV but BSBV is not harmless.Rhizomania is caused by BNYVV but there may be a complicated interaction between BSBV and BNYVV and the vector, Polymyxa betae, which should be paid more attention to.

Introduction

Rhizomania, a severe disease in sugarbeets, was first found in Italy and later in more and more countries in Europe, in Japan and in USA. There has been much confusion about the cause and the occurrence of the disease also after it was shown that a virus, Beet necrotic yellow vein virus (BNYVV), could cause a similar disease (Tamada, 1977). Duffus (1988) reported on other Polymyxa-transmitted viruses involved in the rhizomania complex.Lesemann et al. (1989) made a rough comparison of several Polymyxa-transmitted viruses found in sugarbeets, mainly Beet soil-borne viruses (BSBV), which were all shown to be distinct from BNYVV.

In Sweden rhizomania-like symptoms were found especially in late sown sugarbeets in 1986 and 1987, but it was gradually shown that BNYVV was not the cause. Instead another similiar Polymyxa-transmitted virus of the BSBV-type (86-109) was common in the diseased fields but it also occurred in fields without any obvious disease symptoms (Lindsten, 1989, 1991).

Apparently BSBV is very common in beet fields but has escaped detection as the concentration is uneven and low in older beet plants and therefore difficult to detect by EM, also ISEM, and by sap transmission directly from field beets (Lindsten,1988,1989). It was not until an antiserum to BSBV (86-109) was produced that BSBV was shown to be common in many European countries (Lindsten, 1988, 1991 and unpublished, Bremer et al.1989, Danielsen et al.1992, Prillwitz & Schlösser, 1992) and in USA (Lindsten & Rush, 1993)

Already in my first experiments in a wire netting enclosure with rhizomania-infested soils from Germany, it was found that not only BNYVV but also a second virus, which later was shown to be BSBV, was common and caused mixed infections with BNYVV in the beet plants (Lindsten, 1989). In this report more evidence is given that rhizomania in general seems to have not only BNYVV but also BSBV involved and that a preinfection with the latter virus seems to occur.

Materials and Methods

From soil samples, collected as representative as possible in the fields, 50 g of well airdried soil was used in a soil-water mixture for baiting with two groups of sugarbeet plants mainly according to what is described in Lindsten (1990).

Several sensitive sugarbeet cultivars were tried as bait plants but nowadays mainly Accord is used.As test plants were in general used both Chenopodium quinoa and C. amaranticolor since young plants of the latter can be used to distinguish between BSBV and BNYVV.

Only own produced polyclonal antisera to both BSBV and BNYVV were used (Lindsten 1989).The ELISA procedure was mainly the usual one and readings were taken in a Titertek Multiscan MC at 405 nm 30 and 60 min. after addition of substrate.The diluted plant sap was put in two distantly separated wells on the ELISA-plate giving duplicate readings.

Results

Tests of soils from Germany and France

During 1992 and1993 soil tests with respect to BSBV and BNYVV were carried out from 12 selected German fields with severe rhizomania. Ten samples and in two cases 25 were tested from each field.In nearly all these tests clearly positive ELISA readings (>0.5 at 405 nm after 60 min.) were obtained for BSBV already after 7 days of baiting. In general, much lower readings were obtained for BNYVV and some of the fields showed no evidence of BNYVV after this time of baiting. In cases when transmission was made to C.quinoa and C. amaranticolor both symptoms obtained and ELISA of the inoculated Chenopodium leaves showed only BSBV-infection.

However, ELISA after 14 days of baiting still as a rule gave higher readings for BSBV but now also the readings for BNYVV were clearly positive (values often above 1,0) except in the samples from one field where 8 of 10 did not indicate any occurrence of BNYVV in spite of having ELISA values for BSBV above 2,0. The transmission tests to Chenopodium species after 14 days of baiting showed not only BSBV but also BNYVV or a mixture of both on inoculated Chenopodium leaves.

From the Pithiviers area in France, where rhizomania is a common severe disease in sugarbeets, soils from 6 different fields were collected in the autumn of 1992. Baiting in the usual soil - water mixture and other types of baiting were carried out during the winter 1992-1993. Apparently both the BSBV- and BNYVV- concentrations were high in the Polymyxa population and the ELISA values for BNYVV from all these fields were around or above 1,0 after 60 min. already after 7 days of baiting. However, the values for BSBV were in general still higher.

Tests of soils from USA

In cooperation with Dr. C.M. Rush,Texas Agric. Exp. Station, Bushland, some soil samples mainly from Texas, California and Colorado have been tested with respect to Polymyxa-transmitted viruses in sugarbeets.

From larger samples, selected by Rush, 50 g or 75 g airdried soil were sent to our laboratory and from which 50 g were tested in the usual way with bait plants, by ELISA and by test plants. 23 of 35 fields (66%) from the three mentioned states were found to have BSBV. BNYVV and viruses which are serologically related to BNYVV as Tx 7 were found in less of the samples (34%) but in all these cases, except one, also BSBV could be detected.

The behaviour of the American isolates were rather similar to the European ones.Thus after 7 days of baiting only BSBV could be detected by ELISA and by test plants. However, after 14 days of baiting it was possible to detect also BNYVV and / or Tx 7.

Experiments in progress with soils from various rhizomania fields in Europe.

In a joint program within IIRB (Institut International de Recherches Betteravières) a possible variation in pathogenecity of BNYVV is the objective to be studied and I shall participate in first hand by testing for the occurrence of BSBV. A preliminary test including also BNYVV is shown in Table 1.

The ELISA results for most of the samples are essentially in agreement with those already mentioned and the readings vary especially for BNYVV after 7 and 14 days depending on the concentration.Sample Greece 1 shows unclear and somewhat dubious results in the ELISA readings after 14 days as the second bait plant group was partly rotten. Germany 2 like Greece 1 gave no indication of BSBV and was together with Greece 1 the only one which even after 14 days of baiting failed to infect Chenopodium. It is interesting, but in agreement with earlier

findings that the transmission to Chenopodium spp. after 7 days of baiting only caused BSBV-infection and no BNYVV-infection which was confirmed by ELISA of inoculated leaves.

Table 1 A preliminary test of Polymyxa-transmitted viruses in soils used in a joint IIRB program for pathogenetic studies in rhizomania infested fields in some European countries. ELISA-readings at 405 nm after 60 min.

	ELISA of baitplants and testplant reactions											
	after 7 days of baiting						after 14 days of baiting					
Locality	BSBV	Cq	Ca	BNYVV	Cq	Ca	BSBV	Cq	Ca	BNYVV	Cq	Ca
Netherlands 1	0,51	+	+	-0,04	-	-	0,80	+	+	0,46	+	+
	0,41			0,0 3			0,60			0,32		
Netherlands 2	1,86	+	+	0,33	-	-	1,91	-	-	0,93	+	-
	1,57			0,37			1,52			0,76		
Spain 1	1,00	+	+	0,00	-	-	> 2.0	-	-	0.51	-	-
	0,85			0,03			1,81			0,47		
Spain 2	> 2,0	+	+	0,24	-	-	>2,0	-	-	0,45	+	+
	> 2,0			0,32			1,81			0,77		
Greece 1	0,05	o	o	0,18	o	o	-0.14	o	o	-0,16	o	o
	0,01			0,28			-0.10			0,18		
Germany 1	> 2,0	+	+	0,59	-	-	1,76	+	+	0,66	+	+
	1,74			0,45			1,25			0,57		
France 1	1,45	o	o	0,28	o	o	1,78	+	+	1,19	+	+
	1,18			0,20			1,31			1,08		
France 2	1,90	+	+	0,70	-	-	1,34	+	+	0,64	+	-
	1,75			0,54			1,02			0,45		
Germany 2	0,02	o	o	0,20	o	o	-0,05	o	o	0,61	o	o
	0,02			0,15			-0,04			0,47		
Germany 3	0,05	o	o	0,22	o	o	0,24	+	+	0,94	+	+
	-0,01			0,21			0,21			0,77		
Italy 1	0,30	+	+	0,13	-	-	0,30	+	o	0,17	+	o
	0,38			0,09			0,23			0,21		

+ = symptoms developed and positive ELISA
- = symptoms developed but negative ELISA
o = no symptoms

Discussion

The rather comprehensive tests of soils from Germany, France and USA according to the rapid soil-water mixture method are briefly summarized in the Results. It is shown that BSBV is widespread,likely to occur in high concentrations and together with BNYVV as mixed infections in most rhizomania infested fields.

All these findings suggest in addition that not only mixed infections of BSBV and BNYVV seem to be common in rhizomania fields but also that a preinfection with BSBV is likely. This is also supported by the results in Table 1 with the only exceptions of the isolates from Greece 1 and Germany 2.

Observations of BSBV-and BNYVV infestations in a wire netting enclosure (Lindsten,

1991)and unpublished results of experiments with spread of these viruses with seed potatoes, also suggest that BSBV is more easily spread and becomes established faster both in greenhouses and outside in the fields than BNYVV.

Schlösser (1990) has studied the effect of BNYVV and BSBV on the development of the vector,Polymyxa betae Keskin, and found BSBV to have a promoting effect on the vector in contrast to BNYVV which reduced the cystosori production. Kaufmann et al.(1992) found in greenhouse experiments that BSBV- inoculation of sugarbeets reduced the taproot weight in two cultivars, Rizor and Carla, with about 20% which also may indicate that the resistance to BNYVV found in Rizor does not work for BSBV.Prillwitz & Schlösser (1992)have in other greenhouse experiments found up to 40% taproot reduction caused by BSBV and report that BSBV (BSBV-2) seems to be a part of the rhizomania syndrome.

Personally I have got somewhat contradictory results concerning the damage caused by BSBV (Lindsten, 1988,1989,1991)but still I consider BSBV to cause in general only slight damage in comparison to BNYVV. This is supported by a careful study of an experimental beet field in 1992 in Ressons sur Matz,France, where sugarbeets had been growing for many years with"normal" yields and sugar content. Various tests showed a high concentration of Polymyxa and BSBV in the sugarbeets but no obvious symptoms were found neither on the leaves nor on the roots of the beets. On the other hand, it is wellknown that many symptomless viruses, e.g. PVX, can cause yield reductions up to 10-20% without being detected. As in addition very interesting interactions between BSBV, BNYVV and the vector seem to occur, which may have negative, but possibly also positive, effects for the beet growing,further studies of the rhizomania complex seem to be urgent.In any case further studies of both the damage caused by BSBV and its interactions with BNYVV and the vector seem to be important.

References

Bremer, K.,Hiltunen,L. and Valkonen,J. (1990). Survey of soil-borne virus diseases of sugar beet in Finland and Estonia.In: *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors,Braunschweig,Germany*,pp.17-20.

Danielsen, S.,Mugrabi,M.,Albrechtsen,M.and Thomsen,A. (1992). Rhizomania in sugar beet. Survey for Beet Necrotic Yellow Vein Virus (BNYVV) and Beet Soil Borne Virus (BSBV) in Denmark 1988-1989 and host range of *Polymyxa betae*,BNYVV and BSBV.*Danish J. of Plant and Soil Science* **96**,309-316.

Duffus, J.E. (1988). Soil-borne viruses of the rhizomania complex. In: *Vth International Congress of Phytopathology, Abstracts of Papers*, p.452. *Phytopathological Society of Japan, Kyoto (JP)*

Kaufmann, A., Koenig,R. and Rohloff,H. (1993). Influence of beet soil-borne virus on mechanically inoculated sugar beet.*Plant Path.* **42**,413-417.

Lesemann,D.E., Koenig,R., Lindsten,K. and Henry, C. (1989). Serotypes of beet soil-borne furovirus from FRG and Sweden.*Bulletin OEPP/EPPO Bulletin* **19**,539-540.

Lindsten,K.(1988).Studies concerning rhizomania at Ultuna - a preliminary report. *Betodlaren* **51**, 137 -143. (in Swedish)

Lindsten,K.(1989). Investigations concerning soil-borne viruses in sugarbeet in Sweden.*Bulletin OEPP/EPPO Bulletin* **19**, 531-537.

Lindsten, K.(1990). A rapid method to detect Polmyxa-transmitted beet viruses from soil. In: *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors,Braunschweig,Germany*,pp.13-16.

Lindsten,K.(1991).Report on *Polymyxa*-transmitted beet viruses in Sweden.In: *Proceedings of 2nd International Conf. of sugar beet Rhizomania,Colmar,France* pp 39-44.

Lindsten,K. and Rush,C.M.(1993). First Report of Beet Soilborne Virus in the United States.*Plant Dis* .**00**, 000-000.

Prillwitz,H. and Schlösser,E.(1992). Beet soil-borne virus: Occurence, Symptoms and Effect on Plant Development. *Med.Fac. Landbouww.Rijksuniv. Gent* 57, 295-302.

Schlösser,E.(1990). Rizomania XII. Effect of BNYVV and BSBV on the development of P. betae Keskin.*Med.Fac. Landbouww.Rijksuniv. Gent* 55, 1069-1071.

Tamada,T.(1975). Beet necrotic yellow vein virus. *CMI/AAB Descriptions of Plant Viruses* No.14,4pp.

INTERACTIONS BETWEEN BEET SOIL-BORNE VIRUS (BSBV-2) AND BEET NECROTIC YELLOW VEIN VIRUS (BNYVV)

H. Prillwitz and E. Schlösser
Institut für Phytopathologie und Angewandte Zoologie, Justus-Liebig-Universität, Bismarckstraße 16, D-35390 Giessen, Federal Republic of Germany.

Summary

In Germany, BNYVV and BSBV-2 occur often concomitantly in the same area and in single plants.To test the possibility of interactions between the two furoviruses, the effect of a preinfection of sugar beet plants with one virus on the development of the subsequently inoculated other virus was investigated with two pot experiments. After preinfection with BSBV-2, the BNYVV titer was significantly lower than that where BNYVV had been present in the sugar beet plants from the start of the experiment. The damage caused by the virus was reduced by 50 %. After preinfection with BNYVV, followed by a subsequent inoculation with BSBV-2, the titer of the first virus was again significantly lower. There was, however, no reduction of the tap root weight due to the BNYVV.

Introduction

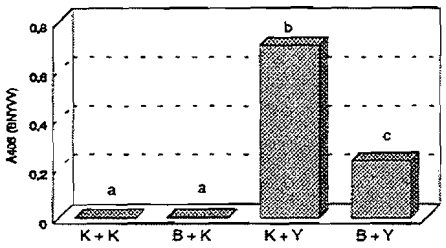
In the classical rizomania areas in Germany, BNYVV and BSBV-2, serotype 2 according to LESEMANN et al. (1989) and HENRY & HUTCHINSON (1989), occur often concomitantly in one plant (PRILLWITZ & SCHLÖSSER 1992, PRILLWITZ 1993). It was therefore tested in pot experiments with sugar beet plants, whether there are interactions between the two furoviruses. Both viruses were inoculated via *Polymyxa betae* KESKIN either singly, or by preinfection with one virus, followed by a subsequent inoculation with the other virus. The experiments were evaluated by determination of the virus titers with ELISA (CLARK & ADAMS 1977, BARBAROSSA et al. 1992) and the tap root weight.

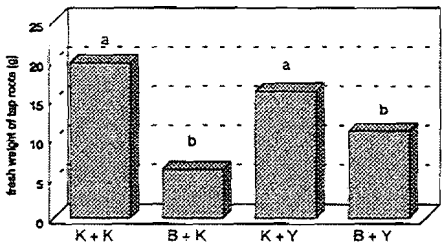
Materials and Methods

For all experiments, sugar beets of the diploid cv. Sandra were used as test plants. The seedlings were precultivated in steamed soil and used when they had reached the 2-leaf stage. Such plants were transplanted into small pots (10 cm) and inoculated by adding inoculum into the planting hole just prior to transplanting. The inoculum constisted of air dried and homogenized sugar beet roots with more than 750 cystosori for each pot. For the check, an equal amount of air dried roots with more than 750 cystosori of virus-free *P. betae* was used.
After a cultivation time of 7 weeks following the first inoculation in small pots (10 cm), the plants were transferred into larger pots (21 cm). For the interaction variants, a second virus inoculation was performed, the single component variants received no further virus inoculation but were supplied with virus-free *P. betae*. The sugar beet plants were then cultivated for further 7 weeks. After a total cultivation time of 14 weeks, the plants were harvested and analyzed for virus titers and fresh weight of tap roots.

Experiment I

In this experiment the effect of a preinfection of young sugar beet plants with BSBV-2 was tested with regard to BNYVV development after a subsequent inoculation.





same letter means not significantly different in the Tukey-test (p=0,05)
n = 25 plants each

		7 weeks culture	+	7 weeks culture
K + K	--->	-----	+	-----
B + K	--->	BSBV-2	+	-----
K + Y	--->	-----	+	BNYVV
B + Y	--->	BSBV-2	+	BNYVV

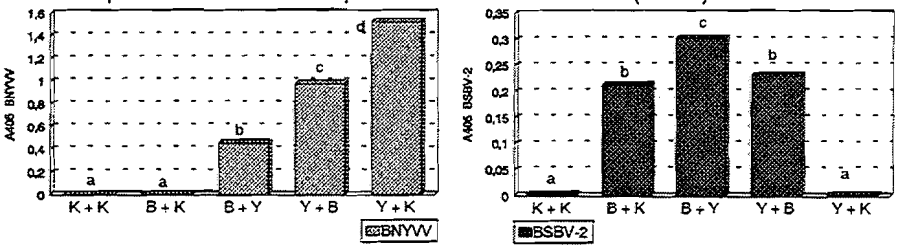
Fig. 1: Titer of BNYVV.

Fig. 2: Fresh weight of tap roots.

After preinfection with BSBV-2 (Fig. 1) the BNYVV titer was significantly lower (B + Y) than in the variant without preinfection with BSBV-2 (K + Y). This means, that a preinfection with BSBV-2 suppressed the development of BNYVV. There were significant differences in fresh weight of tap roots (Fig. 2) between the BSBV-2 variants (B + K, B + Y) and the other variants. It is interesting to note that in this experiment the damage caused by BSBV-2 alone (B + K) was significantly higher than that by BNYVV (K + Y), where the plants were 7 weeks old when they got inoculated with the virus. The significant reduction in fresh tap roots by the BSBV-2 (B + K) in comparison to virus-free plants (K + K) is remarkable and confirms earlier results (PRILLWITZ & SCHLÖSSER 1993a).

Experiment II

In this experiment the variant preinfection with BNYVV (Y + B) was added to the trial.

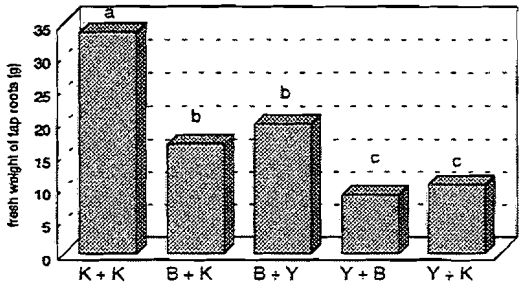


- same letter means not significantly different in the Tukey-test (p=0,05)
- BNYVV: polyclonal (Loewe Biochemica GmbH), positive $A_{405} \geq 0{,}100$
- BSBV-2: monoclonal (BBA Braunschweig), positive $A_{405} \geq 0{,}050$

		7 weeks culture	+	7 weeks culture
K + K	--->	-----	+	-----
B + K	--->	BSBV-2	+	-----
B + Y	--->	BSBV-2	+	BNYVV
Y + B	--->	BNYVV	+	BSBV-2
Y + K	--->	BNYVV	+	-----

Fig. 3: BNYVV and BSBV-2 titer resulting from different inoculation variants (n = 25 plants each).

Also in the this experiment (Fig. 3) the BNYVV titer was significantly lower after preinfection with BSBV-2 (B + Y) as compared to an inoculation with BNYVV alone (Y + K). The BNYVV titer in the variant without BSBV-2 (Y + K) was the highest. Even a subsequent inoculation with BSBV-2 (Y + B) lead to a significantly lower BNYVV titer. Preinfection with BNYVV (Y + B) resulted in a significant lower titer of BSBV-2 as compared to the vice versa variant (B + Y) and was at the same level as BSBV-2 alone (B + K).



same letter means not significantly different in the Tukey-test (p=0,05)

Fig. 4: Fresh weight of tap roots in the different variants.

With regard to the fresh weight of tap roots (Fig. 4), BSBV-2 alone (B + K) caused a reduction of about 50% as compared to the virus-free check (K + K). Contrary to the first experiment, BNYVV alone (Y + K) was significantly more damaging than BSBV-2 alone (B + K). A preinfection with BSBV-2 (B +Y) reduced the damage as compared to preinfection with BNYVV (Y + B). The variant preinfection with BNYVV (Y + B) followed

by BSBV-2 did not differ from BNYVV alone (Y + K). Thus the results can be grouped into 3 different classes. Preinoculation with BSBV-2 resulted in about 50 % losses, whereas preinokulation with BNYVV resulted in yield losses of about 70 % as compared to the virus-free check.

4 Discussion

It has been shown that a preinfection with BSBV-2 suppressed the development of a subsequent inoculation with BNYVV with regard to virus titer and reduction in tap root weight. Vice versa, with preinfection by BNYVV followed by inoculation with BSBV-2 there were not such drastic effects. The BNYVV titer was significantly lowered, but the reduction in tap root weight due to the BNYVV was not diminished. These findings could have epidemiological consequences. A soil temperature ≥ 15 °C seems to be sufficient for a measureable syntheses of BSBV-2 (PRILLWITZ & SCHLÖSSER 1993 b), whereas BNYVV requires apparently higher soil temperatures for longer periods (HILLMANN et al. 1985). Due to a lower temperature requirement, BSBV-2 would develop in young sugar beet seedlings prior to BNYVV, retarding the development of the latter. This could result in a lesser damage due to BNYVV as compared to a situation where BNYVV has been the primary virus. This attractive hypotheses has, however, to be validated under field conditions.

Acknowledgement

The financial support by the "Kuratorium zur Förderung des Zuckerrübenanbaues in Süddeutschland" and the supply of BSBV-2 antiserum by Prof. Dr. R. Koenig are gratefully acknowledged.

References

BARBAROSSA, L., H. J. VETTEN, A. KAUFMANN & D.-E. LESEMANN (1992): Monoclonal antibodies to beet soil-borne virus. Ann. appl. Biol. **121**, 143-150.

CLARK, M. F. & A. N. ADAMS (1977): Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. **34**, 475-483.

HILLMANN,U., W. HESS, I. HORAK & E. SCHLÖSSER (1985): Rizomania IX. Befallszeitpunkt. Ber. **48** I.I.R.B., Winterkongreß Brüssel, 369-384.

HENRY ,C. M. & P. J.HUTCHINSON (1989): Beet soil-borne virus and other beet viruses. Aspects of App. Biol. **22**, 109 - 116.

LESEMANN, D.-E., R. KOENIG, K. LINDSTEN and C. HENRY (1989): Serotypes of beet soil-borne furovirus from FRG and Sweden. EPPO Bulletin **19**, 539 - 540.

PRILLWITZ, H. (1993): Untersuchungen zur Rizomania, mit besonderer Berücksichtigung des beet soil-borne virus (BSBV). Diss. Gießen.

PRILLWITZ, H. & E. SCHLÖSSER (1992): Beet soil-borne virus: occurence, symptoms and effect on plant development. Med. Fac. Landboww. Uni. Gent, **57**, 295 - 302.

PRILLWITZ, H. & E. SCHLÖSSER (1993a): Comparative investigations on the damage of sugar beet by *Polymyxa betae* Keskin, beet soil-borne virus and beet necrotic yellow vein virus. Med. Fac. Landboww. Uni. Gent, **58**, in press.

PRILLWITZ, H. & E. SCHLÖSSER (1993b): Temperature requirements of beet necrotic yellow vein virus (BNYVV) and beet soil-borne virus (BSBV-2), Z. PflKrankh. PflSchutz, in press.

NORDIC ISOLATES OF POTATO MOP-TOP VIRUS, COMPARISON OF REACTIONS WITH MONOCLONAL ANTIBODIES AND OBSERVATIONS ON SPRAING SYMPTOMS IN POTATO CULTIVARS.

Maria Sandgren, Swedish University of Agricultural Sciences, S-750 07 Uppsala

Summary

Potato mop-top virus (PMTV) is common in parts of the potato growing areas in the Nordic countries. Severe symptoms in tubers is a problem especially in cultivars used for the production of crisps. However, in field trials with seed tubers of the same origin, the prevalence of symptoms in some of these cultivars (Lady Rosette and Hulda), seemed to be more frequent when cultivated in Sweden than in Denmark. In tests with a panel of monoclonal antibodies (MAbs) produced against a Scottish isolate of PMTV, Danish isolates reacted with all of the MAbs, whereas Swedish isolates showed limited reactions.

Introduction

Potato mop-top furovirus (PMTV), transmitted by the powdery scab fungus *Spongospora subterranea* (Wallr) Lagerh. (Jones & Harrison, 1969), is one of two viruses known to cause spraing in potato tubers. The other virus giving such symptoms in the tubers is tobacco rattle tobravirus. PMTV is a serious problem in areas of crisp potato and table potato production in the southern and western part of Sweden (Sandgren & Rydén, 1989). The severity of symptoms is dependent on potato cultivar (Calvert, 1968, Rydén et al 1989a and Kurppa, 1989) and climate conditions (Cooper & Harrison, 1973). In the results reported here we found different levels of symptom expression in the same cultivar in trials in three Nordic countries and differencies in reactivity of the PMTV isolates with MAbs.

Materials and methods

Field trials.
Samples of potato tubers from the same stock were planted in Sweden, Denmark and Finland on PMTV infested fields in randomised blocks. The incidence and severity of symptoms was monitored in the tubers after harvest at the Departement of Plant and Forest Protection in Uppsala. The data on spraing severity were analysed statistically using a Duncan test.
Immunosorbent assays.
The monoclonal antibodies (MAbs) were produced against a Scottish isolate of PMTV (Torrance et al 1993). The ELISAs were done following a standard procedure. Antibodies were incubated in 37°C for two hours and samples over night at 5°C. Leaf sap samples were diluted (1/10) in phosphate buffered saline containing 0.1 % Tween and the same buffer including 0.1 % bovine serum albumin was used to dilute the primary and secondary antibodies. Virus isolates were purified as described by Torrance et al (1993) with a few modifications. The ultracentrifugation steps were replaced by two 4%- polyethyleneglycol precipitations each preceeded by extraction with a ½ volume of chloroform. The isolates originated from Swedish, Danish or Finnish potato fields respectively.

Results

The incidence of spraing in the different cultivars is shown in Table 1. The equivalent frequency of spraing in the cultivars Saturna, Lady Rosette and Hulda, when grown in Sweden, has been confirmed in other field trials, irrespective of high or low levels of spraing the actual year (Sandgren, 1993).
The reactions of some MAbs to purified PMTV preparations are shown in Table 2. This test was repeated one month later, with the same virus preparations and Mabs, with a similar result. The absorbance values in ELISAs with PMTV infected leaf sap are shown in Table 3.

Table 1a: Cultivar trials in Denmark (DK) and Sweden (SV) 1989. Percent healthy tubers, Duncan test at the 5%level.
b: Cultivar trials in Denmark (DK), Sweden (SV) and Finland (FIN) 1991. Duncan test as above.

a:

Country	DK	group	SV	group
Cultivar				
Ostara	92	A	96	A
King Edward	90	A	64	B
Ukama	94	A	64	B
Saturna	40	B	66	B

b:

Country	DK	group	SV	group	FIN	group
Cultivar						
King Edward	96	A	99	A	95	A
Ukama	94	A	98	A	81	B
Hulda	79	A	88	B	75	B
Lady Rosette	83	A	80	B	73	B
Saturna	13	B	85	B	47	C

Table 2. Absorbance values (A405) in ELISA tests with purified virus preparations of a Danish PMTV isolate (DK1) and a Swedish PMTV isolate (SV1) respectively. Control was uninfected *Nicotiana bentamiana* leaf preparation.

	Isolate DK1				Control
Dilution	1/50	1/150	1/450	1/1350	1/50
MAbs					
SCR 68	1.2	0.9	0.5	0.2	0.02
SCR 72	0.4	0.1	0.1	0.1	0.02
SCR 73	0.5	0.3	0.2	0.1	0.06
SCR 74	1.4	0.4	0.2	0.1	0.2
SCR 75	2.0	0.6	0.2	0.1	0.04
	Isolate SV1				Control
Dilution	1/75	1/225	1/675	1/2025	1/50
MAbs					
SCR 68	1.4	1.1	0.6	0.2	0.0
SCR 72	0.01	0.01	0.01	0.1	0.02
SCR 73	0.6	0.2	0.1	0.1	0.04
SCR 74	0.04	0.07	0.06	0.08	0.06
SCR 75	0.03	0.03	0.04	0.04	0.06

Table 3. Absorbance values (A405) in three ELISA tests with monoclonal antibodies SCR68, 72-77 and PMTV-infected leaf sap. Origin of the isolates are Danish (DK1), Swedish (SV1) and Finnish (FIN). Values less then 2 times background (healthy control) are set to zero, normal background values were 0.07-0.11.

	isolates	DK1	SV1	FIN
test 1				
	SCR			
	68	2.8	3.0	3.0
	72	0.43	0	0
	73	1.2	1.1	1.7
	74	0.94	0	0
	75	0.80	0	0
	76	0.32	0.28	0.50
	77	0.76	0.62	1.2
test 2				
	68	3.1	3.0	3.0
	72	0.38	0	0
	73	1.5	1.5	1.6
	74	1.4	0	0
	75	1.0	0	0
	76	0.32	0	0
	77	0.40	0	0
test 3				
	68	3.0	3.0	2.9
	72	0.38	0	0
	73	1.5	1.6	1.4
	74	1.4	0	0
	75	1.1	0	0
	76	0.27	0	0
	77	0	0.48	0

Discussion

In the Danish field trials, cv Saturna was seriously affected by spraing whilst the number of tubers with symptoms in the cvs Lady Rosette and Hulda reached a significantly lower level. In Sweden; Saturna, Lady Rosette, Hulda, King Edward and Ukama were all affected by spraing. The reason why the "sensitivity" of the cultivars seems to be different in Sweden compared to Denmark, is not known, but since climatic factors are more similar in the area Denmark - southwestern Sweden compared to southwest vs mideast of Sweden, biotic factors as the fungal vector or the viral genotype should be the first to investigate. It is not known if there exists different forms of *Spongospora subterranea* with different ability to attac different potato cultivars, but the amount of powdery scab-symptoms on the tubers seems not to be correlated with the severity of spraing (Rydén et al 1989b). No potato cultivar is reported to be resistant to *Spongospora subterranea*.

In the tests with virus preparations (Tab 2), the Danish isolate reacted with more of the MAbs than did the Swedish one. The same pattern is obtained with infected leaf sap (Tab 3). In more ELISAs with leaf sap (not shown), three additional Danish isolates could react with MAbs SCR 72, 74 and 75 resp., whilst four additional Swedish and one more Finnish did not. The isolates in leaf sap have been tested in two to six ELISA tests respectively.Mechanical inoculation with Danish isolates have caused necrotic leasions on *Nicotiana tabacum*, while these Swedish or Finnish isolates never have (unpublished results).

The investigations are continued with cDNA techniques to compare PMTV-isolates on genomic level. If it is possible to connect differencies in the genome with the reaction of potato cultivars to infection, such facts will be important in plant breeding. It is not known why isolates from Denmark seem to differ from the ones from Sweden. The might have been introduced from different localities, and thus been different from the beginning. Alternatively the vector, host, soil-type, climate or other factors may have exerted different selection pressure in Sweden and Denmark respectively.

References

Calvert, E.L. 1968. The reaction of some potato varieties to potato mop-top virus. Record of Agricultural Research, Ministry of Agriculture, Northern Ireland 17:31-40.

Cooper, J.I. & Harrison, B.D. 1973. Distribution of potato mop-top virus in Scotland in relation to soil and climate. Plant Pathology 22:73-78.

Jones, R.A.C. & Harrison, B.D. 1969. The behaviour of potato mop-top virus in soil, and evidence for its transmission by Spogospora subterranea. Annales of Applied Biology 63:1-17.

Kurppa, A. 1989. Reaction of potato cultivars to pimary and secondary infection by potato mop-top virus and strategies for virus detection. EPPO Bulletin 19:593-598.

Rydén, K., Lövgren, L. & Sandgren, M. 1989a. Investigations on potato mop-top furovirus in Sweden. EPPO Bulletin 19:579-583.

Rydén, K., Sandgren, M. & Lövgren, L. 1989b. (Potato mop-top virus (PMTV) - occurrence in soil samples and symptom development in some potato cultivars). Växtskyddsnotiser 53:124-129.

Sandgren, M. & Rydén, K. 1989. (Potato mop-top virus - occurrence in Swedish soils and the reaction of some potato cultivars to infection.) Nordisk plantevaernskonference 1989: 301-303.

Torrance, L., Cowan, G.H. & Pereira L.G. 1993. Monoclonal antibodies specific for potato mop-top virus, and some properties of the coat protein. Annales of Applied Biology 122:311-322.

TEMPORAL ASPECTS OF WINTER WHEAT INFECTION BY WHEAT SPINDLE STREAK MOSAIC VIRUS (WSSMV) IN NEW YORK, USA.

J.E. Carroll[1], G.C. Bergstrom[1], and S.M. Gray[2]

Department of Plant Pathology[1] and USDA-ARS[2], 334 Plant Science Building, Cornell University, Ithaca, NY 14853-5908, USA.

Summary

Infection of winter wheat roots by wheat spindle streak mosaic virus (WSSMV) following inoculation by the plasmodiophoraceous vector, *Polymyxa graminis*, is thought to occur principally in autumn during periods of conducive temperatures (4 to 13 °C). Symptom expression occurs in spring and the severity and duration of symptoms depends on the occurrence of temperatures in the conducive range. In order to elucidate the WSSM disease cycle in New York, two susceptible cultivars, 'Frankenmuth' and 'Augusta', were sampled at two week intervals in autumn, once in winter, and at weekly intervals in spring. Roots were assayed by indirect ELISA and whole plants were analyzed to determine the distribution of virus within various tissues. WSSMV incidence increased from 4% in October (30 days after sowing) to 18% in January, and in April rose from 32% to 77%. In May, virus incidence reached a maximum of 82% in roots and 91% in leaves. These data suggested that WSSMV infection may continue to increase during autumn and that virus may replicate during winter leading to maximum virus incidence in May. In October, virus was detected in roots and subcrown internodes of asymptomatic plants and, by mid-November, leaves of such plants also contained WSSMV antigen. Virus continued to be detected in roots and leaves of asymptomatic plants until senescence despite temperatures above the conducive range. *P. graminis* zoosporangia and cystosori were found in virus-infected roots 1 and 2 months after sowing, respectively. Overall, these results indicate that secondary spread and acquisition of WSSMV by the vector may begin 1 and 2 months after sowing and may continue throughout the growing season.

Introduction

Wheat spindle streak mosaic virus (WSSMV), a member of the baymovirus group, is transmitted by *Polymyxa graminis*. In New York, USA, WSSMV is the most prevalent and yield-limiting viral disease of winter wheat (Miller, *et al.* 1991 and Miller, *et al.* 1992) and is currently managed by the use of resistant cultivars. The epidemiology of baymoviruses has not been studied in detail due to the difficulty of experimentation with the diseases caused by these viruses and their vectors (Adams 1990). Slykhuis (1975) found that plants could become infected during late September through October in Ontario, Canada. If infection occurs at this time, it would be expected that WSSMV could be detected in roots from a large proportion of plants in October in New York, USA. Instead, ELISA tests of wheat roots in mid-October indicated that few or no plants contained detectable virus.

