NATO Security through Science Series - C:
Environmental Security

Virus Diseases and Crop Biosecurity

Edited by
J. Ian Cooper
Thomas Kuehne
Valery P. Polischuk

Springer

NATO
OTAN

This publication is supported by:

The NATO Programme for Security through Science

Virus Diseases and Crop Biosecurity

NATO Security through Science Series

This Series presents the results of scientific meetings supported under the NATO Programme for Security through Science (STS)

Meetings supported by the NATO STS Programme are in security-related priority areas of Defence Against Terrorism or Countering Other Threats to Security. The types of meeting supported are generally “Advanced Study Institute” and “Advanced Research Workshops”. The NATO STS Series collects together the results of these meetings. The meetings are co-organized by scientist from NATO countries and scientists from NATO’s “Partner” or “Mediterranean Dialogue” countries. The observations and recommendations made at the meetings, as well as the contents of the volumes in the Series, reflect those of participants and contributors only; they should not necessarily be regarded as reflecting NATO views or policy.

Advanced Study Institutes (ASI) are high-level tutorial courses to convey the latest developments in a subject to an advanced-level audience

Advanced Research Workshops (ARW) are expert meetings where an intense but informal exchange of views at the frontiers of a subject aims at identifying directions for future actions

Following a transformation of the programme in 2004 the Series has been re-named and re-organised. Recent volumes on topics not related to security, which result from meetings supported under the programme earlier, may be found in the NATO Science Series.

The Series is published by IOS Press, Amsterdam, and Springer, Dordrecht, in conjunction with the NATO Public Diplomacy Division.

Sub-Series

A. Chemistry and Biology	Springer
B. Physics and Biophysics	Springer
C. Environmental Security	Springer
D. Information and Communication Security	IOS Press
E. Human and Societal Dynamics	IOS Press

http://www.nato.int/science
http://www.springer.com
http://www.iospress.nl

Series C: Environmental Security

Virus Diseases and Crop Biosecurity

edited by

Ian Cooper

Natural Environment Research Council,
Centre for Ecology and Hydrology,
Oxford, U.K.

Thomas Kühne

Institute of Resistance Research and Pathogen Diagnostics,
Federal Centre for Breeding Research on Cultivated Plants,
Aschersleben, Germany

and

Valery P. Polishchuk

Taras Shevchenko' Kyiv National University,
Ukraine

Published in cooperation with NATO Public Diplomacy Division

Proceedings of the NATO Advanced Research Workshop on
Significance of Virus Diseases for Crop Biosecurity in a Developing
European Community
Kiev Ukraine
4–7 May 2005

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-5297-9 (HB)
ISBN-13 978-1-4020-5297-2 (HB)
ISBN-10 1-4020-5296-0 (PB)
ISBN-13 978-1-4020-5296-5 (PB)
ISBN-10 1-4020-5298-7 (e-book)
ISBN-13 978-1-4020-5298-9 (e-book)

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

www.springer.com

Printed on acid-free paper

CONTENTS

PREFACE

Biosecurity roughly means "safe life" and involves a variety of measures designed to prevent disease – causing agents from entering a region and there being spread.

Food supplies are easy to disrupt and the provision of biosecurity at land-land borders is especially challenging if trade is to be maintained and when very few travellers are subjected to thorough inspection.

Within the context of the NATO sponsored workshop that was held in Kiev, Ukraine during May 4–7, 2005, the pathogens were viruses that infect plants and the region encompassed developing states on the verge of acceding into the European Union. In publishing the papers presented at the workshop, we take this opportunity to thank the sponsors including particularly the NATO Science committee and also the contributors for making the discussions entertaining and beneficial.

Under United Nations FAO auspices, the International Plant Protection Convention aimed to secure common and effective activities against pests and pathogens. Now, most countries party to that convention have laws and regulations in place to sustain agricultural production under natural threat. National plant protection services exist to inspect growing crops and importations and to determine when and how introduced pathogens might be eradicated. The member states of the European Union, through their national [and also the regional plant protection service (The European and Mediterranean Plant Protection Organisation)], advise national governments and develops specific protocols (identification, containment and eradication) that aim at managing pests and pathogens in ways that have minimal impact on trade. The processes are costly and inconvenient. Furthermore, success is not certain. For these and other reasons, the efficiency of established phytosanitary systems are eroding and those who contributed at the workshop highlighted deficiencies that are now in urgent need of remedy.

The savage action in New York and Washington in September 2001 and the instances of anthrax delivery via the U.S. mail caused all types of potentially offensive activity to be assessed or reassessed. Human pathogenic viruses and micro organisms had been identified as of concern whether natural or modified/selected for enhanced virulence and pathogenicity. In some nation states, export controls now exist for a range of vertebrate pathogens. Furthermore, pathogens harmful to bees or the environment more generally are all objects of national or international regulation. However, no similar list of specific plant pathogens is yet internationally agreed despite the potentially

very significant long-term economic impacts coupled with psychological and social disruption.

With a few notable exceptions (e.g. use of a chemical toxin in Japan in support of racial/ethnic/religious and political objectives), non-military human targets seems to have been very uncommon. Furthermore, major terrorist groups have used "traditional" high explosives to obtain publicity and to engender fear in communities that they seek to influence. This does not provide reasonable grounds for complacency. Biological attacks on food supplies, forests or natural vegetation have long been envisaged and must now be considered "likely". Individuals and groups with regional political constituencies are now competent to use a diverse range of pests and pathogens to support their objectives. Living, self-replicating agents, notably viruses and micro organisms with lethal or debilitating outcomes, have been stockpiled and it is generally acknowledged that state-sponsored programmes have "weaponised" fungi (e.g. *Puccinia graminis*) active against plants grown for food. Additionally, *Sugar beet necrotic yellow vein virus* and *Plum pox virus* have been highlighted as candidate agents for use as weapons. Current technology can modify viruses to enhance what nature has already selected. Furthermore, although natural processes tend to result in a balance that moderates the impact of a pathogen in the interests of its persistence, that phenomenon has little relevance when the targets are agricultural crops and not natural self-regenerating populations.

Against this background, it is necessary to raise awareness in the global scientific community without causing undue concern in the lay community. To strengthen the position of scientists (and workers more generally), many nation states impose "health and safety" regulations that cover diverse chemicals and biological agents in line with national laws and also international treaty obligations. Two Conventions under the aegis of United Nations agencies are significant in this regard. The Convention on Biological Diversity was initially signed by 150 government leaders at the 1992 Rio Earth Summit and provides a framework for action bearing on sustainable development and trade. Support for those very broad ranging activities is not universal but has increased somewhat and currently the Convention has188 Parties (168 Signatures). In 2000, the Conference of the Parties to the Convention on Biological Diversity adopted a supplementary agreement known as the Cartagena Protocol on Biosafety. This protocol has a key role to play in matters of biosafety because it seeks to protect biological diversity from potential harm resulting from modern biotechnology (including risks posed by living (genetically) modified organisms). The Protocol enables the establishment of a pool of experts to facilitate the exchange of information. At present, the Cartagena Protocol on Biosafety has 130 Parties (103 Signatures) – but, once again, not all states are formally Party. It is notable that although the United States of America signed

the Biodiversity Convention, that country is neither Party to the Convention nor to the Biosafety Protocol!

Similarly, not all, nation states are party to the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction [the Biological and Toxin Weapons Convention (BTWC) for short]. The BTWC broadened the terms of an existing Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases, and of Bacteriological Methods of Warfare that had been signed at Geneva on June 17, 1925 and was signed at London, Moscow and Washington in 1972. It has laudable aims and facilitates international contacts but offers considerable scope for "interpretation". Thus, for the sake of mankind, the signatory states sought to exclude the possibility of bacteriological (biological) agents and toxins being used as weapons and undertook never to develop, produce, stockpile or otherwise acquire or retain microbial or other biological agents, or toxins in quantities that have no justification for prophylactic, protective or other peaceful purposes – or means of delivery designed to use such agents or toxins. Importantly, each state party to the BTWC agreed not to transfer any of these objects to any other state and to consult one another and to destroy all agents, toxins and means of their delivery while safeguarding the environment. Nevertheless, states party to the BTWC retained rights to participate in the fullest possible exchange of equipment, materials and scientific and technological information for the use of bacteriological (biological) agents and toxins for peaceful purposes.

Not all signatories have enacted appropriate national legislation or operate the same degree of health and safety regulation supported by inspection. There is a constant but slow progression in activities to further the aims of these two conventions and this activity is particularly obvious in the context of genetically modified organisms. Thus, the members of the European Union have agreed regulations (e.g. Directive 2001/18/EC) that require member states, in accordance with the precautionary principle, to ensure that all appropriate measures are taken to avoid adverse effects on human health and the environment (including agricultural production) which might arise from the deliberate releases of genetically modified organisms. Increasing the number and variety of codes of conduct provides important opportunities for education and training of workers including scientists and students in a variety of possible consequences. Undergraduate and postgraduate education programmes (in academic and also industrial settings) can be followed in a climate that supports consideration of ethical issues and helps to identify opportunities for possible misuse of technologies that may be mainly studied because they were expected to offer real potential benefits to society. Scientific research inevitably gives rise to some unexpected findings and the full potential implications are not always appreciated by workers in small intellectually isolates

teams. Fortunately, peer review prior to the funding of research, at later stages in the development of a programme and subsequently during the publication process all provide opportunities for broader appreciation of issues that bear on novelty. There is a clear need for improved guidance to improve the effectiveness of scientific oversight but it is still not easy to identify all issues pertinent to potential uses of knowledge. To this end national academies of science and publishers all have potential roles to play in the process and useful sources are open for inspection (e.g. www.journals.asm.org/misc/Pathogens and Toxins.shtml.; www.brad.ac.uk/acad/sbtwc/briefing/BP 15 2nd series.pdf.; www.sgm.ac.uk/pubs/policy.cfm).

Notwithstanding current obligations under one or other of the conventions, there are concerns that some nation states may be deliberately using students and scientists in training to procure expertise. Furthermore, it is now very easy for molecular geneticists to access scientific literature that describes which genetic sequences have potential utility, can be synthesised and then used to enhance pathogenicity. This scenario is not new but curtailment of information flow (and people) touches on sensitive areas of civil rights and traditional academic freedoms. The best protection against proliferation of biological agents probably is transparency, but covert research in this context is suspected to be not uncommon. Partly as a consequence, the potential hazard to agriculture from biological agents is underrated and is allocated less research support than issues directly bearing on human health. In discussion, many issues were explored that are not reflected in this volume. Contributors were anxious to avoid producing "a terrorist's handbook"!

LIST OF CONTRIBUTORS

Günter, Adam
BioCenter Klein Flottbek,
Phytopathology
University of Hamburg
Ohnhorststrae 18
22609 Hamburg
Germany

Mariano, Cambra
Instituto Valenciano de
Investigaciones Agrarias (IVIA),
Departamento de Protección Vegetal
y Biotecnologia
Carretera Moncada-Náquera km 5
46113 Moncada (Valencia)
Spain

Ian, Cooper
Natural Environment Research
Council,
Centre for Ecology and Hydrology
Mansfield Road
OX1 3SR
Oxford
UK

Tatyana, Erokhina
Shemyakin-Ovchinnikov Institute
of Bioorganic Chemistry,
Russian Academy of Sciences
Miklukho-Maklaya 16/17
117997 Moscow
Russia

Thomas, Kühne
Institute of Resistance Research
and Pathogen Diagnostics,
Federal Centre for Breeding
Research
on Cultivated Plants
Theodor-Roemer Weg 4
06449 Aschersleben
Germany

Sergey, Morozov
A. N. Belozersky Institute of
Physico-Chemical Biology,
Moscow State University
119899 Moscow
Russia

**Daphne, Osborne (deceased
June 16, 2006)**
Oxford Research Unit,
The Open University,
Foxcombe Hall, Boars Hill
OX1 5HR
Oxford UK

Valery, Polishchuk
Taras Shevchenko' Kyiv National
University
Volodymyrska 64
01033 Kyiv
Ukraine

Ivan, Shcherbatenko
Institute of Microbiology and
Virology,
National Academy of Sciences of
Ukraine
Zabolotny 154
03143 Kyiv
Ukraine

Michael, Taliansky
Scottish Crop Research Institute
DD2 5DA
Invergowrie/Dundee
UK

Mike, Thresh
Natural Resources Institute,
University of Greenwich
Chatham Maritime
ME4 4TB
Kent
UK

René, Van der Vlugt
Plant Research International BV.
Droevendaalsesteeg
6700 AA Wageningen
The Netherlands

A SHORT HISTORY OF WARFARE EMPHASIZING THE BIOLOGICAL ASPECTS

Daphne J. Osborne*

1.1. Introduction

Denial of water or food once guaranteed success to an aggressor. If the effects of such denial were fully reversible after capitulation and once the defeated had fled, then the aggressor gained a considerable victory. There is a nice example of this "reversible" activity in The book of Genesis, chapter 26. Isaac, son of Abraham, lived and prospered in the land of the Philistines. This did not please the Philistines, so they routinely blocked up the water wells of the people of Isaac. In order to survive, the people of Isaac kept digging new wells but the Philistine herdsmen attacked these too, so that eventually "the people" of Abraham and Isaac were forced to leave that region. The Philistines did rather well from this victory as they then unblocked all the old and the new wells, none of which had suffered permanent damage.

It is not easy to distinguish terrorist action from warfare but the former tends to be more or less localized and on the smaller scale. In this instance the Philistine herdsmen seemed to have acted as terrorists who targeted a crucial resource but with physical means—not biological. In any event, not all such activity is so readily reversible.

In a historical context there are noteworthy examples of large-scale action against food production with long-term impacts that were not immediately reversible. Thus, in the first century BC, Carthage, in what is now modern Tunisia, was an agricultural and trading area supplying much of the Middle East with wheat and other foods. When the Romans defeated the Carthaginians militarily in 145 BC (razing the city and exporting its occupants into slavery) the victors then destroyed Carthage economically by spreading salt over the land—a form of desertification that is said to have lasted at least 25 years. This activity was state-sponsored warfare and impacted on biological systems but did not depend on biological (replicating) agents. Thus, the term biological warfare is not appropriate although chemical warfare might be.

* Daphne Osborne died on June 16, 2006.

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 1–8.

1.2. Aspects of Biological Warfare—Some Early Examples

During the Middle Ages, attacks on castles or walled cities involved the denying of potable water and untainted food and, as a prelude, when armies faced each other on the field of battle, noise (from drums) was employed to frighten and intimidate opponents. If fresh water springs were within the city walls and enough food had been stored, sieges could last for many months. But when the water sources were outside the walls, then the aggressors polluted them with dead animals making the water undrinkable and ensure the spread of sickness within, whether or not food was still available. From earliest times, catapults, as engines of war, were used to hurl dead and decomposing bodies inside a fortress. When possible, those on the inside hurled the corpses out again onto the besiegers. How far these polluting acts were effective for the introduction of pathogens is not known partly because there were, at the same time diverse conditions that favored the spread of preexisting pests and pathogens.

When the castle or city walls where finally breached by battering rams, catapults or cannon, hostage taking, rape, looting, and the eventual destruction by burning was frequent but the food supply was not usually a specific target (not least because the victors needed food).

1.3. Plagues and Contagion

Until relatively modern times, natural plagues (such as locusts in Egypt, the Black Death of Europe, and Potato Blight in Scotland and Ireland) were not uncommon and were much feared because causes and remedies were unknown. Although wild plants are reservoirs of viruses that threaten agricultural or horticultural crops and some fearful human pathogens are recognized as "emerging" from wild animal reservoirs (e.g. the agents of lethal influenzas and hemorrhagic fevers; Garrett, 1994; McCormick et al., 1996), many plagues have ancient origins.

In many instances, the transmission of pathogens between plants or animals is still not fully understood and it was not until the Second World War that manipulation of the transmission process became possible in a few systems. When the information was available, it became possible for one nation deliberately to generate food shortages.

Even in ignorance of causation and transmission, human health was threatened as early as 1346 when victims of plague (caused by *Yersinia pestis*) were hurled into the city of Katta—one of the fortified Genoese sea ports on the Crimean Coast—that was besieged by the Mongols. The Mongols themselves inevitably risked infection but gained some psychological advantage over their opponents.

Human pathogens causing smallpox, measles, or influenzas were inadvertently introduced with colonization of North America from the 15th century

onward and the importation of slave workers from West Africa subsequently introduced tropical parasites and pathogens (causing, for example, malaria or yellow fever) with their mosquito vectors.

1.4. Intentional Introduction of Pathogens

The first reliably documented intentional use of biological agents for subjugation of indigenous communities occurred in North America during the siege of Fort Pitt in 1763. British forces deliberately used as gifts, blankets, and bedding already contaminated with smallpox scabs. The cost of the items involved was later reimbursed by the British authorities with the approval of Sir Jeffrey Amherst, then commander in chief of the British Forces in America!

The British may well have used smallpox again as a weapon in the American Revolutionary War by identifying civilians with smallpox before sending them to mingle with the opposing troops.

These events strongly suggest state sponsorship of biological warfare [see also the Report of a Royal Society Study Group (1994) and Williams and Wallace (1989)] but it is instructive to consider one aspect of state sponsored chemical warfare that evolved from a "Good Cause." At this point I became personally associated with the activity in the late 1950s and 1960s.

1.5. Novel Aspects of Chemical Warfare

1.5.1. AFRICA

The tse-tse fly (*Glossinia* sp.) carries trypanosomes that are blood parasites that cause sleeping sickness—both in man and animals—with particularly devastating effects in tropical Africa. The flies depend for their survival upon sucking blood and living under shade conditions provided by the trees and shrubs that make up vast tracts of the African bush.

In 1921, it was first proposed that clearing the trees and shrubs around an area that had been freed from the tse-tse would prevent re-invasion of the fly into the land enclosed within. Initially, the clearance was done mechanically with human labor but, in 1936, the trees were killed by applications of arsenic pentoxide or other toxic chemicals. These approaches were only partially successful but there were no further developments until 1949 when there was a revolution in the development of selective herbicides in agriculture. Many of the effective chemical compounds were synthetic analogues of the natural plant hormone, auxin.

G.E. Blackman, the Professor of Agriculture in Oxford at that time, and one of the discoverers of these synthetic auxin-like selective herbicides, suggested to the Colonial Office in London that spraying an area of infested tse-tse

bush with the auxin analogues might cause sufficient defoliation to make regions unsuitable for the adult tse-tse but without actually destroying the trees.

In 1950, the Department of Agriculture in Oxford was commissioned by the British Government to research the use of herbicides, arboricides, and defoliants in a variety of Colonial contexts.

After some encouraging small-scale experiments in Kenya in 1951, the whole area of land of the Waturi peninsular of Lake Victoria, was air-sprayed with a diesel oil formulation of the butyl ester of 2.4.5-trichlorophenoxyacetic acid (the synthetic auxin)—called 245T for short. This chemical was only moderately successful in eliminating tse-tse but the knowledge of how to increase visibility in a tropical forest provided the basis for one of the greatest anti-terrorist defoliation programs ever carried out by the United States military in Vietnam. It came about in the following way.

1.5.2. MALAYA AND COUNTER INSURGENCY

In Malaya, acts of political terrorism became increasingly serious in the 1950s. Insurgents, many infiltrating from the People's Republic of China, were intimidating the local population and matters came to a head in 1952. The British High Commissioner, Sir Henry Gurney traveling in his Rolls Royce out of Kuala Lumpur, was assassinated by terrorists concealed in the dense foliage of the forest trees bordering the road. The British response was to spray (with the 245T) the vegetation on both sides of main roads. Trees were defoliated for a sufficient length of time to reduce cover for terrorists and no more similar assassinations took place (Osborne, 1968; Hallaway and Osborne, 1969).

1.5.3. MALAYA AND ECONOMIC TARGETS FOR CHEMICAL WARFARE AGENTS

Plots of food plants hidden in the forests were targets for herbicides both in Burma at the end of the Second World War and again in Malaya, where insurgents grew rice and food plants in small forest clearings. Spray applications from aircraft were directed at these crops—not the human (insurgent) population directly—and there are parallels now with the activities of drug prevention agencies.

One of the most exciting and possibly successful anti-terrorist activities was in anticipation of bio-terrorist acts targeting the valuable and vast rubber plantations in Malaya. Short-lived spores disperse the fungal disease of leaves caused by *Dothidella ulei*. After the Second World War, with the increase of fast air travel, the British Government feared that insurgents would bring the spores of *D. ulei* from South America (where the fungus was endemic), to threaten Malaya's (infection free) industry.

The British knew that spraying 245T butyl ester would defoliate a rubber tree within 10–14 days without killing the tree, and such defoliation could thereby eliminate the spread of any newly introduced disease agents. Stocks of 245T butyl ester were kept in Malaya for many years to combat such an attack—but in fact, such an attack never developed—or, if it was tried, it was not successful.

It is important to appreciate here, that no long-term or deleterious toxic effects were noted in Malaya after spraying with 245T and this raises the question as to why similar chemical treatment by the USA in Vietnam had devastating results.

1.6. Methods of 245T Synthesis

Synthesis of the most effective defoliant, 245T, (indeed all the biologically active phenoxy acids) is via the chlorinated phenol condensed with chloroacetic acid and then esterified with the appropriate alcohol (Figure1).

Depending upon the conditions of synthesis, dioxins are formed, particularly at high temperatures—and, during the synthesis of 245T, levels of the very toxic dioxin 2,3,7,8-tetrachlorodibenzo-*part*-dioxin (TCDD) increase (Milnes, 1971).

The first dioxins prepared in Germany in 1872 and preparation of TCDD itself in 1957, led to the hospitalization of the laboratory workers involved (Figure2).