A polyclonal antiserum to WSSMV was produced in our laboratory and indirect ELISA was developed for the detection of virus antigen in plants. Indirect ELISA was used to monitor the progress of WSSM in winter wheat plants growing in an area where high levels of WSSM were observed in previous years. The objective of our study was to clarify the etiology of WSSM in New York State.

Materials and Methods

Plant Sample Collection: Two susceptible cultivars of winter wheat, Frankenmuth and Augusta,

were grown in border strips around a plant breeding plot used for WSSMV trials. Sampling began 3 weeks after sowing and continued every 2 weeks in autumn, once in winter, weekly in spring and every 2 weeks in summer. No samples were collected in December, February, or March. At each sampling period five samples from each cultivar were dug from the area of the plot with a history of high disease incidence. Each sample included at least 5 plants and 10 to 15 cm of soil. Twenty-two plants of each cultivar were inspected for symptoms and analyzed for the presence of viral antigen using ELISA.

Sample Preparation: From October to early May, root segments were removed from individual plants and ground 1:10 w/v in 0.1M ammonium citrate buffer pH 6.5 (am. citr. buffer). If root segments tested positive for WSSMV by ELISA, subsamples that had been saved from the same roots were mounted in sterile water on a slide and examined with a compound microscope for *Polymyxa graminis* structures. The root pieces were scored for the presence of plasmodia, zoosporangia, and cystosori. From late May to July, sap expressed from entire root systems was diluted 1:10 v/v in am. citr. buffer. From October to early April, leaf segments from plants of which the roots tested positive for WSSMV were ground 1:10 w/v in am. citr. buffer. From late April to June, leaf samples from all plants were prepared. In October and November, subcrown internodes and stem segments from plants of which the roots tested positive for WSSMV were ground in am. citr. buffer. In July, embryos were excised from grains and ground in am. citr. buffer. After sample preparation, all samples were clarified with 0.5X chloroform and diluted 1:4 in am. citr. buffer.

Serological Assays: Polyclonal antiserum to purified WSSMV was produced in rabbit. Serum was cross-absorbed with healthy wheat sap and the IgG fraction purified by protein-A affinity chromatography. Plant tissue samples were incubated overnight at 4°C. Following 3 washes with phosphate-buffered saline pH 7.4 (PBS) with 0.05% Tween 20, the IgG fraction of WSSMV antiserum (1 mg/ml), diluted 1:3200 in PBS containing 2% nonfat dry milk (PBS-M), was added and incubated 2 hours at 37°C. Plates were washed and goat anti-rabbit alkaline phosphatase conjugate (1 mg/ml), diluted 1:2000 in PBS-M, was added and incubated 2 hours at 37°C. Absorbance values (405 nm) were obtained following a 75 minute incubation at room temperature with phosphatase substrate. All samples were loaded in two wells and all microtiter plates included controls of healthy and WSSMV-infected tissues taken from plants growing in a growth room. Samples were considered positive when their absorbance values were 3X the healthy sap values. The detection limits of the WSSMV antiserum in the indirect ELISA were 3 pg purified virus/μl 1:40 healthy root sap and 50 pg purified virus/μl 1:40 healthy leaf sap.

Temperature Data: Mean daily air and soil temperatures were obtained from the Department of Soils, Crops, and Atmospheric Sciences, Cornell University, Ithaca, NY, for the Game Farm Road weather station, located within 0.5 km from our experimental plot.

Results

WSSMV first was detected 30 days after sowing (October 16) in Frankenmuth plants (4.5%) and 73 days after sowing (November 28) in Augusta plants (9%) (Fig. 1B). By January, incidence of WSSMV rose to 18.2% in Frankenmuth, but 0% incidence was found in Augusta (Fig. 1B). Because the soil was frozen, samples were not obtained in February and March. The first samples in spring (April 2) showed that incidence of WSSMV in plants had increased to 36% in Frankenmuth and 32% in Augusta. Virus incidence continued to rise in April and reached a maximum in May of 82% and 91% in Frankenmuth, and 77% and 60% in Augusta, roots and leaves, respectively, despite temperatures above the conducive range (Fig. 1). In June, incidence of virus fluctuated in roots and leaves, but averaged 39% and 26% in Frankenmuth and 36% and 20% in Augusta, respectively. The final leaf sample taken from flag leaves on June 29, during a period of unusually warm temperatures, gave 59.1% incidence in Frankenmuth and 50% in Augusta. Virus incidence in roots of these plants was only 9.1% and 22.7%, respectively. The mean ELISA absorbance value (positive/healthy), reflecting virus titer in

roots, was 7X healthy in autumn and winter samples and rose to a maximum of 23X healthy in late April when soil temperatures were still in the conducive range (Fig. 1A). In June, mean absorbance values dropped to 8X healthy when temperatures remained above 13°C (Fig. 1A).

In October and November, viral capsid protein was detected in subcrown internodes, as well as roots. Moreover, Frankenmuth plants collected in November and January also contained virus in leaves. Stem and embryo tissues gave negative results. In the first spring sample, 75% of Frankenmuth plants with positive root ELISA contained virus in leaf tissue, while 33% of Augusta contained virus in leaves. Eleven days later (April 13), 100% of Frankenmuth and 78% of Augusta plants that contained virus in root samples also contained virus in leaves. Results from assays of both roots and leaves of all plants indicated that detection of virus was not strictly correlated for individual plants: virus could be detected only in roots, only in leaves, or in both. The frequency of plants with detectable virus in only roots was generally higher than the frequency of plants with detectable virus in only leaves until the final sample (June 29).

Plants were beginning to show WSSM symptoms in mid-April. Yellowing and mosaic were readily apparent from April 20 to May 4, principally on older leaves. However, the distinctive, spindle-shaped, yellow streaks typical of WSSM never developed on emerging leaves. During this time period, soil and air temperatures rose above 13°C on 3 occasions (Fig. 1A).

Virus-infected root segments that were examined for *P. graminis* contained numerous plasmodia and a few zoosporangia in autumn (October and November) samples. January samples contained numerous zoosporangia. Cystosori were found in samples collected November 28 and January 7. Using these fresh root pieces or dissected cystosori, attempts to transmit WSSMV to seedlings growing at conducive temperatures in a growth room failed.

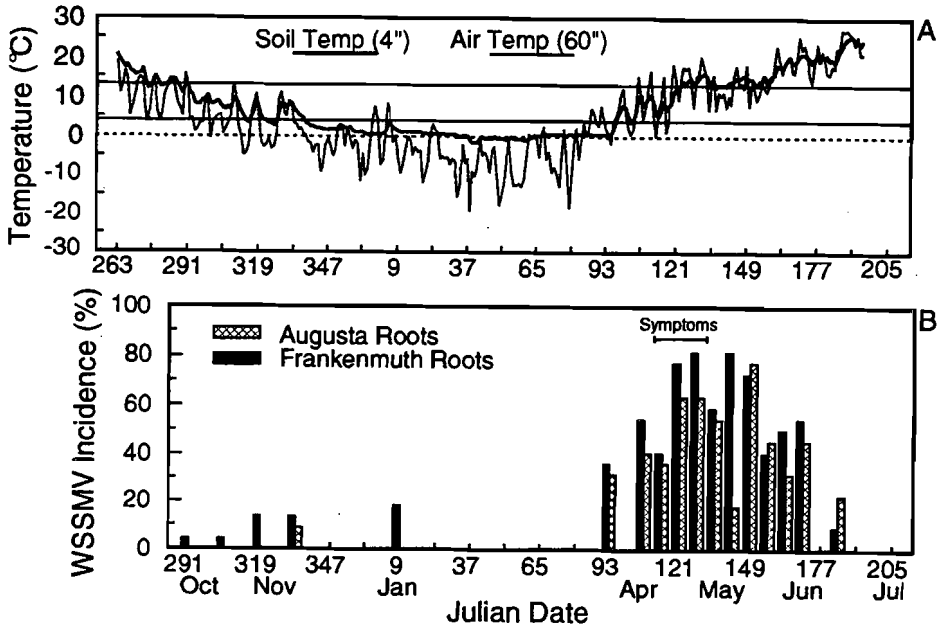


Figure 1. A) Daily mean air (152 cm height) and soil (10 cm depth) temperatures for the 1992/93 growing season, solid horizontal lines delimit the conducive soil temperature range (4 to 13°C). B) Incidence of WSSMV infection expressed as the percentage of plants with root samples testing positive in indirect ELISA on each sampling date for the cultivars Frankenmuth and Augusta.

Discussion

In both roots and leaves, WSSMV was detected earlier in Frankenmuth. Virus was detected in roots of Frankenmuth as early as October (one month after seeding) and incidence continued to rise gradually through January. In contrast, no virus was detected in Augusta roots for the majority of this time period. Thus, Frankenmuth, as compared to Augusta, appeared to become infected earlier by the virus or its roots may have supported earlier and more efficient virus replication. The presence of virus in leaves of Frankenmuth in November and the higher incidence of virus in leaves of Frankenmuth in the first spring sample suggested that virus movement may be more efficient in Frankenmuth than in Augusta.

The incidence of WSSMV in plants increased 2- to 3-fold from January 7 to April 2. Because the soil was frozen during this time, this increase suggests that WSSMV may replicate and move in dormant plants at very low temperatures. Another possibility is that secondary infections by viruliferous zoospores may occur during this time.

Although temperatures in the conducive range can be correlated with symptom expression (Slykhuis 1970) and yield reduction (Miller, *et al.* 1992), temperatures could not correlated with virus incidence in this study. Regardless of soil or air temperature, WSSMV could be detected in asymptomatic plants throughout the growing season. The occurrence of virus in asymptomatic plants may help explain the dramatic yield reductions that have been associated with the disease.

The presence of zoosporangia of *P. graminis* in virus-infected root samples examined in October indicated that secondary spread of WSSMV could be occurring from this time on. Secondary infection by zoospores could help explain the increase in virus incidence between January and early April. In addition, the presence of cystosori as early as November in virus-infected roots indicated that the vector could acquire virus in autumn for primary infections in subsequent years. Cystosori become most abundant in roots by late spring (Ledingham 1939) and our data showed that WSSMV is found in roots throughout this period allowing opportunities for continued acquisition of virus by vector resting spores. Our results demonstrate that virus could be acquired by the vector for primary and secondary inoculum during the majority of the growing season. If acquisition of the virus by the vector occurs at low frequency, as reported by Adams, *et al.* (1988), then this would maximize opportunities for virus spread and acquisition.

References

Adams, M.J. 1990. Epidemiology of fungally-transmitted viruses. Soil Use and Management. 6:184-189.

Adams, M.J., Swaby, A.G., and Jones, P. 1988. Confirmation of the transmission of barley yellow mosaic virus (BaYMV) by the fungus *Polymyxa graminis*. Annals of Applied Biology 112:133-141.

Ledingham, G.A. 1939. Studies on *Polymyxa graminis*, n. gen. n. sp., a plasmodiophoraceous root parasite of wheat. Canadian Journal of Research C 17:38-51.

Miller, N.R., Bergstrom, G.C., and Sorrells, M.E. 1992. Effect of wheat spindle streak mosaic on yield of winter wheat in New York. Phytopathology 82:852-857.

Miller, N.R., Bergstrom, G.C., and Gray, S.M. 1991. Identity, prevalence, and distribution of viral diseases of winter wheat in New York in 1988 and 1989. Plant Disease 75:1105-1109.

Slykhuis, J.T. 1975. Seasonal transmission of wheat spindle streak mosaic virus. Phytopathology 65:1133-1136.

Slykhuis, J.T. 1970. Factors determining the development of wheat spindle streak mosaic caused by a soil-borne virus in Ontario. Phytopathology 60:319-331.

EFFECTS OF SOME FACTORS ON SYMPTOM EXPRESSION OF LETTUCE BIG-VEIN VIRUS IN LETTUCE (*LACTUCA SATIVA*)

J A Walsh

Department of Plant Pathology & Weed Science, Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, U.K.

Summary

A nutrient film technique (NFT) system was developed to infect lettuce plants with an isolate of *Olpidium brassicae* carrying lettuce big-vein virus (LBVV) and to examine the effect of some biotic and abiotic factors on symptom expression of the virus. The developmental stage of lettuce plants (5 true leaves) and duration of exposure to inoculum (4 wk) in the NFT system that induced most severe symptoms were determined. Despite previous reports that low temperatures favour symptom expression, the most severe symptoms were induced in the shortest time at 18°C. Successively reducing light levels from 487 $\mu E\ m^{-2}\ s^{-1}$ to 150 $\mu E\ m^{-2}\ s^{-1}$ increased the severity of symptoms markedly. There was no clear effect of pH on symptom expression. When lettuce plants were inoculated in the NFT system and subsequently transferred to a growth room operating at 18°C with light levels of 133 $\mu E\ m^{-2}\ s^{-1}$ and photoperiods of at least 10 hr it was possible to induce severe big-vein symptoms all year round. It is suggested that a combination of temperatures higher than those previously implied and low light levels are key factors in inducing conspicuous symptoms.

Introduction

Lettuce big-vein is a serious disease of field (Tomlinson & Faithfull, 1979) and glasshouse grown lettuce crops (Tomlinson & Faithfull, 1980) in the U.K. The main symptoms are vein-banding accompanied by crinkling and distortion of leaves. The economic importance of the disease is a result of the unsightliness of the foliage, which depresses market value (Zink & Grogan, 1954), delayed head formation, decreased head size and a reduced proportion of harvestable plants (Ryder, 1980). After many years of research the pathogenic agent causing big-vein was confirmed as a virus (Kuwata, *et al*, 1983; Vetten, *et al*, 1987; Huijberts, *et al*, 1990). It is transmitted by the obligately parasitic soil-borne fungus, *Olpidium brassicae* (Tomlinson & Garett, 1962).

Teakle (1988) cited temperature, moisture, pH, soil structure, light and chemicals as factors affecting LBVV or its vector *Olpidium brassicae*. "Low" but unspecified temperatures are reported to favour symptom expression (Ryder 1980; Campbell, *et al*, 1980). From experiments conducted *in vitro*, pH conditions in the range of 6 to 8 have been found to be most conducive for the transmission of viruses by zoospores of *O.brassicae* (Kassanis & Macfarlane, 1964; Teakle & Gold, 1964). Germination of zoosporangia of *O.brassicae* was inhibited at pH 5.2 (Tomlinson & Garrett, 1964). Nothing has been published on the effect of light on LBVV symptom expression although light has been shown to have an inhibitory effect on the encystment of zoospores of *O.brassicae* (Beever & Fry, 1970). The adverse effect of chemicals and fungicides on *O.brassicae* was reviewed by Tomlinson (1988). There is little published information on the effect of biotic factors on lettuce big-vein disease.

This paper describes a systematic investigation into the effect of some abiotic and biotic factors on symptom expression in lettuce plants infected by LBVV.

Materials and Methods

Virus and vector isolates and their maintenance. The virus and viruliferous *O.brassicae* isolates were those described by Tomlinson & Faithfull (1979) and were known to induce characteristic symptoms. All experiments were carried out with lettuce cv. Lobjoits Green.

Virus transmission. Zoospores were obtained by steeping lettuce roots infected by *O. brassicae* in cold tap water containing 0.05M glycine (Teakle & Gold, 1964) and 10g l^{-1} sucrose. Small lettuce seedlings were placed in the zoospore suspension and left overnight at 4°C. The following day the seedlings were inserted into rockwool blocks (36 x 36 x 40mm) and placed in plastic

guttering on a slight slope with a recirculated nutrient solution flowing through them. This miniaturised nutrient film (NFT) system was located in a glasshouse compartment maintained close to 18°C by heating in winter and shading and air conditioning in summer. Infection was maintained by placing healthy lettuce plants in rockwool blocks between infected plants for 4 weeks.

Effect of period of exposure to inoculum and subsequent incubation temperature. Lettuce seeds were sown into rockwool blocks (in plastic trays containing nutrient solution) and maintained in a virus-free glasshouse until they developed 4 true leaves. They were then moved to the NFT system described previously for periods of 1, 2, 3, 4 or 5 wk with infected plants inserted between each lot of 5 test plants. After inoculation, plants were moved to Saxcil constant environment (C.E.) cabinets at 14°C, 16°C or 18°C, with a photoperiod of 10hr and a light intensity of 300 μE m^{-2} s^{-1}. Illumination was provided by "Colour 35" fluorescent tubes and tungsten bulbs. Plants were kept in guttering and irrigated with nutrient solution. The date on which symptoms appeared was recorded and at the end of the experiment symptom severity was scored on a scale of 0-5, where 0 represents no visual symptoms; 1, mild symptoms on lower leaves; 2, good symptoms on lower leaves; 3, good symptoms on lower leaves and mild symptoms on upper leaves; 4, good symptoms on most leaves and 5, severe symptoms on most leaves, sometimes with leaf distortion.

The effect of light intensity. Seedlings were raised and inoculated as before and then placed in Saxcil C.E. cabinets at 18°C, with a photoperiod of 10 hr and light intensities of 487, 383, 246, or 150 μE m^{-2} s^{-1}. As only two cabinets were available simultaneously, comparisons were carried out in the following pairs 487/383, 383/246, and 246/150 μE m^{-2} s^{-1}. Each comparison was tested twice with forty plants in each cabinet on each occasion.

The effect of pH. Plants were inoculated at the 5 true-leaf stage in the NFT system in the glasshouse at 18°C. Two lengths of guttering were linked to each of three tanks of nutrient solution, at pH5, pH6 or pH8. These nutrient solutions were recirculated, their pH checked at regular intervals and renewed every week. After 4 wk, plants were transferred to an illuminated growth-room at 18°C with a photoperiod of 10 hr and a light intensity of 123 μE m^{-2} s^{-1}.

Statistical analyses. Plants were examined twice weekly, the dates on which symptoms appeared were recorded and at the end of the experiments symptom severity was recorded as described earlier. All experiments were fully randomised and analyses of variance were carried out on symptom scores and the days to symptom expression.

Results

Effect of period of exposure to inoculum and subsequent incubation temperature Only five plants (2%) failed to develop big-vein symptoms and there were no positional effects. There were significant effects of both period of exposure to inoculum and temperature on the time taken for symptoms to appear but no significant interaction occurred between these treatments. Plants developed symptoms most rapidly at 18°C and after a 4 wk. exposure to inoculum (Table I). Temperature but not exposure time to inoculum significantly affected the severity of symptoms (Table I). Plants kept at 18°C after inoculation developed more severe symptoms than those kept at the lower temperatures of 14°C and 16°C (Table I).

Table I The effect of period of exposure to inoculum & subsequent incubation temp. on symptom expression in lettuce infected by LBVV

	Incubation temperature (°C)	Period of exposure (weeks): 1	2	3	4	5
Mean symptom score	14	1.64	1.33	1.33	1.63	1.86
	16	1.28	1.03	0.91	1.39	1.21
	18	3.11	3.04	2.55	2.37	2.53
		L.S.D. (P = 0.05) = 0.57				
Mean days to symptom expression	14	43.9	47.7	44.7	36.7	43.1
	16	46.1	41.0	38.1	35.4	46.1
	18	37.1	35.6	38.0	33.9	43.5
		L.S.D. (P = 0.05) = 6.6				

The effect of light intensity. Lower light intensities induced more severe symptoms more quickly than higher intensities. The effect on symptom severity was greater than that on the time taken for symptoms to be expressed.
The effect of pH. No consistent trends were observed in this experiment. Plants irrigated with nutrient solution at pH 8 developed LBVV symptoms more slowly than those irrigated with nutrient solution of lower pH. Plants in nutrient solution of pH 5 developed more severe symptoms than plants in nutrient solution of pH 6.

Discussion

Results from experiments on the effect of temperature on symptom expression of LBVV infection in lettuce confirm previous reports of its importance. The temperature at which most severe symptoms were observed most rapidly (18°C) is at the top of the range (14-18°C) reported for severe lettuce big-vein disease by Westerlund *et al.*, (1978) and towards the top of the range (10-20°C) reported for severe *O.brassicae* infection by Fry & Campbell (1966). In practice the suggestion that "low" or "cooler" temperatures favour symptom expression leads to some confusion since these are relative terms; in California where the observations were made (Ryder, 1979; Campbell *et al.*, 1980), ambient temperatures are in general higher than in the UK.

Different periods of exposure to inoculum of one week or more had little or no effect on symptom severity or the time taken for symptoms to appear, perhaps because of secondary infection by zoospores from zoosporangia produced after the initial inoculation making external infection from other plants less important.

Despite many years of research on LBVV and *O.brassicae* it is surprising that the effect of light intensity has never been investigated. At the times of the year when big-vein symptoms are most prominent, not only are the temperatures cooler than summer when big-vein symptoms are less evident but light intensities are relatively low. The experiments reported here demonstrate for the first time that the lowering of light intensity reduced the time taken for symptom expression, and dramatically increased symptom severity. It has been shown that transmission of tobacco necrosis virus (TNV) by *O.brassicae* was reduced in the light when compared with transmission in the dark. This was thought to be due to the inhibitory effect of light on encystment of zoospores on the root surface. Excessive light together with higher temperatures have been implicated in reduced disease symptoms of plants infected by several viruses transmitted by fungi including: TNV, cucumber necrosis virus and melon necrotic spot virus (Teakle, 1988). Slykhuis (1970) found that plants infected by wheat spindle streak mosaic virus, which is transmitted by the soil-borne fungus *Polymyxa graminis*, developed more conspicuous symptoms at 10,000 lux than at 15,000 or 20,000 lux.

The most severe symptoms were observed when plants were infected at the 5-8 true leaf stage. Although LBVV symptoms can be induced rapidly by zoospore inoculation of much younger lettuce seedlings, such small seedlings may recover as they grow. This may explain why more severe symptoms were seen in plants that were older when inoculated. Conversely, if plants are infected at a much later stage during commercial cultivation they may mature and be harvested before symptoms are expressed.
The lack of a clear cut effect of pH on LBVV symptom expression may be explained by a tendency for the solutions at pH 5 and 6 to drift upwards to pH 7.4 and 7.6 respectively. There appeared to be a tendency for lower pH values to favour symptom expression. Kassanis & Macfarlane (1964) showed that transmission of TNV by *O.brassicae* was favoured by increasing the pH value of the nutrient solution used in transmission experiments in the range pH 4.5 to 7. However, as TNV is carried externally on zoospores of *O.brassicae* and LBVV is thought to be carried internally, pH may effect transmission of the two viruses differently. According to Teakle (1988) pH appears to act mainly through its effect on vector fungi and pH of 6-8 has been found to be most suitable for *in vitro* experiments on the transmission of viruses by zoospores of *O.brassicae*.

Optimum conditions for symptom expression by lettuce infected with LBVV have been determined and it is now possible to induce severe symptoms of the disease throughout the year. It is probably the combination of higher

temperature (up to 18°C) and low light intensity that are the key factors in the expression of severe LBVV symptoms. These findings will allow experiments which rely on symptom expression, such as screening of lettuce and related species for resistance to LBVV and testing chemicals and potential biocontrol agents for big-vein control, to be carried out throughout the year and in a more reliable and reproducible manner than has previously been possible.

References

BEEVER,J.E. & FRY,P.R. (1970). The effect of light on the transmission of tobacco necrosis virus by *Olpidium brassicae*. *Virology* **40**, 357-362.

CAMPBELL,R.N., GREATHEAD, A.S. & WESTERLUND, F.V. (1980). Big-vein of lettuce: Infection and methods of control. *Phytopath* **70**, 741-746.

FRY,P.R. & CAMPBELL,R.N. (1966). Transmission of a tobacco necrosis virus by *Olpidium brassicae*. *Virology* **30**, 517-527.

HUIJBERTS,N., BLYSTAD,D.R. & BOS,L. (1990). Lettuce big-vein virus: mechanical transmission and relationships to tobacco stunt virus. *Ann. appl. Biol.* **116**, 463-475.

KASSANIS,B. & MACFARLANE,I. (1964). Transmission of tobacco necrosis virus by zoospores of *Olpidium brassicae*. *J. gen. Microbiol.* **36**, 79-93.

KUWATA,S., KUBO,S., YAMASHITA,S. & DOI,Y. (1983). Rod-shaped particles, a probable entity of lettuce big-vein virus. *Ann. Phytopath. Soc. Japan* **49**, 246-251.

RYDER,E.J. (1979). Effects of big vein resistance and temperature on disease incidence and percentage of plants harvested of crisphead lettuce. *J. Am. Soc. Hortic. Sci.* **104**, 665-668.

RYDER,E.J. (1980). Studies on sources and nature of big vein resistance in lettuce and progress in breeding resistant cultivars. pp 78-85 in Proceedings of Eucarpia Meeting on Leafy Vegetables, Littlehamptom, 11-14 March, 1980. Glasshouse Crops Research Institute, 137pp.

SLYKHUIS,J.T. (1970). Factors determining the development of wheat spindle streak mosaic caused by a soil-borne virus in Ontario. *Phytopath.* **60**, 319-331.

TEAKLE,D.S. (1988). The effect of environmental factors on fungus-transmitted viruses and their vectors. In Developments in Applied Biology 2: *Viruses with fungal vectors*, pp.167-179. Ed. J.I.Cooper & M.J.C. Asher, Association of Applied Biologists, Warwick, 335pp

TEAKLE,D.S. & GOLD,A.H. (1964). Prolonging the motility and virus transmitting ability of *Olpidium* zoospores with chemicals. *Phytopath.* **54**, 29-32.

TOMLINSON,J.A. (1988). Chemical control of *Spongospora* and *Olpidium* in hydroponic systems and soil. In, Developments in Applied Biology 2: *Viruses with fungal vectors*, pp.293-303. Ed. J.I. Cooper & M.J.C. Asher, Association of Applied Biologists, Warwick, 335pp.

TOMLINSON,J.A. & FAITHFULL,E.M. (1979). Effects of fungicides and surfactants on the zoospores of *Olpidium brassicae*. *Ann. appl. Biol.* **93**, 13-19.

TOMLINSON,J.A. & FAITHFULL,E.M. (1980). Studies on the control of lettuce big-vein disease in recirculated nutrient solutions. *Acta Hortic.* **98**, 325-332.

TOMLINSON,J.A. & GARRETT,R.G. (1962). Role of *Olpidium* in the transmission of big vein disease of lettuce. *Nature, Lond.* **194**, 249-250.

TOMLINSON,J.A. & GARRETT,R.G. (1964). Studies on the lettuce big-vein virus and its vector *Olpidium brassicae* (Wor.) *Ann. appl. Biol.* **54**,45-61

VETTEN,H.J., LESEMANN,D.E. & DALCHOW,J. (1987). Electron microscopical and serological detection of virus-like particles associated with lettuce big vein disease. *J. Phytopath.* **120**,53-59.

WESTERLUND,F.V., CAMPBELL,R.N. & GROGAN,R.G. (1978). Effect of temperature on transmission, translocation, and persistence of the lettuce big-vein agent and big-vein symptom expression. *Phytopath.* **68**, 921-926.

ZINK,F.W. & GROGAN,R.G. (1954). The inter-related effects of big-vein and market price on the yield of head lettuce. *Plant Dis. Rep.* **38**, 844-846.

DEVELOPMENT OF MOLECULAR METHODS FOR THE IDENTIFICATION OF *POLYMYXA* SPECIES

E. Ward, E.S. Mutasa[1], M.J. Adams, C.R. Collier and M.J.C. Asher[1].

AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts, AL5 2JQ, UK and [1] Broom's Barn Experimental Station, Bury St. Edmunds, Suffolk, IP28 6NP, UK.

Summary

Molecular methods are being developed to simplify and improve the detection of *Polymyxa* and discriminate between species (*P.betae* and *P.graminis*) and isolates. DNA was extracted from zoospores, resting spores and roots infected with isolates of *P.betae* and *P.graminis* and analysed using Southern hybridization, PCR methods and CHEF pulsed field gel electrophoresis. There were some clear differences between *P.betae* and *P.graminis*, and at least 2 distinct subgroups within *P.graminis* but so far this has not been correlated with any other property.

Introduction

Polymyxa species are obligate parasites of plant roots and are important as vectors of plant viruses. Screening for *Polymyxa* infection currently involves skilled and tedious microscopic examination and there is a need to develop more reliable and quicker methods of detection. Molecular techniques offer the possibility of doing this and of distinguishing between isolates with different host plant or virus specificities.

Materials and Methods

Growth of Polymyxa and DNA Preparations

Isolates of *Polymyxa* were propagated on plant roots in sand culture (Adams *et al.*, 1986). Standard methods (e.g. Lee & Taylor, 1990) were used to extract DNA from *Polymyxa* with slight modifications to the initial stages of the protocol depending on the starting material (zoospores, resting spores, or plant roots infected by *Polymyxa*). Zoospores have only a thin outer membrane which was disrupted simply by mixing with the lysis buffer. Resting spores were first purified from the plant material containing them by blending with detergent, straining through cheesecloth and then centrifuging. The resulting pellet was resuspended in lysis buffer and the cells disrupted by shaking with Ballotini balls for 5 minutes. *Polymyxa*-infected root material was freeze dried and then ground to a fine powder before adding lysis buffer and continuing with the DNA extraction.

CHEF Pulsed Field Electrophoresis

Zoospores of *P.graminis, P.betae* and *Olpidium brassicae* were collected by centrifugation and suspended in molten agarose (1.2% low melting point agarose, 125mM EDTA) with ProteinaseK at 2mg ml^{-1}. The final concentration of zoospores in the mixture was 10^5 - 10^6 ml^{-1}. The mixture was then set in a mould at 4°C to form plugs that were then incubated at 50°C in NDS buffer (1% SDS, 10mM Tris pH8, 450mM EDTA). The plugs were then washed in several changes of 50mM EDTA over an 8 hour period with a final soaking overnight. Plugs were stored at 4°C in fresh 50mM EDTA until needed.

The chromosomes were separated on the Biorad CHEF-DR electrophoresis apparatus using 0.5X TBE buffer (45mM Tris, 45mM boric acid, 1mM EDTA) and Ultra pure grade agarose. A range of conditions were used with various voltages, run times, switch times and agarose concentrations.

Southern Hybridizations

Restriction digestions, gel electrophoresis and Southern blotting of genomic DNA using hybridization with digoxigenin labelled probes (Boehringer-Mannheim) were done by standard methods (Ward & Gray, 1992; Mutasa *et al.*, 1993).

PCR and Digestion of Amplified DNAs

Each PCR reaction contained 100pmol each primer, 2.5 units of *Taq* polymerase, reaction buffer

a level of about 10ng total genomic DNA for heavily infected roots). Negative controls, that showed no hybridisation to the probe were: healthy sugar beet roots, contaminating microorganisms isolated from the sugar beet rhizosphere (including algae, protozoa, bacteria and other fungi), *Polymyxa graminis*, *Olpidium brassicae, Plasmodiophora brassicae* and a range of over 20 mycelial fungi commonly associated with soil.

The screening procedure (using labelled total genomic DNA) that identified the clone was selective for repetitive DNA sequences and we have confirmed that the sequence is present on at least 4 *P.betae* chromosomes by Southern blots of CHEF gels (Mutasa *et al.*, 1993).

Ribosomal DNA Polymorphisms - RFLPs generated from PCR-amplified DNA.

Ribosomal DNA amplified using primers ITS4 and ITS5 was digested with several different restriction enzymes to find those useful in identification. The enzymes *Hha*I and *Dde*I cut the DNA several times and gave differences between the species (Fig. 2) so these were tested on 4 *P.betae* and 13 *P.graminis* isolates. Both enzymes allowed discrimination between *P.graminis* and *P.betae*. All the *P.betae* isolates tested had identical patterns but the *P.graminis* ones could be subdivided into 2 groups of related isolates (Table 1).

Table 1 *Polymyxa* isolates used and results of RFLP analysis of their rDNAs. The patterns obtained (A - F) are shown in Fig. 3.

ISOLATE	SOURCE	*Dde*I	*Hha*I
Polymyxa betae			
F41	sugar beet, Norfolk, UK	A	D
F68	sugar beet, Yugoslavia	A	D
F62	sugar beet, Suffolk, UK,	A	D
BB1	sugar beet, Suffolk, UK,	A	D
Polymyxa graminis			
F1	barley, Beds, UK	B	E
F5	barley, Suffolk, UK	B	E
F6	barley, Herts, UK	B	E
F24	*Poa* sp., Hants, UK	B	E
F32	barley, Sunstedt, Germany	B	E
F38	barley, Glos, UK	B	E
F40	barley, Xiaoshan, China	B	E
F42	barley, Cambs, UK	B	E
F60	barley, Oxon, UK	B	E
F36	wheat, PEI, Canada	C	F
F43	barley, Gramat, France	C	F
F51	oats, Kent, UK	C	F
F53	wheat, Ottawa, Canada	C	F

Any contaminating DNA in the sample (eg. from algae, plant or other fungi) may also be amplified by the primers used and contribute to the patterns seen. Plant DNA poses no problem because RFLP tests on DNA extracted from infected and healthy roots showed that the amplified plant bands and their digestion products were different from those of *P.betae* and *P.graminis* and could easily be distinguished by agarose gel electrophoresis (Fig. 3).

Discussion

Developing molecular techniques to study *Polymyxa* is more difficult than for typical 'mycelial fungi', mostly because the fungus cannot yet be grown in pure culture away from all contaminants. It is difficult therefore to obtain DNA that is derived from *Polymyxa* only. In addition, the fungus grows slowly (about 1 month for zoospores and 3-4 months for resting spores) and yields of spores may be small.

Nevertheless, we now have several techniques to analyse these organisms. Pulsed field gel electrophoresis techniques such as CHEF are potentially very powerful tools in the study of the molecular genetics of microorganisms (Mills & McCluskey, 1990). The method separates individual chromosomes which allows assignment of genes or other probes to particular chromosomes, analysis of genetic variation and production of chromosome-specific libraries. With these aims in mind we have been developing the technique for analysis of *Polymyxa* species and now have protocols that can be used to separate their chromosomes. We have a probe (pPbKES-1) that is specific for *Polymyxa betae* that we hope to be able to use in screening programs to replace the tedious microscopy currently used. We also have a method for studying variation between the species and isolates of *Polymyxa* using RFLP analysis of PCR-amplified ribosomal DNA. Preliminary results suggest that there are some clear differences between *P.betae* and *P.graminis*, and that there are at least 2 distinct subgroups within *P.graminis* but so far this has not been correlated with any other property.

(20mM Tris pH8, 1.5mM $MgCl_2$, 25mM KCl) and 100ng fungal DNA. Incubations were done in a Perkin Elmer Cetus thermal cycler for 25 cycles of 94°C for 1 minute, 42°C for 2 min and 74°C for 3 min. The base sequences of the primers used, which anneal to consensus regions of fungal (and plant) ribosomal DNA were (White *et al.*, 1990): ITS4, TCCTCCGCTTATTGATATGC; ITS5, GGAAGTAAAAGTCGTAACAAGG. Amplified DNA (5-8μl, approximately 0.5μg) was digested, without further purification, with restriction enzymes, the products were separated on gels containing 2% Nusieve GTG agarose (Flowgen) + 1% standard agarose and the DNA stained with ethidium bromide.