Because phenoxy acetic acids in the United Kingdom were almost exclusively used as selective herbicides on crops that humans would eventually eat, the synthesis procedures were deliberately designed to minimize dioxins levels. As a result, TCDD was a very tiny component of the 245T in use before the Vietnam War.

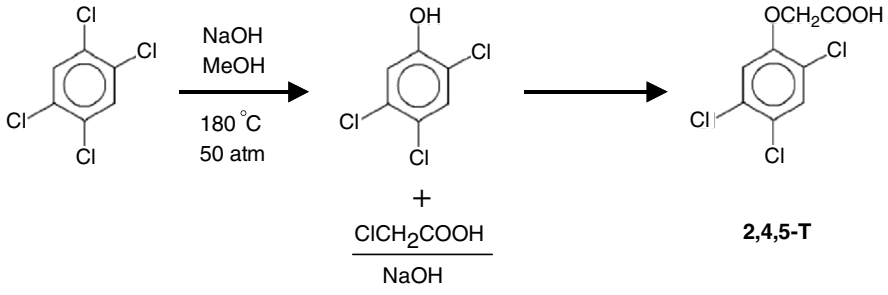


Figure 1. Normal preparation of phenoxy acids. Synthesis of 2,4,5-T. For industrial preparation, 1,2,4,5-tetrachloro-benzene is hydrolyzed under basic conditions to 2,4,5-trichlorophenate. 2,4,5-T is formed in a subsequent reaction with chloroacetic acid (see above). Phenoxy acids are then esterified with the appropriate alcohols

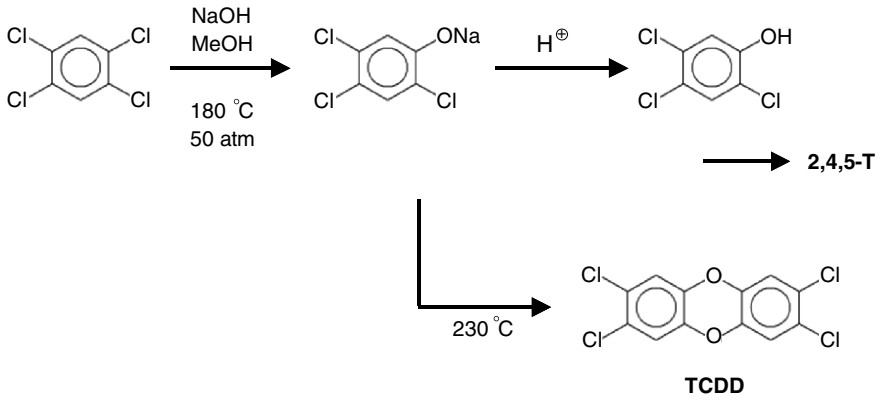


Figure 2. Conditions of phenoxy acid synthesis with enhanced TCDD formation. The formation of Dioxin TCDD

For defoliating the jungles surrounding the Ho Chi Min trail, or mangroves along the Mekong River in Vietnam, a different chemical composition (Agent Orange) was used and purity of the active ingredient (245T) was not considered to be necessary. The drive for quantity rather than quality of product resulted in much greater amounts of TCDD and other dioxins in Agent Orange than in formulations of 245T used in the United Kingdom.

1.7. Mangroves

The regeneration of mangroves along the river borders took a particularly long time because mangroves undergo continuous development of the embryo from the time of egg fertilization until the establishment of a new individual seedling after the ripe and mature fruit has dropped into the river. Dehydration of the fruit at any time stops DNA replication and kills the embryo within. As a result, attempts at reforestation with collected seed were generally unsuccessful (Osborne and Berjak, 1997).

1.8. Changing Views on Biological Warfare

Although chemical and biological warfare against humans became [internationally] less acceptable during the Cold War years, assaults on economic plants became increasingly appealing and, by 1969, it has been estimated that the USA had stockpiled 30,000 tonnes of wheat stem rust (*Puccinia* sp.) spores and a tonne of rice blast (*Piricularia* sp.) spores. The Soviet Union was thought to have stockpiled wheat stem rust and pathogens for maize and rice and it is suspected that Iraq developed a wheat smut bomb!! Plant pathogenic

viruses and genetically modified microorganisms having enhanced expression of innate (or extraneous) toxins and pathogens harmful to bees or fish are not always explicit in the diverse lists of controlled agents but in many states there are "catch-all" clauses in legislation and guidance notes governing the production and distribution of agents with a potential for use as biological weapons.

A deliberate use of microorganisms (or toxins derived from living organisms) to induce death or disease in plants may, theoretically, be carried out with no direct harm to the human population. However, the targeting of key agricultural industries is likely to undermine confidence and potentially causes destabilization of a Government. The epidemic of foot and mouth disease in sheep and cattle in the United Kingdom during February 2001 directly caused the postponement of national elections, had durable and very substantial cost implications and radically changed the patterns of farming. The earlier (1967–1968) epidemic in the United Kingdom was estimated to have had a direct cost of £35 million, largely attributed to compensation for slaughter, valuation, cremation/deep burial, and decontamination. The indirect costs attributable to loss of income from the slaughtered animals and disruption of production, marketing, and trade provide scope for wide divergence of view but were in the region of £100 million.

There are real fears that pathogens which harm animals used for food or draught have potential for use by terrorists but it is not so easy to introduce an effective agricultural plant pathogen, whether fungal, bacterial, or viral. The transmission dynamics are key but, in addition, plant pathogens are generally sensitive to their environmental conditions and viruses often need specific vector biotypes.

1.9. Means of Delivering Pathogens

Agricultural crops are extensive and stationary and do not require very sophisticated targeting. Although largely beyond the scope of this chapter, it is noteworthy that attempts to deliver plant pathogens sometimes seem bizarre (e.g. bomblets holding spore-coated feathers that, on detonation, float down onto crop fields, and paper balloons containing spores that would burst over the enemies' food plants). Nevertheless, it is very likely that any nation holding stocks of biological agents has adequate and tested means of delivery (see Guillemin, 2005).

1.10. The Future

Biosecurity in a developing state of the expanded European Union is the theme of this meeting and my quick survey suggests that there is a real threat

from terrorists using approaches developed for warfare or for wholly benign purposes. It is impossible for a scientist to anticipate all the uses to which a discovery might be put; the inventor of the wheel could not anticipate its use for transportation of artillery, rockets, planes, or bombs. Analogously, the development of herbicides used for tse-tse fly management (and human benefit) is far removed from the durable human harm that accompanied use of Agent Orange in Vietnam. Since 1936, when the Japanese Army established a unit with the innocuous title "Epidemic prevention and Water Supply Unit" to develop biological warfare systems for use against people and also crops (e.g. Williams and Wallace, 1989), states have stockpiled a range of biological agents for the same purposes. I do not doubt that food and forest crops are still targets. Delivery of the pathogens does not need much sophistication or training. For the reasons outlined by Cooper, Cambra, and van der Vlugt (this volume), it is by no means certain that even the most developed states in Europe will be able to respond effectively to such challenges unless rapid diagnosis is ensured and effective management follows a terrorist attack, from whatever direction such assaults might arise.

Acknowledgments

The author acknowledges the generous assistance of G.B. Carter (Personal communications, Defence Scientific and Technology Laboratory, Porton Down, UK) and Holly Huber (Oxford Research Unit, Open University, UK) in the preparation of this manuscript.

References

Garrett, L., 1994. The Coming Plague, Farrer, Straus and Giroux, New York.

Guillemin, J., 2005. Biological Weapons, Columbia University Press, New York.

Hallaway, H.M., and D.J. Osborne, 1969. Ethylene, a factor in defoliation by auxins, Science, 163, 1067–1068.

McCormick, J.B., S. Fisher-Hoch, and L.A. Horvitz, 1996. Level 4: Virus Hunters of the CDC, Turner Publishing Inc., Atlanta.

Milnes, M.H., 1971. Formation of 2,3,7,8-tetrachlorodibenzodioxin by thermal decomposition of sodium 2,4,5-trichlorophenate, Nature, 232, 395–396.

Osborne, D.J., 1968. Defoliation and defoliants, Nature, 219, 564–567.

Osborne, D.J., and P. Berjak, 1997. The making of mangroves: The remarkable pioneering role played by seeds of *Avicennia marina*, Endeavour, 21, 143–147.

The Royal Society Report on Scientific Aspects of control of biological weapons, 1994.

Williams, P. and D. Wallace, 1989. Unit 731, Hodder and Stoughton, Sevenoaks, Kent.

CROP VIRUSES AND VIRUS DISEASES: A GLOBAL PERSPECTIVE

J. M. Thresh

2.1. Introduction

Viruses were distinguished as a separate group of plant pathogens in the 1890s, as a consequence of pioneering studies in Russia and the Netherlands (Bos, 2000). They have since received much attention from plant pathologists and more recently from molecular biologists. Nevertheless, the information available on the distribution, prevalence, and importance of plant viruses and the diseases they cause is still incomplete and any attempt to present a global perspective is fraught with difficulties. The main problems are

- The very wide range of crops and agro-ecologies that must be considered to provide a comprehensive overview of the global situation. Inevitably, the overall research effort has been inadequate in relation to the magnitude of the problems encountered.
- The patchy and irregular distribution of plant virologists and facilities in the different regions of the world. In some countries there is a long tradition of plant virology, utilizing well-equipped laboratories and trained personnel. Other countries are in a much less satisfactory situation and the information available is correspondingly limited or almost entirely lacking. Moreover, there are language and cultural barriers to the dissemination of research findings.
- The decreasing attention being given in recent years to the applied aspects of plant virology. In many developed countries funds, personnel, and resources are being withdrawn or diverted to molecular biology and biotechnology because crop productivity and food production are no longer perceived as a high priority. By contrast, research in developing countries is being curtailed, in part because of a misconception by donors and policy-makers that many of the findings already available are not being adopted or utilized effectively by farmers. This is because of the failure or limitations of extension services and the inadequate funds allocated.
- The lack of data on the prevalence of plant virus diseases and the losses they cause. The commonly held view that losses have increased in recent decades is plausible and supported by general observations in diverse agro-ecologies and on many different crops. However, there are few data to confirm or deny the supposition and enable an informed opinion.

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 9–32.

Despite these and other difficulties it is possible to make generalizations and inferences on the status of plant viruses and virus diseases, as presented in the following sections. These are relevant and apposite in any further discussion on the implications of enlarging the European Community and on the importance of plant virus diseases and their possible rôle in bioterrorism.

2.2. Viruses as Plant Pathogens

Charles Darwin (1859) wrote in *The Origin of Species* "we see beautiful adaptations everywhere and in every part of the organic world." His comment arose from observations on ectoparasites of birds, but applies equally to plant pathogens. These are now known to comprise a very wide range of fungi, bacteria, algae, protozoa, rickettsias, phytoplasmas, spiroplasmas, viruses, and viroids.