Results

CHEF

A variety of conditions were investigated but so far none have been found that separate all the chromosomes from one another. However, the technique has allowed a minimum estimate of the number of chromosomes and their sizes, and we have been able to see differences between the *P.graminis* and *P.betae* isolates tested (Fig. 1). For *P.betae* (isolate F41) three bands were detected in

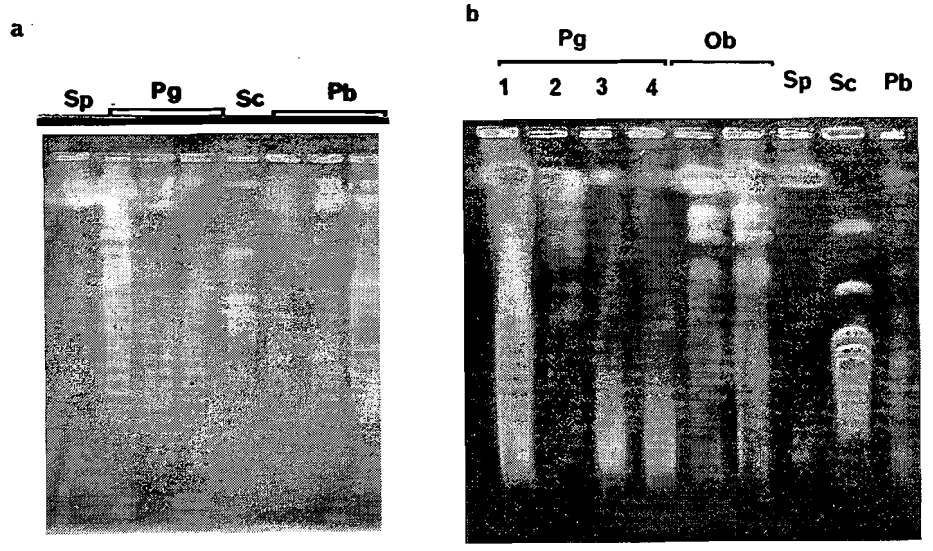


Fig. 1 Pulsed field gels of *Polymyxa* chromosomes. Sp = *Schizosaccharomyces pombe*, Pg = *P. graminis* (F42 in Fig. 1*b*, lane 2, otherwise F1), Sc = *Saccharomyces cerevisiae*, Pb = *P. betae* (F41), Ob = *Olpidium brassicae*. Fig. 1*a*, 0.7% agarose, 100v, switch time 300 s, run time 46 h; Fig. 1*b*, 0.7% agarose, 75v, switch times ramped from 1000 to 300 s over 96 h.

the 2 - 5 Mb range and 6 bands in the 0.85 - 1.1 Mb range (Fig. 1*a*). Three isolates of *P.graminis* were tested (F1, F40 and F42). For F1 and F40 there were three clear bands in the 2 - 5 Mb range and there appeared to be a number of other bands in the 0.2 - 1 Mb range that could not be resolved clearly (Fig. 1*b*). For F42 it was possible to resolve 12 bands ranging from 0.9 - 5 Mb. The method has also been used to locate one of our probes on the *P.betae* chromosomes (discussed below).

A specific DNA Probe for P.betae (pPbKES-1)

A *P.betae* genomic DNA fragment of 1.9kb has been cloned from infected sugar beet roots and shown to be specific to *P.betae* (Mutasa *et al.*, 1993). The probe can detect *P.betae* in roots (down to

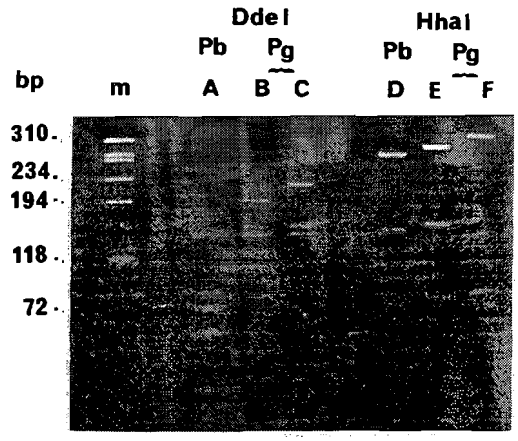


Fig. 2 RFLP analysis of *Polymyxa* isolates using PCR-amplified rDNAs. Lanes A and D = *P. betae* F41, B and E = *P. graminis* F1, C and F = *P. graminis* F53. Letters A-F correspond with RFLP types in Table 1. Lane m is the size marker ϕX174 *Hae*III.

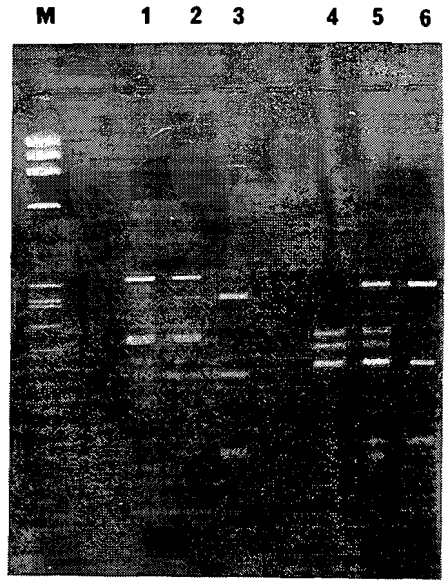


Fig. 3 RFLP analysis (using *Hha*I) of DNA from *Polymyxa* and *Polymyxa*-infected roots using PCR-amplified rDNA. 1 = sugar beet, 2 = sugar beet infected with *P. betae* F68, 3 = *P. betae* F41, 4 = barley, 5 = barley infected with *P. graminis* F24 and 6 = *P. graminis* F1. Lane M is the size marker ϕX174 *Hae*III.

References

Adams, M.J., Swaby, A.G. and MacFarlane, I. (1986). The susceptibility of barley cultivars to barley yellow mosaic virus (BaYMV) and its fungal vector, *Polymyxa graminis*. *Ann. Appl. Biol.* **109**:561-572.

Lee, S.B. and Taylor, J.W. (1990). Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols. A guide to methods and applications*. Eds M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, Academic Press, San Diego. pp.282-287.

Mills, D. and McCluskey K. (1990). Electrophoretic karyotypes of fungi: the new cytology. *Molecular Plant-Microbe Interactions*. **3**:351-357.

Mutasa, E.S., Ward, E., Adams, M.J., Collier, C.R., Chwarszczynska, D.M. & Asher, M.J.C. (1993). Sensitive DNA-based detection of *Polymyxa betae* in sugar-beet roots. Submitted to *Physiol. & Mol. Plant Path.*

Ward, E. and Gray, R.M. (1992). Generation of a ribosomal DNA probe by PCR and its use in identification of fungi within the *Gaeumannomyces-Phialophora* complex. *Plant Path.* **41**:730-736.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols. A guide to methods and applications*. Eds M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White. Academic Press, San Diego. pp.315-322.

RAPD ANALYSIS OF *POLYMYXA BETAE.*

Alice L.Pilgeram and James E. Duffus

USDA ARS. Sugarbeet Research Unit. 1636 East Alisal Street, Salinas, CA 93905. USA.

Summary

The variability of *Polymyxa betae* was evaluated using PCR and RAPD analysis . Total DNA was isolated from *Polymyxa*-infected and non-infected beet tissue and amplified using the polymerase chain reaction. DNA was amplified with PCR primers specific for the ITS sequence of the ribosomal DNA and with several RAPD primers (random amplified polymorphic DNA).

Introduction

Beet necrotic yellow vein virus (BNYVV) is vectored by the soil-borne fungus, *Polymyxa betae* Keskin (reviewed in Abe, 1987). Although different isolates of *P. betae* are morphologically indistinguishable, they may parasitize the roots of different host plants (Barr and Asher, 1992; Gerik and Duffus,1988). Differences in host ranges can even be observed among isolates from the same original host species.

The coding regions of ribosomal DNA (rDNA; 16S-like, 28S-like, and 5.8S) are highly conserved sequences even among distantly related organisms. In contrast, sequences of the internal transcribed spacer regions (ITS) of the rDNA are frequently variable in fungal isolates from a single genus and in some cases, from isolates within a single species (White *et al.*, 1990). The coding and ITS regions of the rDNA have been isolated and sequenced from numerous organisms using the polymerase chain reaction and used to estimate genetic diversity and/or similarity within a population.

Randomly amplified polymorphic DNA (RAPD) analysis has also been used to differentiate isolates of plant pathogenic fungi (Haemmerli ,1992; Guthrie, 1992). The DNA of interest is amplified using random oligonucleotide primers and the resulting amplification products resolved on agarose or acrylamide gels. The DNA banding patterns of different samples have proven useful for differentiating between closely related organisms.

DNA isolated from *P. betae*-infected plant tissue and *P.betae* zoospore preps have been amplified using specific PCR primers and non-specific RAPD primers. Amplification of DNA from individual cystosori has been unsuccessful using either PCR or RAPD.

Materials and Methods

Fungal Isolates: All isolates of *Polymyxa betae* were originally obtained from infected plant tissue or infested soil. Freshly infected beet tissue was obtained by planting susceptible seedlings into sterile sand mixed with infested soil or dried root tissue. The presence or absence of *P. betae* in root tissue was determined by observing roots under a compound microscope.

DNA isolation: Flash frozen roots or leaves (liquid N_3) or lyophilized zoospore pellets were ground to a fine powder using a mortar and pestle. DNA was then extracted and purified using a total DNA CTAB extraction method (Doyle and Doyle, 1990). Individual

cystosori were isolated using a single-ascospore isolation technique developed for *Gaeumannomyces graminis* (unpublished). The cystosori were suspended in whitefly DNA extraction buffer (5 mM Tris HCl (pH 8.0), 0.5 mM EDTA, 0.5% Triton X-100, 1 mg/mL proteinase K) and incubated in a thermocylcer to 65C for 15 minutes (lysis), followed by 95C for 10 minutes (enzyme inactivation) (Gawel and Bartlett, personal communication). PCR amplification: DNA (0.01 μl - 5.0 μl) was added to a 100 μl PCR mixture (1X PCR buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM NTP, 50 pM primer ITS 1, 50 pM primer ITS 2 or ITS 4, and 1-2 U Taq polymerase (Promega)). Amplification was performed in a thermocylcer programmed for 3 cycles of 3 min at 94C, 1 min at 45C, 3 min at 72C; 35 cycles of 1 min at 93C, 1 min 45C, 3 min at 72C; 1 cycle of 10 min at 72C. Reaction products were resolved by electrophoresis in 2% agarose gels or 6-12% acrylamide gels. RAPD: DNA (0.01 μl - 5.0 μl) was added to a 100 μl RAPD mixture (1X PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM NTP, 0.05 pM RAPD primer (Operon Technologies), and 1-2 U Taq polymerase (Promega)).Amplification was performed in a thermocylcer programmed for 1 cycle of 2 minutes at 94C; 40 cycles 30 sec at 94C, 1 min at 35 C, 1 min at 72C.Reaction products were resoled by electrophoresis in 2 % agarose gels or 6-12% acrylamide gels.

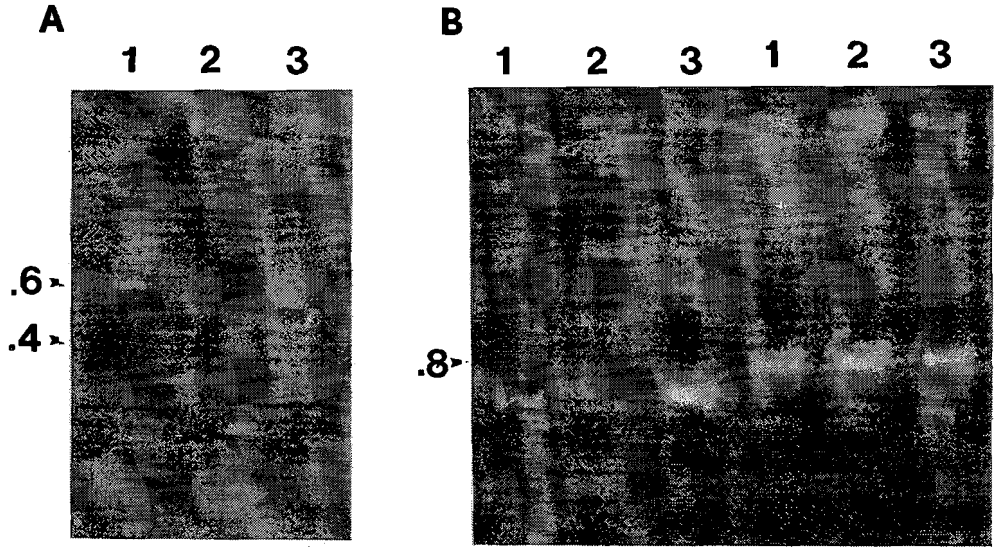


Fig.1 A. PCR amplification of the ribosomal DNA ITS region in total DNA isolated from sugarbeet tissue. B. RAPD amplification of total DNA. Lanes 1-3 were amplified with primer AA-04 (AGGACTGCTC) and lanes 4-6 were amplified with RAPD primer AA-19 (TGAGGCGTGT). 1. USH11 (healthy tissue); 2. SC4 (USH11 inoculated with diseased root tissue, *P. betae* was not observed); 3. SC4++ (USH11 inoculated with diseased root tissue, *P. betae* cystosori were observed).

Results and Discussion

PCR amplification DNA from non-infected *Beta vulgaris* roots (USH11) using primers specific for the ITS region of rDNA resulted in a single DNA amplification product (~ 0.6 Kb)(Fig. 1). An additional amplification product (~ 0.4 Kb) was observed in roots infected with *P. betae*. Additional products were occasionally observed when DNA from non-sterile plants (soil/root inoculated or field) was amplified (Fig. 1) and may represent ITS sequences from other soil fungi such as *Olpidium* or other strains of *Polymyxa*. The relative intensities of the DNA products is variable and may correlate with the ratio of plant DNA to fungal DNA in a given DNA preparation.

RAPD banding patterns were highly variable (Fig. 1). Differences in RAPD amplification products from *P.betae*-infected sugarbeet plants and non-infected plants were not consistently obvious, even when ITS analysis clearly showed a difference (Fig 1).

References

Barr, K.J. and M.J.C. Asher (1992). The host range of *Polymyxa betae* in Britain. Plant Pathol. 41:64-68

Doyle, J.I. and J.I. Doyle (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Gerik, J.S. and J.E. Duffus (1988). Differences in vectoring ability and aggressiveness of isolates of *Polymyxa betae*. Phytopathology 78:1340-1343.

Guthrie, P.A.I., Magill, C.W., Frederiksen, R.A, and G.N. Odvody (1992). Random amplified polymorphic DNA markers: a system for identifying and differentiating isolate of *Colletotrichum graminicola*. Phytopathology 82:832-835.

Haemmerli, U.A., Brandle, U.E., Petrini, O., and J.M. McDermott (1992). Differentiation of isolates of *Discula umbrinella* (Teleomorph: *Apiognomonia errabunda*) from beech, chestnut, and oak using randomly amplified polymorphic DNA markers. Mol. plant-microbe interact. 5:479-483.

White, T.J., Bruns, T., Lee, S. and J. Taylor (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phlyogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. Academic Press.

ZOOSPORE ENCYSTMENT OF *OLPIDIUM BRASSICAE* IS TRIGGERED BY FUSION BETWEEN THE BODY AND FLAGELLUM MEMBRANE

C. Hiruki

Department of Plant Science, University of Alberta, Edmonton, Alberta T6G 2P5, Canada.

Summary

Different stages of encystment of *Olpidium brassicae* on roots of young tobacco seedlings were investigated by scanning and transmission electron microscopy. During the encystment, fusion was evident between the zoospore body and flagellum membranes. The significance of this observation was discussed in light of the theories pertaining to "reeling in" and "wrap-around" processes.

Introduction

Olpidium brassicae is an efficient vector of certain plant viruses. It was assumed that tobacco necrosis virus (TNV) particles were taken up during postulated axonemal withdrawal by the so-called "reeling in" process (Temmink and Campbell, 1969b; Temmink et al., 1970; Temmink, 1971). However, no definitive proof has been presented for the actual mode of encystment, although a different interpretation of this phenomenon has been presented (Alderson and Hiruki, 1977). This is due, in part, to the fact that encystment, when it occurs, is completed in a very short period of time, which makes it extremely difficult to study.

The purpose of this study is to shed light on the process of encystment which is believed to be involved in virus transmission by this interesting fungus. This report provides ultrastructural evidence for membrane fusion during the "retraction" of the flagellum by zoospores of *O. brassicae*.

Materials and Methods

Fungus Culture:
O. brassicae used was a tobacco isolate maintained in 'Bright Yellow' tobacco (*Nicotiana tobacum* L.) seedlings grown in microincubators under controlled environment (Hiruki, 1969; Hiruki and Alderson, 1976), and zoospore isolation was performed as reported earlier (Alderson and Hiruki, 1977).

Scanning Electron Microscopy (SEM):
For free zoospores, the suspension of zoospores was placed on a Millipore filter (50 nm pore size) and fixed in osmium tetroxide vapour and critical-point dried from 85% amylacetate. For zoospores attached to tobacco roots, samples were fixed after 10 and 15 min. of incubation of zoospores with 10-day-old tobacco seedlings at room temperature. Fixation was either in 1% osmic acid in distilled water for 30 min., or in 2% glutaraldehyde plus 2% formaldehyde in 0.01 M phosphate pH 7.0 for one hour followed by osmium tetroxide. Fixed roots were dehydrated through an ethanol series and critical-point dried. Dried specimens were mounted on stubs using low resistance contact cement, coated with carbon and gold, and examined in a scanning electron microscope, Cambridge Stereoscan S4, operated at 20 kV and 15-30° tilt.

Transmission Electron Microscopy (TEM):
Both free zoospores and root samples fixed in aldehydes and osmium tetroxide were embedded in Araldite after dehydration through ethanol and passage into propylene oxide. Sections were cut on a Reichart ultramicrotome, collected on formvar coated grids, stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope, Philips 200, operated at 60 kV.

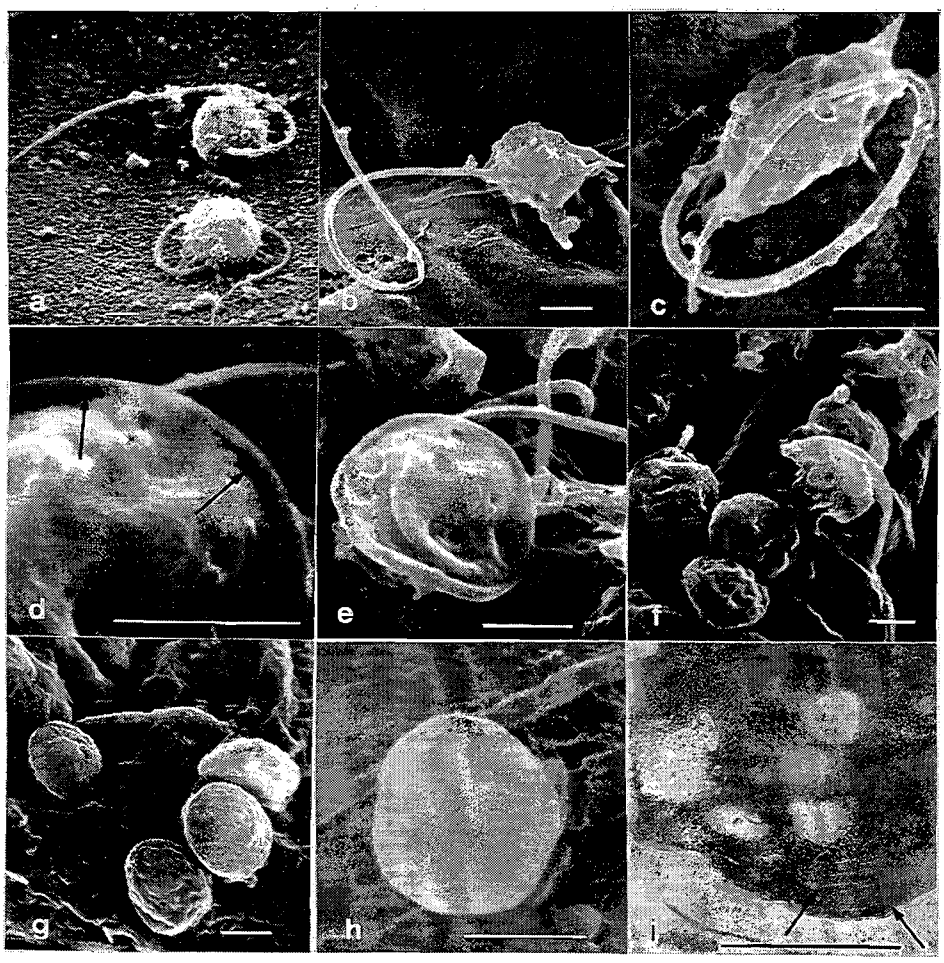


Fig. 1. Zoospores of Olpidium brassicae, free (a) or attacned to tobacco root and in different stages of flagellum retraction (b - h), scanning electron micrographs. (i) transmission electron micrographs of a thin section of an encysted zoospore attacned to tobacco root. (After P.G. Alderson and C. Hiruki). Bar, 1 µm

Results

In the SEM, each free zoospore of *O. brassicae* possessed a round body, 2-3 µm in diameter, with a single whiplash flagellum with a tapered end, 14-17 µm in length (Fig. 1a).

When the roots of young tobacco seedlings were exposed to *Olpidium* zoospores in suspension, the zoospores were found attached to the root surface, in particular in the zone of elongation. In the SEM, after a 10 min. incubation when the zoospores were found attached to the root surface, the flagellum was still present in them (Fig. 1b), and some had their flagella in the process of coiling (Fig. 1c). A ridge outline around some zoospores was seen where apparent encystment was occurring by membrane fusion (Fig. 1d arrows). In many cases, merging appeared to occur from the base to the tip of the flagellum (Fig. 1e,f) and was completed with the fusion of the entire length of the flagellum with the body membrane (Fig. 1g). In some cases, however, membrane fusion appeared to take place rather haphazardly at the points of contact between the body and flagellum membrane, leaving portions of the flagellum membrane unfused, although it might eventually come into contact with the body membrane.

During of encystment, a new membranous material was seen laid over the body membrane (Fig. 1d arrows). As encystment and membrane fusion approached a final stage, the entire cyst body appeared to become firm and began to be transformed from a discoid (Fig. 1g) to a spheroid (Fig. 1h). After encystment, the axonemal fibrils (Fig. 1i arrows) within the cytoplasm lacked a surrounding membrane.

Discussion

During encystment of *O. brassicae,* the flagellum wraps around the body of a zoospore. The entry of axonemal fibres into the body was evidenced in the ultrastructure of encysted zoospores in this study as well as in earlier publications (Lesemann and Fuchs, 1970; Temmink and Campbell, 1969; Lange and Olson, 1976). The fate of the axonemal membrane is not understood and is postulated differently. Lesemann and Fuchs (1970) stated that the axonemal membrane was incorporated into the plasmalemma, while Temmink and Campbell (1969b), in favor of the "reeling in" process, suggested that a "knot of wound-up membranes" which they found in the cytoplasm of the encysted zoospore was the remnants of axonemal membrane accumulated during the course of encystment.

Our SEM and TEM observations suggest the following events (Fig. 2): (1) the zoospore is transformed into an amaeboid body after attaching to the root surface, (2) the flagellum wraps around the body of the zoospore, (3) the flagellum flattens and its membrane spreads over the body but is not fused, (4) the flagellum membrane starts fusing at the points of contact with the body membrane, and (5) the axonemal fibres are deeply embedded in the cytoplasm that is covered with cyst membrane, and the cyst regains its roundedness, presumably due to an increase in cytoplasmic pressure. At this time the cyst wall is formed as the result of membrane fusion.

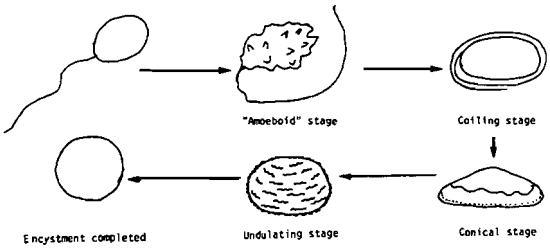


Fig.2. Observed processes of encystment in Olpidium brassicae zoospores

The occurrence of membrane fusion in *Olpidium* zoospores is not surprising since the body and flagellum membranes of free zoospores are continuous (Lesemann and Fuchs, 1970; Temmink and Campbell, 1969a, 1969b; Lange and Olson, 1976; Barr and Hartmann, 1977). Roth and Shigenaka (1964) demonstrated a comparable fusion of membranes during the resorption of cilia by certain protozoa. After

attachment of the zoospores to the surface the flagellum wraps around the body. The stimulus for membrane fusion must result from at least two factors, as suggested earlier (Alderson and Hiruki, 1977), i.e., adhesion of the zoospore body to the root surface, and wrapping of the flagellum which brings the membranes into closed proximity, a prerequisite for membrane fusion. The adhesion seems to be a requirement since free zoospores do not show this membrane fusion. Coiling of the zoospore flagellum that takes place prior to the wrap-around process may suggest a similar change is occurring in the integrity of axonemal fibrils. Further factors which are unknown at present may also be involved since fusion does not occur frequently between the flagellum and body membranes of different zoospores attached to roots. The irregular outline, often reminiscent of that of amaeba bodies, was found in zoospores undergoing membrane fusion. The occurrence of this transitory state of the membranes may suggest that a dynamic change in membrane configuration is taking place during this particular time. This is also clearly suggested in Fig. 1h, which illustrates the fluid nature of the membrane during encystment. If membrane fusion is involved in virus uptake, it would be much more efficient than the "reeling in" process proposed earlier (Temmink and Campbell, 1969b; Temmink et al., 1970; Temmink, 1971). Although the hypothetical "reeling in" process was preferred by these authors to the "wrap-around" process, there seems no particular reason to believe that virus adsorption to the axonemal sheath (membrane) is followed only by the pulling in of the axonemal fibrils. Published electron micrographs, in fact, clearly show virus particles abundantly attached to both the zoospore body and the axonemal membrane (Temmink et al., 1970). Therefore, it may be suggested that in the process of membrane fusion, virus particles attached to both the body and the flagellum membranes may more effectively be taken up into the zoospore cytoplasm.

Understanding of the exact role of the zoospore membrane fusion in the uptake and subsequent release of virus particles will require further studies with more refined techniques, including immunofluorescence experiments. Such an investigation is in progress.

References

Alderson, P.G. & Hiruki, C. (1977). Scanning electron microscopy of zoospores of *Olpidium brassicae*, free or attached to tobacco roots. *Phytopathol. Z.* **90**, 123-131.

Barr, D.J.S. & Hartmann, V.E. (1977). Zoospore ultrastructure of *Olpidium brassicae* and *Rhizophlyctis rosea*. *Can. J. Bot.* **55**, 1221-1235.

Hiruki, C. (1969). An incubator useful for culturing *Olpidium brassicae* for transmission of plant viruses. *Virology* **39**, 333-334.

Hiruki, C. (1987). Recovery and identification of tobacco stunt virus from air-dried resting spores of *Olpidium brassicae*. *Plant Pathol.* **36**, 224-228.

Hiruki, C. (1991). The fine structure of germinating resting spores of *Olpidium brassicae*. *Proc. Japan Acad.* 76B, 89-92.

Hiruki, C. & Teakle, D.S. (1987). Soil-borne viruses of plants. In *Current Topics in Vector Research*, Vol. 3, (Ed. K.F. Harris), Springer-Verlag, NY. pp. 177-215.

Koch, W.J. (1968). Studies of the motile cells of chytrids. V Flagellar retraction in posteriorly uniflagellate fungi. *Amer. J. Bot.* 55, 841-859.

Lange, L. & Olson, L.W. (1976). The zoospore of *Olpidium brassicae*. *Protoplasma* **90**, 33-45.

Lesemann, D.E. and Fuchs, W.H. (1970). Elektronenmikroskopische Untersuchung über die Vorbereitung der Infektion in encystierten Zoosporen von *Olpidium brassicae*. *Arch Mikrobiol.* **71**, 1-19.

Roth, L.E. & Shigenaka, Y. (1964). The resorption of Cilia. Z. Zellforsch. *Mikrosk. Anat.* **64**, 19-24.

Temmink, J.H.M. (1971). An ultrastructural study of *Olpidium brassicae* and its transmission of tobacco necrosis virus. *Meded. Landbouwhogeschool Wageningen* **71-6**, 1-135.

Temmink, J.H.M. & Campbell, R.N. (1969a). The ultrastructure of *Olpidium brassicae*. II. Zoospores. *Can. J. Bot.* **47**, 227-231.

Temmink, J.H.M. & Campbell, R.N. (1969b). The ultrastructure of *Olpidium brassicae*. III. Infection of Host Roots. *Can. J. Bot.* **47**, 421-424.

Temmink, J.H.M., Campbell, R.N. & Smith, P.R. (1970). Specificity and site of in vitro acquisition of tobacco necrosis virus by zoospores of *Olpidium brassicae*. *J. Gen. Virol.* **9**, 201-213.

INCIDENCE OF INFECTION WITH *SPONGOSPORA SUBTERRANEA*, THE VECTOR OF POTATO MOPTOP VIRUS, IN FOUR WILD POTATO SPECIES.

E. Mäkäräinen[1], H. Rita[2], E. Teperi[3], and J. P.T. Valkonen[1]

[1]Department of Plant Production, P.O.Box 27, Viikki, and [2]Department of Forest Resource Management, P.O.Box 24, FIN-00014 University of Helsinki, Finland; [3]Institute of Plant Protection, Agricultural Research Center, FIN-31600 Jokioinen, Finland.

Summary

The incidence of infection with *Spongospora subterranea* was studied in the non-tuber bearing *Solanum brevidens* and *S. etuberosum* and the tuber bearing *S. acaule*, *S. sucrense* and potato cvs. Olympia and Pito. Shoot cuttings were grown in soil naturally infested with *S. subterranea*, or the roots were inoculated with a zoospore suspension. Logit models were used to analyze the data. The incidence of *S. subterranea* was higher in plants inoculated with zoospore suspension than in those grown in infested soil (odds ratio (OR) 10.65). Ageing of the inoculum reduced the incidence of infection in the plants (OR 0.30) without altering the interspecific differences. The ORs of infection (compared to cv. Olympia) were 0.07, 0.29, 0.60 and 2.88 for *S. acaule, S. sucrense, S. brevidens*, and *S. etuberosum*. Only *S. acaule* was significantly more resistant to infection than cv. Olympia. No infection was detected in cv. Pito.

Introduction

Powdery scab caused by the fungus *Spongospora subterranea* (Wallr.) Lagerh. (*Plasmodiophoromycetes*) is distributed worldwide and can significantly reduce the quality and yield of potato (*Solanum tuberosum* L.) (Karling, 1968; Hughes, 1980). Besides being an important primary pathogen of potato, *S. subterranea* is the vector of potato moptop virus (PMTV) which causes growth reduction in potato plants and spraing in the tubers (Jones & Harrison, 1969). PMTV has become important in potato in Northern Europe (Jones & Harrison, 1972; Ryden et al., 1986; Kurppa, 1989).

The incidence of infection with *S. subterranea* varies among potato cultivars and genotypes, probably reflecting genetic variability in the resistance to infection (Hughes, 1980; Kirkham, 1986; Ganns et al., 1987; Jellis et al., 1987; Christ & Weidner, 1988; Wastie et al., 1988; Diriwächter & Parberry, 1991). Many wild potato species have been extensively used as a source of disease resistance in potato breeding (Ross, 1986; Hawkes, 1990). However, little information is available on the infection of the wild *Solanum* spp. with *S. subterranea*.

The incidence of root infection with *S. subterranea* was investigated in the tuber bearing species *S. acaule* Bitt. and *S. sucrense* Hawkes and in the non-tuber bearing species *S. brevidens* Phil. and *S. etuberosum* Lindl. in experimental conditions. Because both tuber bearing and non-tuber bearing species were included, the infection with *S. subterranea* was detected in roots instead of tubers. Logistic models being currently developed (Collett, 1991) were used to analyze the data.

Materials and Methods

Plant material and culture:

S. acaule CIP 761366, *S. etuberosum* CIP 761058 and *S. sucrense* CIP 761351 were obtained as true potato seed (TPS) from the International Potato Center (CIP), Lima, Peru. *S. brevidens* CPC 2451 (Commonwealth Potato Collection, Scotland, UK) was maintained as *in vitro* clone. Pathogen-tested tubers of the potato cvs. Olympia and Pito were obtained from the Seed Testing Institute, Helsinki, Finland. The plants were clonally multiplied by shoot cuttings and grown in washed sand in the glasshouse

Inoculation and detection:

Two-week-old plants were inoculated with *S. subterranea* either by growing them in naturally infested soil or with a suspension of zoospores released from tomato roots (*Lycopersicon esculentum* Mill. cv. Ida). The field soil sample was collected from a potato field in Köyliö in southwestern Finland and stored at 4°C. The soil was air-dried for 2 wk and used for inoculation (Jones & Harrison, 1969). Plastic pots were filled with the infested soil (700 cm^3), which was moistened, and the rooted cuttings were transplanted into the pots. The isolate of *S. subterranea* for zoospore inoculation was obtained from a potato tuber lot (cv. Saturna) grown in Pieksämäki in eastern Finland. The resting spores were purified and the fungus was baited into tomato roots (Merz, 1989). The fungal culture was maintained in the roots of tomato grown at 15°C as described below. The zoospore suspensions were prepared by incubating infected roots of tomato cv. Ida in 0.035 % Shultz's nutrient solution (N:P:K = 10:7:8) at room temperature for 2 h. Aliquots of 5 ml were pipeted onto the roots of the test plants grown in washed sand in plastic pots (700 cm^3). The plants were watered daily with 0.035 % Schultz's nutrient solution and grown under the illumination of fluorescent lamps at 15 °C (RH = 85 %) in a growth chamber. Five plants of each species were inoculated with field soil and five more with zoospore suspension and grown at 2000 lux as descibed above. The experiment was repeated one year later, except for that the plants were grown at 5000 lux. The roots were harvested 6 wk post-inoculation, rinsed under tap water, and cut into 1-cm-long pieces. Five randomly taken root pieces of each plant were stained for the detection of sporangia of *S. subterranea* as described by Kormanik & McGraw (1983), mounted onto a glass slide for observation under light microscope, and scored either infected (1) or non-infected (0).

Statistical analysis:

Logistic models are suitable for modelling and analysing data presented in the form of proportions (Collett, 1991). Their logic of statistical inference is based on the binary nature of the response variable and these models thus offer natural parameter interpretations. The central statistic to describe differencies in proportions is odds ratio. In logistic models, logarithms of odds ratios are modelled. The response variable in the present study was the proportion of the succesfully inoculated plants. In order to adjust the proportions of the successful infections for the effect of the experimental conditions and the method of inoculation, these two factors (both with two levels) were first included in the model. After this adjustment, the effect of varieties and their possible interactions between the method of inoculation were tested.

Results and discussion

The numbers of plants with detected *S. subterranea* infection are presented in Table 1.