Viruses and viroids are exclusively obligate parasites, with the simplest structural and physicochemical features of all pathogens, yet they resemble true living organisms in displaying great diversity, versatility, and adaptability in exploiting varied habitats and different modes of perennation and spread. Some notable features of plant viruses are listed below and many are also characteristic of viroids. The latter are a separate group of sub-microscopic systemic pathogens that are distinguished from viruses by their mode of replication, smaller genome, and lack of coat or other protein components.

- Plant viruses have limited ability to enter intact host cells and mainly depend on insect, mite, nematode, or fungus vectors to gain entry.
- Viruses usually invade their host plants systemically from the initial entry sites. Infections that remain localized in the roots or leaves can occur, but seldom have very deleterious effects.
- Viruses usually persist throughout the life of systemically infected plants and so pass to vegetative propagules and, in some instances, to a proportion of the pollen and seed produced. Viruses are seldom eliminated naturally from systemically infected plants and there is no recovery phenomenon equivalent to the immunological response of mammals.
- Few plant viruses remain viable for long outside living tissues and their survival is largely dependent on the availability of a continuous sequence of hosts. Thus there are likely to be close co-evolutionary relationships between viruses and their host plants. This possibility has received only limited attention, although it is apparent that plant viruses are seldom lethal to their host(s) and this would threaten their long-term survival.
- Some plant viruses can infect their insect vectors and so persist for long periods in vector populations, and in some instances through many generations, even in the absence of susceptible host plants.

- Viruses as obligate intracellular parasites largely avoid competition with other microorganisms. However, important interactions occur between dissimilar viruses and between different strains of the same virus. For example, "dependent" viruses are known that are not transmissible by vectors in the absence of a distinct "assistor" virus and a few "satellite" viruses only multiply in the presence of an appropriate unrelated "helper" virus. Moreover, mild strains of virus usually protect plants from the severe effects of virulent strains of the same or closely related virus species and this phenomenon can be exploited to assess relationships and to provide an effective means of control.
- Viruses can be spread widely by vectors or other means and sometimes influence plant populations far beyond the usual range of gene flow in pollen or seed (Thresh, 1983a). This is apparent from experience with cereal yellow dwarf viruses that can be spread northwards over distances of hundreds of kilometres by aphids dispersing from southern USA to cereal-growing areas of the northern states and of Canada (Irwin and Thresh, 1988). There is also evidence of a northward movement of cereal and other aphid species from mainland Europe to Scandinavia (Wiktelius, 1977). Moreover, the rice brown planthopper (*Nilaparvata lugens*) migrates even longer distances from tropical and sub-tropical areas of South-East Asia, where rice is grown throughout the year, to temperate areas of China and Japan where growth is seasonal (Thresh, 1983a).
- Differences in host range and in the susceptibility or response of plants to infection suggest that viruses act as selective, discriminating pathogens that exert important ecological effects on competition within and between species in natural plant communities and also in crop stands.
- The severity of the damage caused by viruses is seldom closely related to virus content. Some viruses reach uniformly high concentrations yet have barely detectable effects, whereas others that occur in much smaller amounts, or mainly in phloem tissue are extremely damaging.

These distinctive features of plant viruses and viroids explain their great economic importance. They also influence the "natural history" of viruses and the population dynamics of the diseases they cause. Before discussing these topics it is appropriate to consider some aspects of crop plants and the habitats they provide as hosts of viruses and other pathogens.

2.3. Host Populations

Biologists considering the population dynamics of animal pests or weeds of crops can utilize the experience and concepts derived by the many ecologists

studying plants and animals in natural habitats. Crop pathologists seldom have this advantage because there have been few quantitative studies of fungal pathogens in natural stands of the type discussed by Dinoor and Eshed (1987) from their experience in Israel. There is even less information on plant pathogenic bacteria and viruses, which is a serious limitation of the literature. It has led to a biased "agrocentric" perspective in plant pathology that has until recent decades largely overlooked the profound differences between crops and wild vegetation (Browning, 1981). These differences are discussed by Burdon and Shattock (1980), who emphasize how many crops are grown in monoculture and more or less synchronously in dense, regular stands of uniform genotype.

Virtually all published epidemiological data on virus spread were obtained from observations on such plantings, and with a few notable exceptions there has been an emphasis on particularly important diseases of mainly arable crops. For this reason and because virus incidence is usually expressed in percentages, there is a tendency to ignore the great differences between arable and perennial tree crops in plant population per unit area and in the duration and type of habitat they provide.

2.3.1. HOST DIVERSITY

A feature of the earliest forms of agriculture is that many different species were exploited (Harlan, 1976). They included both herbaceous and woody species of very diverse habit and growth characteristics. Initially, the crops grown were those that originated within the region, but there has long been an extensive traffic in plants, seeds, and vegetative propagules. This occurred during human migrations and military campaigns and through the activities of missionaries, explorers, colonialists, plant collectors, and commerce. One consequence has been that many crops are now grown extensively far from their region of origin and introduced crops predominate in several continents and are grown almost exclusively in Australasia. This has been an important factor in enabling crops to escape from co-evolved pathogens and arthropod pests, but has also led to new disease problems as introduced hosts first encounter viruses that are already established in indigenous species (Buddenhagen, 1977; Mitchel and Power, 2003).

An important feature of traditional cropping systems is that there is extensive use of many landraces that were selected locally by farmers and often grown in complex mixtures (Smithson and Lenné, 1996). Moreover, this form of diversity was often supplemented by the common practice of incorporating one or more intercrops (Trenbath, 1975). The implications of both varietal and crop diversity have received considerable attention from researchers in recent years as cropping systems have become specialized and increasingly

dependent on a restricted range of cultivars that are usually grown singly and without intercrops. The consequent loss of diversity is now seen as being a serious flaw of modern practices, as it was important in providing crops with a substantial degree of resilience that enabled farmers to overcome, at least in part, the deleterious effects of pests, diseases, and other biotic or environmental constraints. This is because tolerant crops and genotypes partially compensate for the impaired growth of their more seriously affected neighbors.

Attempts are now being made to exploit the benefits of diversity, although this may not be readily compatible with the use of herbicides and other modern cropping practices and the requirements of commercial markets for highly uniform produce. Diversity is also being utilized in the search for virus- and vector-resistant varieties and substantial collections of genotypes have been assembled at the main International Crop Research Centers and elsewhere (Thurston, 1977). The use of host plant resistance has obvious advantages in controlling virus and other diseases compared with the use of pesticides (Buddenhagen, 1983). It is particularly important to identify multiple types of resistance for use in developing resistant varieties that are durable, in the sense that they do not "break down" quickly or readily due to the emergence of resistance-breaking virus strains (Harrison, 2002).

2.3.2. HOST GENETIC VULNERABILITY

There are many examples in the plant pathology literature of varieties that are extremely susceptible to infection with a particular disease and which express very severe symptoms. If such varieties are widely promoted and adopted because of their quality or other favorable characteristics there is a risk that serious disease outbreaks will occur (Thresh, 1990). A notable example occurred when the high-yielding Indian variety of sugarcane NCo310 was introduced to Queensland, Australia (Ryan, 1998). The variety was initially very successful and so suitable for milling that it was soon grown almost exclusively by many growers. However, a very damaging epidemic of *Fiji disease virus* affecting sugarcane occurred in the 1970s in areas where hitherto it had been of only limited importance. Additional control measures were introduced, but the problem was not overcome until NCo310 was replaced by less susceptible varieties.

An earlier example of genetic vulnerability occurred in Ghana and elsewhere in West Africa where cacao production was based initially on plantings of the introduced Amelonado variety from South/Central America (Thresh et al., 1988). This variety was grown successfully for a time until it became apparent in the 1930s and 1940s that it was extremely vulnerable to what became known as *Cacao swollen shoot virus*. Millions of Amelonado trees died

within 2 years of infection and more than 193 million have been removed by the authorities in continuing attempts to restrict spread (Ampofo, 1997). More recently, the farmer-selected Ebwanateraka variety of cassava was severely affected during the 1990s epidemic of cassava mosaic disease in Uganda and neighboring parts of western Kenya (Otim-Nape et al., 2000). The variety was popular with farmers because it was high yielding and had other favorable attributes and so it was being grown almost exclusively at the time in several of the most important cassava-growing districts. The epidemic caused severe food shortages and farmers responded by switching to other crops and to local varieties of cassava that were less vulnerable than Ebwanateraka. Farmers have also adopted the officially released mosaic-resistant varieties which now account for a substantial part of the current production.

From these and other experiences there are obvious advantages to be gained from a detailed evaluation of varieties under a wide range of conditions and for several years, before they are recommended and adopted widely by farmers. It is particularly important to avoid undue reliance on a single variety or small group of related varieties of similar genetic background. However, the threat posed by hitherto seemingly minor diseases is not always appreciated by farmers or researchers, or may become apparent only when a vulnerable variety is introduced and grown extensively. There is also a reluctance by breeders, agronomists, and horticulturists to discard productive high-yielding genotypes because of defects that are regarded initially as unimportant. A more stringent and enlightened attitude is required if current practices are to be improved so as to avoid releasing additional unduly vulnerable varieties. For this reason there are benefits of "negative selection" in crop improvement programs, by which the most susceptible and least productive varieties and breeding lines are discarded. This practice is adopted by breeders and also intuitively by farmers, who may have little or no knowledge of the pathogen involved. Much can be achieved in this way, even when there is no "positive selection" for some form of host plant resistance.

2.3.3. HOST LONGEVITY

Many of the crops grown from seed are harvested within only a few weeks (e.g. groundnut and lettuce) or months (e.g. brassicas and cereals) from planting. Thus, there is only a restricted opportunity for viruses to become prevalent and the only ones likely to be damaging are those that can spread rapidly or persist very effectively between successive crops. The opportunity for spread is greater in biennial crops and greatest with long-lived woody perennials (Vanderplank, 1949). These include tree crops such as cacao, cherry, citrus, coconut, grapevine, oil palm, peach, and walnut, for which the time-scale of

epidemics is relatively unimportant. This is because they can develop over many years and ultimately cause serious damage, even though annual spread is slow and mainly localized (Thresh, 1983b).

2.3.4. VEGETATIVE PROPAGATION

The importance of host longevity as an epidemiological factor has been greatly enhanced by the widespread adoption of vegetative propagation to provide the usual planting material of many important woody and herbaceous crops. This long-established practice may involve inter-grafting rootstocks and scions of different species or variety and is likely to become increasingly important with an extension of *in vitro* culture and micropropagation techniques.

Vegetative propagation greatly facilitates crop establishment and the exploitation of superior genotypes, but the epidemiological implications are profound. This is because grafting is a very effective means of transmitting viruses and of creating new virus/host combinations and virus mixtures. Moreover, viruses and their vectors can be widely disseminated within or on vegetative propagules into entirely new regions and over distances far greater than those traversed by natural means. Virus-infected plants so introduced into plantings become particularly dangerous foci of infection, as they occur from the outset and tend to be randomly scattered, which facilitates further spread (Thresh, 1983b). Furthermore, viruses may build up over successive cycles of vegetative propagation, leading to the progressive degeneration of stocks. This has long been familiar to potato and temperate fruit growers, and also occurs with such tropical crops as avocado, banana, cassava, citrus, pineapple, sweet potato, taro, and yams.