The odds ratios describing the differences between species were formed with respect to cv. Olympia (Table 1). Only the odds ratio for *S. acaule* was statistically different from unity (= 1). As the values of odds ratio (and the upper conficende interval (C.I.) value) less than the unity indicate more resistance to *S. subterranea* than in cv. Olympia, only *S. acaule* can be considered as more resistant. Interestingly, *S. acaule* has previously been found resistant to *S. endobioticum*, which also belongs to the family *Plasmodiophoromycetes* (Hawkes, 1990). Few sporangia of *S. subterranea* were detected in the roots of *S. sucrense*, although the number of infected plants was not significantly less than in the potato cv. Olympia, and no infection was found in the potato cv. Pito (data not shown). Great numbers of sporangia were detected in the roots of infected *S. brevidens*, *S. etuberosum* and potato cv. Olympia (data not shown).

Table 1. The numbers of plants which contained sporangia of *S. subterranea* in at least one root piece out of the total of five examined.

	Plants grown in infested soil		Plants inoculated with zoospore suspension					
	Fresh inoculum	Inoculum stored for one year	Fresh inoculum	Inoculum stored for one year	Σ	%	o.r.[1]	C.I.[2]
S. acaule	0/5	0/4	1/3	0/5	1/17	6	0.07	0.01 - 0.77
S. brevidens	1/5	0/4	4/5	1/5	6/19	32	0.60	0.12 - 2.85
S. etuberosum	1/4	2/5	5/5	3/5	11/19	58	2.88	0.61 - 13.66
S. sucrense	1/5	0/5	1/5	2/5	4/20	20	0.29	0.06 - 1.55
S. tuberosum								
cv. Olympia	1/5	0/5	3/4	3/5	7/19	37	1.00	
cv. Pito	*	0/5	*	0/5	0/10	0		

* not tested; [1]odds ratio; [2]confidence interval

The effects of both experimental conditions and method of inoculation were statistically significant. The odds ratio between the incidences of infection in the two replicate inoculations using infested soil and zoospore suspension was 0.30 (C.I.) 0.10 - 0.92) meaning that the overall incidence of infection was significantly higher in the first inoculation than in the second one with both methods. Perhaps the inoculum potential of *S. subterranea* was reduced or the increased illumination made the plants physiologically more resistant to infection. The odds ratio between the methods was 10.65 (C.I. 3.11 - 36.46). The inoculation using a suspension of zoospores resulted in a greater number of infected plants than the inoculation by growing the plants in naturally infested soil. These differences could be due to soil suppressiveness affecting inoculum potential (Merz, 1989), a higher density of the infective units (zoospores) in the suspension than in the infested soil, or differences between the virulence of the isolates (Karling, 1968). The addition of the varieties into the model improved the fit significantly (improvement in the deviance 16.29, degrees of freedom (d.f.) 3, p-value 0.003). The fit of this model was acceptable (deviance 10.80, d.f. 13, p-value 0.623). The addition of the interactions between method of inoculation and the species and varieties into the model did not improve the fit.

It is an interesting subject of further studies to examine whether the low and high occurance of PMTV in cvs. Pito and Olympia, respectively, in the field (Kurppa, 1989; A. Hassi pers. com.), is due to low and high occurance of *S. subterranea*. Neither of these cultivars suffers particularly from powdery scab and the occurance of *S. subterranea* in them has not been studied in natural conditions.

References

Christ, B.J. & R.J. Weidner, 1988. Incidence and severity of powdery scab on potatoes in Pennsylvania. *Am. Potato J.* 65: 583-588.

Collett, D., 1991. Modelling binary data. Chapman & Hall, Cornwall, UK. 369 pp.

Diriwächter, G. & D.G. Parberry, 1991. Infection of potato by *Spongospora subterranea*. *Mycol. Res.* 95: 762-764.

Ganns, P.T., W.D. Carson & M.B. Bishop, 1987. The susceptibility of potato cultivars to powdery scab caused by *Spongospora subterranea* (Wallr.) Lagerh. *J. Natl. Inst. Agric. Bot.* 17: 337-343.

Hawkes, J.G., 1990. The Potato. Evolution, biodiversity and genetic resources. Smithsonian Institution Press, Washington, 259 pp.

Hughes, I.K., 1980. Powdery scab (*Spongospora subterranea*) of potatoes in Queensland: occurence, cultivar susceptibility, time of infection, effect of soil pH, chemical control and temperature relations. *Aust. J. Exp. Agric. Animal Husb.* 20: 625-632.

Jellis, G.J., P.S. Phul & N.C. Starling, 1987. Evaluation of potato germplasm for resistance to powdery scab (*Spongospora subterranea*). Tests of Agrochemicals and Cultivars 8 (1987). *Ann. Appl. Biol.* 110 (Supplement).

Jones, R.A.C. & B.D. Harrison, 1969. The behaviour of potato mop-top virus in soil, and evidence for its transmission by *Spongospora subterranea* (Wallr.) Lagerh. *Ann. Appl. Biol.* 63: 1-17.

Jones, R.A.C. & B.D. Harrison, 1972. Ecological studies on potato mop-top virus in Scotland. *Ann. Appl. Biol.* 71: 47-57.

Karling, J.S., 1968. The Plasmodiophorales. Hafner Publ., New York, 256 pp.

Kirkham, R.P., 1986. Screening for resistance to powdery scab disease of potatoes. *Aust. J. Exp. Agric. Animal Husb.* 26: 245-247.

Kormanik, D.P. & A.C. McGraw, 1983. In: Methods and principles of mycorrhizal research. (Ed. N.C. Schenck). Phytopath. Soc., St. Paul, Minnesota, USA, 244 p

Kurppa, A., 1989. The distribution and incidence of potato mop-top virus in Finland as determined in 1987 and on the variation of disease symptoms in infected potatoes. *Ann. Agric. Fenn.* 28: 285-295.

Merz, U., 1989. Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution-culture system. *EPPO Bull.* 19: 585-592.

Ross, H., 1986. Potato breeding - problems and perspectives. J. Plant Breed. Suppl. no. 13. Paul Parey, Berlin, 132 pp.

Ryden, K., B. Erikson & V. Insunza, 1986. Rostringar hos potatis orsakade av potatismopptoppvirus (PMTV). (Spraing in potato tubers caused by potato mop-top virus) *Växtskyddsnotiser* 50: 97-102.

Wastie, R.L., P.D.S. Caligari & S.J. Wale, 1988. Assessing the resistance of potatoes to powdery scab (*S. subterranea* (Wallr.) Lagerh.). *Potato Res.* 31: 167-171.

EPIDEMIOLOGICAL ASPECTS OF POWDERY SCAB OF POTATOES CAUSED BY *SPONGOSPORA SUBTERRANEA*

U. Merz

Plant Pathology Group, Institute of Plant Sciences, Swiss Federal Institute of Technology, 8092 Zurich, Switzerland

Summary

The importance of powdery scab for Swiss potato growers has increased seriously despite strict seed certification laws concerning disease levels. This suggests that infested soil may be the main inoculum source. To gain more information about the current geographical distribution of the pathogen, a survey of soil samples (78) from potato-producing areas was made using a bioassay. More than 70% of the samples - mostly from below 600 maS - were infested with *Spongospora subterranea* (*S.s.*). The fungus was found in places with no history of powdery scab, which shows that S.s. is more widespread than previously expected. A comparison of infection scores from tuber samples (cv "Agria") against levels of soil infectivity showed that the risk of tuber infection is significant only when infectivity is high. Among the different cultural measures, ploughing in spring, often following pasture as a pre-crop, was correlated with fields with powdery scab problems. In laboratory experiments with the same bioassay, it was confirmed that inoculum density is high in highly infested soils (>500 cystosori/g). The fungus has no dormancy: resting spores from freshly harvested tubers gave high rates of root infection. Root exudates stimulated resting spore germination; the causal factor is not host-specific. The nature of spore germination and its role in epidemiology is discussed.

Introduction

In the past, powdery scab disease of potatoes was considered to be most important in the hilly regions between 650 and 800 maS, that means sites of traditional seed production in Switzerland. For some years, there has been an increasing incidence of powdery scab in the lowlands (Winter und Winiger, 1983). In 1991, the disease caused, often for the first time, severe problems, in particular, for growers of cv. "Agria". The cause of the epidemic is unknown. An increase in the occurrence of powdery scab has been observed also in other countries (Scandinavia, Pakistan, USA: pers. comm.; England: Harrison et al, 1993).

The initial inoculum for the disease in a particular field may arise from fungal resting spores that were already present in the soil or imported either with seed or with manure, soil or sludge. However, considering the low tolerance of powdery scab on seed potatoes in Switzerland - not more than 1% of tubers with more than 5 lesions - the most likely cause is existing soil infestation which is a common occurrence but the inoculum density is normally low. Under optimal conditions however (cool and wet, susceptible variety, poor crop rotation, heavy soil), such densities may be sufficient to cause epidemics.

In this context, Wild (1930), working with powdery scab, made her field trials mostly below 500 maS in the Swiss lowlands. Scabbed potatoes were harvested at all sites even where clean seed had been used. Based on her findings Wild stated that the fungus was more widespread than she had expected.

It is generally accepted that a soil, once infested, stays infectious for a long period. But we do not have information on the distribution of *S. s.* in soils of different regions. The epidemiology of the fungus has not yet been studied so, for example, we do not know the minimum level of inoculum necessary to start an epidemic. Soil infestation in relation to cropping history was investigated only by Winter and Winiger (1983). The stimulus for resting spores germination is still unknown: if spores germinate

spontaneously, why then can we get a massive tuber attack on fields not cropped with potatoes for more than 10 years? On the other hand, how can the wide host range of *S. s.* be explained (Würzer, 1965; Jones and Harrison, 1972), if the spores need a specific stimulus?

At present, direct control of the fungus is not possible. Indirect control has to be concentrated on prevention of spread of soil infestation. To obtain basic information, a survey was made of the occurrence of the pathogen in soils, using a bait-plant bioassay, and of disease on tuber samples from 78 selected farms mostly located in the Swiss lowlands in potato-producing areas .

For the development of new biocontrol measures, information about internal or external factors controlling resting spore germination is essential. The bioassay was also used therefore to start a basic study on these factors. Some results will be presented in this report.

Material and Methods

Soil survey:

Soils were sampled mainly in July 1991 from fields cropped with potato cv. "Agria". A full sample consisted of 20 sub-samples along the diagonals of the field. A 3cm-layer was always removed first to try to avoid possible solarisation effects. The soil samples (average volume = 2 l) were air-dried at room temperature for about 3 weeks and then stored at 15°C. Soil infectivity was assessed by using a modified solution culture test system (Merz, 1989). The mixed soil was suspended in 0.5l nutrient solution (NS) and incubated for 9 days; the baiting period was 1 day. The test was repeated three times for each field.

Scoring tuber samples :

The tuber samples, taken from three plants per field, were stored at 2°C, then washed and scored for disease incidence (scale 0-8), using a standard series of photographs by Winter und Winiger (1983). In doubtful cases, lesions were checked under the microscope. Finally, a disease index was calculated (= average score x percentage of diseased tubers) with the following scale: >0-100=little, >100-400=medium and >400-800=heavy attack.

Artificially infected soil:

In a dilution series, untreated, noninfested soil was mixed with a decreasing quantity of cystosorus inoculum (2000 cyst./g soil, 1000/g and 500/g), obtained from older tubers of cv. "Agria" as described in Merz (1989), and baited, together with a highly infested field soil, as described above.

Resting spore germination:

For checking dormancy, cystosorus inoculum was obtained from heavily scabbed and freshly harvested potato tubers and also baited in the same way as the soil samples. Inoculum from older tubers was used in a bioassay to test the influence of root exudates. The incubation period here was 10 days. The resting spores were then sampled on a 10µ Millipore filter, resuspended in 1l NS and the 'exudate' plants were added. After 5 hours, the resting spores were separated from the NS by a 7µ filter and the tomato bait plants added to the filtrate for another 4 hours. Exudate plants were 4 week old tomatoes and wheat, grown in quartz sand with NS. For this experiment the NS was buffered with MOPS at a pH of 6.2 (0.01M).

Results

From the total of 78 soils tested, 74% were infested. About the same percentage (70%) was found among farms below 600 maS (Fig.1) with the following ranking distribution of bait plant root infection: 12x1, 15x2, 10x3 and 11x4. Disease symptoms were found on tubers of 37 samples although most samples scored below 100. With little to moderate soil infectivity (range >0-2), only a few of the corresponding samples with a small number of lesions were found. Tuber infection was more frequent

in the infectivity range >2-4, showing a marked increase above 3 (Fig.2), but one third of the samples in the highest range of soil infectivity were still disease-free.

Almost 50% of the farms with infested soil samples did not have problems with powdery scab before the introduction of cv. "Agria". In 1991, more than one third of the 78 farms had powdery scab problems at harvest. Heavy tuber attack occurred only on those fields where soil infectivity was high. Ploughing in spring, often following pasture as a pre-crop, was correlated with fields with powdery scab problems. The percentage of farms with powdery scab problems was higher where imported seed was planted. Irrigation showed no influence on disease occurrence.

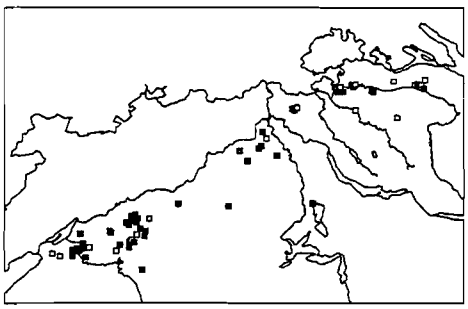


Fig. 1 Geographical distribution of infested (filled square) and non-infested soils of selected farms below 600 maS.

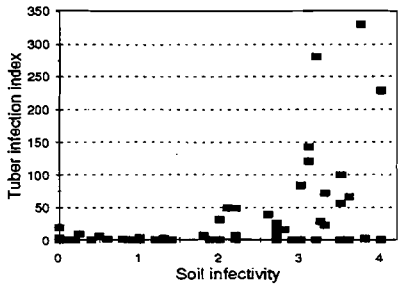


Fig. 2 Relationship of soil infectivity to infection of young potato tubers by *S.s.*.

The intensity of bait plant root infection of the naturally highly infested field soil ranked between the infection from soils artificially inoculated with 500 and 1000 cystosori/g soil respectively (Fig. 3).

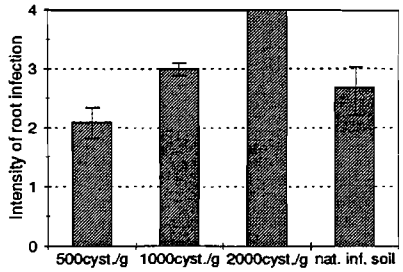


Fig. 3 Intensity of bait plant root infection by *S.s.*of three artificially and one naturally infested field soil (mean of 3 rep.).

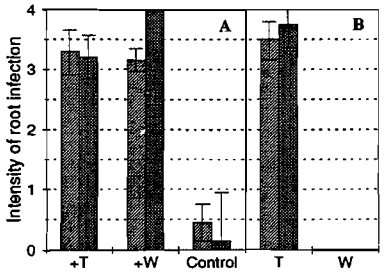


Fig. 4 A) Influence of tomato (+T) and wheat (+W) root exudates on bait plant root infection by *S.s.* **B)** "exudate" plant root infection (results of 2 experiments are shown).

In an earlier bioassay it was found that a 3-day incubation of cystosori together with roots of tomato plants raised the pH of the unbuffered NS from 4.3 to 6.2 and increased the intensity of bait plant root infection. With buffered NS and a modified bioassay, the effect on bait plant root infection was still present (Fig. 4A). A 5h-incubation of cystosori together with roots of tomato or wheat was sufficient to increase significantly the intensity of root infection. A high level of root infection was also found on the tomato "exudate"-plants but not on wheat (Fig. 4B). Cystosori inoculum from tubers, taken at harvest time, infected the roots of tomato bait plants (rating=3).

Discussion

The results from the survey show that *S.s.* is more widespread in soils, especially in the lowlands, than previously expected. Assessment of tuber samples from the fields and, even worse, disease levels at harvest, represent only poorly the distribution of the pathogen. Taking these findings together with the statement by Wild (1930) about the unexpected occurrence of *S.s.*, it may be that the fungus is endemic in the soils of potato-producing areas as, for instance, *Polymyxa betae* is in regions where sugar beet is cropped (Häni *et al.*, 1985). The risk of tuber infection is significant only when soil infectivity is high. However, with the increasing use of a highly susceptible variety, probably the most important factor, disease problems may develop rapidly from soils with initially low infestation. Other factors, such as poor crop rotation, may contribute to this development.

The results from the survey also support the theory that soil is the most important inoculum source. Bait-tests of individual soils could thus provide substantial help for disease management. An open question is still the role of apparently healthy seed tubers with sporeballs containing lenticels (Diriwächter and Parbery, 1991) in the epidemiology of the fungus. A more detailed control of seed, especially when imported, should be considered.

Resting spores from young tubers were not dormant in contrast to the results of Diriwächter (1981). Cystosori from older tubers released many more zoospores when incubated together with roots of "exudate" plants. However, the effect was not host-specific and, indeed, wheat, even though it is a non-host (Fig. 4B), stimulated spore germination. This result suggests a possible survival startegy as postulated by MacFarlane (1952) for *Plasmodiophora brassicae*: Some of the resting spores in soil may germinate spontaneously whereas others require a stimulus which need not be host-specific. MacFarlane (1952) obtained disease reduction on cabbage plants following the use of a grass, *Lolium perenne*, as a green manure. With winter rape as a pre-crop, Winter and Winiger (1983) obtained a significant reduction of powdery scab on potato tubers. Because inoculum density is high in highly infested soils - more than 500 cystosori/g soil consisting of 500-1000 single spores - the time needed between potato crops for decline of soil infectivity towards zero is expected to be at least 5 years. This would explain the occurrence of powdery scab in regions with intensive potato production. In those cases where problems occurred on fields not cropped with potatoes for more than 10 years, survival of *S.s.* on an alternative host has to be considered. The fact that powdery scab occurred more often following ploughing in spring supports this suggestion. According to White (1954) overwintering of zoospores without a host is not possible.

The soil survey and the research on the nature of germination stimuli in exudates will be continued.

References

Diriwächter, G. and D.G.Parbery, 1991. Infection of potato by *Spongospora subterranea*. Mycol. Res. **95**: 762-764.

Häni A., C.H.Högger, U.Merz und H.Meyer, 1985. Vorkommen der Rhizomaniakrankheit, ihres Vektors *Polymyxa betae* sowie des Rübenzystennematoden, *Heterodera schachtii*, an Zuckerrüben in der Schweiz. Mitt. Schweiz. Landw. **85**: 52-58.

Harrison J.G., E.A.Rees, H.Baker and R.Lowe, 1993. Detection of spore balls of *Spongospora subterranea* on potato tubers by enzyme-linked immunosorbent assay. Plant Pathol.**42**: 181-186.

Jones, R.A.C. and B.D. Harrison, 1972: Ecological studies on potato mop-top virus in Scotland. Ann. appl. Biol. **71**: 47-57.

MacFarlane, I., 1952. Factors affecting the survival of *Plasmodiophora brassicae* Wor. in the soil and its assesment by a host test. Ann. appl. Biol. **39**: 239-256.

Merz, U., 1989. Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution-culture test system. EPPO Bulletin **19**: 585-592.

White, N.H., 1954. The use of decoy crop in the eradication of certain soilborne plant diseases. Austr. J. Sci. **17**: 18-19.

Wild, N., 1930: Untersuchung über den Pulverschorf der Kartoffelknollen *Spongospora subterranea* (Wallr.) Johnson. Phytopath. Z. **1**: 367-452.

Winter, W. und F.A. Winiger, 1983: Einfluss verschiedener Fangpflanzen sowie von Kalk und Kalkstickstoff auf die Bodenverseuchung mit *Spongospora subterranea*, dem Erreger des Pulverschorfes. Mitt. Schweiz. Landw. **31**: 190-206.

Würzer, B., 1965, Ergänzende Untersuchungen über den Pulverschorf der Kartoffel und dessen Erreger *Spongospora subterranea* (Wallr.) Lagerh. Verlag E. Ulmer, Stuttgart.

VIRUS - VECTOR INTERACTIONS IN THE RIZOMANIA SYNDROME

H. Prillwitz and E. Schlösser

Institut für Phytopathologie und Angewandte Zoologie, Justus-Liebig-Universität, Bismarckstraße 16, D-35390 Giessen, Federal Republic of Germany.

Summary

The rizomania syndrome on sugar beets is governed by the vector *Polymyxa betae* KESKIN, the beet necrotic yellow vein virus (BNYVV) and the beet soil-borne virus (BSBV-2), respectively. To obtain information on possible interactions, the roots of virus-free and virusinfected sugar beets were shaved off, followed by an evaluation of the development of *P. betae* in the newly formed roots. The two viruses affected their common vector differently. As compared to virus-free sugar beets, the BNYVV reduced the cystosori significantly by 56,9 %, while the BSBV-2 increased the cystosori significantly by 51,7 %. This differential reaction has considerable epidemiological implications.

Introduction

Beet necrotic yellow vein virus (BNYVV) and beet soil-borne virus (BSBV-2), serotype 2 according to LESEMANN et al. (1989) and HENRY & HUTCHINSON (1989) as well as their fungal vector *Polymyxa betae* KESKIN are all biotrophic, competing for space, nutrients and energy. For this reason, interactions between these components of the rizomania syndrome can be anticipated. One possibility would be a competition between the two viruses, which has already been observed (PRILLWITZ 1993, PRILLWITZ & SCHLÖSSER 1993a).The second possibility would be an effect of the viruses on their common vector *P. betae*. Such interactions has also been found (GERIK & DUFFUS 1988, SCHLÖSSER 1990). The following experiment was conducted to validate the differential response of *P. betae* to the two viruses.

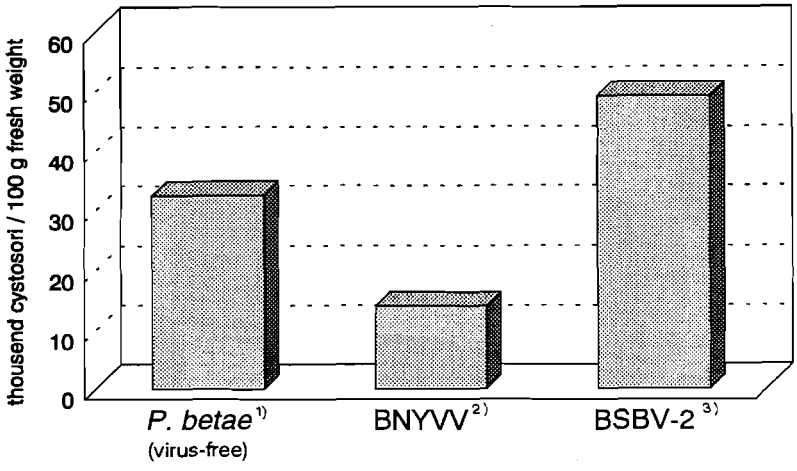
Material and method

Sugar beet plants of the cv. Lena were precultivated in steamed soil and transplanted in pots (14 cm) with infested soil, when they reached the 2-leaf stage. These plants were grown for 5 months either in BNYVV infested soil (origin: PR China), BSBV-2 infested soil (origin: Einbeck) or in soil containing virus-free *P. betae* (origin:

Reichenberg). After complete removal of all lateral roots and the rhizodermal layer with a knife and carefully cleaning by repeated washings, the beets were planted in autoclaved sand and inoculated with cystosori of virus-free *P. betae* (> 750 per pot). All leaves except the innermost leaflets were removed. During the first 3 days the sugar beet plants were watered with a 2% TMTD-solution. The following 2 weeks only sterile water was used. After 6 weeks of cultivation, representative root samples were analyzed for BNYVV and BSBV-2 by ELISA (CLARK & ADAMS 1977, BARBAROSSA et al. 1992). The number of cystosori of *P. betae* in the root sap, obtained with a Pollähne press, was determined by counting 5 replicates of each sample with a Fuchs-Rosenthal counting chamber.

Results

The problem in this experiment was the high mortality rate especially, in the variant with BNYVV preinfection. From 50 shaved plants only 5 sugar beets survived. In the BSBV-2 variant 27 plants died, while no plants were lost in the virus-free *P. betae* variant.



significantly different in the Scheffeé-test (p=0,05)

1) n = 50 2) n = 5 3) n = 23

Fig. 1: Effect of virus-free *P. betae*, BNYVV and BSBV-2 on the subsequent colonization by *Polymyxa betae*.

The differences between the variants were significant despite the high mortality in the BNYVV variant. With virus-free *P. betae* twice as much cystosori were counted as in the BNYVV variant. The number of cystosori in the BSBV-2 variant was one and a half times higher than in the *P. betae* variant.

Discussion

SCHLÖSSER (1990) reported a competition between BNYVV, BSBV and *P. betae.* In his experiment preinfection with BNYVV reduced significantly the colonization of subsequently inoculated *P. betae,* as compared to roots preinfected with virus-free *P. betae* or BSBV-2. Roots preinfected with BSBV-2 resulted in a higher cystosori formation as compared to the virus-free check, but this difference was not significant.
This experiment was repeated using the method described by SCHLÖSSER (1990). Here, roots preinfected with BNYVV showed again a significantly lower colonization of *P. betae* in the newly formed roots as compared to the virus-free check. But this time the positive effect of BSBV-2 on the development of *P. betae* was significant. Thus, both viruses had a significant but opposite influence on their common fungal vector in newly formed roots. BNYVV suppresses and BSBV-2 promotes the development of *P. betae.* One possible reason for the supression of the fungus by BNYVV could be the different degree of damage on sugar beet plants by the two furoviruses. In pot and field experiments the damage caused by the BNYVV was significantly higher than that by BSBV-2 (PRILLWITZ & SCHLÖSSER 1993b). BNYVV damages sugar beet roots drastically. The roots die, while the plant react with a continuous proliferation of lateral roots finally resulting in the formation of a "root-beard". As an obligate biotrophic fungus, *P. betae* needs intact roots. Thus, the BNYVV destroys the basis for fungal development. This agrees with field observations. In sugar beets serverely infected with BNYVV with pronounced "root-beards" collected in autumn, high virus titers but hardly any cystosori of the fungal vector can be found (HILLMANN 1984). The reason for the promotion of *P. betae* by the BSBV-2 is unknown.

Acknowledgement

The financial support by the "Kuratorium zur Förderung des Zuckerrübenanbaues in Süddeutschland" and the supply of BSBV-2 antiserum by Prof. Dr. R. Koenig are gratefully acknowledged.

References

BARBAROSSA, L., H. J. VETTEN, A. KAUFMANN & D.-E. LESEMANN (1992): Monoclonal antibodies to beet soil-borne virus. Ann. appl. Biol. **121**, 143-150.

CLARK, M. F. & A. N. ADAMS (1977): Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. gen. Virol. **34**, 475-483.

GERIK, J. S. & J. E. DUFFUS (1988): Differences in vectoring ability and agrressiveness of isolates of Polymyxa betae. Phytopathology **78**, 1340 - 1343.

HENRY ,C. M. & P. J.HUTCHINSON (1989): Beet soil-borne virus and other beet viruses. Aspects of App. Biol. **22**, 109 - 116.

HILLMANN, U. (1984): Neue Erkenntnisse über die Rizomania an Zuckerrüben mit besonderer Berücksichtigung bayerischer Anbaugebiete. Diss. Gießen.

Lesemann, D.-E., R. Koenig, K. Lindsten and C. Henry (1989): Serotypes of beet soil-borne furovirus from FRG and Sweden. EPPO Bulletin 19, 539 - 540.

PRILLWITZ, H. (1993): Untersuchungen zur Rizomania, mit besonderer Berücksichtigung des beet soil-borne virus (BSBV). Diss. Gießen.

PRILLWITZ, H. & E. SCHLÖSSER (1993a): Interactions between beet soil-borne virus (BSBV-2) and beet necrotic yellow vein virus (BNYVV). IWGPVFV, Montreal 1993.

PRILLWITZ, H. & E. SCHLÖSSER (1993b): Comparative investigations on the damage of sugar beet by Polymyxa betae Keskin, beet soil-borne virus and beet necrotic yellow vein virus. Med. Fac. Landboww. Uni. Gent, **58**, in press.

SCHLÖSSER, E. (1990): Rizomania XII. Effect of BNYVV and BSBV on the development of *Polymyxa betae* Keskin. Med. Fac. Landbouww. Rijksuniv. Gent **55**, 1069 - 1071.

A SUMMARY OF RESEARCH ON WATERCRESS YELLOW SPOT VIRUS AND ITS FUNGAL VECTOR *SPONGOSPORA SUBTERRANEA F.* SP. *NASTURTII*

J A Walsh and C M Clay

Plant Pathology and Weed Science Department, Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK

Summary

Watercress yellow spot virus has been a problem in watercress production in the UK for the past 10 years. Yellow and chlorotic blotches appear on the leaves of severely affected plants rendering them unmarketable. Although Koch's postulates have not been fulfilled, all the evidence to date supports the assumption that the crook root pathogen (*Spongospora subterranea* f. sp. *nasturtii*) acts as a vector of WYSV. This fungus causes an important disease of watercress in its own right and is the most important pathogen affecting watercress in the UK. In a survey of 16 watercress farms, both diseases were found to be endemic in all regions of the UK where the crop is produced. WYSV particles are isometric with a diameter of 37.2 - 38.3 nm, sediment at 133.8S and have a buoyant density of 1.335 g/cm^3 in caesium chloride. The molecular weight of the nucleic acid (which is thought to be ssRNA) was estimated to be 1.6 x 10^6 Daltons and that of the coat protein 43,500 Daltons. Although WYSV has a number of properties in common with viruses in the tombusvirus, dianthovirus and carmovirus groups, its host range, large diameter, instability of particles, difficulty of mechanical transmission and lack of serological relationships suggest only a distant relationship with any of these groups.

Introduction

Watercress yellow spot virus (WYSV) was first described infecting watercress (*Rorippa nasturtium-aquaticum*) in France in 1960 (Spire, 1962). It was first found in the UK in Kent in 1983 (Tomlinson and Walsh, 1984) and was subsequently identified in crops in Dorset and Hampshire (Walsh *et al.*, 1989), the main regions of UK watercress production. Symptoms range from discrete bright yellow spots 2-4 mm in diameter to irregular shaped, bright yellow blotches up to 8 mm in diameter occurring primarily on the leaf veins. Symptoms are rarely seen in the summer months. They start to appear in October, are most severe in winter and spring until April/May and seem to be more common and more severe when plants are under any form of stress.

Transmission experiments suggest that WYSV is transmitted by motile zoospores of the crook root pathogen *Spongospora subterranea* f. sp. *nasturtii* but other means of transmission have not been excluded (Walsh *et al.*, 1989). Further circumstantial evidence linking *S. subterranea* f.sp. *nasturtii* and WYSV was provided by Walsh and Phelps (1991). Crook root is an important pathogen of watercress in its own right and has been reported to be a major limiting factor for watercress production in Europe and the USA. In the UK, the fungus has been controlled since the 1950's by the application of zinc to the spring water supplying watercress beds (Tomlinson, 1960). Recently, zinc has been shown to be toxic to freshwater shrimps (*Gammarus pulex*) (Martin and Holdich, 1986) and zinc emissions from

watercress beds have been implicated in the reduction of these shrimps in rivers and streams downstream. Consequently, alternative non-chemical methods of control are being sought, primarily through genetic resistance to *S. subterranea* f.sp. *nasturtii* and WYSV.

The original description of WYSV (Spire, 1962) reported the virus particles to be 27 nm in diameter and provided little further information on properties, structure and composition of particles. Since then, we have found the diameter of particles to be 37-38 nm in a range of stains and report here further information on the virus.

Materials and Methods

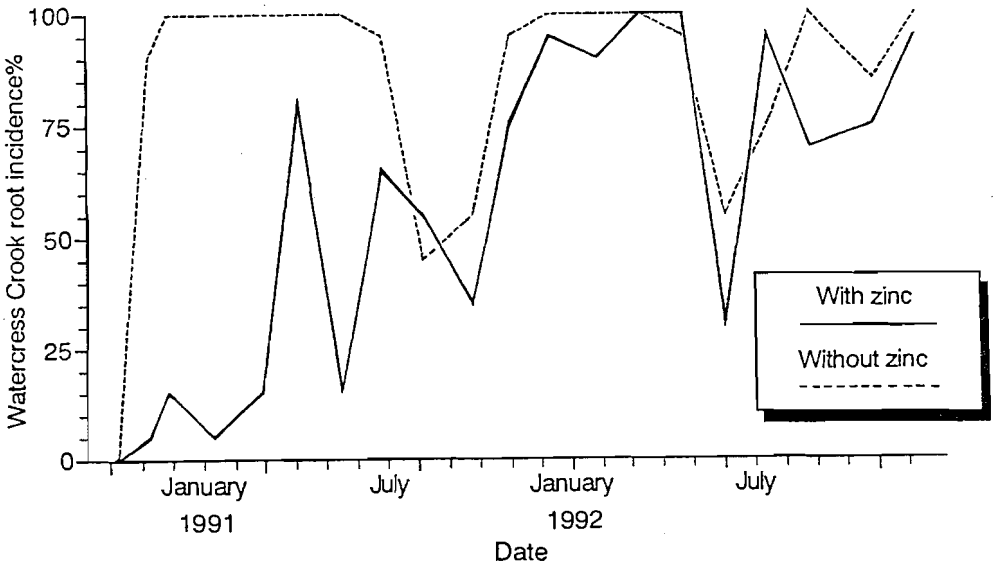
Disease detection. WYSV was detected by ELISA tests on whole plants as described by Walsh *et al.* (1989). The presence of *S. subterranea* f.sp. *nasturtii* was determined visually as described by Walsh and Phelps (1991).
The incidence of crook root and WYSV on UK watercress farms. Sixteen watercress farms were visited between September 1991 and February 1992 and where possible 10 plants were sampled from the middle region of 15 watercress beds at each site. The presence of crook root and WYSV in individual plants was determined as described above.
The incidence of crook root and WYSV in watercress beds, monitored over a period of 27 months. Every six weeks between October 1990 and December 1992, 10 watercress plants were sampled at random across the width of watercress beds in each of three regions: the inlet region where the spring water enters, the middle and the outlet regions. Four beds were sampled, two of which were treated with zinc and two were untreated. The presence of crook root and WYSV in plants was determined as before.
Virus properties. Virus measurements were made as described by Walsh *et el.* (1989). Buoyant density and sedimentation coefficient were determined as described by Govier (1985). The molecular weights of the coat protein of WYSV was determined as described by Thompson *et al.* (1988) and of the nucleic acid as described by Edwards *et al.* (1985).

Results

The incidence of crook root and WYSV on UK watercress farms. Of the 16 farms visited, virus infection of watercress plants was detected at all but two. Crook root was found at all sites. Of 203 beds sampled, 152 (75%) contained plants infected by the virus and 183 (90%) contained plants infected by the crook root fungus. Of all the plants sampled at random from the beds at all sites, 47% were infected by the virus and 58% by crook root.
The incidence of crook root and WYSV in watercress beds monitored over a period of 27 months. Over the 27 months, the percentage infection of plants in the different regions of the beds varied dramatically. Generally, infection levels were highest in the lower (outlet) region of the beds, lowest in the upper (inlet) region of the beds and intermediate in the middle of the beds. Zinc treatments were only applied to the two treated beds in the winter months and tended to reduce infection levels of both diseases. When zinc treatments ceased, infection levels increased. During the summer months a rapid decline in the levels of infection of both pathogens was observed when there was a combination of reduced water flow through the beds and high ambient temperatures. Infection levels increased in the following autumns. The incidence of WYSV closely followed that of

the crook root fungus. The incidence of crook root in the bottom (outlet) region of the beds is illustrated in Fig. 1.