Viruses can ultimately become prevalent in vegetatively propagated crops, even if the growth cycle is of short duration and virus spread is slow. Inevitably there has been selection of virus-tolerant genotypes that grow and yield satisfactorily, despite virus infection. Selection has occurred unwittingly with many crops and it is notable that some temperate fruit cultivars have been grown successfully for more than 100 years, even though all available clones have become totally infected with one or more viruses. The practice of replacing degenerated stocks of potato with healthier ones of new varieties from less severely affected areas also emerged empirically and led to many subsequent certification schemes in attempts to improve overall health status (Hollings, 1965).

2.3.5. HOST MOBILITY

Those comparing the epidemiology of plant and animal pathogens emphasize the immobility of rooted plants compared with the mobility and complex social

behavior of higher animals. This attitude is fully justified, as the differences are indeed great and have a crucial impact on "contact rates" between host and pathogen and the opportunities for virus spread. Nevertheless, there is a tendency to underestimate the epidemiological significance of plant movement, whether this occurs naturally or due to man.

Many viruses can be disseminated efficiently and far, in or on the seed of crops, weeds or wild plants and this is particularly important in the epidemiology of viruses with slow-moving ectoparasitic nematode vectors (Murant, 1981). Furthermore, there is an extensive and expanding traffic in crop seed, seedlings, or vegetative propagules due to the activities of commercial growers, nurserymen, international companies, and researchers, and to the use of regions with a favorable climate or cheap labor to produce plants and plant material for export and sale for cultivation elsewhere.

Another aspect of plant mobility is the use of seed beds or nurseries to raise plants for subsequent transplanting to wider spacings at cropping sites that are sometimes remote from the source of the planting material. For example, there is an extensive traffic in plants and plant material between Africa and Europe and also from South/Central America to North America and Europe. Virus spread can occur before or after transit and new or extended opportunities occur as the plants are handled, or when they become mixed during collection, handling, grading, distribution, and transplanting. This facilitates mechanical transmission and also disrupts any groups of infected plants so that new "contacts" are established with healthy neighbors and further spread occurs. Transplants are used widely with rice, tobacco, tomato, and many other crops to make the most effective use of the land, irrigation water, and growing seasons available. This may permit sequential cropping, or involve raising plants in glasshouses or plastic structures before transfer outside. Such practices are becoming increasingly widespread as methods of production become specialized and this further exacerbates disease problems (Thresh, 1982; Bos, 1992).

2.3.6. CROP GROWING SEASONS

Crops may be grown in continuous overlapping sequence in the lowland humid tropics, where the climate is suitable for growth throughout all or much of the year. This enables viruses and their vectors to become endemic, in the sense that they are always present and encounter few problems of survival or perennation. Elsewhere, inadequate rainfall or low temperatures lead to seasonal growth and there are distinct and sometimes prolonged breaks between successive crop-growing seasons. Virus spread is then greatly restricted and the severity of the dry season or of winters at higher latitudes has a crucial influence on the prevalence of many vector-borne viruses. Such information

can be utilized in forecasting disease epidemics based on pre-planting rainfall or temperature records (Thresh, 1986).

Seasonal crops that are only briefly vulnerable and accessible may largely escape infection, or become infected at such a late stage of growth that losses are insignificant. Any serious outbreaks that develop are likely to be due to viruses with exceptional epidemiological "competence," using this term for the ability of pathogens to sustain the continuous sequence of infection necessary for their survival (*sensu* Crosse, 1967). This can be due to local perennation, or the capacity to colonize and then exploit new habitats quickly and sometimes from afar. The degree of competence required becomes less when the natural growing season is prolonged, or extended by irrigation or some form of protected cropping (Thresh, 1982). This facilitates the perennation of viruses and their vectors and can lead to serious problems, as apparent from experience with tropical rice. The problems are particularly acute in areas such as Bali, Indonesia, where rice is grown in mainly small plantings and in overlapping sequence throughout the year. This maintains the cycle of infection that is difficult to break and in these circumstances rice tungro disease has become endemic. There is a similar situation with cassava in many countries of sub-Saharan Africa and also where market garden and ornamental crops are grown in sequence throughout the year.

2.4. Strategies of Virus Spread

Considering the great range of crop species and habitats, it is hardly surprising that there is equivalent diversity amongst viruses in host range, means of spread, and perennation. Cereal yellow dwarf and several other important viruses are "specialists" in an ecological sense, as they have a very restricted host range amongst a few closely related species, or they are transmitted by only one or a few vector species of the same taxonomic group. By contrast, *Cucumber mosaic virus* and *Alfalfa mosaic virus* are examples of "generalists" as they have a very wide host range, and are transmitted by many different aphid species. Generalist viruses have less opportunity than specialists to become closely adapted to particular hosts or vectors in ways that facilitate survival.

Similar considerations could account for the great differences between viruses in mode of spread by contact, through pollen and/or seed and by arthropod, nematode, or fungus vectors. No one virus is known to be transmitted by each of these routes and it is unlikely that the typically small viral genomes can carry sufficient information for this to be possible. Thus each virus utilizes only one or a combination of some of the possible modes of spread to achieve an effective distribution of inoculum over short distances

within plantings and also over greater distances between localities. This is of great epidemiological importance in enabling viruses to reach and then exploit new habitats as they arise (Vanderplank, 1963) The actual methods employed greatly influence the pattern and sequence of virus spread, and hence the overall dynamics of disease progress. They are discussed in detail elsewhere (Thresh, 1974, 1978, 1983b) and are not considered further here.

2.5. The Different Types of Plant Virus Disease

Plant virus diseases can be grouped and categorized in different ways. For example, this can be done according to the taxonomic status of the virus or viruses responsible. Another approach is "operational" and based on the mode of spread, type of vector and mechanism of transmission. For the purposes of this paper a distinction is made between diseases caused by indigenous viruses and those due to introduced "exotics."

2.5.1. INDIGENOUS VIRUSES

These can be illustrated by reference to those causing diseases of three important tropical food crops: rice, maize, and cassava. The main virus diseases of each of the three crops are restricted in distribution to either one or two of the three continents where the crop is grown widely(Table 1). The assumption is that the virus or viruses responsible are indigenous and spread into the crop from wild hosts, or remained associated with the crop as it was domesticated from wild progenitors. For example, it is known that each of the three main viruses of rice infects wild rice species, or other members of the Gramineae from which spread occurred to rice when the crop was domesticated or introduced from elsewhere. Each of the three rice viruses is restricted to one

TABLE 1. The regional distribution of the main virus diseases of rice, maize, and cassava

Virus disease	Africa	Asia	South/Central America
Rice hoja blanca	–	–	+
Rice tungro	–	+	–
Rice yellow mottle	+	–	–
Maize streak	+	–	–
Maize rayado fino	–	–	+
Cassava mosaic	+	+	–
Cassava brown streak	+	–	–
Cassava frog skin	–	–	+

continent and their importance is likely to have increased during the 20th century with an intensification of cropping practices. There is a similar situation with cassava which was first introduced to Africa from South America in the 16th century and to India in the 18th century. Once in the Old World cassava encountered indigenous cassava mosaic viruses that were already present in Africa or Asia and these now cause serious problems (Swanson and Harrison, 1994). Maize was also transported by the Portuguese from South America to Africa, where it first encountered *Maize streak virus*, which infects a wide range of indigenous African grass species.

Groundnut rosette and several other African viruses have a similar evolutionary "background" and this has important implications for those concerned with crop improvement and crop protection projects. Inevitably, problems arise when exotic germplasm is first introduced to a region for crop diversification and plant breeding purposes and it is exposed to viruses not previously encountered and for which there is little or no inherent resistance. This has been an important factor in cassava breeding programs in Africa and India where little use has been made of South American genotypes because of their vulnerability to Old World cassava mosaic viruses (Porto et al., 1994).

Another implication of the limited distribution of many viruses is the importance of quarantine controls on the movement of plant material between and sometimes within countries to prevent or at least decrease the risk of further dissemination of viruses and their vectors. This need for effective quarantine procedures is widely recognized, but not always easy to implement because of technical problems and the difficulty in controlling the continually increasing movement of people and plant material for trade, tourism and crop improvement purposes. These trends and attempts to eliminate trade barriers and statutory controls partially negate the improvements that have been made in quarantine procedures due to the introduction of new and sensitive methods of pathogen detection that also facilitate a greatly increased throughput of samples.

The problems that have been encountered in enforcing quarantine controls have led to the suggestion that they are of limited value because pests and pathogens will eventually become established in all the areas where agroecological conditions are suitable. However, this "inevitability concept" is unduly pessimistic and there are cogent arguments for enforcing quarantine controls to delay the introduction of pests and pathogens for as long as possible and to provide the opportunity to introduce resistant varieties and make other contingency arrangements for use should the need arise (Hewitt and Chiarappa, 1997; Kahn, 1989). These objectives have been promoted by the UN Food and Agriculture Organization which has published guidelines for the quarantine procedures to be used with some of the most important crops (Frison and Putter, 1989).

2.5.2. INTRODUCED VIRUSES

An inevitable consequence of the long history of traffic in plants and plant material is that viruses and virus vectors have been introduced to completely new areas. Many have become established and caused severe problems in areas sometimes far from those where they originated. Sugarbeet curly top disease in the south-west region of USA provided an early example. Sugarbeet, *Sugarbeet curly top virus* and the leafhopper vector [*Neoaliturus* (formerly *Circulifer*) *tenellus*], and many of the herbaceous weed hosts are all considered to have been introduced from the Old World. They are likely to have been present in or on the fodder beet carried as animal feed on ships traveling from the Mediterranean in the 19th century, or even earlier. Virus, vector, and weed hosts thrived in the new environment and initially threatened the viability of beet production in the region until curly top-resistant varieties were introduced (Bennett, 1971).

Citrus tristeza virus causes an even more widespread disease as it has been introduced in plants or in budwood to almost every country where *Citrus* species are grown (Bar-Joseph et al., 1981). Serious losses have ensued, as reported in Africa, the Americas, and Spain. Moreover, these losses continue as new virus strains or aphid vectors reach new areas (see section 2.7. pp. 23–24). *Banana bunchy top virus* has also been widely disseminated in vegetative propagules and it has been reported recently for the first time in Pakistan, Hawaii, and New Caledonia.

Many other viruses of vegetatively propagated crops including *Solanum* potato, sweet potato, hop (*Humulus lupulus*), temperate and tropical fruits, and ornamentals have a similarly wide distribution. This is due to the inadvertent traffic in infected propagules and despite the adoption of quarantine controls. Similar problems arise with seed-borne viruses and this explains the cosmopolitan distribution of several damaging viruses including *Alfalfa mosaic virus*, *Bean common mosaic virus*, *Cucumber mosaic virus*, *Soybean mosaic virus*, and *Tomato mosaic virus*. *Tobacco mosaic virus* also has a very wide distribution due to the traffic in smoking and chewing tobacco. When this is prepared from infected leaves the hands or clothing of farm workers can become contaminated and transfer inoculum to crop stands during routine cultural operations.

Tomato yellow leafcurl virus: Israel provides a particularly notable example of a virus that has extended its geographic range in recent years. When introduced to Spain it largely displaced the leafcurl virus of tomato that occurred previously (Sánchez-Campos et al., 1999). The virus also spread to the Dominican Republic which was attributed to the introduction of infected seedlings from the Mediterranean (Polston et al., 1994). Infection has since become prevalent in many other parts of Central and North America and has caused serious losses.

2.6. Cropping Systems

There is abundant evidence, and from different regions of the world, of the way in which virus disease problems are influenced by the cropping practices adopted (Thresh, 1982). These have changed repeatedly over the millennia and continue to do so as agriculture has evolved from the initial phase of hunting/gathering food to the adoption of the latest technological innovations. The current situation is extremely complex because in some regions traditional practices have been retained and there is a continued reliance on shifting "slash and burn" cultivation, locally selected landrace varieties and animal traction or human labor. Moreover, there is little or no use of artificial fertilizers, pesticides, or herbicides and crops are usually grown in small areas and in complex mixtures. In other areas the traditional practices of small-holder subsistence farmers have been largely abandoned with the adoption of the latest techniques by large-scale commercial enterprises. However, there are also many areas in which a dual system of traditional and modern methods is operated by different sectors of the farming community.

Some of the contrasting features of traditional and modern practices are set out inTable 2. Many of the innovations have provided the opportunity to greatly increase productivity in terms of land and labor requirements. Nevertheless, there have also been undesirable consequences due to the increased risk of pest and disease attack. Such problems attracted particular attention following the outbreaks of rice brown planthopper (*Nilaparvata lugens*) and rice tungro virus disease and other pests and diseases that were encountered in the 1960s and 1970s during the early years of the "green revolution" in rice production in South-East Asia (Thresh, 1989a). These and other problems were associated with the increased intensity of production that was facilitated by improvements

TABLE 2. Contrasting features of traditional and modern methods of crop production: adapted from Jones (1981)

Feature	Traditional	Modern
Fields	Small, irregular	Large, regular
Crop species	Often intermixed	Usually single
Cultivars	Often intermixed, usually landraces	Usually grown singly, usually specially bred
Propagules	Own-grown or produced locally	Usually specially bred, usually purchased, seldom produced locally
Inorganic fertilizers	Seldom used	Used routinely
Herbicides/pesticides	Seldom used	Often used
Rotations	Much use of bush or other fallow	Limited use of fallow
Traction	Mainly human/animal	Mechanical

in water supply and management and the introduction of new, dwarf, short-duration, day length-insensitive varieties of rice that respond to applications of nitrogenous fertilizer without lodging. It also became apparent that the use and misuse of pesticides had led to an upsurge in populations of *N. lugens* that previously had been regulated by natural enemies and largely unimportant (Kenmore et al., 1984).

These developments stimulated intense international debate on the rôle of cropping practices in facilitating disease spread and on the need to enhance crop productivity without the concomitant disadvantages of increased damage by pests and diseases. Attention was also drawn to the merits of varietal diversity and on the most appropriate means of utilizing pesticides without risk to the environment, natural enemies, or human health. The debate on these issues has become even more cogent with the realization of the need to meet the increased demands of a burgeoning human population and to do so in a sustainable manner and despite a decrease in the rural work force due to urban migration, industrialization, and the ravages of HIV/AIDS (Cockcroft, 2004). There are no easy solutions to these formidable problems and difficulties, but plant virologists could make an important contribution by collaboration with others concerned with crop protection, plant breeders, agronomists, and horticulturists to devise effective means of exploiting the advantages of technological innovations without incurring the losses now caused by virus diseases.

2.7. Changes in Disease Prevalence

There are difficulties in assessing possible long-term changes in the occurrence and prevalence of virus diseases. This is due to the overall paucity of survey data and also because of problems in interpreting reports of a completely "new" disease, or of the apparent "spread" of a known disease into a new area. Such reports may be reliable and reflect a new development, or they could simply be due to the acquisition of expertise, or of an effective means of virus detection where none existed previously. Moreover, some now well-known virus diseases were for long regarded as being due to other causes. Hop nettlehead, black-currant reversion, apple mosaic, cereal yellow dwarf, rice tungro, sugarbeet yellows, banana streak, and carrot motley dwarf diseases are all examples of this type and there are others.

Despite these difficulties, there is abundant evidence from different agro-ecological regions of big changes that have occurred in the distribution and prevalence of several well-known diseases. Rice tungro disease in South-East Asia is a particularly important example as discussed earlier. The emergence of tungro and other diseases was influential in drawing attention to the hazards

associated with an intensification of cropping practices. This was associated with the displacement of many traditional locally selected landraces of rice by a relatively small number of specially bred cultivars, some of which were vulnerable to infection (Thresh, 1989a). Rice yellow mottle and maize streak in many countries of sub-Saharan Africa and rice hoja blanca in South/Central America are other diseases that have become prevalent following an intensification of cropping practices.

In Europe, there is clear evidence of the spread of *Plum pox virus* northwards and westwards from the Balkan countries that were known to be affected in the early decades of the 20th century (Thresh, 1980). This expansion was due to the activity of the aphid vectors and also to the inadvertent dissemination of infected planting material. Consequently, there have been serious problems in plum, peach, and other stone fruit crops and the virus has been reported recently in the Americas (Gildow et al., 2004) and also in China.

Sugarbeet rhizomania is another virus disease known to have spread across Europe from the southern areas that were first affected and it too has been reported recently in North America (Asher, 1999). Spread has been facilitated by the ability of the virus to remain infective for years in the resting spores of the fungus vector (*Polymyxa betae*). The spores are carried in soil, on crop debris, and along water courses and drainage channels and the spores can also be disseminated in contaminated stocks of seed and seed potatoes. This is likely to be the means by which the fungus-borne *Rice stripe mosaic virus* spread from Africa and became established in South America (Morales et al., 1999) The two barley mild mosaic viruses have similar epidemiologies and they have also been transported widely across Europe in recent decades. Losses in the UK have been enhanced because of changes in cropping practices and the trend to sow early in the autumn rather than in the spring (Adams, 1991).

Other recent virus disease problems have been due to the movement of virus vectors into new areas. The brown citrus aphid (*Toxoptera citricidus*) is the most efficient vector of *Citrus tristeza virus* and its movement northwards in recent years from South America into the Caribbean region, Mexico, and the southern states of USA has led to serious problems (Yokomi et al., 1994). The aphid has been reported recently in Europe and now poses a threat to citrus production in the Mediterranean region (M. Cambra, personal communication).

Additional problems due to the introduction of vectors have occurred in Australia and New Zealand, following the first reports of two legume aphids in these countries (Ashby et al., 1979; Bishop et al., 1982). Moreover, the soybean aphid (*Aphis glycine*) has been found recently for the first time in North America (Onstad et al., 2005; Venette and Ragsdale, 2004) where it is expected to enhance the spread of *Soybean mosaic virus* and other viruses of soybean. Problems due to new introductions have occurred already in

groundnut, tomato, and ornamental crops due to the continued spread of the western flower thrips (*Frankliniella occidentalis*) from North America to Europe and Australasia (Baker et al., 1993).

Such introductions are attributed to the continually increasing traffic in plant material. This also accounts for the wide dissemination of the damaging "B biotype" of the whitefly *Bemisia tabaci* (also known as *B. argentifolia*). It is a direct pest and also a virus vector and severe disease problems caused by whitefly-borne viruses have been encountered in tomato and other vegetable crops in many Asian countries and also in South/Central/North America and the Mediterranean Region (Brown and Bird, 1992; Brown, 1994; Morales and Anderson, 2001). Control has been difficult, despite the widespread use of insecticides which creates hazards to the environment and to human health. Additional problems are that insecticide-resistant strains of *B. tabaci* have developed and natural enemies tend to be killed more readily than the whiteflies targeted, which leads to a resurgence of vector populations.

These developments largely explain why whitefly-borne viruses have become so important in recent years and in such a wide range of crops. However, an additional reason is that particularly damaging virus strains or strain combinations have arisen. A notable example is the occurrence of the Uganda recombinant strain of *East African cassava mosaic virus* that is associated with the current pandemic now affecting large areas of East and Central Africa and causing very serious losses (Zhou et al., 1997; Otim-Nape et al., 2000). Other novel recombinants have caused severe problems in cotton in Pakistan and in tomato in Europe and the Americas (Padidam et al., 1999).

2.8. The Equilibrium Concept

Viruses as obligate parasites have complex inter-relationships with their host(s), but a dynamic equilibrium is to be expected between pathogen virulence and host response if a virus is to survive in the long term without annihilating its host(s). Clearly, an increase in the abundance and availability of the host population will facilitate virus spread. Favorable environmental conditions for virus and vector will enhance this effect and lead to an increased risk of damage. However, the extent to which this occurs will depend on the prevalence and vulnerability of the host population and the response to infection. There may be periods when the virus causes little or no damage and its survival is in jeopardy. At other times infection may be so prevalent and damaging as to endanger the host.

The different factors involved and their inter-relationships are illustrated diagrammatically inFigure 1, which shows how damage can be avoided by host evasion, resistance, or tolerance, or some combination of these three features.

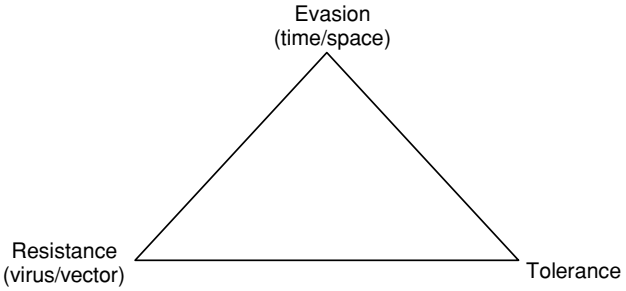


Figure 1. Diagrammatic representation of the ways in which plants avoid the harmful effects of plant viruses by some combination of resistance, tolerance, and evasion in time or space. Total reliance on evasion in time or in space is positioned at the apex. Similarly, total reliance on resistance to the virus or vector is on the extreme left and reliance on tolerance is on the extreme right. Combinations of any two of the three factors are positioned as appropriate along one of the three sides. A combination of all three factors occurs within the triangle and is placed according to their relative importance

Clearly, evasion in time or in space is not an option with long-lived perennials, or with crops that are grown widely and intensively with little or no break between seasons. Losses then become inevitable unless resistant or tolerant varieties are grown, or other effective control measures are adopted. However, the problem can be expected to abate if there is a decrease in host abundance, or environmental conditions become less favorable and this can lead to cycles of increasing and decreasing host populations associated with corresponding cycles of disease prevalence (Buddenhagen, 1977). The outcome is a dynamic equilibrium between "pathogen pressure" and host response(Figure 2).