Fig. 1. The incidence of crook root infection of plants in watercress beds with and without zinc treatments between October 1990 and December 1992



Virus properties. Virus particles are isometric with a mean diameter of 37.2 - 38.3 nm. They sedimented at 133.8S in 0.01 m phosphate buffer, pH 7.4 and banded in CsCl at a density of 1.335 g/cm^3. The molecular weight of the coat protein was estimated to be 43,500 Daltons and the nucleic acid 1.6 x 10^6 Daltons (thought to be ssRNA).

Discussion

It was interesting that no virus-infected plants were found at two of the watercress farms sampled. At one of these sites the absence of WYSV was probably partly due to the low incidence of crook root at this site; with an overall infection level of 11%, it was much lower than any of the other sites. This site was the only one visited where zinc treatments were applied throughout the whole year. At the other site where no virus-infected plants were found, very high levels of crook root were detected. This suggested that *S. subterranea* f.sp. *nasturii* at this site was not transmitting WYSV.

The relative incidences of crook root and WYSV in different regions of watercress beds agree with earlier findings; the distribution of virus-infected plants was coincident with that of crook root (Walsh *et al.*, 1989), and levels of WYSV and crook root were lower in zinc-treated beds than in untreated beds (Walsh, 1990). Certain cultural practices may provide components of an integrated control programme. When combined with zinc treatment such practices are likely to be even more effective.

WYSV has a number of properties in common with viruses in the tombusvirus, dianthovirus and carmovirus groups. However, host range (very restricted), large diameter of particles (37-38 nm), instability of particles, difficulty of mechanical transmission and lack of serological relationships, suggest only a distant relationship with any of these groups. WYSV is also different from dianthoviruses in that no second major RNA species has been detected.

The close association between WYSV and the crook root pathogen in natural infections in watercress beds and in experimentally infected plants, provides further circumstantial evidence for the crook root pathogen acting as vector of WYSV. If this is the case, it is the first record of a plasmodiophoromycetes transmitting an isometric virus.

References

Edwards, M.-L., Cooper, J.I., Massalski, P.R. and Green, B. (1985). Some properties of a virus-like agent found in *Brachypodium sylvaticum* in the United Kingdom. *Plant Path.* 34: 95-104.

Govier, D.A. (1985). Purification and partial characterisation of beet mild yellowing virus and its serological detection in plants and aphids. *Ann. appl. Biol.* **107**: 439-447.

Martin, T.R. and Holdich, D.M. (1986). The acute lethal toxicity of heavy metals to peracarid crustaceans (with particular reference to fresh-water Asellids and Gammarids). *Water Res.* 20: 1137-1147.

Spire, D. (1962). Etude de la maladie des taches jaunes du cresson (*Nasturtium officinale* R.BR.) *Annls. Epiphyt.* 13: 39-45.

Thompson, S., Fraser, R.S.S. and Barnden, K.L. (1988). A beneficial effect of trypsin on the purification of turnip mosaic virus and other potyviruses. *J. Virol. Methods* 20: 57-64.

Tomlinson, J.A. (1960). Crook root of watercress. A review of research. *N.A.A.S. q. Rev.* 49: 13-19.

Tomlinson, J.A. and Walsh, J.A. (1984). Virus diseases of watercress *Rep. Natn. Veg. Res. Stn.* for 1983, pp 88-89.

Walsh, J.A. (1990). The epidemiology of watercress yellow spot virus. German Phytomedical Society Series, Band 1, 173-176, Ulmer, Stuttgart. Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors. Braunschweig, Germany, August 21-24, 1990.

Walsh, J. A., Clay, C. M. and Miller, A (1989). A new virus disease of watercress in England. *EPPO Bull.* 19: 463-470.

Walsh, J.A. and Phelps, K. (1991). Development and evaluation of a technique for screening watercress (*Rorippa nasturtium - aquaticum*) for resistance to watercress yellow spot virus and crook root fungus (*Spongospora subterranea* f.sp. *nasturtii*). *Plant Path.* 40: 212-220.

FLAME CHLOROSIS, A NOVEL, SOIL-TRANSMITTED, VIRUSLIKE DISEASE OF SPRING CEREALS AND WILD GRASSES IS LINKED TO *PYTHIUM* SPP.

S. Haber[1], D.J.S. Barr[2], J. Chong[1], A. Tekauz[1], and W. Kim[1]

[1]Agriculture Canada Research Station, Winnipeg MB R3T 2M9; [2]Centre for Land and Biological Resources Research, Ottawa ON K1A 0C6, CANADA

Summary

Flame Chlorosis (FC) is a viruslike, soil-transmitted disease primarily affecting spring barley and wheat, but also oat (Haber *et al.* 1991), triticale (Haber *et al.* 1993) and panicoid grasses (Haber & Harder 1992) in Manitoba, Canada. Characteristic "flame"-like chlorosis appears as early as the one-leaf stage yet healthy seedlings transplanted from sterile medium into infective soil never develop symptoms (Haber *et al.* 1991), and FC plants often have symptom-free tillers uninfected with FC-specific RNA (Haber *et al.* 1992). Since these observations suggest that the viruslike agent of FC might be soil-transmitted to leaf and root initials during early germination rather than to established root systems, *Pythium* spp. were investigated as possible FC vectors. Nucleic acid extracts of *Pythium* isolates from FC soils hybridized to FC-RNA probes, and mycelial suspensions of these isolates, added to sterilized soil, transmitted FC to seedlings grown from barley seeds placed in the soil. Flame chlorosis is the first example of a plant virus disease transmitted by *Pythium*.

Introduction

The early evidence that FC was soil-transmitted prompted the search for a fungal vector (Haber *et al.* 1990). Examinations of zoosporic fungi isolated from FC- and non-FC soils in Manitoba found no association between FC and *Polymyxa graminis* Led., the fungal vector of all previously identified soil-transmitted virus diseases of cereals, but a clear association between FC and both *Olpidium brassicae* Wor. and *Lagena radicicola* Vanterpool and Led. (Haber *et al.* 1991). *O. brassicae*, although not reported to vector any soil-borne virus that infects cereals, transmits tobacco stunt, lettuce big vein, and tobacco necrosis and satellite viruses (Hiruki & Teakle 1987). *L. radicicola* is not known to transmit any plant virus, but resembles *O. brassicae* in its life cycle and shares properties with known fungal vectors, and was therefore also considered as a candidate vector (Haber *et al.* 1991).

Several aspects of FC differ profoundly from known soil-transmitted cereal virus diseases: a) FC is naturally transmitted to spring, rather than winter, cereals (Adams 1991); b) symptoms appear as early as the one-leaf stage; c) FC is consistently associated with multiple species of double-stranded RNA but virions are never detected, suggesting a closer resemblance to some mycovirus infections than to any known plant virus infection (Haber *et al.* 1990; Chong & Haber 1992). These observations suggest that FC vector relations might, therefore, also be fundamentally different from those of the known soil-borne cereal viruses transmitted by *P. graminis*. Rather than releasing zoospores that enter host plant root systems, a candidate FC vector should readily infect leaf and root initials in the earliest stages of seed germination. *Pythium* spp. meet this requirement. We report here evidence from nucleic acid hybridizations and transmission experiments that *Pythium* isolates from FC soils are indeed vectors of the FC viruslike agent.

Materials and Methods

Isolation of Pythium*:*

Pythium spp. and other filamentous fungi were isolated from barley seedlings. Seed was germinated for two days at 20-22 C and buried in soil for one week at 6-10 C. Seedlings were then rigorously washed free of soil and sections were plated on 2.5% V8 agar with 200 mg/L streptomycin sulfate.

Multiplication of mycelium:

Pythium inoculum was produced in V8-dextrose liquid medium, or in Czapex-Dox broth, in 2 L conical flasks. After autoclaving, several plugs from a 3-day old culture growing on potato dextrose agar were added to each flask. Flasks were incubated with aeration at room temperature (20-24 C) and ambient light for 5 to 8 days. After incubation, mycelium was either: a) filtered through Whatman #31 filter paper to produce a mycelial mat for extraction of nucleic acid; or b) chopped to produce a homogeneous suspension for use as inoculum.

Transmission to barley seedlings:

Barley seeds were manually dehulled, surface-sterilized by soaking for 20 min in 10% (v/v) household bleach/0.1% Tween-20, washed several times with sterile, distilled water, soaked for 4 h in sterile water before being slashed in the plumule (Zhang *et al.* 1991), placed on moist, sterile filter paper in Petri plates, and then pre-germinated in the dark at 20 C. The seeds were transferred to sterilized soil in 10 cm fibre pots, and 100 mL of mycelial suspension was immediately added to each pot; seedlings transferred to pots without added mycelium served as controls. The seedlings were then grown in cabinets on a cycle of 14 h light at 12 C followed by 10 h dark at 5 C.

Dot-blot assay for FC-specific RNA:

Mycelial mats (cf. above) were lyophilized, and nucleic acid was extracted (Kim *et al.* 1990). After spectrophotometric quantitation, the mycelial nucleic acid's specific hybridization to FC-RNA was titrated in a dot-blot assay (Haber *et al.* 1992). Leaf disks were sampled from transmission controls and *Pythium*-incubated seedlings with putative symptoms, and probed with FC-RNA (Haber *et* al. 1992).

Results and Discussion

Pythium *isolates from FC soils:*

Among the fungi isolated from barley seedlings, several isolates were morphologically similar to a virulent *Pythium* found recently in Alberta, Canada (Huang *et al.* 1992), and were selected for further investigation. Additional tests including analyses of isozyme polymorphism performed on two of these isolates showed they were both a sexually sterile form of *P. ultimum* Trow var. *ultimum* (data not shown).

FC-RNA in Pythium *extract:*

Nucleic acid purified from cultured mycelium of certain *Pythium* isolates from FC soils hybridized stringently with FC-RNA (Fig. 1, Table 1). The hybridization signal increased with amount of *Pythium* nucleic acid loaded; FC-RNA was ca. 10^{-6} of total nucleic acid (Fig. 1) for isolate SH 1 and ca. 10^{-3} for SH 12 (not shown), an isolate of *P. arrhenomanes* Drechs. (Table 1). Two isolates of *P. irregulare* Buisman shown earlier to contain abundant dsRNA (Kim et al. 1990) did not hybridize with FC-RNA probes (Fig.1, Table 1).

FC transmission mediated by Pythium*:*

Depending on experiment, 5-25% of wounded seeds placed in soil inoc-

ulated with FC-positive *Pythium* isolates gave rise to seedlings with FC symptoms in which the chlorotic areas contained FC-RNA (Fig. 2); seeds placed in soil inoculated with FC-negative *Pythium* isolates (Table 1) or in non-inoculated soil gave rise to symptomless seedlings that lacked FC-RNA. In 1.5 mm disks sampled from leaf areas with the most advanced FC symptoms, FC-RNA was as much as 0.1% of the tissue dry mass, indicating that FC-RNA introduced by *Pythium* had replicated extensively in barley host seedlings.

We are currently examining *Pythium* isolates from diverse geographic origins, particularly those from cereal crops. Such studies will help address the question whether FC-related sequences are more widespread in *Pythium* populations, and possibly shed light on the origin of the flame chlorosis virus-like agent.

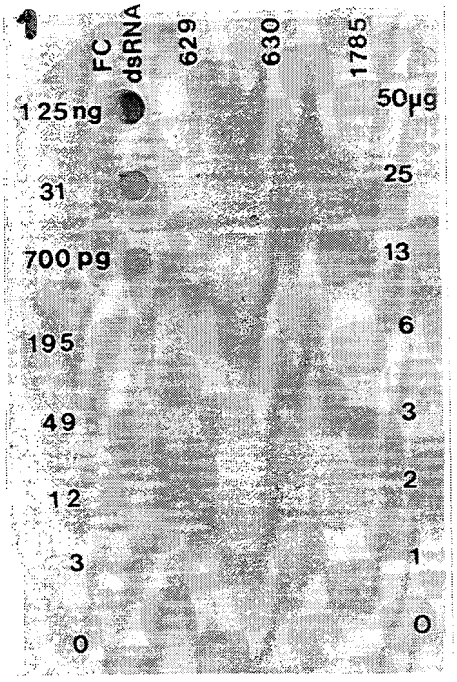


Figure 1. Dot-blot hybridization to labelled FC-RNA. FCdsRNA was purified from FC-affected leaf tissue. Tracks 629, 630 and 1785 represent dilution series of purified total nucleic acid extracted, respectively, from *Pythium* isolates BR 629, BR 630 and SH 1 listed in Table 1.

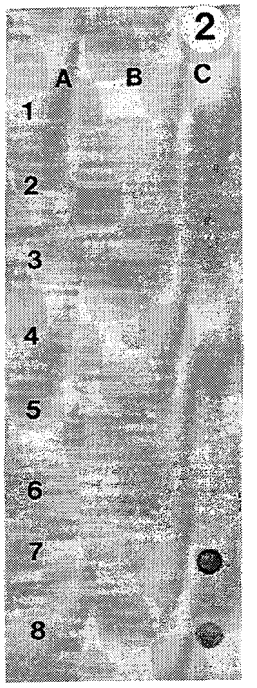


Figure 2. FC-RNA in leaf disks from seedlings grown in *P.ultimum* SH 1- or in non-inoculated soil. A1-A5, yellow streak (SH 1); A6-A8, chlorotic spot (SH 1); B1-B5, oldest chlorosis (SH 1); B6, edge of streak (A1); B7, centre of B1; B8, early chlorosis (A5); C1, green tissue (control soil); C2-C3, early chlorosis (A2); C4-C6, negative control barley (grown separately); C7-C8, FC-positive control (field transplant with characteristic symptoms).

Table 1. Hybridization of *Pythium* nucleic acid extracts with FC-RNA in dot-blot assay

Isolate No.	Taxon	Plant host	Geographic origin	FC at site	Hybridization with FC-RNA
BR 629	*P.irregulare*	cucumber	Edmonton, Alberta	(-)	(-) }abundant
BR 630	*P.irregulare*	cucumber	Edmonton, "	(-)	(-) }ds RNA
SH 1	*P.ultimum* G1*	barley	Foxwarren, Manitoba	(+)	(++)
SH 5-C	*P.ultimum* G1	barley	Niverville "	(+)	(+)
SH 7	*P.ultimum* G1	barley	Niverville "	(+)	(+)
SH 9	*P.ultimum* G2	barley	Niverville "	(+)	(+)
SH 12	*P.arrhenomanes*	barley	Minnedosa "	(+)	(++++)
SH 14	*P.ultimum* G1	barley	Minnedosa "	(+)	(+/-)
SH 20	*P.ultimum* G1	wheat	Niverville "	(+)	(+)
SH 21	*P.ultimum* G1	barley	Niverville "	(+)	(+)

* G1 and G2 are morphologically different globose forms of vegetative mycelium and are designations that distinguish these asexual isolates.

References

Adams, M.J. (1991). Transmission of plant viruses by fungi. Ann. appl. Biol. 118:479-492.

Chong, J. and Haber, S. (1992). Cytological alterations associated with flame chlorosis, a novel viruslike disease of barley, wheat and oat. Phytopath. 82:815-821.

Haber, S. and Harder, D.E. (1992). Green foxtail (*Setaria viridis*) and barnyard grass (*Echinochloa crusgalli*), new hosts of the virus-like agent causing flame chlorosis in cereals. Can. J. Plant Pathol. 14:278-280.

Haber, S., Kim, W., Gillespie, R., and Tekauz, A. (1990). Flame chlorosis: a new, soil-transmitted virus-like disease in barley in Manitoba. J. Phytopath. 129(4):245-256.

Haber, S., Barr, D.J.S., and Platford, R.G. (1991). Observations on the distribution of flame chlorosis in Manitoba and its association with certain zoosporic fungi and the intensive cultivation of cereals. Can. J. Plant Pathol. 13:241-246.

Haber, S., Wakarchuk, D.A., Cvitkovitch, S.E., and Murray, G. (1992). Diagnosis of flame chlorosis by detection of disease-specific RNA with digoxigenin-labeled RNA probes. Plant Dis. 76:590-594.

Haber, S., Prasar, S., and Murray, G. (1993). Triticale confirmed as a host of the viruslike agent causing flame chlorosis in cereals. Plant Dis. 77:536.

Hiruki, C. and Teakle, D.S. Soil-borne viruses of plants. Pages 177-215 in K.F. Harris, ed. Current Topics in Vector Research. Springer-Verlag, New York, 1987.

Huang, H.C., Morrison, R.J., Muendel, H.-H., Barr, D.J.S., Klassen, G.R., and J. Buchko. (1992). *Pythium* sp. "group G", a form of *Pythium* causing damping-off of safflower. Can. J. Plant Pathol. 14:229-232.

Kim, W.K., Mauthe, W., Hausner, G., and Klassen, G.R. (1990). Isolation of high molecular weight DNA and double-stranded RNAs from fungi. Can. J. Bot. 68:1898-1902.-

Zhang, L., Zitter, T.A., and Lulkin, E.J. (1991). Artificial inoculation of maize white line mosaic virus into corn and wheat. Phytopath. 82:397-400.

IMMUNOGOLD CYTOCHEMISTRY DEMONSTRATES THE PRESENCE OF MAIZE DWARF MOSAIC VIRUS INSIDE *PUCCINIA SORGHI* UREDIOSPORES CONFIRMING THE VECTOR ABILITY OF THIS FUNGUS.

M.B.von Wechmar and M.A. Jaffer

Department of Microbiology, University of Cape Town, Private Bag, Rondebosch, 7700, South Africa.

Summary

Maize dwarf mosaic potyvirus (MDMV) has previously been shown to be transmitted by *Puccinia sorghi* urediospores. The presence of virus could be demonstrated on the surface of urediospores by indirect immunofluorescence, but it was not clear whether transmission was incidental by surface contamination of the urediospore with virus, or whether a more intimate relationship existed between the rust fungus and the virus, thus facilitating virus transmission. Immunogold cytochemistry on ultrathin sections of *P. sorghi* urediospores from MDMV-infected plants (fungus vectored virus infection) with MDMV-B specific antiserum, provides evidence for the presence of MDMV particles inside the urediospore matrix mainly inside lipid appearing bodies and in the spore germ tube. These structures were absent in controls. The active vector ability of *P. sorghi* urediospores to transmit MDMV has important epidemiological implications. This is the first report presenting evidence of the presence of MDMV inside the *P. sorghi* urediospore and confirms the vector ability of the fungus reported previously.

Introduction

Maize dwarf mosaic virus strain B (MDMV-B) infection was maintained in a plant growth room on maize co-infected with *Puccinia sorghi*. Urediospores dropping off and/or washing off onto healthy maize seedlings placed below older plants caused seedlings to become MDMV-B infected. The *P. sorghi* fungus maintained itself on the same maize plants thus causing the maize plant to be infected with two obligate parasitic pathogens. The cycle of virus and fungus infection was maintained over five years in a plant growth room. A virus-free *P. sorghi* infection was maintained in the same growth room in a location upstream of air currents and the flow of the automatic humidifier. Throughout the entire period the control *P. sorghi* isolate remained virus-free. Investigations into the vector ability of *P. sorghi* urediospores and the presence of virus on the urediospore surface were reported previously (von Wechmar *et al*, 1992). The efficiency of virus transmission and the strong immunofluorescence of urediospores in uredia with virus specific antisera (von Wechmar *et al*, 1992) suggested that the virus had a more firm association with the fungal spore. In this paper we describe the detection of MDMV inside the urediospore and the urediospore germ tube, and in maize tissue sections with immunogold cytochemistry.

Materials and methods

Preparation of maize infected with P. sorghi and MDMV-B:
The conditions for growing maize seedlings, maintaining the virus-free and the virus-infected fungus , and the MDMV-B isolate used, were the same as reported previously (von Wechmar *et al*, 1992). A new cycle of infection was started by sap-inoculating maize seedlings (cv. Potchefstroom Pearl susceptible to MDMV-B and *P. sorghi*) with MDMV-B and co-infecting the same plants with urediospores from the virus-free source. Urediospores were suspended in distilled water containing 0.01% Tween-20 and the spore suspension dropped into leafwhorls of young plants. A high relative humidity of about 70% was maintained in the plantroom by an automatic humidifier to facilitate the fungus infection in the leaf whorls. Urediospores developing on the virus-infected plants were allowed to infect a new set of seedlings to establish a fungus-vectored MDMV-B infection (von Wechmar *et al* 1992). Leaf tissue with young uredia were collected for embedding. Mature urediospores were collected by tapping free-falling spores into glass petri dishes. Leaf sections with uredia and mature

urediospores were also collected from the virus-free *P. sorghi* infected maize plants. Urediospores were germinated prior to fixing and embedding (Erasmus, 1982).

Preparation of thin sections and immunocytochemistry:

Embedding, thin sectioning and immunogold labelling were described previously (Laubscher *et al*, 1992). The anti-MDMV-B-IgG used was the same as was used in the earlier investigation and was shown to be MBMV-B specific (no reaction with MDMV-A) (von Wechmar *et al*, 1992). Prior to using the anti-MDMV-B-IgG on the thin sections, its specificity for the MDMV-B isolate was re-confirmed by immuno-specific electron microscopy (ISEM). A non-related potyvirus antiserum (ie zucchini yellow mosaic virus, (ZYMV)) was used as a control.

Results

The specificity of the anti-MDMV-B-IgG for the virus is illustrated by strong decoration of virions with antibodies (Fig. 1b). Fig. 1c illustrates a urediospore germ tube with IGL throughout the tube matrix. Fig 1g illustrates a germ tube prepared by indirect immunofluorescent labelling. Dense IGL is visible inside a hypha (Fig. 1d) in leaf tissue. The hyphal tip is clearly visible. In sections through germinated urediospores, IGL occurs mainly in lipid bodies scattered throughout the spore matrix (Fig. 1e). The arrangement of the IGL follows the curvature of the filamentous poty virions illustrating the specificity of IGL detection. Urediospores from non-virus infected control plants show less lipid bodies and no IGL (Fig. 1f). The matrix of these spores also appear more uniform (Fig. 1f). Sections of leaf tissue from control plants with uredia (no virus infection) show no IGL in the cell wall or cytoplasm (not shown). Differences observed between thin sections of control plants and sections of MDMV-infected plants cannot be ascribed to differences in age, as in both cases spores from early maturing uredia were collected. A section through virus infected leaf tissue illustrates IGL in the cell wall and cytoplasma (Fig. 2a). A similar section prepared with antiserum to ZYMV, is illustrated in Fig. 2(b). No IGL was detected.

Discussion

Specific IGL was detected in the urediospore germ tube, fungal hypha *in situ* and in lipid bodies inside the urediospore. No IGL was detected in control urediospores or leaves of plants hosting control *P. sorghi*. Detection of MDMV-B in *P. sorghi* urediospores and the spore germ tube in particular indicate that the virus is present in spores originating from MDMV infected source plants and could explain their efficiency in vectoring the MDMV potyvirus.

The high concentration of virus shown to be present in individual spores (Fig. 1e) and in the germ tube (Fig. 1c) and the efficiency with which urediospores vector MDMV (von Wechmar *et al*, 1992), raises the question whether the obligate fungal parasite with its unique survival mechanism also serves as host to MDMV. This aspect should be investigated.

Considering the fact that *P. sorghi* is an obligate parasite having evolved with its open-pollinated hosts in Latin America (Hooker, 1985), it is feasable to assume that the interaction of *P. sorghi* with MDMV evolved at the same time, particularly in view of the limited host range of both *P. sorghi* and MDMV. Where two obligate parasites share one host it is essential that a mutualistic tolerance exists in order to survive.

Discussing the defense of maize against rust fungi in the center of evolution, Wahl *et al* (1984) refer to an earlier analysis by Borlaug (1972). They quote from his work as follows: "Although one or the other of these rusts, *P. sorghi* or *P. polysora* is commonly found infecting nearly every plant of maize throughout its natural host range in Mexico, Central America and Northern South America, infection seldom occurs in sufficient intensity to cause appreciable damage, except rarely and locally" where the equilibrium existing between hosts and pathogens is upset. Borlaug concluded that a host-parasite balance conditioned by general resistance "*is established on the basis of both latitude and elevational environments resulting in harmonius survival of host and pathogen with little damage being done to either*". It is probable that MDMV could have played a part in this host-parasite balance. In the case of *P. tritici* vectoring brome mosaic virus (BMV) (von Wechmar, 1980; Erasmus and von Wechmar,

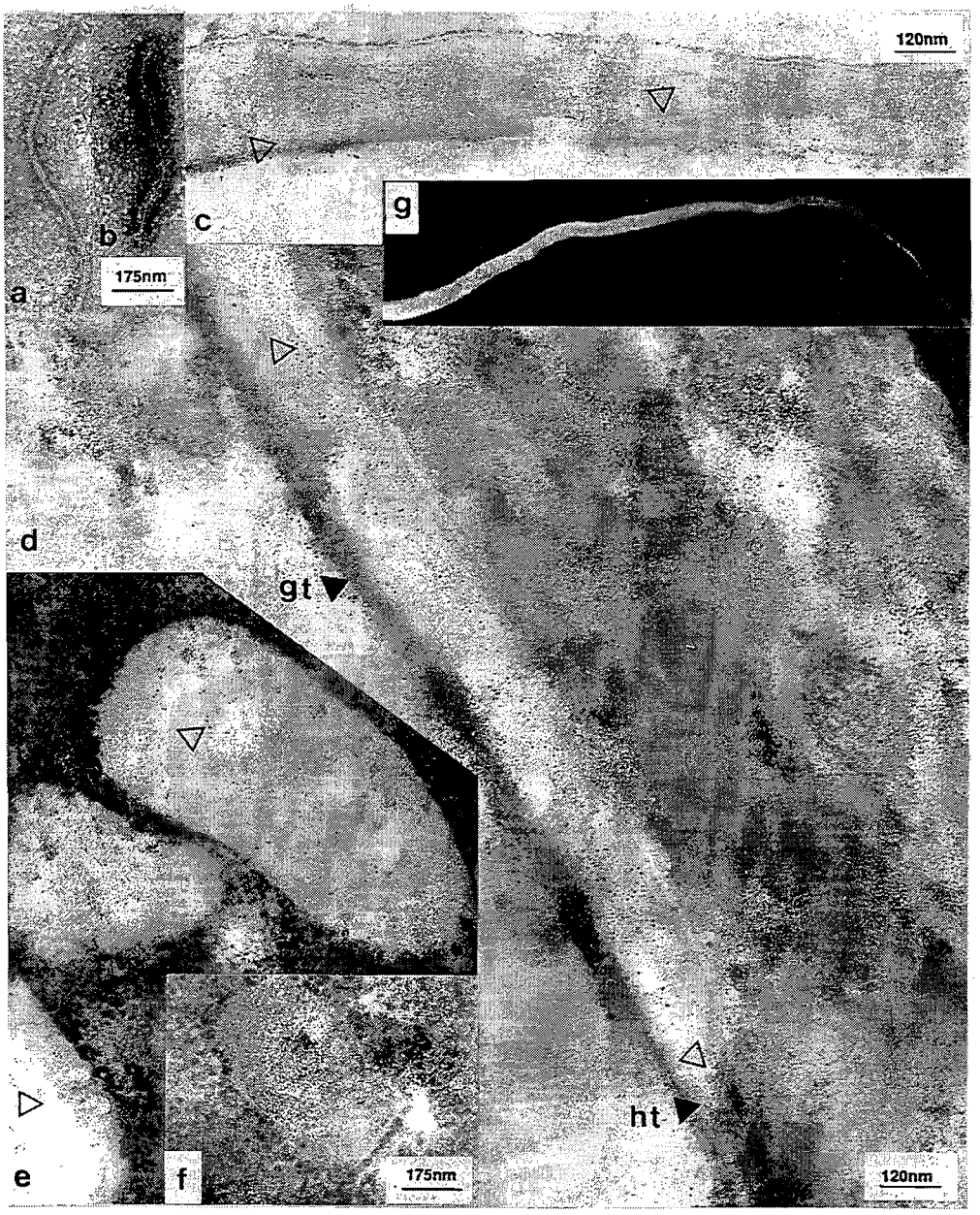


Fig. 1. MDMV-B particles: (a) negative stain, (b) ISEM with anti-MDMV-B-IgG. (c) MDMV-B positive urediospore germ tube on leaf surface and (d) in leaf tissue IGL with anti-MDMV-B IgG (ht, hyphal tip; gt, germ tube). (e) MDMV-B positive urediospore and (f) control urediospore IGL with MDMV-B-IgG. Note difference in structure of matrix. △ shows position of IGL. (g) As in (c) but immunofluorescent labelled with anti-MDMV-B-IgG.

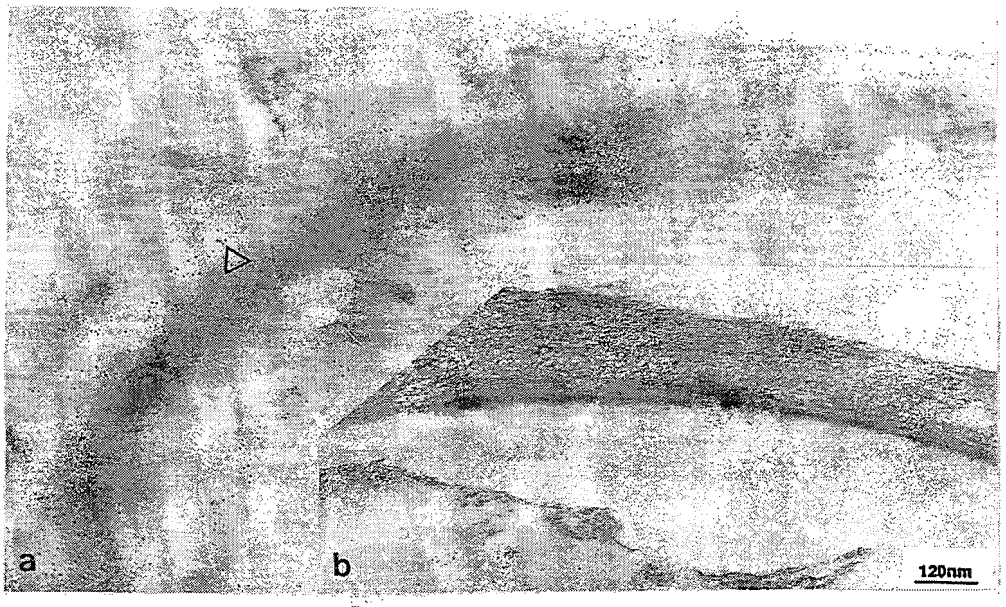


Fig. 2. MDMV-B vectored infection of maize leaf with IGL (a) with anti-MDMV-B IgG (b) anti-ZYMV-serum (negative control).

1983a), the presence of the BMV in the host, changed the fungus reaction type from severe to less severe (Erasmus and von Wechmar, 1983b), thus limiting the destructive action of the fungal obligate parasite.

Earlier work by Erasmus (1982) and Erasmus *et al* (1983) showed the presence of BMV inside urediospores of *P. tritici*. Two different viruses have thus been shown to be vectored by *Puccinia* urediospores. Surely more similar interactions exist. A better understanding of these fungal-virus associations may cast light on complex epidemiological situations.

References

Erasmus, D. S. (1982). The association of bromegrass mosaic virus (BMV) with *Puccinia graminis tritici*. MSc Thesis, University of Cape Town, p177.

Erasmus, D. S., and von Wechmar, M. B. (1983a). The association of brome mosaic virus and wheat rusts: I Transmission of BMV by urediospores of wheat stem rust and leaf rust. *J. Phytopathol.* **108**:26-33.

Erasmus, D. S., and von Wechmar, M. B. (1983b). Brome mosaic virus reduces susceptibility of wheat to stem rust (*Puccinia graminis tritici*). *Plant Dis* **67**:1196-1198.

Erasmus, D. S., Rybicki, E. P. and von Wechmar, M. B. (1983). The association of brome mosaic virus and wheat rust: II Detection of virus in/on urediospores. *J. Phytopathol.* **108**:34-40.

Hooker, A. L. (1985). Corn and Sorghum rusts. In: *The Cereal Rusts* **Vol II**. pp207-236.

Laubscher, J. M., Jaffer, M. A., and von Wechmar, M. B. (1992). Detection by immunogold cytochemical labelling of aphid lethal paralysis virus in the aphid *Rhopalosiphum padi* (Hemiptera:Aphididae). *J. of Invertebrate Pathology* **60**:40-46.

von Wechmar, M. B. (1980). Transmission of brome mosaic virus by *Puccinia graminis tritici*. *Phytopathol. Z.* **99**:289-293.

von Wechmar, M. B., and Knox, E. (1988). Distribution of maize dwarf mosaic virus strain A and B in South African maize, sweet corn, sorghum, several grasses and sugarcane. In: Proceedings of the 8th South African Maize Breeding Symposium. Department of Agriculture, Technical Communication, No **222**, p44-48.

von Wechmar, M. B., Chauham, R., and Knox, E. (1992). Fungal transmission of a potyvirus: urediospores of *Puccinia sorghi* transmit maize dwarf mosaic virus. *Arch Virol* [**Suppl. 5**]:239-250.

Wahl, I., Anikster, Y., Manisterski, J., and Segal, A. (1984). Evolution at the center of origin. In: *The Cereal Rusts* **Voll**. pp39-77.

MUCOR CIRCINELLOIDES VAN TIEGHEM F. LUSITANICUS (BRUDERLEIN) SCHIPPER HOSTS AND VECTORS TOBACCO NECROSIS VIRUS.

M. B. von Wechmar, M. A. Jaffer, and M. Purves

Department of Microbiology, University of Cape Town, Private Bag Rondebosch, South Africa

Summary

The association of tobacco necrosis virus (TNV) with a Mucorales-type fungus (*Mucor circinelloides* van Tieghem f. lusitanicus (Bruderlein) Schipper was reported previously. Immunogold labelling (IGL) utilising TNV-A strain specific antisera prepared against TNV-Braz isolated and multiplied in plants, and TNV-Apt isolated from a bacterial culture grown from mineral apatite rock and multiplied (1X) in plant, was used to detect the presence of TNV in thin sections of the fungal hypha and the yeast morph. IGL was detected in lipid bodies and the cell-wall of hyphae and inside the yeast matrix, cell-wall and external mucous layer and in bacteria present in young yeast. Longevity studies of the fungus in the mycelial morph showed that it survived well at -20° and -70°C, the TNV remaining infectious. At 4-6°C and room temperature the yeast morph only survived after one year and in a one-year old culture bacteria grew containing infectious TNV. Fungicides had no apparent effect on the mycelial morph, but triggered the yeast morph and the release of bacteria. Ciliates were found to assist with the release of TNV from fungal tissue. *M. circinelloides* appears to be a host to TNV generated by bacteria having a mutualistic arrangement with the fungus.

Introduction

Following on the discovery of the association of *Mucor circinelloides* van Tieghem f. lusitanicus (Bruderlein) Schipper with Tobacco necrosis virus (TNV) (von Wechmar *et al*, 1990), twenty other disease problems in crops were investigated where TNV was found to be present in association with fungi and/or bacteria (von Wechmar *et al*, 1991a, 1993a,b). Disease symptoms were not typical for virus infection. Fungi and TNV were also isolated from plant nutrients (von Wechmar *et al*, 1992) and from soil and irrigation water (von Wechmar *et al*, 1991b). In one case TNV was found to be generated in cultures of mineral apatite rock containing bacteria (Jaffer and von Wechmar, 1993 and unpublished results). In a separate study ciliates were found in association with bacteria and TNV in avocado leaves (Jaffer *et al*, 1993 and von Wechmar *et al*, 1993a). These findings stimulated further work on the association of TNV with fungi and bacteria. In this paper we provide evidence for virus export from mycelial and yeast morphs of the fungus, the survival of TNV in association with the fungus, attempts at controlling the fungus hosting TNV, and micro-epidemiology with ciliates.

Materials and Methods

Preparation of thin sections and immunocytochemistry: M. circinelloides growing in the mycelial and in the yeast morph were embedded for thin sectioning and immunogold labelling (IGL), (Laubscher *et al*, 1992). The mycelial culture was grown from sporangiospores on a shallow layer of potato dextrose (PD) and the yeast morph subcultured from a two-year old PD stock-culture (4-6°C) onto PD-agar containing sodium azide (0.0017%).

Antisera production: TNV-Braz was isolated from Passiflora (von Wechmar *et al*, 1991a), propagated in tobacco, the virus isolated and sucrose gradient purified. TNV-Apt was isolated from culture fluid prepared of mineral apatite rock in PD (von Wechmar unpublished results). Concentrated bacteria culture fluid was inoculated onto *Chenopodium quinoa*, the virus isolated, and sucrose gradient purified. Antisera were raised in rabbits. Specificity of antisera to TNV-M.circ. and to TNV-Apt, was checked in Western blots,in immunospecific electron microscopy (ISEM) and with immunogold labelling (IGL).

Longevity of fungus stock cultures: Aliquots of *M. circinelloides* cultures hosting TNV-M.circ. (von Wechmar *et al*, 1990) were stored at -70°C and -20°C in 50% glycerol, in PD at 4-6°C and at room temperature for two years. Survival of fungus was tested by subculturing to PD-agar and virus

infectivity assayed on detached leaves at one and three months, and one and two years after start. Experimental work was done with -20°C cultures.
Fungicide treatment: A broad range of commercial fungicides were tested. A more detailed examination was done with a granular formulation of benzimadazole at 0.5g, 0.05g and 0.005g/100ml PD inoculated with mycelial culture. Survival was assessed by subculturing to PD-agar and nutrient agar.
Micro-epidemiology: Control-ciliates in water culture containing PD were added to fungus mycelial culture. One week later the liquid phase was concentrated by ultracentrifugation and tested for infectivity, TNV particles and for virus protein in Western blotting.
Infectivity assays: Detached leaves of *C. quinoa, Phaseolus vulgaris, Nicotiana tabaccum* cv. Xanthi and *N. benthamiana* were used. Inoculated leaves were maintained on wet towel paper and covered with plastic.

Results

Antisera utilised in IGL studies bound strongly to virus protein of TNV-M.circ and TNV-Apt in Western blots (result not shown) and to TNV-Apt (1Xplant) in IGL and ISEM (Fig. 1a & b). In Fig. 1c IGL is visible in the distended hyphal wall and in underlying cytoplasmic regions showing signs of disturbance compared to a normal appearing hyphal wall (inset). A higher density of IGL was noted in lipid-rich droplets in the stroma (Fig. 1d). The conidiospore in Fig 1e illustrates a mucous layer specked with IGL. Fig. 2(a) illustrates IGL in the cell wall of a young yeast cell. In an older cell (Fig. 2b) IGL is visible in the disintegrating cytoplasmic matrix and the start of mucous shedding from the surface. Dense IGL is visible on mucous released from an older cell (Fig. 2c). Immunofluorescence of the yeast morph is shown in Fig. 2d. IGL studies confirm earlier results obtained with immunofluoresce (von Wechmar *et al*, 1990, von Wechmar and Jaffer, 1990, von Wechmar *et al*, 1991c).

The fungus maintained at -70° and -20°C retained its ability to grow as mycelium and continued to host infectious TNV for two years (longest time tested). Cultures at 4-6°C and room temperature lost their ability to produce mycelium after one year and continued in the yeast morph (Cihlar, 1985). One culture at room temperature produced only bacteria on subcultures. Virus isolated from the yeast morph and the bacteria was infectious to plants. Fungicides tested had no apparent effect on the fungus. Benzimadazole treated cultures tested after one year subcultured to PD-agar showed active growth of the yeast morph and the presence of Bacilli-like bacteria. The mycelium in the control treatment with no fungicide was dead. Ciliates thrived on mycelium with spores and appeared larger compared to control cultures. Electron microscopy revealed a high count of TNV particles in the ciliate culture concentrate and infectivity assay on *C. quinoa* and other leaves produced many lesions providing evidence for the release of infectious TNV.

Discussion

Immunocytochemistry provides evidence for the firm association of TNV with the dimorphic fungus *M. circinelloides*. Export of virus is visible through the distended hyphal wall and more clearly in the ageing yeast cell wall with TNV being sloughed off with the mucous layers. The entrapment of TNV in the mucous explains difficulties experienced previously with the release of infectious virus from culture fluid. The fungus itself is not a pathogen but harbours a virus that can be pathogenic to higher plants. Fungicides designed to hit plant pathogenic fungi apparently do not affect the mycelial morph of the fungus, but initiate the "switch on" of the yeast morph and the release of bacteria which appear to have a mutualistic arrangement with the fungus. The yeast morph appears to generate and export TNV actively and has been found inside plant tissue (unpublished results). Circumstantial evidence indicates that *M. circinelloides* harbours bacteria which generate a virus similar if not identical to the TNV-Apt isolated from mineral apatite rock (Jaffer and von Wechmar, 1993). The discovery of a bacterial host for TNV supports views that it may indeed behave like a RNA phage under certain circumstances (Fraenkel-Conrat, 1988). Ciliates found in water and in plant tissue appear to play a function in releasing TNV and may explain the presence of free infectious virus particles recovered from water sources (von Wechmar *et al*, 1991b). The question whether *M. circinelloides* is a host or a vector is debatable. Our current view is that it fulfills both rolls.

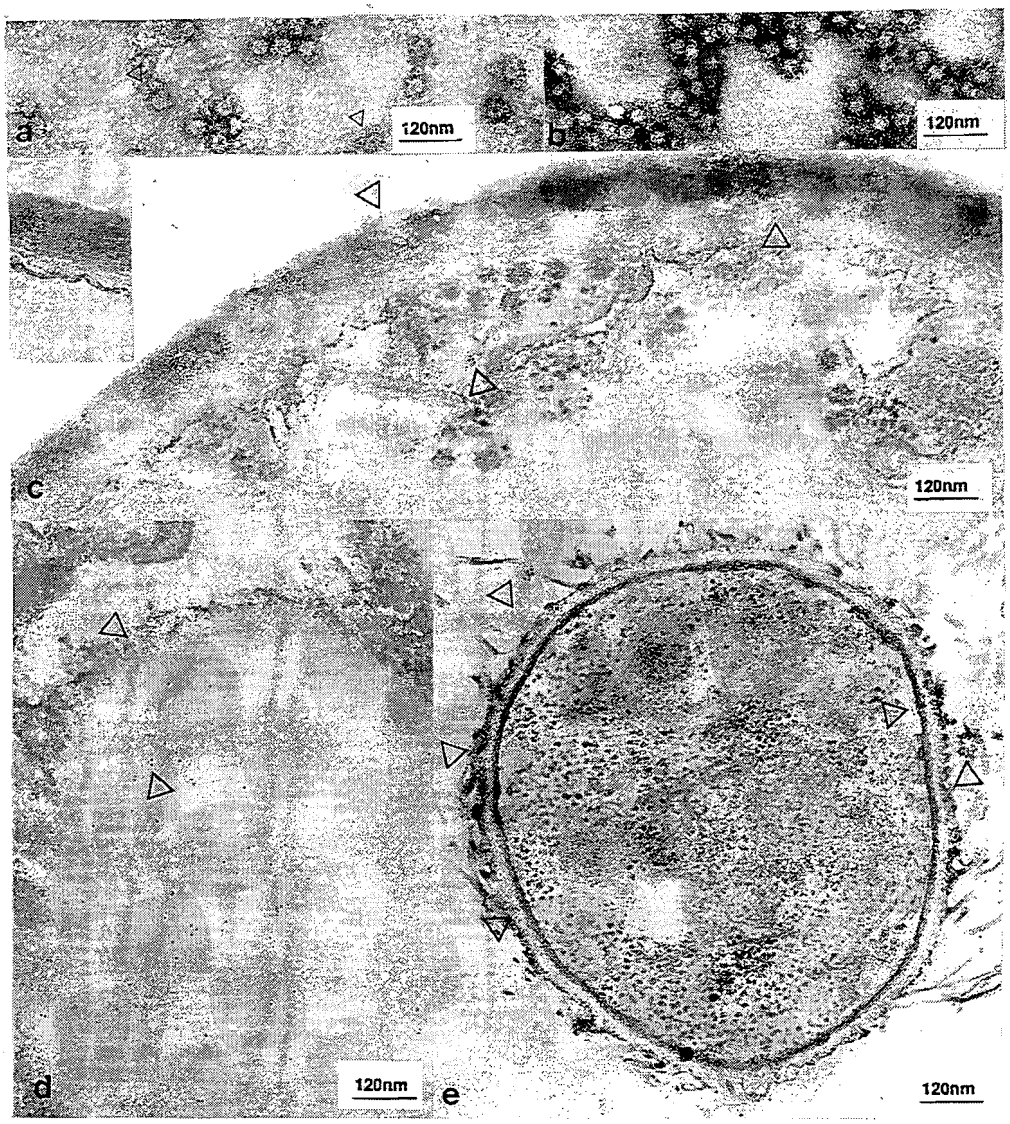
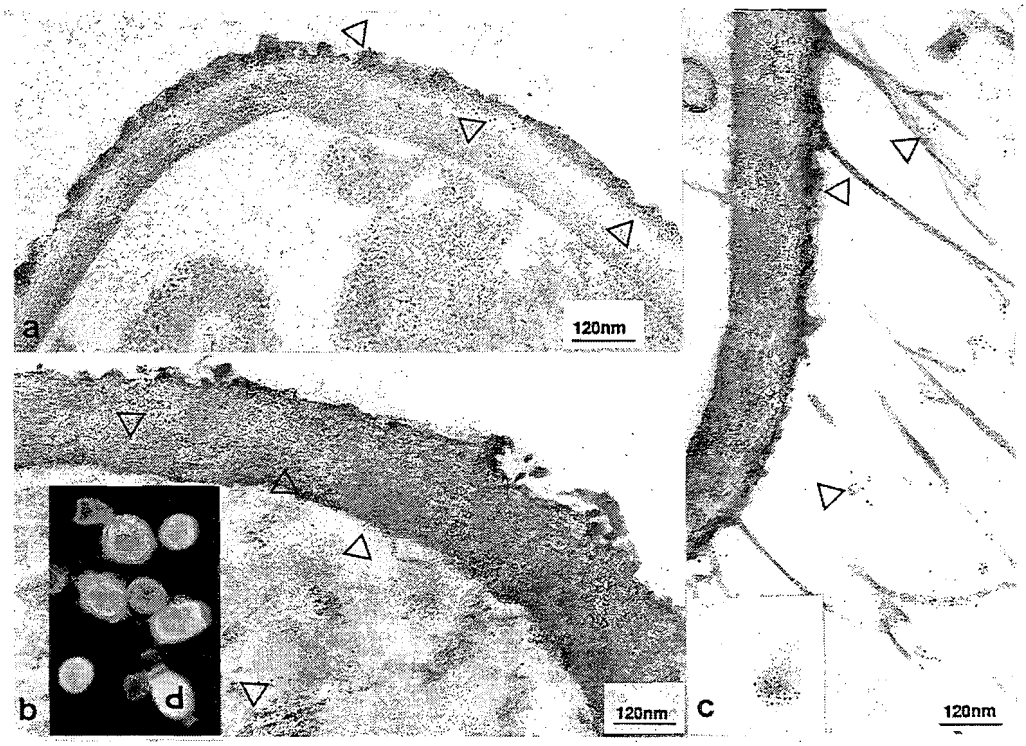


Fig 1. (a). TNV-Apt isolated from *C. quinoa* labelled with anti-TNV-Braz and IGL and (b) ISEM with anti-TNV-Apt. (c) & (d) Thin sections of *M. circinelloides* hyphae and (e) a conidia-spore IGL with anti-TNV-Apt (c & e) and anti-TNV-Braz (d). △shows position of IGL.

Fig. 2 (a - c) Thin sections of the yeast morph IGL with anti-TNV-Apt (a & c) and anti-TNV-Braz (b). (d) Immunofluorescent labelling of the yeast morph (von Wechmar *et al*, 1990).
△shows position of IGL.



References

Cihlar, R. L. (1985). Morphogenesis and dimorphism of Mucor. In: *Gene Manipulations in Fungi* (J.W. Bennett and L.L. Lasure eds), Academic Press, Inc, pp449-467.

Fraenkel-Conrat, H. (1988). Tobacco necrosis, Satelite Tobacco necrosis and related viruses. In: *The Plant viruses* (R. Koenig ed.), Plenum Press, New York. Vol. 3:147-161.

Jaffer, M. A., and von Wechmar, M. B. (1993). Immunocytochemistry detects tobacco necrosis virus in spores of Bacillus-type bacteria cultured from mineral apatite rock. Communications. *Electron. Microsc. Soc. South Afr.* **23**: (In press).

Jaffer, M. A., von Wechmar, M. B., and Purves, M. (1993). Detection of tobacco necrosis virus associated with Bacillus-type bacteria in thin sections of avocado leaf tissue by immunogold cytochemistry. Communications. *Electron. Microsc. Soc. South Afr.* **23**: (In press).

Laubscher, J. M., Jaffer, M. A., and von Wechmar, M. B. (1992). Detection by immunogold cytochemical labelling of aphid lethal paralysis virus in the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae). *J. of Invertebrate Pathology* **60**:40-46.

von Wechmar, M. B., and Jaffer, M. A. (1990). Identification of Plant Pathogenic viruses in Fungal Hyphae by Immunofluorescence and Electron Microscopy. Communications. *Electron. Microsc. Soc. South Afr.* **20**:129-130.

von Wechmar, M. B., Purves, M., Lindsey, S., and Jaffer, M. A. (1990). Fungus, a host of maize infecting tobacco necrosis virus. Proceedings: First Symposium of the International Working Group on Plant Viruses with Fungal Vectors. *German Phytomedical Society Series.* **1**:149-152.

von Wechmar, M. B., Collett, H., Motyer, S., and Purves, M. (1991a). Tobacco Necrosis Virus - a common virus disease of Passiflora in South Africa. Abstract, *J. S. Afr. Soc. Hort. Sci.***1**:8.

von Wechmar, M. B., Purves, M., Illing, C., and Lindsey, S. (1991b). Identification of plant pathogenic viruses in soil and irrigation water. Abstract, *Phytophylactica* **23**:94.

von Wechmar, M. B., Jaffer, M. A., and Purves, M. (1991c). Detection of tobacco necrosis virus in ultra-thin sections of fungal hyphae by immuno-cytochemistry. Communications. *Electron. Miscrosc. Soc. South Afr.* **21**:173-174.

von Wechmar, M. B., Purves, M., Jaffer, M. A., and Willment, J. (1992). Fertilizer and dolomitic lime contaminated with fungal propagules harbouring tobacco necrosis virus, causes endophytic plant disease. Abstract, *J. S. Afr. Soc. Hort. Sci.* **2**:Suppl. 11.

von Wechmar, M. B., Purves, M., and Jaffer, M.A. (1993a). Examination of graft transmissible dwarfing agents in avocado. Abstract, *J. S. Afr. Soc. Hort. Sci.* **3**:28.

von Wechmar, M. B., Purves, M., and Jaffer, M. A. (1993b). The cause of freckle spots on papaya fruit: virus, fungus, or both? Abstract, *J. S. Afr. Soc. Hort. Sci.* **3**:12.

TRANSMISSION OF SEVEN CUCURBIT VIRUSES BY HOST-SPECIFIC STRAINS OF OLPIDIUM RADICALE. R. N. Campbell & S. T. Sim, Department of Plant Pathology, University of California, Davis, CA 95616

Twelve isolates of O. radicale belonging to three host specific strains (Campbell & Sim. 1992. Phytopath. 82:1098) were tested in all combinations with seven viruses for in vitro acquisition and transmission to roots of watermelon seedlings, a common host for all fungal strains. Five isolates of the cucumber strain transmitted cucumber leaf spot virus (CLSV), cucumber necrosis tombusvirus (CNV), melon necrotic spot carmovirus (MNSV) and squash necrosis virus (SqNV) but not cucumber soil borne virus (CSBV), petunia asteroid mosaic tombusvirus (PAMV), or tobacco necrosis necrovirus (TNV). Five isolates of the melon strain transmitted MNSV; varied in ability to transmit CLSV, CNV, and SqNV; and did not transmit CSBV, PAMV, or TNV. Two isolates of the squash strain transmitted CSBV, MNSV, and SqNV; were variable in transmission of CNV; and did not transmit CLSV, PAMV, or TNV.

MECHANISMS OF RESISTANCE TO *POLYMYXA BETAE* IN WILD *BETA* SPECIES

M.J.C. Asher and K.J. Barr

IACR Broom's Barn Experimental Station, Higham, Bury St Edmunds, Suffolk IP28 6NP, UK

Summary

The resistance of *Beta patellaris* and *Beta procumbens* to *Polymyxa betae* has been examined to determine at what stage during the infection process resistance is expressed. Fluorescence microscope studies of labelled zoospores have shown that these attach to and appear to infect the root hairs of both resistant and susceptible (*B. vulgaris*) hosts. However, no further plasmodial development was observed in light and electron microscope studies of resistant spp., suggesting that resistance is expressed at a very early stage. The high level of partial resistance observed in a population of *B. maritima* is also being examined and introgressed into sugar beet.

Introduction

Polymyxa betae, an obligate parasite of sugar-beet roots and the vector of beet necrotic yellow vein virus (BNYVV) is found in almost all soils where the crop is grown (Payne & Asher, 1990). The fungus spreads from plant to plant by means of motile zoospores (Keskin, 1964) and survives in the soil for long periods in the form of thick-walled resting spores or cystosori (Abe & Tamada, 1986).

All varieties of sugar beet (*Beta vulgaris*) are susceptible to the fungus (Bolz and Koch, 1983; Paul, 1990) as were the species *B. perennis* and *B. orientalis* tested by Fujisawa and Sugimoto (1979). However, *B. patellaris* and *B. procumbens*, and hybrids between these species and *B. vulgaris*, have been found to exhibit almost complete resistance to *P. betae* (Fujisawa & Sugimoto, 1979), sufficient to prevent or greatly inhibit virus entry and multiplication (Paul *et al.*, 1991; Paul *et al*, 1992). Examination of monosomic addition lines in *B. vulgaris* has shown that the vector resistance in *B. procumbens* is largely controlled by genes on two of the nine chromosomes (Paul *et al.*, 1992). We have used artificial inoculation methods and time-course studies to examine the stage at which resistance is expressed in these wild species. In addition, the behaviour of zoospores on the roots of *Beta* species has been studied by means of fluorescence staining and microscopy.

Partial resistance to the fungus has been observed in some populations of the wild species, *B. maritima* (Fujisawa & Sugimoto, 1979; Asher & Barr, 1990). This species is of interest to plant breeders as it readily hybridizes with *B.vulgaris* and the results of further inbreeding and selection within a resistant population are reported here.

Resistance in B.patellaris and B.procumbens

Root colonization:

Techniques developed to examine the progress of infection of *B.vulgaris* by *P.betae*, following exposure of roots to zoospore suspensions (Barr, 1992), were also used with the wild species. Zoospore inoculum was derived from mature zoosporangia within 17 day old *B.vulgaris* seedlings 10 days after inoculation (Barr & Asher, 1992). These heavily infected donor seedlings were co-incubated with 7 day old seedlings of the wild species in 5 ml quarter-strength Hoaglands nutrient solution for 15h at room temperature. 7 day-old *B.vulgaris* seedlings were included as controls. Following inoculation, seedlings were potted up individually in sterilized sand and grown on in a controlled environment room at 20°C with 16h light. Plants were sampled daily for two weeks following inoculation and 2 cm root segments fixed, dehydrated and embedded in Araldite CY 212 prior to sectioning (Barr & Asher, in press). Semi-thick sections were stained using the methylene blue-azure II-basic fuchsin staining procedure (Buczacki & Moxham, 1979) and examined by light microscopy.

In *B.vulgaris*, the first plasmodia were observed in epidermal cells 48h after inoculation. Thereafter, extensive infection of the epidemis occurred, with the production of zoosporangia in the majority of cells by 8 days after inoculation (Fig 1a; from Barr & Asher, in press). In later sections zoosporangia were found in progressively deeper layers within the cortex and resting spores (cystosori) were observed 14 days after inoculation. In contrast, seedlings of *B.patellaris* and *B.procumbens* sampled

at 4 and 7 days after inoculation showed no evidence of *P. betae* infection in epidermal or cortical cells in any of the many hundreds of sections examined (Fig 1b; Barr & Asher, in press).

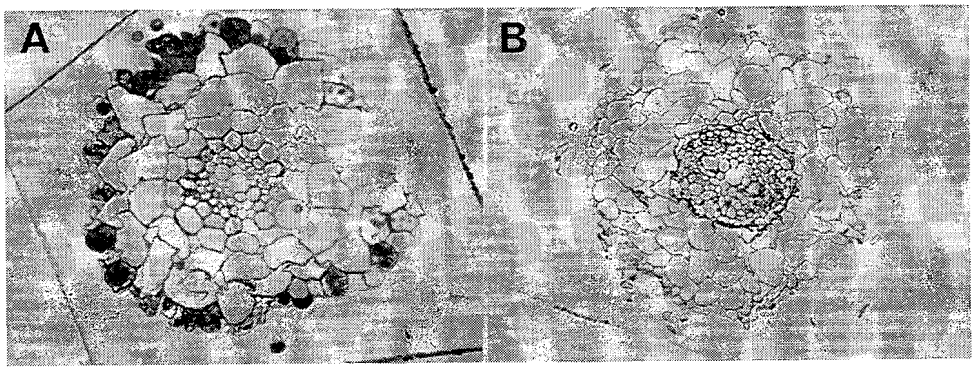


Fig. 1 Tranverse section of lateral root of (a) *B. vulgaris* 8 days after inoculation and (b) *B. procumbens* 7 days after inoculation with *P. betae*.

Zoospore attraction and infection:
The behaviour of zoospores on the roots of susceptible and resistant species was examined using the lipophilic fluorochrome stain, 3,3' dihexyloxacarbocyanine iodine [$DiOC_6(3)$] (Duckett & Read, 1991). Seedlings roots were exposed to freshly released zoospores for 3-4h and then stained for 15 min. The stained material was mounted in water and examined microscopically using a blue excitation filter (450-490 nm) and a 520 nm barrier filter (Barr & Asher, in press). Brightly fluorescing zoospores were seen attached to the root hairs and epidermal cells on the lateral roots of all species (Fig 2); there was no evidence that fewer spores had been attracted to the roots of resistant hosts.

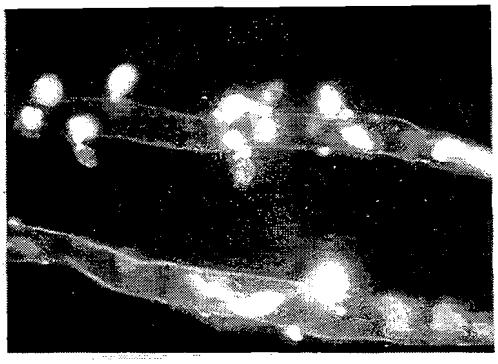


Fig. 2 Fluorescent stained zoospores of *P. betae* attached to root hairs of *B. patellaris*.

Also, in all cases some attached zoospores appeared to have discharged their contents, with only the outer envelope remaining. Corresponding to the point of attachment of these empty zoospores, brightly fluorescing material, presumed to be of fungal origin, could be seen within the root hair (see Fig 2). This suggests that zoospores are able to infect both susceptible and resistant *Beta* species.

Resistance to B. maritima

The identification of highly resistant segregants in a wild population of *B. maritima* (BB19) exhibiting partial resistance to *P. betae* was reported at the First Symposium (Asher & Barr, 1990). Three of these highly resistant segregants of BB19 were subsequently intercrossed in isolation and 140 randomly selected progeny from these plants screened for resistance to *P. betae* in a glasshouse test. All progeny were grown, along with *B. vulgaris* as controls, in naturally infested soil at 20°C for 6 weeks and the roots then examined microscopically. The level of infection was scored on a 0-5 scale of increasing severity according to the density of fungal resting spores in the fibrous roots. In contrast to the *B. vulgaris* population, the frequency distribution of disease severity was strongly skewed towards resistance. Six plants appeared to be completely free of the fungus and have been cloned for further inbreeding.

At the same time crosses were made between three highly resistant segregants of *B. maritima* BB19 and a male-sterile line of *B. vulgaris*. 67 F1 progeny from this male sterile line and sample populations from the *B. maritima* and *B. vulgaris* parents were assessed for *P. betae* resistance in a preliminary glasshouse test. Fig 3 illustrates the frequency distribution of resistance observed in each of these populations. The F1 hybrid population exhibited the complete range of reactions, from highly resistant to highly susceptible, with a mean disease score value intermediate between the two parents. One fully resistant hybrid, with no evidence of fungal infection, was obtained from the 67 screened.

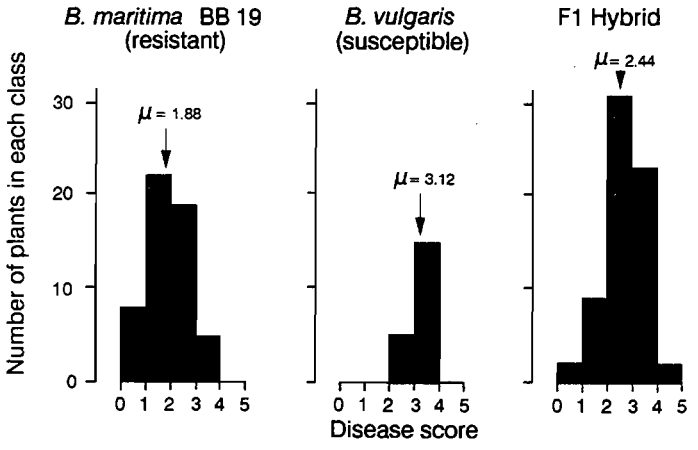


Fig. 3 Frequency distribution of resistance to *P. betae* in populations of *B. maritima* BB19, *B. vulgaris* (male sterile line) and F1 hybrids between them.

Discussion

The failure to observe early stages in the life cycle of *P. betae* in thin sections of *B. patellaris* and *B. procumbens* roots confirms and extends the earlier observations of Fujisawa and Sugimoto (1979) and Paul *et al.* (1991) who reported the absence of cystosori in these species. It suggests that resistance is operating at a very early stage in the infection process. Nevertheless, it appears from the studies of zoospore attachment that the root hairs and epidermal cells of these wild species are susceptible to initial infection. If so, the resistance mechanism may be due to the failure of the introduced fungal cytoplasm to develop into a recognizable plasmodium, possibly involving a hypersensitive host response. In the presence of viruliferous *P. betae* populations the occurrence of initial infection may allow some entry of the virus, since low levels of BNYVV were apparently detected in these species by Paul *et al.* (1991). Whether such resistance, which is under relatively simple genetic control (Paul *et al.*, 1991), would be sufficient to prevent damage from rhizomania disease if transferred to sugar beet remains to be explored. Progress with this is likely to be slowed by the difficulty of hybridizing these wild species with *B. vulgaris*.

Preliminary results following the inbreeding and hybridization of the partial resistance derived from *B.maritima* BB19 demonstrate that highly resistant segregants can be selected from both the parent population and hybrids between *B. maritima* and *B. vulgaris*. Again, the level of vector resistance needed to prevent significant entry or multiplication of the virus needs to be established in these resistant hybrids. Also, given that the resistance appears, from preliminary studies, to be quantitatively inherited, a more rapid screening method needs to be devised to increase the efficiency of selection in a breeding programme. Work is in progress to develop such methods.

References

Abe, H. & Tamada, T. (1986). Association of beet necrotic yellow vein virus with isolates of *Polymyxa betae* Keskin. *Ann. Phytopath. Soc. Japan* **52**, 235-247.

Asher, M.J.C. & Barr, K.J. (1990). The host range of *Polymyxa betae* and resistance in *Beta* species. *Proc. First Symp. Int. Working Group on Plant Viruses, Braunschweig*, Eugene Ulmer & Co. Stuttgart. pp. 65-68.

Barr, K.J. (1992). Aspects of host-parasite relationships of *Polymyxa betae*. *Ph.D. thesis*. University of East Anglia. 109 pp.

Barr, K.J. & Asher, M.J.C. (1992). The host range of *Polymyxa betae* in Britain. *Plant Pathology* **41**, 64-68.

Bolz, G. & Koch, F. (1983). Aussichten der Resistenz-(Toleranz) Züchtung in Rahmen der Bekämpfung der Rizomania. *Gesunde Pflanzen* **35**, 275-278.

Buczacki, S.T. & Moxham, S.E. (1979). A triple stain for differentiating resin-embedded sections of *Plasmodiophora brassicae* in host tissues under the light microscope. *Trans. Brit. Mycol. Soc.* **72**, 311.

Duckett, J.G. & Read, D.J. (1991). The use of the fluorescent dye, 3,3' dihexyloxacarbocyanine iodide, for selective staining of ascomycete fungi associated with liverwort rhizoids and encoid mycorrhizal roots. *New Phytol.* **118**, 259-272.

Fujisawa, I. & Sugimoto, T. (1979). The reaction of some species in *Beta Patellares, Corollinae* and *Vulgares* to rhizomania of sugar beet. *Proc. Sugar Beet Res. Assocn. of Japan* **21**, 31-38.

Keskin, B. (1964). *Polymyxa betae* n. sp. ein Parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugen en twicklung der Zuckerrübe. *Arch. Mikrobiol.* **49**, 348-374.

Paul, H. (1990). Genetic control of rhizomania in sugar beet (*Beta vulgaris*). *Proc. First Symp. Int. Working Group on Plant Viruses, Braunschweig*, pp. 79-82.

Paul, H., Henken, B. & Alderlieste, M.F.J. (1991). Resistance to rhizomania in sugar beet. *In*. Biotic Interactions and Soil-borne Diseases (Eds A.B.R. Beemster, G.J. Bollen, M. Gerlagh, M.A. Ruissen, B. Schippers & A. Tempel). Elsevier, Amsterdam. pp. 95-100.

Paul, H., Henken, B., de Bock, Th. S.M. & Lange, W. (1992). Resistance to *Polymyxa betae* in *Beta* species of the section *Procumbentes* , in hybrids with *B. vulgaris* and in monosomic chromosome additions of *B. procumbens* in *B. vulgaris*. *Plant Breeding* **109**, 265-273.

Payne, P.A. & Asher, M.J.C. (1990). The incidence of *Polymyxa betae* and other fungal root parasites of sugar-beet in Britain. *Plant Pathology* **39**, 443-451.

VARIATION IN THE LEVEL OF INFECTION WITH *POLYMYXA BETAE* AND ITS EFFECT ON INFECTION WITH BEET NECROTIC YELLOW VEIN VIRUS IN BEET ACCESSIONS OF THE SECTIONS *BETA* AND *COROLLINAE*

H. Paul, B. Henken, O.E. Scholten, Th.S.M. De Bock and W. Lange

DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, 6700 AA Wageningen, The Netherlands

Summary

Some variation in the level of infection by *Polymyxa betae* occurred in beet accessions of the section *Beta*, but no high levels of resistance to *P. betae* were found. A few accessions had low concentrations of beet necrotic yellow vein virus (BNYVV). High levels of resistance to *P. betae* and BNYVV were found in *Beta* species of the section *Corollinae*. Low numbers or even the absence of cystosori of *P. betae* often did not result in low virus concentrations. Resistance to *P. betae* seemed to have little effect on the infection with BNYVV and perspectives of its use in breeding sugar beet with resistance to rhizomania are limited.

Introduction

Rhizomania in sugar beet is caused by beet necrotic yellow vein virus (BNYVV). The virus is transmitted by the soil-borne fungus *Polymyxa betae* Keskin. Resistance to *P. betae* could be used as a means to breed sugar beet with resistance to rhizomania. In *Beta vulgaris* subsp. *maritima* (L.) Arcang. of the section *Beta* resistance to both BNYVV (Whitney, 1989) and *P. betae* (Fujisawa and Sugimoto, 1979; Asher and Barr, 1990) was reported. Resistance to *P. betae* was also found in *Beta* species of the sections *Procumbentes* and *Corollinae* (Fujisawa and Sugimoto, 1979).

Using *B. procumbens* Chr. Sm. and monosomic chromosome additions of *B. procumbens* in *B. vulgaris* with high levels of resistance to *P. betae*, some effect of the resistance to *P. betae* on the infection with BNYVV was found (Paul *et al.*, 1992b). In the present study, the relationship between the level of infection with *P. betae* and the level of infection with BNYVV is investigated, using accessions of the sections *Beta* and *Corollinae*.

Materials and Methods

Experiment 1. Plant material consisted of the susceptible sugar beet cultivar Regina, the cultivar Rima, with partial resistance to BNYVV, and several accessions of *B. vulgaris* subsp. *vulgaris* and *B. vulgaris* subsp. *maritima*. Holly-1-4 is a homozygous BNYVV resistant selection from the Holly material (Lewellen *et al.*, 1987). WB42 and WB52 have dominantly, simply inherited resistance to BNYVV (Whitney, 1989).

Experiment 2. Various accessions of the section *Corollinae* were tested for resistance. Dr Lothar Frese from the German-Dutch Beta Genebank is acknowlegded for sending most of the seed samples of the *Corollinae* species.

Seedlings were inoculated in a mixture of sand and soil containing *P. betae* with BNYVV (Paul *et al.*, 1992a). In the first experiment, half the number of plants were

sampled after four weeks and the other half after five weeks. Plants of the second experiment were sampled after four weeks. Roots were homogenised in phosphate buffered saline (PBS) in a ratio 1:20 (w/v), using a Polytron mixer. Resting spores (cystosori) were counted to estimate the level of infection with *P. betae* and ELISA was applied to determine concentrations of BNYVV in the roots (Paul *et al.*, 1992a; 1992b). Plants with estimated virus concentrations below 4 ng/ml were considerd to be free of virus. Only a part of the results of both experiments is given. For a full description of the results, see Paul (1993).

Results

Experiment 1. Both between and within accessions variation was found for the numbers of cystosori (Table 1). None of the plants was free of cystosori and most plants were highly infected with *P. betae*. The lowest number of cystosori was found in the accession BM-8-80-209. Large differences in average virus concentrations between accesions were found, but variation was also present within accessions. More than one plant without virus were found in Rima, Holly-1-4, WB42, and WB52. In one plant of BMA-P9-983 no virus could be detected. Similar results were found at both sampling times. Investigations in the effect of variation in the level of infection with *P. betae* on the infection by BNYVV were difficult, because of the presence of resistance to BNYVV in some accessions. Within the accessions, no effect of variation in the level of infection by *P. betae* on the infection with BNYVV could be found.