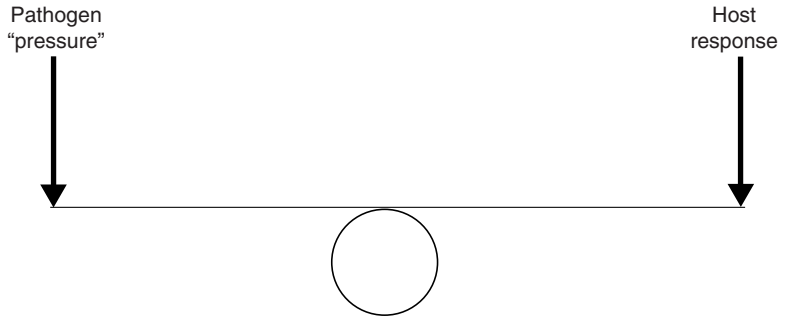


Figure 2. Diagrammatic representation of the dynamic equilibrium between pathogen "pressure" and host response. The equilibrium is disrupted in favor of the pathogen if there is an increase in virulence, or environmental conditions become more favorable for disease development and vector multiplication. Conversely, the balance is tipped in favor of the host if it becomes less abundant, or more dispersed, or more resistant to infection

The "equilibrium concept" illustrated diagrammatically in Figure 2 is consistent with the widely held view of ecologists that pests and diseases are seldom prevalent or sustained in natural populations in which mutual adaptation and co-evolution can occur (Harper, 1977; Zadoks and Schein, 1979). Moreover, the concept can be used to consider the impact of cropping practices and other features that contribute to an increased prevalence of disease. On this view the introduction of new crops, viruses, or vectors, or of novel host cultivars, virus strains, or vector biotypes, can be seen as "ecological perturbations" that disrupt the dynamic equilibrium between host and pathogen until such time as mutual adaptation occurs and the equilibrium is restored. Furthermore, it is apparent that one of the overall objectives of crop production strategies should be to maintain the equilibria established in nature and adopt means of enhancing crop production without the concomitant disadvantages. When equilibria are disrupted they should restored with the least possible delay. Clearly, these are demanding requirements but ones that must be achieved if crop production is to meet the demands of an increasing human population and it is to be done in an environmentally acceptable and sustainable manner.

2.9. Plant Viruses as Agents of Bioterrorism

In recent years attention has been given to the threat posed by the deliberate introduction of plant pathogens not already present in a country, or of novel and particularly aggressive strains of pathogens already present. The scope for this form of bioterrorism and the risks posed are discussed in a recent review (Madden and Wheelis, 2003) and by other contributors to this volume. However, the risks are likely to be less than those posed by highly infectious viruses, or other pathogens of humans or livestock (Gewin, 2003). Moreover, wind-borne plant pathogenic fungi seem to present a greater threat than plant viruses, that usually spread less rapidly and require an animal or fungus vector.

From the foregoing sections it is clear that plant viruses *could* be used as agents of bioterrorism and that there are several possibilities of causing severe outbreaks by inducing damaging ecological perturbations. One is to introduce a virus or viruses to an area where they are not already present. This requires considerable epidemiological knowledge on the current distribution of suitably damaging viruses and on the possibility of establishing them in the "target" region and of causing sufficiently severe losses to arouse serious concern. Moreover, an effective means of "delivery" is required that enables the viruses to establish quickly and soon become prevalent.

These are exacting requirements and ones that are not likely to be met by viruses, other than those with effective fungus or arthropod vectors. Even with these viruses a substantial amount of time, expertise, and extensive facilities

would be required to build up adequate stocks of infective vectors for release on a sufficiently large scale to ensure their establishment and rapid impact over a wide area.

Another difficulty is that although plant viruses may cause substantial economic damage they seldom have such devastating effects as to imperil food security, livelihoods, and human health. This is particularly true in developed countries which can compensate for any losses incurred by imports purchased from elsewhere. Developing countries are at much greater risk because they are more dependent on local produce for food supplies and on export crops as an important source of family income, employment, and foreign currency earnings. In all areas there are opportunities for terrorists to exploit the ignorance of the general public on plant pathological issues. This makes it easy to provide misleading information and initiate unease and even panic or hysteria, especially by targeting fresh fruit or vegetable crops intended for immediate consumption.

Similar considerations apply to the other two possibilities of bioterrorism. One is to introduce particularly damaging strains of a plant virus or viruses that is/are already present, but having relatively benign effects. The scope for adopting this approach is apparent from the devastation caused by the recombinant strain of a cassava mosaic virus that appeared naturally and is now causing food shortages in large regions of East and Central Africa (Otim-Nape et al., 2000). Problems have also been caused by particularly virulent strains of *Citrus tristeza virus* (Bar-Joseph et al., 1981) and by novel strains of *Sugarcane mosaic virus* that seriously damage cultivars selected for their resistance to the strain(s) occurring previously (Thresh, 1989b). Clearly, considerable expertise will be required to select the most appropriate viruses for this approach and to develop suitable strains by selection from those occurring naturally, or following some sort of genetic manipulation.

The other possibilities are to introduce an entirely new vector or a novel biotype of an existing vector. The consequence could be very damaging, as evident from the apparent ease with which the western flower thrips, the brown citrus, and other aphids and the "B-biotype" of *B. tabaci* have become established recently in new areas (pp. 23–24). However, there is again a requirement for expertise, rearing facilities, an effective means of introduction and sufficient time for the vectors to become established and build up damaging populations.

The various possibilities of bioterrorists deploying plant viruses or their vectors against crops must be evaluated in relation to the possible alternatives. These are to use fungal or bacterial pathogens of crops which are less dependent than viruses on vector intermediaries and are more readily cultured, stored, and disseminated. However, plant pathogens generally seem to pose a lesser threat than pathogens of humans and livestock. These agents would

undoubtedly have a greater and more immediate impact on public sentiment, attitudes and actions, especially if reinforced by an effective propaganda campaign designed to initiate panic and an irrational behavior and response.

2.10. Consequences of an Enlarged European Community

The creation and subsequent enlargement of the European Community has had an important influence on national economies and the effects on agriculture, agricultural research, the rural work force, and the trade in agricultural produce have been particularly great. Additional changes will be inevitable following any further enlargement by incorporating more countries of Eastern Europe. This will lead to increased movement of people between countries for tourism and also for trade. There will also be increased movement of seeds, plants, and plant produce associated with the increased size of the community, the wider range of produce and agro-ecologies available and the decrease in trade and cultural barriers.

These changes will increase the risk of disseminating viruses and virus strains, vectors, and vector biotypes to entirely new areas. This is apparent from past experience in Europe and elsewhere and the risks will be greatly increased because of the opportunity for movement between countries of Western Europe and those to the east and especially those which have long-standing links with western Asia and the Caucasus. There will be additional consequences of the inevitable increase in international traffic in seeds and vegetative propagules which means that there will be an opportunity to deploy varieties in areas far from those in which they were selected and lead to possible "new encounters" between hosts and pathogens.

A feature of the existing European Community is that individual countries seek to exploit whatever advantages of physical environment, situation, labor costs, or economy they possess to produce the most appropriate crops for sale in the current highly competitive environment. Inevitably, this trend will continue in an enlarged community and lead to further changes in the distribution of both agricultural and horticultural crops and in the means and intensity of crop production. These developments and associated socio-economic changes in the patterns of trade and crop utilization are likely to have an impact on the prevalence of pests and diseases. It is important that such changes are monitored so that any undesirable consequences can be mitigated to avoid undermining the benefits of a more competitive agricultural economy.

A further consequence of an enlarged European Community will be the opportunity for closer international collaboration between countries which historically have had only limited contact. Language and cultural barriers will become less restrictive than previously and there will be increased scope

for the interchange of experience, expertise, and research findings. Moreover, countries in which there is no strong tradition of plant virology and a paucity of facilities and expertise will be able to benefit from access to other European countries which are in a more favorable position. Some of these countries have had a long history of providing support for plant virology in developing countries of Africa and to a lesser extent East and South-East Asia and South/Central America. This support has included the provision of facilities, equipment, supplies, project funds and the opportunity for post-graduate training, post-doctoral fellowships, study tours, training courses, and sabbaticals.

There are well-established precedents that can be followed by an enlarged European Community and it is important that this is done to make the best use of the resources and skill available during a period when budgets are so constrained that a duplication of facilities would be undesirable and difficult to achieve. Inevitably there will be problems in funding an increased program of international collaboration but there are good reasons for doing so and great benefits to be gained.

References

Adams, M.J., 1991. The distribution of barley samples yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) in UK winter barley 1987–1990, Plant Pathol., 40, 53–58.

Ampofo, S.T., 1997. The current swollen shoot virus disease situation in Ghana, in Proceedings 1st International Cocoa Pests and Diseases Seminar, Accra, Ghana, 1995, pp. 175–178.

Ashby, J.W., P.B. Teh, and R.C. Close, 1979. Symptomatology of subterranean clover red leaf virus and its incidence in some legume crops, weed hosts and certain alate aphids in Canterbury, New Zealand, N.Z. J. Agric. Res., 22, 361–365.

Asher, M., 1999. Sugar beet rhizomania: the spread of a soilborne disease, Microbiol. Today, 26, 120–122.

Baker, C.R.B., I. Barker, P.W. Bartlett, and D.M. Wright, 1993. Western flower thrips: its introduction and spread in Europe and rôle as a vector of tomato spotted wilt virus. BCPC Monograph 54, Plant Health and the European Economic Community, pp. 355–360.

Bar-Joseph, M., C.N. Roistacher, S.M. Garnsey, and D.J. Gumpf, 1981. A review on tristeza, an ongoing threat to citriculture, Proc. Int. Soc. Citricult., 1, 419–423.

Bennett, C.W., 1971. The curly top disease of sugarbeet and other plants, Monograph 7, American Phytopathological Society, St. Paul, Minnesota, 81 pp.

Bishop, A.L., P.J. Walters, R.H. Holtkamp, and B.C. Dominiak, 1982. Relationships between *Acyrthosiphon kondoi* and damage in three varieties of alfalfa, J. Econ. Entomol., 75, 118–122.

Bos, L., 1992. New plant virus problems in developing countries: A corollary of agricultural modernization, Adv. Virus Res., 38, 349–407.

Bos, L., 2000. 100 years of virology: From vitalism via molecular biology to genetic engineering, Trends Microbiol., 8, 82–87.

Brown, J.K., 1994. Current status of *Bemisia tabaci* (De Genn. lete) as a plant pest and virus vector in agroecosystems world-wide, FAO Plant Prot. Bull., 42, 3–32.

Brown, J.K., and J. Bird, 1992. Whitefly-transmitted geminiviruses and associated disorders in the Americas and the Caribbean basin, Plant Dis., 76, 220–225.