Table 1. Average numbers of cystosori of *P. betae* and virus concentrations, with 95% confidence intervals, in accessions of the section *Beta*, after a test period of four and five weeks (entries are $\log_{10}$ of the data; original data in cystosori/mg root and ng/ml)(n=8)

	Four weeks		Five weeks	
Accession	Number of cystosori	Virus concentration	Number of cystosori	Virus concentration
B. vulgaris				
subsp. *vulgaris*				
Regina	2.25 ± 0.33	2.49 ± 0.22	2.43 ± 0.08	2.64 ± 0.25
Rima	2.30 ± 0.31	1.65 ± 0.46	2.51 ± 0.15	1.43 ± 0.73
Holly-1-4	2.34 ± 0.17	1.18 ± 0.47	2.46 ± 0.16	1.01 ± 0.49
subsp. *maritima*				
BM-8-80-209	1.75 ± 0.33	2.57 ± 0.13	2.03 ± 0.38	2.68 ± 0.23
BMH-CHL1	2.04 ± 0.35	2.60 ± 0.21	2.34 ± 0.19	2.62 ± 0.18
WB42	2.31 ± 0.21	0.40 ± 0.28	2.65 ± 0.14	0.44 ± 0.41
BMA-P9-983	2.35 ± 0.24[1]	2.31 ± 0.51[1]	2.35 ± 0.20	1.85 ± 0.67
WB52	2.39 ± 0.14	0.33 ± 0.32	2.61 ± 0.13	0.47 ± 0.31

[1] n=5

Experiment 2. Large variation for the numbers of cystosori and for virus concentrations were found between and within accessions (Table 2). Various accessions of different species had plants with a few or without cystosori. The latter plants were found in the accessions 18253, 17822 and 58248 of *B. corolliflora* Zos., 61241 of *B. lomatogona* Fisch. and Mey. and WB65 of *B. macrorhiza* Stev. Also various accessions of different species had plants with low virus concentrations or no virus. Plants without virus were found in the accessions 18253 and 61227 of *B. corolliflora*, 61218, 17913 and 17967 of *B. intermedia* Bunge and WB23, 61191 and WB5 of *B. lomatogona*. In Fig. 1, the data of the individual plants of four accessions are given as illustration of the various situations that occurred. Low numbers or even the absence of cystosori often did not result in low virus concentrations. A significantly positive correlation between the number of cystosori and virus concentration was only found for the *B. macrorhiza* accession WB65 ($r=0.85$; $P<0.05$).

Table 2. Average numbers of cystosori of *P. betae* and virus concentrations, with 95% confidence intervals, in accessions of the section *Corollinae*, after a test period of four weeks (log_{10} of the data; original data in cystosori/mg root and ng/ml)(n=6)

Accession	Number of cystosori	Virus concentration	Accession	Number of cystosori	Virus concentration
B. vulgaris subsp. *vulgaris* Regina	2.35 ± 0.20	2.42 ± 0.18	*B. corolliflora* BGRC 18253	0.44 ± 0.66	1.52 ± 0.96
			BGRC 61227	1.10 ± 0.58	1.12 ± 1.12
			BGRC 17822	1.42 ± 0.95	2.56 ± 0.29
B. intermedia			BGRC 58248	1.43 ± 0.85	2.15 ± 0.48
BGRC 61218	1.53 ± 0.61	2.21 ± 1.30			
BGRC 17913	1.86 ± 0.34	0.37 ± 0.33	*B. lomatogona*		
BGRC 17967	2.46 ± 0.17	0.42 ± 0.53	BGRC 61241[3]	1.63 ± 1.30	2.04 ± 1.40
			WB23	2.17 ± 0.89	0.67 ± 0.47
B. macrorhiza			BGRC 61191[1]	2.35 ± 0.27	0.91 ± 0.94
WB65	1.06 ± 1.01	2.22 ± 0.48	WB5[2]	2.41 ± 0.29	0.70 ± 0.33

[1] n=3; [2] n=4; [3] n=5

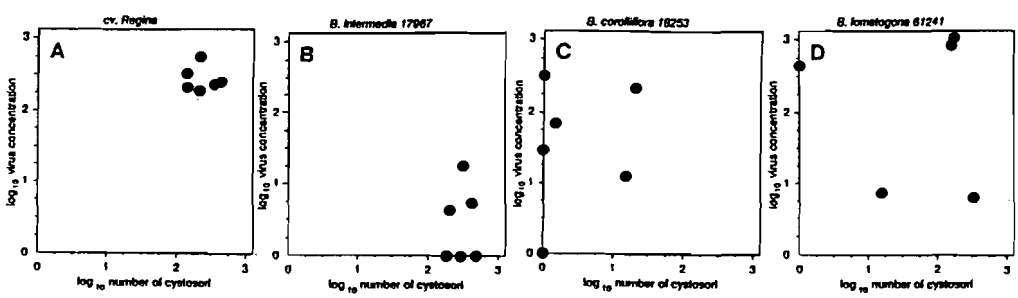


Fig. 1. Variation in the level of infection by *P. betae* and BNYVV. A. Cultivar Regina, with high numbers of cystosori and high virus concentrations; B. *B. intermedia* 17967, with high numbers of cystosori and low virus concentrations; C. *B. corolliflora* 18253, with low numbers of cystosori and low and high virus concentrations; D. *B. lomatogona* 61241, with low and high numbers of cystosori and low and high virus concentrations.

Discussion

Within the accessions of the section *Beta*, no high levels of resistance to *P. betae* were found and no effect of variation in the number of cystosori on the level of infection with BNYVV could be demonstrated. Resistance to *P. betae* was present in *Corollinae* species, thus confirming the results of Fujisawa and Sugimoto (1979). The results of these investigators suggested an absolute resistance to *P.betae*, but the present results indicate high levels of partial resistance. Within the *Corollinae* species also high levels of resistance to BNYVV were found, which has not been reported before. Resistance to *P. betae* did not always result in low virus concentrations. There was only little evidence for an effect of resistance to *P. betae* on the level of infection with BNYVV. Such an effect has been reported from a study with chromosome addition plants of *B. procumbens* in *B. vulgaris*, in which the average numbers of cystosori were significantly correlated with the average virus concentrations, but correlations within accessions were not significant (Paul et al., 1992b). Based on the present results it is expected that quantitatively inherited resistance to *P. betae*, as found in *B.vulgaris* subsp. *maritima* (Asher and Barr, 1990) will have no substantial effect on the infection with BNYVV. Cultivars with resistance to rhizomania can be obtained with resistance to the virus, without resistance to the vector. However, resistance to *P. betae* could help to reduce the inoculum level in the soil and retard the spread of the disease.

References

Asher, M.J.C., and Barr, K.J. (1990). The host range of *Polymyxa betae* and resistance in *Beta* species. In: R. Koenig (Ed.) Proceedings first symposium of the international working group on plant viruses with fungal vectors, Braunschweig, German Phytomedical Series Vol. 1 Eugen Ulmer, Stuttgart, p. 65-68.

Fujisawa, I., and Sugimoto, T. (1979). The reaction of some species in *Beta Patellares, Corollinae* and *Vulgares* to rizomania of sugar beet. Proceedings of the Beet Research Association of Japan 21: 31-38.

Lewellen, R.T., Skoyen, I.O., and Erichsen, A.W. (1987). Breeding sugarbeet for resistance to rhizomania: evaluation of host-plant reactions and selection for and inheritance of resistance. Proceedings 50th Winter Congress IIRB, Brussels, p. 139-156.

Paul, H., 1993. Studies on resistance to *Polymyxa betae* and beet necrotic yellow vein virus in beet. Ph.D. Thesis Wageningen Agricultural University (in press).

Paul, H., Henken, B., and Alderlieste, M.F.J. (1992a). A greenhouse test for screening sugar-beet (*Beta vulgaris*) for resistance to beet necrotic yellow vein virus (BNYVV). Netherlands Journal of Plant Pathology 98: 65-75.

Paul, H., Henken, B., De Bock, Th.S.M., and Lange, W. (1992b). Resistance to *Polymyxa betae* in *Beta* species of the section *Procumbentes*, in hybrids with *B. vulgaris* and in monosomic chromosome additions of *B. procumbens* in *B. vulgaris*. Plant Breeding 109: 265-273.

Whitney, E.D. (1989). Identification, distribution and testing for resistance to Rhizomania in *Beta maritima*. Plant Disease 73: 287-290.

COMPARISON OF SEROLOGY AND A REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ASSAY FOR EVALUATING HARD RED WINTER WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS FOR REACTION TO WHEAT SOILBORNE MOSAIC.

R. E. Pennington, J. L. Sherwood, and R. M. Hunger

Plant Pathology Department, Oklahoma State University, Stillwater, OK 74078-9947, USA

Summary

Serology is commonly used to detect wheat soilborne mosaic virus (WSBMV) when evaluating cvs for resistance to WSBM. Reverse transcription (RT)-polymerase chain reaction (PCR) assays are a sensitive alternative, but their utility in cultivar evaluation has not been examined. ELISA and RT-PCR were compared over two growing seasons for detection of WSBMV in susceptible (Sage and Vona) and resistant (Hawk, Newton, and Tam 101) cvs. Using RT-PCR, the two distinct WSBMV RNAs were detected individually and WSBMV was detected sooner after planting than by ELISA. However, evaluation of resistance, as an inhibition of virus spread to the foliage of resistant cultivars, was more pronounced when assessed by ELISA. Thus, RT-PCR is a useful supplement, but not a substitute, for ELISA and symptomatology in evaluating cultivars for reaction to WSBM.

Introduction

Wheat soilborne mosaic virus (WSBMV) causes wheat soilborne mosaic (WSBM), a major disease of hard red winter wheat (*Triticum aestivum* L.) in the Plains States (Brakke, 1987). Long (281 X 20 nm) and short (142 X 20 nm) particles of the virus contain, respectively, 7090 base (RNA1) and 3593 base (RNA2) components of a bipartite, RNA genome (Shirako and Wilson, 1993). The short particle is approximately twenty times more prevalent in infected tissue than is the long particle but both particles, or their RNAs, are required for infection (Shirako and Brakke, 1984). The virus is transmitted by the soil fungus *Polymyxa graminis* Ledingham (Brakke et al., 1965).

Efforts to control WSBM have focused on development of resistant cultivars. Since the roots of both susceptible and resistant cultivars can be colonized by *P. graminis* (Kucharek et al., 1974; Larsen et al., 1985), resistance is likely directed at the virus rather than the vector. The detection of virus and viral antigen in foliage of both resistant and susceptible plants following spring growth suggests that resistance involves more than inhibition of viral replication. Results obtained using ELISA (Myers et al., 1993) suggested that resistance is due to a temperature-modulated inhibition of virus movement. Alternative methods are needed to detect virus and viral components to test and refine this hypothesis.

We recently examined the utility of a reverse transcription (RT)-polymerase chain reaction (PCR) based assay as a supplement to ELISA in the study of the host-virus interaction (Pennington et al., 1993). Here we report results of a two year study on use of an RT-PCR assay to assess WSBMV in field-planted hard red winter wheat .

Materials and Methods

Cultivars: The WSBM-susceptible cultivars Vona, Sage, and Tam 101; and the WSBM-resistant cultivars Hawk and Newton were used. The reaction to WSBM of these cultivars has been previously characterized (Hunger et al., 1991).

Field study: In the 1991 growing season, pots (25 cm diameter X 45 cm) were filled with soil from a WSBMV-infested field west of Stillwater, OK and buried to the rim in this field. For each cultivar, five to ten seeds were sown in each of 20 pots (80 pots total). Pots were arrayed randomly in the field and seed was planted on 30 September 1991. Plants were thinned to 3 to 5 plants/pot after 4 wk. In the 1992 growing season, seeds were sown (0.5 gm/ft) in drill strips spaced 2 ft apart.

Sampling dates during the 1991 and 1992 growing seasons were as indicated in Table 1. In the 1991 growing season, plants from two to three randomly selected pots of each cultivar were removed at each sampling date. In the 1992 growing season groups of three to five plants were removed from two to three randomly selected locations within rows of each cultivar. Plants within each pot or from each group were pooled, cleansed of soil, and separated at the crown into root and foliar sub-samples. Within 24 hrs, root and foliar sub-samples were ground separately in liquid nitrogen and further sub-divided for analysis by ELISA and isolation of viral RNA.

ELISA: Virus was detected by ELISA as previously described (Hunger et al., 1991). A_{405nm} values ≥ 0.10 were considered positive for WSBMV.

Viral RNA isolation and RT-PCR assay: Viral RNA was isolated as described by Langeveld et al. (1991) with minor modifications as outlined in Pennington et al. (1993). Production of cDNA from viral RNA templates was performed using "Superscript" reverse transcriptase (Gibco BRL, Gaithersburg, MD) in 40 µl reaction volumes containing 1 µl sample. Reaction conditions were those suggested by the supplier. Priming was with downstream PCR primers specific for WSBMV RNA1 (1-1020-) or RNA2 (2-860-).

PCR amplification was performed in 0.5 ml "GeneAmp" tubes (Perkin Elmer Cetus, Norwalk, CT) using 1 µl cDNA reaction mixture, 25 pmoles each of a selected upstream and a selected downstream primer (Oligos Etc. Inc., Wilsonville, OR), and 0.25 units *Taq* polymerase (Perkin Elmer Cetus) in 50 µl reaction mixtures prepared as suggested by the supplier. For PCR amplification of WSBMV RNA1, primer 1-1020- was used in conjunction with either primer 1-361 + or primer 1-501 +. For amplification of WSBMV RNA2, primer 2-860- was used in conjunction with either primer 2-341 + or 2-481 +. The 5′ to 3′ sequences of primers used were as follows: AACGCGGCACACATAGTTTT (1-361 +), GTGCATTGTTGCTGTCCCAC (1-501 +), CGAAAGTCTTAGTAAGATAT (1-1020-), TAAATAAAGGTTACACTGGT (2-341 +), ATGCTTAATGGCGTGAGTAA (2-481 +), and CTCGAACCTTCCCATTTCAA (2-860-). Thermocycling was performed in a PTC-100 model 60 (MJ Research, Inc., Watertown, MA) programmed as follows: 94 C for 1 min; followed by 40 cycles of 90 C for 30 sec, 55 C for 45 sec, and 70 C for 45 sec; and 70 C for 3 min. PCR products were analyzed by electrophoresis and ethidium bromide staining.

Results

In the 1991 and 1992 seasons, WSBMV coat protein was detected by ELISA in the minority of root samples taken in or before November and in the majority of root samples taken in December or later (Table 1). Resistant and susceptible cultivars did not differ, in this regard, during the 1991 season. During the 1992 season, however, coat protein was detected in 3 of 6 root samples of resistant cultivars taken in December but was not detected in root samples collected in January. WSBMV RNA 2 was detected by RT-PCR in all ELISA-positive root samples and in the majority ELISA-negative root samples taken in November or earlier. Attempts to detect by RT-PCR viral RNA in soil infested with *P. graminis* have been unsuccessful (T.M.A. Wilson, personal communication, Pennington and Sherwood, data not shown).

During both growing seasons, WSBMV coat protein was not detected in shoot

samples of resistant cultivars and only rarely in shoot samples of susceptible cultivars taken before December. By December of the 1991 season and January of the 1992 season, coat protein was detected in half or more of the shoot samples of susceptible cultivars but none of the shoot samples of resistant cultivars. RNA 2 was detected in all the ELISA-positive shoot samples of susceptible cvs and in the majority of ELISA-negative shoot samples of susceptible and resistant cultivars taken in December or later. During the 1991 season, RNA 2 was detected only rarely in shoot samples collected on the October and November samplings but in the majority of shoot samples taken during these months in the 1992 season.

RNA2 was detected rarely in shoots unless the corresponding roots were positive by ELISA (pairing of root and shoot samples not shown). In root and shoot samples of resistant and susceptible cultivars taken during the 1991 season, WSBMV RNA1 was detected by RT-PCR in the majority of samples shown to contain RNA2 but rarely in the absence of RNA2 (data not shown).

Table 1. Detection of WSBMV RNA2 by RT-PCR and WSBMV coat protein by ELISA in shoot and root samples of susceptible (Vona, Sage, Tam 101) and resistant (Hawk, Newton) cvs of hard red winter wheat from a field with a history of soilborne mosaic. Planting dates were 30 September 1991 and 25 September 1992. Numbers separated by slashes are (left to right) number of samples positive by ELISA, number of samples positive by RT-PCR and total number of samples. NT = sample not taken. Samples positive in ELISA = $A_{405} \geq 0.10$. Samples positive in RT-PCR had appropriately sized bands visible by agarose gel electrophoresis and ethidium bromide staining.

Sampling date	Susceptible cultivars				Resistant cultivars			
	Vona		Sage		Hawk		Newton	
1991 season	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
16 October	1/3/3	0/1/3	1/2/3	0/0/3	0/0/3	0/0/3	0/2/2	0/1/2
2 November	1/3/3	0/1/3	0/3/3	0/0/3	1/2/2	0/0/3	0/3/3	0/0/2
4 December	3/3/3	2/3/3	3/3/3	1/3/3	2/2/2	0/0/3	3/3/3	0/3/3
	Vona		Tam 101		Hawk		Newton	
1992 season	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
15 October	0/1/2	0/1/2	0/2/2	0/2/2	NT	0/0/2	0/0/1	0/1/2
4 November	2/3/3	0/1/3	1/1/1	1/1/1	0/2/2	0/0/2	0/3/3	0/1/3
4 December	2/2/2	0/2/3	2/2/2	0/2/2	1/3/3	0/2/2	2/3/3	0/1/3
18 January	3/3/3	3/3/3	3/3/3	3/3/3	0/3/3	0/1/3	0/3/3	0/3/3

Discussion

In the 1991 season, WSBMV coat protein was detected in the roots of resistant and susceptible cultivars at approximately the same time following planting in a field with a history of severe WSBM. In the 1992 season, detection of coat protein in the roots of resistant cultivars was delayed and reduced relative to the susceptible cultivars. In both experiments, WSBMV RNA2 was detected by RT-PCR in all ELISA-positive root samples and in most ELISA positive root samples of both susceptible and resistant cultivars taken at the

October and November samplings. As we have been unable to detect viral RNA in samples of *P. graminis*-infested soil, the RT-PCR results indicate that viral RNA was present in the roots up to 7 wk before coat protein could be detected by ELISA.

Coat protein was not detected in the foliage of resistance cultivars during either growing season which is consistent with previous results suggesting resistance is due to inhibition of upward virus movement (Myers et al., 1993). However, the limited detection of coat protein in the roots of resistant cultivars in the second field experiment suggests a mechanism other than inhibition of systemic movement may also play a factor in resistance. The detection of RNA2 in the foliage of susceptible cultivars confirms previous findings that the resistance is not absolute (Myers et al., 1993).

RNA2 is reportedly 20 times more prevalent than RNA1 in infected tissue (Shirako and Brakke, 1984). Consistent with this, fewer samples tested positive for RNA1 than for RNA2 when assayed during the 1991 season. Susceptible and resistant cultivars did not differ noticeably in this regard suggesting that resistance is not targeted differentially at either the long or the short WSBMV particles or their RNAs.

We have shown that RT-PCR assay is useful for detection of WSBMV and provides important information not obtainable using ELISA alone on the WSBMV-wheat interaction. However, the detection of WSBMV by RT-PCR, but not by ELISA, in a large number of shoot samples of resistant cultivars indicates that RT-PCR is a useful supplement but not a substitute for the ELISA-based approach, used over several years, to evaluate resistance to WSBMV in hard red winter wheats.

References

Brakke, M. K. (1987). Wheat diseases of wheat. Pages 585-624 in: Wheat and Wheat Improvement. 2nd edition, E.G. Heyne, ed., Agronomy Monograph 13. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, WI.

Brakke, M. K., Estes, A. P., and Schuster, M. L. (1965). Transmission of soil-borne wheat mosaic virus. *Phytopathology* **55**:79-86.

Hunger, R. M., J. L. Sherwood, E. L. Smith , Armitage, C. R. (1991). Use of symptomatology and the enzyme linked immunosorbent assay to facilitate breeding for resistance to wheat soilborne mosaic virus. *Crop Sci.* **31**:900-905.

Kucharek, T. A., Walker, J. H., and Barnett, R. D. (1974). Effect of cultivar resistance and soil fumigation on soilborne wheat mosaic virus in Florida. *Plant Dis. Reptr.* **58**:878-881.

Langeveld, S., Dore, J. -M., Memelink, J., Derks, A., van der Vlugt, C., Asjes, C., and Bol, J. (1991). Identification of potyviruses using the polymerase chain reaction with degenerate primers. *J. Gen. Virol.* **72**:1531-1539.

Larsen, H. J., Brakke, M. K., and Langenberg, W. G. (1985). Relationship between wheat streak mosaic virus and soilborne wheat mosaic virus, disease resistance, and early growth of winter wheat. *Plant Dis.* **69**:857-862.

Myers, L. D., Sherwood, J. L., Siegrist, W. C., and Hunger, R. M. (1993). Temperature-influenced virus movement in expression of resistance to soilborne wheat mosaic virus in hard red winter wheat (*Triticum aestivum*). *Phytopathology* **83**:548-551.

Pennington, R. E., Sherwood, J. L., and Hunger, R. M. (1993). A PCR-based assay for wheat soilborne mosaic virus in hard red winter wheat. Plant Dis. **77**: In press.

Shirako, Y., and Wilson, T. M. A. (1993). Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* **195**:16-32.

OCCURENCE AND RECURRENCE OF SYMPTOMS OF AUGUSTA DISEASE CAUSED BY TOBACCO NECROSIS VIRUS IN TULIP

C.J. Asjes, Bulb Research Centre, PO Box 85, 2160 AB Lisse, The Netherlands

Summary

The Augusta disease caused by tobacco necrosis virus (TNV) in tulip variably occurred at high rate in recent years. The symptoms affected by weather conditions throughout the season were described. The symptom development was largely delayed at the time of flowering in tulips grown in sandy soil, whereas it was prolonged in plants in heavy loam till the end of season. Primarily infected symptomless plants of a susceptible cultivar were diseased at high rate in the following season. The recurrence of symptoms in progeny bulbs planted fairly late in autumn was less than in early-planted bulbs. The recurrence of symptoms in early-planted bulbs with symptomless infection in plants grown in sand in the previous season and in heavy loam in the current season was evident but still at half of the rate at least in the culture in heavy loam in both years. The recurrence of symptoms in different rates in tulips forced to flower in winter was fairly variable but largely due to the difference in susceptibility of cultivars. The effect of conditions on the incidence of symptoms of Augusta disease and possible loss of TNV was discussed.

Introduction

Tobacco necrosis virus (TNV) infects a wide range of plant species. Symptomless infection often occurs in nature. Systemic symptoms are known in tulip for long. A syndrome 'Augusta disease' named by its incidence in cv. Queen Augusta in the Netherlands in 1928 (De Bruyn Ouboter and Van Slogteren, 1949) was caused by tobacco necrosis virus (Kassanis, 1949; Van Slogteren and Visscher, 1967). The disease occurred worldwide, e.g., in Britain (Mowat, 1970), Denmark (Lange, 1976) and Japan (Nahata et al., 1988). In the Netherlands in the late eighties the incidence in some regions with tulips grown in heavy soil types indicated the need to resume the study of the disease. In this paper data about the occurrence and recurrence of symptoms under different field conditions, and the impact of TNV in tulip forced to flower out of season, will be reported.

Material and methods

Material. Comparable samples of tulip bulbs (c.50-100; 9-11 cm in circumference) were used to observe the symptoms in the field. 200 bulbs (10-11 cm) of growers' lots were used for forcing. Some bulbs did not grow.
Soil. The sandy soil (4% silt) was of fine texture with the water level at 50-60 cm below soil surface. The heavy loam (36% silt; 23% lutum) was of suitable texture to grow tulips.
Culture and forcing of tulips. Bulbs were planted in the field in autumn (October/ November). In (late) March the plants emerged from soil. The flowering occurred in late April (4th week) and first half of May. The lifting of bulbs was due in early July.
Temperature treatments were applied to bulbs to force them to flower in winter, viz., set 1: 20°C till September 12(9-12), 9°C till 10-20 before planting in boxes, 7°C till 11-10 and 5°C till 01-02 in cooled storage compartments. Boxes were taken into the greenhouse to produce flowers in c.4 weeks; set 2: 20°C till 10-15, 17°C before planting at 11-25, 5°C till 02-18, and -1°C till 03-18.

Results

Symptoms. In the field plants were stunted and distorted early after the emergence from soil. This particularly occurred in bulbs planted early in autumn before October 15, as well as at high rates in secondarily infected bulbs of susceptible cultivars, e.g., Angelique, Apricot Beauty, etc., in the field, and during the forcing of bulbs for cut flowers. This severe symptom of 'dwarfing' of plants while other tulips fairly rapidly increased in height, is known as the 'old Augusta disease'. The plants being normal in height could develop symptoms later on, particularly during and after flowering. The syndrome of 'mild' symptoms, e.g., large oval necrotic spots, was named 'summer or late Augusta disease'.

Syptoms appeared in a diversity of fairly wide chlorotic, brown-necrotic streaks, or fairly large elliptical, or oval, or round necrotic spots in the leaves. Initially dark-brown tissue surrounded grey-necrotic central areas. Flowers showed tiny streaks, particularly along the petal edges. The flowers were malformed in severely infected plants of susceptible cultivars. Diseased plants died prematurely. These leaf symptoms in forced tulips developed within a few weeks and were albeit complete during flowering. Newly formed bulbs of field plants showed somewhat sunken waxy spots which became brown and necrotized. The bulb symptoms on outer and inner scales developed in severity during storage. However, no symptoms in bulbs developed at all in many cultivars.

Mild winter- and spring-weather conditions in 1989 and 1990 appeared favourable to induce high rates of severe and mild symptoms. Frost periods in the winter 1990-1991 were favourable for the development of severe symptoms of dwarfing in plants early in spring, but the development of mild symptoms was delayed later on. Dry and fairly hot weather in May 1992 inhibited mild symptom development in the field altogether.

Appearance of symptoms

Table 1 shows that in tulip cv. Angelique planted in sand severe symptoms developed before flowering, while in cvs. Blenda and Capri hardly any symptom developed. Lots of bulbs in heavy loam produced high disease rates of mild symptoms after flowering, particularly if planted early.

Table 1: Augusta-diseased plants in secondarily infected tulips in 1990 planted in 2 soil types at 2 dates in 1989

Cultivar	diseased plants before/after flowering total							
	planted October 15				planted November 15			
	sand		heavy loam		sand		heavy loam	
Angelique	38/2	40	12/63	75	27/7	34	1/0	1
Blenda	0/0	0	0/13	13	0/0	0	7/10	17
Capri	0/1	1	1/60	61	0/0	0	2/10	12

Recurrence of symptoms

Primary infection. In 1988 in tulip cv. Angelique symptoms developed favourably throughout the season. Bulbs of plants with mild symptoms were seperately lifted from adjacent symptomless plants. The replanting of those bulbs at October 15 in heavy loam gave 82 and 60% plants with symptoms, whereas the rates were 73% and 1%, respectively, if planted in sand.

Secondary infection. Lots of tulip cvs. Angelique (16 x 50 bulbs) and Miss Holland (60 x 50 bulbs) grown in sand rated on average 33 and 60% plants with symptoms, and in heavy loam in the following year 76 and 93%, respectively, under favourable conditions for symptom development.

Table 2 shows that overall rates of severe and mild symptoms in c.100% primarily infected bulbs of tulip cv. Angelique recur at lower rate than

from the secondarily infected, which was the more so in cv. Don Quichotte grown in sand. The disease rates were much lower in bulbs grown in sand than in heavy loam. The rates in secondarily infected bulbs planted at November 15 were lower than obtained at October 15. The bulbs of tulip cv. Don Quichotte which showed symptoms, were lost after the replanting.

Table 2: Recurrence of Augusta disease in progeny bulbs in 1990-1991 of primarily and secondarily infested crops of tulip (100%) in 1989-1990.

Cultivar	Bulb symptoms	Replanting in soil type in 1990	% symptoms in 1991 primary at 10-15-1990	secondary infection at 10-15	11-15-1990
Angelique	no	heavy loam	65	85	31
Angelique	no	sand	23	30	17
Don Quichotte	no	heavy loam	64	39	7
Don Quichotte	yes	heavy loam	none recovered	-	-
Don Quichotte	no	sand	1	2	2
Don Quichotte	yes	sand	none recovered	-	-

Table 3 shows that in 1990 again the rates of severe and mild symptoms were decreased by the culture in sand compared to that in heavy loam, like by the late planting. The replanting of bulbs from sand in heavy loam prominently induced the increase in disease rate, but still at half of the rate of the early-planted lots from loam in loam consecutively. The mean rate of symptoms in consecutive crops in heavy loam were similar in the early-planted bulbs but differed considerably, however, only determined in the late-planted lots of two cultivars in 1989.

Table 3: Recurrence of Augusta disease in tulips grown in sand and heavy loam planted at two dates in 1989, and the progeny in heavy loam in 1990.

Cultivar	% plants with symptoms in 1991 planted 10-15-1989				planted 11-15-1989				number
	sand 1990	loam 1991	loam 1990	loam 1991	sand 1990	loam 1991	loam 1990	loam 1991	of lots
Angelique	7	27	79	88	4	41	-	-	4
Apricot Beauty	17	36	32	90	17	46	-	-	2
Attila	0	45	62	25	0	34	-	-	1
Blenda	0	32	27	19	0	17	8	44	3
Capri	1	18	45	37	1	9	8	48	2
Don Quichotte	0	6	-	-	0	1	15	21	2
Inzell	5	19	-	-	0	29	36	33	1
Miss Holland	10	86	88	88	5	87	-	-	1
Mean	5	30	54	60	4	30	13	38	16

Table 4 shows the overall rates of severe and mild symptoms in forced tulips. The symptom rates were high in cvs. Angelique, Apricot Beauty, and Dreamland, fairly high in cv. Capri, and low in cvs. Blenda, Inzell, Prominence, Snow Star, and Yellow Present. The rates in forced lots and in those grown in sand in the field were largely comparable, while occasionally at very high level in lots grown in heavy loam in 1991. Variable loss of disease rate in 1991 compared to those estimated in fields of growers in 1990 occurred.

Table 4: Recurrence of Augusta disease in tulips forced into cut flowers in winter and in bulbs in sand and heavy loam in the field in 1990-1991.

Cultivar	Number of lots	% symptoms of Augusta disease field 1990	forcing in winter set 1	set 2	field in 1991 sand	heavy loam
Angelique	22	60	19	17	20	67
Apricot Beauty	6	70	28	19	13	83
Blenda	4	50	1	2	2	32
Capri	6	67	9	5	10	35
Dreamland	1	100	18	18	11	73
Inzell	9	46	3	2	2	31
Prominence	7	67	2	2	2	8
Snow Star	2	35	0	1	0	6
Yellow Present	2	47	0	0	1	35

Discussion

The development of symptoms of Augusta disease proved to be variable due to different conditions, e.g., primary or secondary infection, susceptibility of cultivars, planting of bulbs in light and heavy soil early or late in autumn, and the impact of weather conditions throughout or in parts of the growing season. The absence of symptom development may be associated with too little build-up of virus concentration. The prosperous growth of infected plants in heavy soil in which the retaining capacity of moisture will be better than in sand, may particularly foster mild symptoms after flowering in early-planted bulbs under favourable weather conditions. The susceptibility of cultivars particularly expressed in symptoms of dwarfed plants in the field and during forcing seems an unchangeable characteristic.

The incidence of symptomless plants in the field either primarily or secondarily infected did not preclude that symptoms developed in the following year. The culture in consecutive years in sand and unfavourable conditions to develop mild symptoms particularly after flowering in tulips planted in heavy soil may indicate a natural way to get rid of tobacco necrosis virus in the progeny bulbs on the long run.

References

De Bruyn Ouboter, M.P., and Van Slogteren, E., 1949. Het Augustaziek der tulpen, een virusziekte van het tabaksnecrosetype. Tijdschrift over Planteziekten (Netherlands Journal of Plant Pathology) 55: 262-267.

Kassanis, B. 1949. A necrotic disease of forced tulips caused by tobacco necrosis viruses. Annals of Applied Biology 36: 14-17.

Lange, L. 1976. Augusta disease in tulips. Field experiments concerning tobacco necrosis virus and its vector Olpidium brassicae. Tidskrift Planteavl 80: 153-169.

Mowat, W.P., 1970. Augusta disease in tulip - a reassessment. Annals of Applied Biology 66: 17-28.

Nahata, K., Kusaba, T., and Mukobata, H., 1988. Studies on the ecology and control of tulip virus diseases. Bulletin of the Toyama Agricultural Research Centre 2: 1-132.

Van Slogteren, D.H.M., and Visscher, H.R., 1967. Transmission of tobacco necrosis virus, causing 'Augusta' disease to the roots of tulip by zoospores of the fungus Olpidium brassicae (Wor) Dang. Mededelingen Rijksfaculteit voor Landbouwwetenschappen, Gent 32: 927-938.

DETECTION AND QUANTIFICATION OF VIRUSES AND THEIR VECTORS IN SOIL

M. J. Adams

Plant Pathology Department, AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, UK

Summary

A convenient assay for fungally-transmitted viruses and their vectors in soil would be useful for quarantine tests, for epidemiological studies and for advising farmers on future cropping. Bait plant methods using serial soil dilutions and most probable number analysis, similar to those described by others, were used to detect and quantify *Polymyxa graminis* and BaMMV from plots of a field experiment designed to investigate the effects of rotation. The results illustrate the variability of such labour-intensive procedures. The requirements of a reliable bioassay are listed; they include a good choice of dilution series and reproducible temperature and soil moisture conditions. The importance of testing the fit to the mathematical model used is illustrated and emphasised. Mathematical considerations show that the method has inherent limitations in precision. Attempts to develop a more rapid, direct, assay have been hindered by the small populations of the vectors in soil and by difficulties in detecting viruses in samples contaminated by soil. All assay methods face serious sampling problems if used on a field scale.

Introduction

For those fungally-transmitted viruses that survive within their vector resting spores in soil for long periods (mostly those with plasmodiophorid vectors), a convenient assay for them and their vectors in soil would be useful for quarantine tests, for epidemiological studies and for advising farmers on future cropping. Because the virus vectors are obligate parasites of plant roots, some methods commonly used by plant pathologists to quantify soil-borne pathogens are not available and the methods used to date have all been based on bioassays (bait plant methods). This paper describes some experience with bioassays and summarises the principles that need to be observed when they are used. Some attempts to develop a more rapid, direct assay are also described and prospects for such a test discussed.

Bioassay

Bioassays were used to assess populations of *Polymyxa graminis* (total and with barley mild mosaic virus, BaMMV) from plots of a field experiment designed to investigate the effects of rotation on inoculum levels, disease and yield (Adams *et al.*, 1993). Soil samples from each plot were dried, crushed, sieved (2mm) and stored at 15-20°C before preparing a dilution series by mixing with sterile sand. Each dilution was distributed to ten cells of a divided seed tray (4 x 10 rows; cell capacity 50 ml) and one barley seedling sown in each cell. Dilutions of 1/20 to 1/4860 with a dilution ratio of 3 were used. After sowing, the plants were grown in a glasshouse at 15-20°C. The watering regime was designed to encourage *P. graminis* infection, with 2 weeks at or near saturation beginning one week after sowing (Maraite, Goffart and Bastin, 1988). Seven weeks after sowing, the plants were washed free of soil and sand and the presence or absence of *P. graminis* in the roots assessed by looking for the brown resting spore clusters under a dissecting microscope. Samples of root from each plant were crushed in a Pollähne sap press (Behncke & Co., Hannover) and were frozen and stored at -20°C for up to 3 wk before testing for the presence or absence of BaMMV by ELISA (Adams, 1991). The most probable numbers (MPN) of propagules of *P. graminis* (total and with virus) were estimated using the Maximum Likelihood Program (MLP) on the Rothamsted VAX computer (Ross, 1987).

Inoculum level assessments in soil after growing susceptible barley, resistant barley or wheat for

Table 1 MPNs of *Polymyxa graminis* (nos/ml soil with 95% confidence limits in brackets), after 3 years of cropping with susceptible barley (S), resistant barley (R), or wheat (W)

Crop sequence	Total	+ virus
S,S,S	30.3 (17.9-51.2)	10.5 (5.4-20.4)
R,S,S	26.6 (19.6-35.7)	38.6 (17.9-83.1)
R,R,S	25.5 (15.2-42.8)	8.9 (2.1-37.5)
R,R,R	19.3 (10.5-35.6)	3.8 (0.9-16.4)
W,S,S	23.1 (13.6-39.5)	8.7 (3.8-19.9)
W,W,S	19.0 (10.6-34.0)	8.7 (3.6-21.1)
W,W,W	15.5 (9.1-26.4)	2.7 (1.5-5.1)
Total	24.0 (19.6-29.5)	10.0 (7.0-14.2)

3 years at one of the sites (Table 1) illustrate the variability of the data. No significant differences related to pre-cropping treatment were detected. Such experiments require large amounts of space and are very time-consuming. If they are to be accurate and reproducible, a number of conditions must be fulfilled.

(a) Choice of dilution factor, numbers of steps and replication. Dilution factors less than 10 should be chosen to help stabilise the standard errors and maximum accuracy can be achieved by uneven replication, with larger numbers in the centre of the series (Finney, 1952) provided that a suitable method of analysis for such data is available. The advantage of this is illustrated in Fig. 1, using artificially created data sets analyzed by the computer program of Clarke & Owens (1983). Uneven replication and a smaller dilution ratio gives smaller and more stable standard errors over the range of populations likely to be of interest. (b) Careful mixing of soil. (c) Reproducible, disease-conducive, growing conditions. This is particularly difficult to achieve because of the need to provide similar soil moisture conditions in all treatments. (d) The root system should explore all the available soil. Pot volumes should be small to optimise this. (e) A single unit of inoculum should cause disease. (f) Accurate assessment of infection. Interpretation of ELISA data can be rather subjective. (g) Suitable method of analysis, which should include three steps:

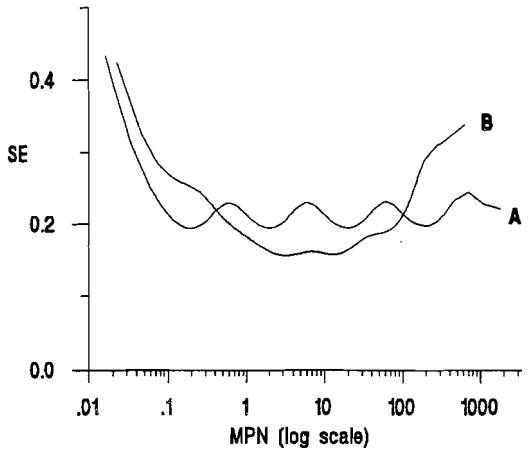


Fig. 1 Standard errors of log MPN for sets of dilution data from a total of 30 pots. A = dilution ratio of 10, 5 steps and 6 replicates. B = dilution ratio of 5, 7 steps and uneven replication (2,2,7,8,7,2,2 pots).

(i) estimation of the most probable number: of the two different methods available, the maximum likelihood estimation is more accurate than one based on the tables of Fisher and Yates (1963), which is acknowledged to be only 88% efficient. Sets of tables have now been largely superseded by computer programs for these calculations. A simple BASIC program for IBM-compatible PCs which can analyze data with uneven replication is that of Clarke and Owens (1983), while MLP is both more complex and more flexible; (ii) estimation of the standard error (of log MPN) and derivation of confidence limits. Some computer programs provide this information, or the formula of Cochran (1950) can be used to obtain an average (and apparently fairly conservative) estimate; (iii) testing the fit of the statistical model used: this is often neglected but can be of great importance. Tables and computer programs are generally based on the assumption of random distribution of infective propagules (the Poisson model) but,

especially with computer programs, MPN values and confidence limits may be produced for sets of data that depart very strongly from this assumption. The goodness of fit to the statistical assumptions is quite separate from examining the standard errors. Various ways of testing this fit have been used, including the range test of Stevens (1958), examining the data for unlikely dilution patterns (e.g. Scott and Porter, 1986) and a X^2 test for goodness of fit of the data to the mathematical model fitted.

The importance of testing the statistical model can be illustrated by data on *P. graminis* from the field trial mentioned above. These data (Fig. 2) show a very poor fit to the Poisson distribution ($X^2 = 887.8$, 5df), which assumes completely random dispersion, but a much better one to the negative binomial ($X^2 = 2.21$, 4df), which is an alternative model in the MLP computer program and which allows for some degree of clumping. MPN values calculated on the basis of the Poisson distribution would, in this case, have been mathematically correct but would have seriously underestimated the population (0.62/ml soil; compared with 15/ml for the negative binomial model) and would have been statistically and biologically meaningless. Some explanation needs to be sought where data are not a good fit to the expected random distribution. Erratic values (e.g. 10, 6, 1, 5, 0 in a 5-step series with 10 replicates) might suggest poor mixing of the soil samples, while an extended range (as seen in the example above) could arise from fragmentation of the infective units (root pieces containing several resting spore clusters or break up of the resting spore clusters themselves) during dilution. Similar results might be obtained by diluting soil if this also diluted factors in the soil inhibitory to the vector or created conditions (e.g. of aeration) increasingly favourable for the fungus.

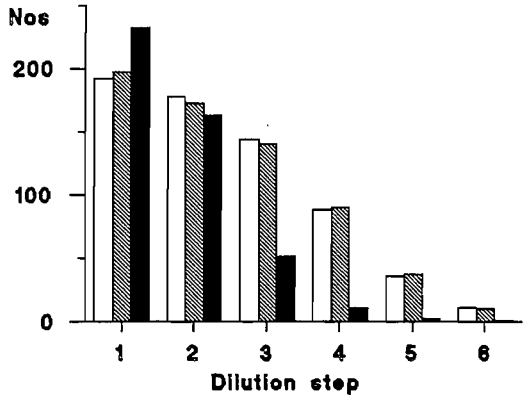


Fig. 2 Numbers of pots with *Polymyxa graminis* (open) and expected from negative binomial (cross-hatched) and Poisson (solid) distributions. Data combined from 21 plots of field experiment: individual plots usually showed the same trend

Purely mathematical considerations show that the MPN method can never be very precise with the numbers of replicates that could reasonably be used: the formulae and tables of Cochran (1950) show that a precision substantially less than an order of magnitude would be very difficult to achieve. The precision of the calculation does not justify quoting MPN values with more than two significant figures.

Direct assay

Direct assays of fungally-transmitted viruses and their vectors in soil have two obstacles to overcome. First, the resting spores of the fungus must be extracted from the soil and, perhaps, enumerated. Takahashi and Yamaguchi (1987; 1989) have reported a fluorescent staining method for directly counting numbers of resting spores of *Plasmodiophora brassicae* in soil suspensions and for assessing their viability. Their method appears to be suitable for populations in the range 10^3-10^5/g soil. This, however, is much greater than the populations reported for *Polymyxa* spp., which are typically 1-50/g soil, and their methods would not therefore be applicable unless the spores could first be substantially concentrated. Because the fungus vector resting spores are presumably associated with organic matter, the organic fraction of soils infested with *Polymyxa* have been extracted by wet sieving. This has proved to be very laborious and, compared with the original soil, organic fractions have been only slightly enriched with *Polymyxa* as judged by bioassay. A more promising approach has been to use density separation of a soil/detergent mixture, with a low speed centrifugation over a Percoll cushion. Spores from artificially amended soils can be concentrated and counted in a light microscope, but further refinement

is needed to detect the numbers expected in naturally-infested soils. The second obstacle is to test spores for the presence of virus. Spores could be broken to release their contents by shaking with glass beads but, although such techniques as amplified ELISA and especially PCR may have the required sensitivity to detect virus, they are inhibited by soil components, perhaps especially humic acids. Further refinement of the spore purification procedure and advances in virus diagnosis will be needed before the direct assay can become a possibility.

Conclusions

Bioassays can provide a reasonably sensitive method for detecting fungally-transmitted viruses and their vectors in soil. Experiments take several weeks to complete and quantification of inoculum requires much effort, which becomes prohibitive when many samples need to be processed. Provided the conditions described above are observed, reliable results can be obtained but the difficulties of standardising growing conditions and virus assays mean that these results should be treated as relative, rather than absolute measurements of inoculum. For the time being, there is no adequate alternative to this approach and new methods need to be sought. However, all assay methods face serious sampling problems if used on a field scale.

Acknowledgements

I thank Miss C. R. Collier for technical assistance and Miss S. J. Welham and Mr G. J. S. Ross for statistical advice. I also thank Drs D. R. Jones (ADAS, Bridgets), T. M. O'Neill (ADAS, Boxworth) and S. A. Hill (Central Science Laboratory, Harpenden) who were collaborators in the field experiment and the UK Home-Grown Cereals Authority for financial support for it.

References

Adams, M. J. (1991). The distribution of barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) in UK winter barley samples, 1987-1990. *Plant Path.* **40**:53-58.

Adams, M. J., Jones, D. R., O'Neill, T. M., and Hill, S. A. (1993). The effect of cereal break crops on barley mild mosaic virus. *Ann. Appl. Biol.* **123**: in press.

Clarke, K. R., and Owens, N. J. P. (1983). A simple and versatile micro-computer program for the determination of "most probable number". *J. Microbiol. Meth.* **1**:133-137.

Cochran, W. G. (1950). Estimation of bacterial densities by means of the "most probable number". *Biometrics* **6**:105-116.

Fisher, R. A., and Yates, F. (1963). *Statistical tables for biological, agricultural and medical research*, 6th edition. Harlow, Essex: Longman. 146 pp.

Finney, D. J. (1952). *Statistical method in biological assay.* London: Charles Griffin. 661 pp.

Maraite, H., Goffart, J. P., and Bastin, V. (1988). Development of a quantitative method for assessment of *Polymyxa graminis* Led. inoculum potential in soils. In: *Integrated Crop Protection in Cereals* (Eds R. Cavalloro and K. D. Sutherland) pp.259-266. Rotterdam: A.A.Balkema.

Ross, G. J. S. (1987). MLP manual. Numerical Algorithms Group, Oxford.

Scott, J. C., and Porter, F. E. (1986). An analysis of the accuracy of a plant infection technique for counting rhizobia. *Soil Biol. Biochem.* **18**:355-362.

Stevens, W. L. (1958). Dilution series: a statistical test of technique. *J. Roy. Stat. Soc. Ser. B* **20**:205-214.

Takahashi, K., and Yamaguchi, T. (1987). An improved method for estimating the number of resting spores of *Plasmodiophora brassicae* in soil. *Ann. Phytopath. Soc. Japan* **53**:507-515.

Takahashi, K., and Yamaguchi, T. (1989). Assessment of pathogenicity of resting spores of *Plasmodiophora brassicae* in soil by fluorescent microscopy. *Ann. Phytopath. Soc. Japan* **55**:621-628.

DISTRIBUTION OF PEANUT CLUMP VIRUS (PCV), A VIRUS WITH HIGH SYMPTOM VARIABILITY

M. Dollet[1], J. Dubern[1], F. Waliyar[2], S.K. Manohar[1]

(1) Laboratoire de Phytovirologie des Régions Chaudes (LPRC), CIRAD-ORSTOM, BP 5035, 34032 MONTPELLIER, Cedex 1, FRANCE.
(2) ICRISAT Sahelian Centre, BP 1024, NIAMEY, NIGER

Summary

In 1974, Peanut Clump disease was present only in two very localized places in West Africa: Area of Bambey in Senegal and one agricultural research station in Burkina Faso. Following a number of surveys made in the 80s up to 1991, Peanut Clump Virus (PCV) was detected in Côte d'Ivoire, Mali, Niger and Benin. In Senegal, the virus is now widely distributed from the Senegal river to the frontier of Gambia. Transmission of PCV through seeds is partly responsible for the increased spread of the disease. Abundance of PCV in agricultural research stations, or in seed-gardens shows the importance of seed transmission. Existence of infected soils is another factor of dissemination of the virus. Symptoms induced by PCV in a given variety of groundnut vary from classical stunting with small dark green leaves, to normal sized plants with different light leaf symptoms such as line pattern, specking and a great variety of other foliar symptoms. Therefore, PCV is very difficult to diagnose in the field.

Introduction

Peanut Clump was described for the first time in Senegal in 1931 by Trochain, in a very limited groundnut growing area between Diourbel and Bambey. The disease remained localized in this region up to the beginning of the 70s, when it was reported at the Saria agricultural station, Burkina Faso (Germani and Dhery, 1973). At that time, the disease was described by a unique symptomatology: severe plant stunting, with small, dark green leaves and short internodes. It was on plants with such symptoms that PCV was identified then characterized (THOUVENEL *et al.*, 1976). DOLLET *et al.* (1976) showed that sorghum, was a natural PCV host and that the virus was transmitted via the soil from infected sorghum roots. *Polymixa graminis* is the presumed vector of PCV (THOUVENEL and FAUQUET, 1981).

At the beginning of the 80s, one of us (J.D.) observed viral leaf symptoms which had not yet been described in Senegal. Surveys were then carried out in Senegal from 1986 to 1990, in Niger (1989) and Burkina Faso and Mali (1991).

Materials and Methods

In Senegal, one or two groundnut fields were visited every ten km. In Burkina Faso and Mali, 4 to 5 fields were surveyed around every 50 km. Samples consisted of 1 to 3 branches placed in a carefully sealed plastic bag and put into a portable ice-box. In Montpellier, the samples were grafted onto a single variety of groundnut -69101-. The plants were kept in a climatic chamber at a temperature of 29-30°C during the day and 24-25°C at night.

Soil (10 to 50 cm horizon) was collected and taken to the ORSTOM nematology laboratory in Dakar. Seeds of the Florunner groundnut variety from the United States (where there is no PCV) were sown 4 to a pot.

The existence of PCV was checked successively by 1) physical inoculation on *Chenopodium amaranticolor*, 2) Leaf-Dip examination under the electron microscope and 3) possible serological test with polyclonal antibodies by micro-precipitation.

Results

In Senegal, PCV was found not only in the region of Bambey, but also in the Cap Vert Region, the Thies Region, in Siné Saloum and on irrigated land along the Senegal river. PCV was present on research stations (Bambey, Thyssé-Kaymor), but also in smallholdings (Pout, Mbour) or large plantations (Kirene) (Fig. 1).

In Burkina Faso, PCV was found at Saria and Kamboinsé to the North of Ouagadougou. Clump symptoms have been reported in the Koupéla region and between

Bobo Dioulasso and Niangoloko. To the north of Ouagadougou, numerous smallholdings are affected by PCV (G. Konate, personal communication) (Fig. 2).

In Mali, PCV was identified for the first time at the Cinzana research and seed multiplication station near Ségou. Numerous cases of stunting were observed between Koutiala and Bamako and to the South of Bamako, though the virus was not identified (Fig. 3). These PCV "non-identifications" in stunted groundnut plants were due either to graft death during the first attempts at grafting, or to non-transmission of the symptoms observed in the field.

In Niger, the surveys carried out primarily by ICRISAT revealed the existence of clump in the Maradi region and near Niamey. During these various surveys, PCV was identified in the stunted groundnut plants with typical clump symptoms, but also in normal sized plants without short internodes but with various leaf symptoms: chlorotic patches or rings more or less in the form of an eyespot, geometric, angular, yellow line patterns, yellow specking, yellow mosaic, green blotches (Fig. 4). These symptoms were sometimes very slight or localized solely on the oldest leaves hidden by the tuft of younger leaves and PCV therefore escaped detection. It worth noting that the greatest symptomatological variability is found at research stations.

The detection of groundnut plants infected by PCV 3 weeks after sowing, in a plot at Ndiongo (Senegal river) in which neither groundnut nor sorghum had been grown before led us to test soil infectivity in a glasshouse in Dakar. The groundnuts sown in this soil were contaminated by PCV, which showed that the inoculum was present in the soil, despite no prior groundnut or sorghum cultivation.

Discussion

PCV has spread from pinpoint localization in the 70s to widespread dispersal virtually throughout Senegal and into several West African countries.

Detection of this dispersal was accompanied by the discovery of extensive symptomatological variability. Several explanations can be considered. The first is transmission of the virus by seeds. Such transmission may be all the more difficult to avoid in that certain groundnuts affected by PCV sometimes reveal very few, or atypical symptoms. Finally, it may be that the inoculum (vector-virus) exists in numerous soils, probably due to wild grasses. The first investigations carried out on these grasses showed that several of them were infected by PCV (unpublished results). In the traditional cropping system, traditional groundnut-sorghum rotations in sub-Sahelian countries are undoubtedly propitious to inoculum multiplication.

These surveys, conducted since 1986, therefore opened up new horizons in the study and understanding of PCV. Extensive serological variability has already been discovered (HUGUENOT *et al.*, 1989, MANOHAR *et al*, 1993 a), along with substantial genomic variability (MANOHAR *et al*, 1993 b). Finally, however, it should be noted that not all stunted groundnut plants harbour PCV. We have discovered at least three viruses (two flexuous and one spherical) associated with stunting symptoms (unpublished results).

References

Dollet, M., Fauquet, C., Thouvenel, J.C. (1976). *Sorghum arundinaceum*, a natural host of Peanut Clump Virus in Upper Volta. *Plant. Dis. Reptr.* 60: 1076-1080.

Germani, G., Dhery, M. (1973). Observations et expérimentations concernant le rôle des nématodes dans deux affections de l'arachide en Haute Volta: la "chlorose" et le "Clump". *Oléagineux* 28: 235-242.

Huguenot, C., Givord, L., Sommermeyer, G., Van Rengenmortel, M.H.V. (1989). Differentiation of Peanut Clump Virus serotypes by monoclonal antibodies. *Res. Virol.* 140: 87-102.

Manohar, S.K., Dollet, M., Dubern, J., Gargani, D. (1993)a. Studies on variability of Peanut Clump Virus: symptomatology and serology. (submitted for publication).

Manohar, S.K., Guilley, H., Dollet, M., Richards, K., Jonard, G. (1993)b. Nucleotide sequence and genetic organization of Peanut Clump Virus RNA2 and partial characterization of deleted forms. *Virology.* 195: 33-41.

Thouvenel, J.C., Dollet, M., Fauquet, C. (1976). Some properties of Peanut Clump Virus, a newly discovered virus. *Ann. Appl. Biol.* 84: 311-320.

Thouvenel, J.C., Fauquet, C. (1981). Further properties of Peanut Clump Virus and studies on its natural transmission. *Ann. Appl. Biol.* 97: 99-107.
Trochain, J. (1931). La "lèpre" de l'arachide au Sénégal. *Rev.Bot.Appl. et d'Agric.Trop.* 11: 330-334.

Acknowledgements

We would like to thank Messrs. Dhery, Beaujard, Pariselle, Mortreuil (Senegal), Konate (Burkina), and Soumano (Mali) for their work in the surveys.

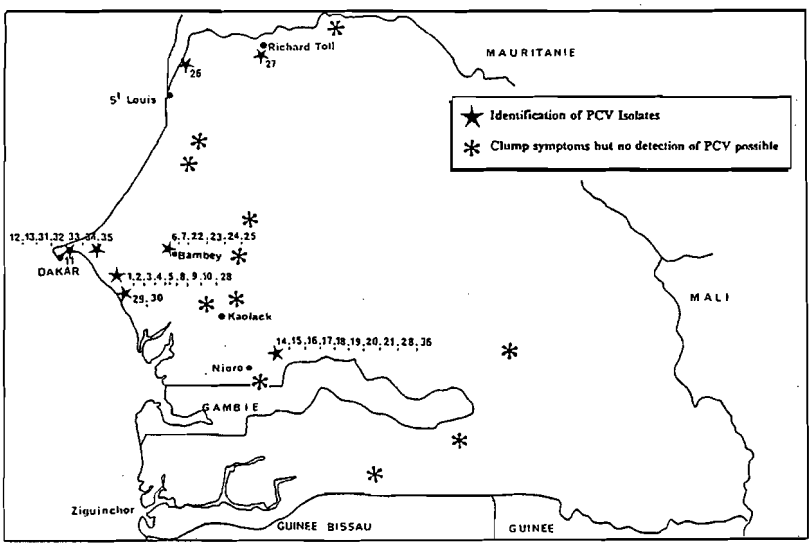


Fig. 1 PCV distribution in Senegal in 1990. Numbers represent each isolate collected during the surveys.

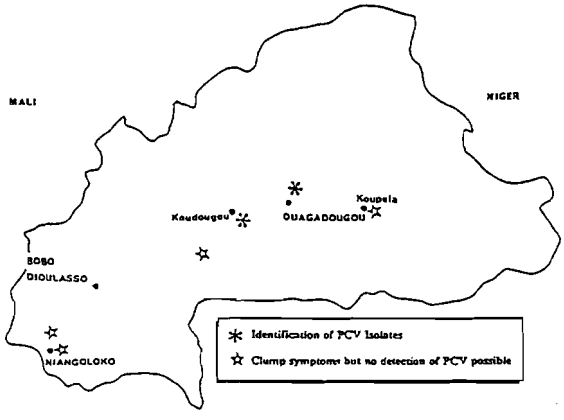


Fig. 2 PCV distribution in Burkina Faso in 1991.

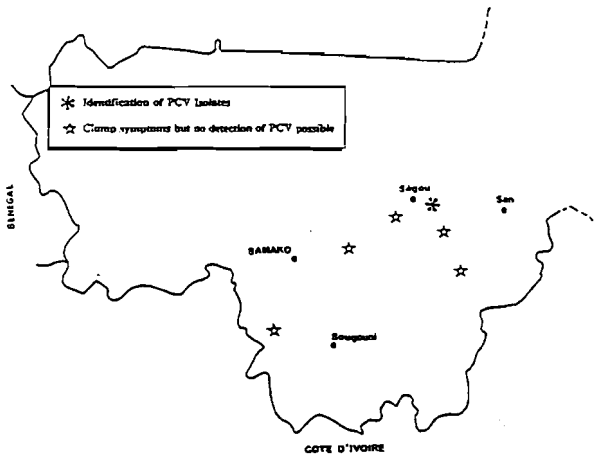


Fig. 3 PCV distribution in Mali in 1991.

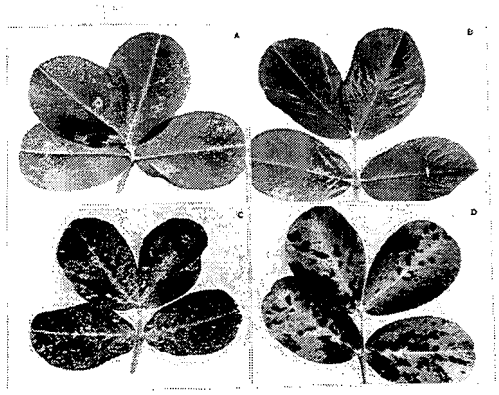


Fig. 4 Examples of PCV symptom variability observed on the variety 69101. A chlorotic rings, B yellow angular line pattern, C yellow specking, D green blotches.

EFFECT OF FLUAZINAM ON INFECTION OF SUGAR BEETS BY *POLYMYXA BETAE*, VECTOR OF BEET NECROTIC YELLOW VEIN VIRUS.

H. Uchino, K. Kanzawa and T. Tamada[1]

Research Center, Nippon Beet Sugar Mfg. Co., Ltd., Obihiro, and [1] Hokkaido Central Agricultural Experiment Station, Naganuma, Hokkaido, Japan.

Summary

In Japan, sugar beet growers have mostly used a transplanting system in their cultivation. Rhizomania damage to transplanted beets has been much less than to directly sown beets. The effects of fluazinam on preventing infection by *P. betae* in bed soil was tested by detection of BNYVV by ELISA in roots of sugar beet seedlings grown for 10 or 20 days in treated soil samples. BNYVV was detected from rootlets in infested soils treated with fluazinam below 10 ppm(W/W), but not in treated above 25 ppm. Moreover, it wasn't detected from rootlets in infested soil kept in a greenhouse for 3 months after treatment at 50 ppm. Fluazinam seemed to restrain both germination of resting spores and infection by *P. betae* zoospores. It was also proved by field trial that fluazinam is practically effective for controlling rhizomania in the nursery.

Introduction

The first outbreak of rhizomania in Japan was reported in 1972 (Kanzawa and Ui, 1972). It was proved in the next year that the causal agent was beet necrotic yellow vein virus (BNYVV) and the vector of BNYVV was *Polymyxa betae* Keskin (Tamada and Baba, 1973). The paper pot transplanting system is used for 97 % of Japanese sugar beet cultivation. Rhizomania damage of the transplanted beets is less than that of the directly sown beets (Bürcky, 1987). However, attention must be paid in transplanting when infested soil is used as nursery bed soil. Soil fumigants such as methylbromide and D-D are effective for controlling rhizomania (Kanzawa, 1969. Kanzawa, 1972. Henry et. al., 1992. Martin and Whitney, 1990. Schaüfele, 1987), but common fungicides have not been effective. We tested effects of fungicides against rhizomania by the screening method which detected BNYVV by ELISA from bait plants transplanted into the soil samples treated with chemicals (Uchino and Kanzawa, 1993). As a result, fluazinam was regarded as the most effective fungicide, and we report on the effects of its fungicide on resting spores and zoospores of *P. betae*.

Materials and Methods

Screening method of chemicals: Efficacy of chemicals was studied by this method to detect BNYVV in soil (Uchino et. al., 1990). 130 g of sterilized quartz sand was put in a polyethylene cup (capacity 275 ml) with a few small holes at the bottom. 100 g of infested soil mixed with fluazinam (0 - 100 ppm(W/W)) was put on the sand in the cup. Three to five one-month old sugar beet seedlings were transplanted into the soil sample as bait plants. Each cup was set in a small tray. It was incubated in a growth chamber at 25 C with a photoperiod of 16 hrs., and water was added when needed. The presence of BNYVV in the roots was tested by ELISA 10 or 20 days after incubation.

Persistency of efficacy in soil: Soil samples mixed with fluazinam (0 - 50

ppm(W/W)) were held in a greenhouse at 25 C in sunshine for 3 months. Water was often added to keep the moisture of soil samples in the greenhouse. The presence of BNYVV in roots of bait plants planted in the soil samples was ascertained with the above method.

Efficacy against the germination of resting spores: Sugar beet seedlings inoculated with *P. betae* were grown in test tubes with draining holes for 40 days. They were soaked in the fluazinam solutions (0 - 50 ppm), and zoospores were collected from the draining hole after 4 hrs.. The number of zoospores was counted in a Burker-Turk hemacytometer by microscopical observation.

Efficacy to zoospores: Zoospores were collected using the test tube culture system (Abe and Tamada,1987). Zoospores were kept at the rate of 1.1 x 10^4 spores/ml in fluazinam solutions (0 - 10 ppm) for 1 minute. One-month old seedlings were inoculated with the solutions at the rate of 5 ml/plant after treatment. Seedlings were grown in a growth chamber at 25 C with a photoperiod of 16 hr. for one month. Microscopical observation was used for detection of *P. betae* and ELISA was used for detection of BNYVV in rootlets after incubation.

Field trial: Rhizomania infested soil was used as the nursery bed soil. Fluazinam (0 - 50 ppm) was incorporated into the bed soil, and paper pots were filled with the treated soil. Sugar beets were sown in them on March 26 and were grown in a plastic film house. Each of 3 replicates of seedlings were transplanted into a non-infested field in a randomized block design on May 6. Root yield and sugar content were measured on October 13.

Results

Our results showed that BNYVV wasn't detected from seedlings in infested soils treated with fluazinam above 25 ppm, but it was detected from seedlings in soil treated below 10 ppm (Table 1). Moreover, BNYVV was detected at 25 ppm but not at 50 ppm, when infested soil was kept in the greenhouse for 3 months after treatment (Table 2).

The number of zoospores germinated from resting spores after soaking in 5 ppm fluazinam solution decreased to half the level of non-treatment. It decreased greatly when resting spores were soaked in 25 and 50 ppm solutions (Table 3). When the germinated zoospores were kept in a solution above 5 ppm for 1 minute and beet seedlings were inoculated with them, no infection of fungal vector was observed, and BNYVV wasn't detected (Table 4). When fluazinam was incorporated into the infested nursery bed soil, and the seedlings were transplanted in the non-infested field, rhizomania symptoms didn't appear for plants from 25 ppm and 50 ppm treatment, but a slight

Table 1. Detection of BNYVV from bait plant roots in infested soil treated with fluazinam by the screening method.

Concn. of fluazinam (ppm(W/W))	Absorbance value[a)]	
	10days	20days
50	0.00	0.03
25	0.02	0.00
10	0.09	0.94
5	0.08	1.40
0	>2.00	1.98

a)Means of 3 replicates.
Absorbance value of bait plants was measured 10 and 20 days after transplanting in infested soil treated with fluazinam.

Table 2. Detection of BNYVV from bait plant roots in infested soil kept in glass house for 3 months after treatment with fluazinam.

Concn. of fluazinam (ppm(W/W))	Absorbance value[a)]	
	10days	20days
50	0.00	0.00
25	0.01	1.15
10	0.11	>2.00
0	>2.00	>2.00

a)Means of 3 replicates.
Absorbance value of bait plants was measured 10 and 20 days after transplanting in the infested soil kept in a greenhouse for 3 months.

Table 3. Number of zoospores germinated from resting spores which soaked in fluazinam solutions for 4 hrs..

Concn. of fluazinam (ppm)	No. of zoospores[a)] germinated (x 10^3 spores/ml)
50	1.6
25	1.6
10	3.3
5	4.2
0	11.3

a)Means of 5 replicates.
Zoospores were counted using the Burker-Turk hemacytometer.

Table 4. Number of infected seedlings, when the beet seedlings were inoculated with the zoospore solution treated with fluazinam[a)].

Concn. of fluazinam (ppm)	No. of plants observed *P. betae*	No. of plants detected BNYVV
25	0/10	0/10
10	0/10	0/10
5	1/10	0/10
1	2/10	4/10
0	10/10	10/10

a)Zoospores which were kept in fluazinam solutions for 1 minute were used as inoculum. Each number was counted 40 days after inoculation by microscopical observation or by ELISA.

Table 5. Effect of fluazinam treatment of infested nursery bed soil[a)].

Concn. of fluazinam (ppm(W/W))	Rate of diseased beets (%)	Sugar yield[c)] (t/ha)
50	0.8 bc	8.52 a
25	0.0 c	8.67 a
10	4.2 b	8.23 a
0	99.2 a	0.14 b
STEAM[b)]	0.0 c	8.67 a

a)The treated seedlings were transplanted in the non-infested field in a randomized block design. One plot was 14.4 m^2.
b)Steam sterilized soil was used as nursery bed soil.
c)Means followed by the same letter within a column are not significantly different (p=0.05) according to Duncan's multiple range test.

symptom occurred at 10 ppm treatment. In contrast, severe symptoms were found in plants not treated. At harvest sugar yields of fluazinam treatment were the same as with plants from the sterilized bed soil (Table 5).

Discussion

Our results suggest that fluazinam possibly restrained infection by *P.*

betae and controlled rhizomania. BNYVV wasn't detected by the screening method from bait plants in infested soils treated with fluazinam above 25 ppm by our screening method. Moreover, its effect at 50 ppm was kept up for 3 months in the greenhouse.

The number of zoospores which germinated from resting spores was decreased by soaking in the fluazinam solution at the rate of above 5 ppm. Both *P. betae* and BNYVV were absolutely undetected in rootlets inoculated with zoospores which were kept in solutions above 10 ppm. It has been reported that fluazinam has suppressed mycelial growth of *Sclerotinia minor* classified to *Ascomycotina Discomycetes* (Smith et. al., 1991). We conclude that fluazinam restrain the germination of resting spores and the infection of sugar beet rootlets by zoospores of *P. betae* classified to *Myxomycota Plasmodiophoromycetes*. It hasn't been clear, however, whether fluazinam has a fungicidal effect against resting spores of *P. betae*.

We tried to control rhizomania in the nursery, as Japanese growers have mostly used the transplanting system with paper pots for cultivation of sugar beets. It was confirmed that fluazinam could control rhizomania in the nursery by soil incorporation above 25 ppm. We regard fluazinam to be a practically effective fungicide. This fungicide has a distinctive merit:beet seeds can be sown into nursery bed immediately after its treatment, which is not the case with soil fumigants.

References

Abe, H. and Tamada, T.(1987). A test tube culture system for multiplication of *Polymyxa betae* and beet necrotic yellow vein virus in rootlets of sugar beet. *Proc. Sugar Beet Res. Asso. Japan* 29: 34-38.

Bürcky, K.(1987). Zuckerertrag nach Pflanzen Vorkultivierter Zuckerrüben auf BNYVV-verseuchten Feldern. *Zuckerind.* 112: 812-814.

Henry, C. M., Bell, G. J. and Hill, S. A.(1992). The effect of methyl bromide fumigation on rhizomania inoculum in the field. *Plant Path.* 41: 483-489.

Kanzawa, K.(1969). Abnormal growth of sugar beets (A provisional name) 2. On the sterilization of the prepared soil for the paper-pot-nursery. *Bull. Sugar Beet Res. Asso. Japan* Suppl. 11: 48-55.

Kanzawa, K.(1972). Studies on rhizomania of sugar beet. 3. On the effects of several fungicides and nematocides on the control of rhizomania in soil prepared for the paper-pot nursery. *Bull. Sugar Beet Res. Asso. Japan.* Suppl. 14: 35-42.

Kanzawa, K. and Ui, T.(1972). A note on rhizomania of sugar beet in Japan. *Ann. Phytopath. Soc. Japan.* 38: 434-435.

Martin, F. N. and Whitney, E. D.(1990). In-bed fumigation for control of rhizomania of sugar beet. *Plant Disease* 74: 31-35.

Schäufele, W. R.(1987). Versuche zur Chemischen Bekämprung von *Polymyxa betae. IIRB 50th. Winter congress, Brüssel*: 97-110.

Smith, F. D., Phipps, P. M. and Stipes, R. J.(1991). Agar plate, soil plate, and field evaluation of fluazinam and other fungicides for control of Sclerotinia minor on peanut. *Plant Disease* 75: 1138-1143.

Tamada, T. and Baba, T.(1973). Beet necrotic yellow vein virus from rizomania affected sugar beet in Japan. *Ann. Phytopath. Soc. Japan* 39: 325-332.

Uchino, H., Abe, H., Tamada, T. and Kanzawa, K.(1990). A method to detect rhizomania in soil. *Proc. Japan Soc. Sugar Beet Technol.* 32: 86-93.

Uchino, H. and Kanzawa, K.(1993). Screening of fungicides with test plant method against rhizomania of sugar beets. *Ann. Phytopath. Soc. Japan* 59: 90(abst.).