Browning, J.A., 1981. The agro-ecosystem—natural ecosystem dichotomy and its impact on phytopathological concepts, in Pests, Pathogens and Vegetation, edited by J.M. Thresh, Pitman, London, pp. 159–172.

Buddenhagen, I.W., 1977. Resistance and vulnerability of tropical crops in relation to their evolution and breeding, Ann. NY Acad. Sci., 287, 309–326.

Buddenhagen, I.W., 1983. Plant breeding or pesticides to narrow the yield gap? Proc. 10th Int. Congr. of Plant Prot., Brighton, UK, pp. 803–809.

Burdon, J.J., and R.C. Shattock, 1980. Disease in plant communities, Appl. Biol., 5, 145–219.

Cockcroft, L., 2004. Current and projected trends in African agriculture: implications for research strategy, in Plant Virology in Sub-Saharan Africa, edited by J.d'A. Hughes and B.O. Ed. Odu, Conference Proceedings, IITA, Ibadan, Nigeria, pp. 172–188.

Crosse, J.E., 1967. Plant pathogenic bacteria in the soil, in The Ecology of Soil Bacteria, edited by T.R.G. Gray and D. Parkinson, University Press, Liverpool, pp. 552–572.

Darwin, C., 1859. The Origin of Species, John Murray, London, 703 pp.

Dinoor, A., and N. Eshed, 1987. The analysis of host and pathogen populations in natural ecosystems, in Populations of Plant Pathogens, edited by M.S. Wolfe and C.E. Caten, Blackwell Scientific, Oxford, UK, pp. 75–88.

Frison, E.A., and C.A.J. Putter, 1989. FAO/IBPGR Technical Guidelines for the Safe Movement of Germplasm, Food and Agriculture Organisation of the United Nations/International Board for Plant Genetic Resources, Rome.

Gewin, V., 2003. Agriculture shock, Nature (Lond.), 421, 106–108.

Gildow, F., V. Damsteegt, A. Stone, W. Schneider, D. Luster, and L. Levy, 2004. Plum pox in North America: identification of aphid vectors and a potential rôle for fruit in virus spread, Phytopathology, 94, 868–874.

Harlan, J.R., 1976. The plants and animals that nourish man, Sci. Am. 235, 89–97.

Harper, J.L., 1977. The Population Biology of Plants, Academic Press, London, 892 pp.

Harrison, B.D., 2002. Virus variation in relation to resistance-breaking in plants, Euphytica, 124, 181–192.

Hewitt, W.B., and L. Chiarappa, 1977. Plant Health and Quarantine in International Transfer of Genetic Resources, Cleveland, Ohio, CRC Press.

Hollings, M., 1965. Disease control through virus-free stock, Annu. Rev. Phytopathol., 3, 367–396.

Irwin, M.E., and J.M. Thresh, 1988. Long-range aerial dispersal of cereal aphids as virus vectors in North America, Phil. Trans. R. Soc. Lond. B, 321, 421–446.

Jones, R.A.C., 1981. The ecology of viruses infecting wild and cultivated potatoes in the Andean region of South America, in Pests, Pathogens and Vegetation, edited by J.M. Thresh, Pitman, London, pp. 89–107.

Kahn, R. P., 1989. Plant Protection and Quarantine, Vol. 1–3, Boca Raton, Florida, CRC Press.

Kenmore, P.E., F.O. Cariño, C.A. Perez, V.A. Dyck, and A.P. Gutierrez, 1984. Population regulation of the rice brown planthopper (*Nilaparvata lugens* Stål) within rice fields in the Philippines, J. Plant Prot. Trop., 1, 19–37.

Madden, L.V. and M. Wheelis, 2003. The threat of plant pathogens as weapons against US crops, Annu. Rev. Phytopathol., 41, 155–176.

Mitchell, C.E. and A.G. Power, 2003. Release of invasive plants from fungal and viral pathogens, Nature (Lond.), 421, 625–627.

Morales, F.J. and P.K. Anderson, 2001. The emergence and dissemination of whitefly-transmitted geminiviruses in Latin America, Arch. Virol., 146, 415–441.

Morales, F.J., E. Ward, M. Castaño, J.A. Arroyave, I. Lozano, and M.J. Adams, 1999. Emergence and partial characterization of rice stripe necrosis virus and its fungus vector in South America, Eur. J. Plant Pathol., 105, 643–650.

Murant, A.F., 1981. The role of wild plants in the ecology of nematode-borne viruses, in Pests, Pathogens and Vegetation, edited by J.M. Thresh, Pitman, London, pp. 237–248.

Onstad, D.W., S. Fang, D.J. Voegtlin, and M.G. Just, 2005. Sampling *Aphis glycines* Homoptera: Aphididae) in soybean fields in Illinios, Environ. Entomol., 34, 170–177.

Otim-Nape, G.W., A. Bua, J.M. Thresh, Y. Baguma, S. Ogwal, G.N. Ssemakula, G. Acola, B. Byabakama, J. Colvin, R.J. Cooter, and A. Martin, 2000. The Current Pandemic of Cassava Mosaic Virus Disease in East Africa and its Control, Chatham, UK, Natural Resources Institute, 100 pp.

Padidam, M., S. Sawyer, and C.M. Fauquet, 1999. Possible emergence of new geminiviruses by frequent recombination, Virology, 265, 218–225.

Polston, J.C., D. Bois, C.A. Serra, and S. Concepción, 1994. First report of a tomato yellow leafcurl-like geminivirus in the Western Hemisphere, Plant Dis., 78, 831.

Porto, M.C.M., R. Asiedu, A. Dixon, and S.K. Hahn, 1994. An agroecologically-orientated introduction of cassava germplasm from Latin America into Africa, in Tropical Root Crops in a Developing Economy, edited by F. Ofori and S.K. Hahn, Proceedings 9th Symposium International Society for Tropical Root Crops, pp. 118–129.

Ryan, C.C., 1998. Epidemiology and control of Fiji disease virus of sugarcane, Adv. Dis. Vector Res., 5, 163–176.

Sánchez-Campos, S., J. Navas-Castillo, R. Comero, C. Soria, J.A. Díaz, and E. Moriones, 1999. Displacement of tomato yellow leafcurl virus (TYLCV)-Sr by TYLCV-Is in tomato epidemics in Spain, Phytopathology, 89, 1038–1043.

Smithson, J.B., and J.M. Lenné, 1996. Varietal mixtures: a viable strategy for sustainable productivity in subsistence agriculture, Ann. Appl. Biol., 128, 127–158.

Swanson, M.M. and B.D. Harrison, 1994. Properties, relationships and distribution of cassava mosaic geminiviruses, Trop. Sci., 34, 15–25.

Thresh, J.M., 1974. Vector relationships and the development of epidemics: The epidemiology of plant viruses, Phytopathology, 64, 1050–1056.

Thresh, J.M., 1978. The epidemiology of plant virus diseases, in Plant Disease Epidemiology, edited by P.R. Scott and A. Bainbridge, Blackwell Scientific Publications, Oxford, pp. 79–91.

Thresh, J.M., 1980. The origin and epidemiology of some important plant virus diseases, Appl. Biol., 5, 1–65.

Thresh, J.M., 1982. Cropping practices and virus spread, Annu. Rev. Phytopathol., 20, 193–218.

Thresh, J.M., 1983a. The long-range dispersal of plant viruses by arthropod vectors, Phil. Trans. R. Soc. Lond. B., 302, 497–528.

Thresh, J.M., 1983b. Progress curves of plant virus disease, Adv. Appl. Biol., 8, 1–85.

Thresh, J.M., 1985. Plant virus dispersal, in The Movement and Dispersal of Agriculturally Important Biotic Agents, edited by D.R. MacKenzie, C.S. Barfield, G.G. Kennedy, R.D. Berger, and D.J. Taranto, Claitor's Publishing Division, Baton Rouge, pp. 51–106.

Thresh, J. M., 1986. Plant virus disease forecasting, in Plant Virus Epidemics: Monitoring, Modelling and Predicting Outbreaks, edited by G.D. MacLean, R.G. Garrett, and W.G. Ruesink, Academic Press, Sydney, pp. 359–386.

Thresh, J.M., 1989. Insect-borne viruses of rice and the green revolution, Trop. Pest Manage., 35, 264–272.

Thresh, J.M., 1990. The battle of the genes, in Recognition and Response in Plant-Virus Interactions, edited by R.S.S. Fraser, NATO, ASI Series, Series H: Cell Biology, Vol. 41, pp. 93–121.

Thresh, J.M., G.K. Owusu, A. Boamah, and G. Lockwood, 1988. Ghanaian cocoa varieties and swollen shoot virus, Crop Prot., 7, 219–231.

Thurston, H.D., 1977. International crop development centers: A pathologist's perspective, Annu. Rev. Plant Pathol., 15, 223–247.

Trenbath, R.B., 1975. Diversify or be damned?, Ecologist, 5, 76–83.

Vanderplank, J.E., 1949. Vulnerability and resistance to harmful plant viruses: a study of why the viruses are where they are, South Afr. J. Sci., 46, 58–66.

Vanderplank, J.E., 1963. Plant Diseases: Epidemics and Control, Academic Press, London.

Venette, R.C. and D.W. Ragsdale, 2004. Assessing the invasion by soybean aphid (Homoptera: Aphididae): Where will it end, Ann. Entomol. Soc. Am., 97, 219–226.

Wiktelius, S., 1977. The importance of southerly winds and other weather data on the incidence of sugar beet yellowing viruses in southern Sweden, Swed. J. Agric. Res., 7, 89–95.

Yokomi, R.K., R. Lastra, M.B. Stoetzel, V.D. Damsteegt, R.F. Lee, S.M. Garnsey, T.R. Gottwald, M.A. Rocha-Peña, and C.L. Niblett, 1994. Establishment of the brown citrus aphid (Homoptera: Aphididae) in Central America and the Caribbean basin and transmission of citrus tristeza virus, J. Econ. Entomol., 87, 1078–1085.

Zadoks, J.C., and R.D. Schein, 1979. Epidemiology and Plant Disease Management, Oxford University Press, New York, 427 pp.

Zhou, X., Y. Liu, L. Calvert, C. Munoz, G.W. Otim-Nape, D.J. Robinson, and B.D. Harrison, 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination, J. Gen. Virol., 78, 2101–2111.

PLANT VIRUSES IN EUROPEAN AGRICULTURE: CURRENT PROBLEMS AND FUTURE ASPECTS

René A.A. van der Vlugt

3.1. Introduction

Plant viruses are an important group of plant pathogens in agriculture worldwide. In Europe, they cause considerable economic damage in different crops including vegetables, grains, and ornamentals. As an example: in the Netherlands the annual costs associated with *Tulip mosaic virus* in flower bulbs culture are estimated at 9 million Euros. However, over the last 10–15 years this group of plant pathogens has received relatively little attention, especially in comparisons to fungal pathogens and insects.

Several developments in European agriculture have contributed to this. Over the last decades the general focus on agriculture has shifted from the production of food and a contribution to the economy, toward the impact agriculture can have on the environment. Obviously this especially related to the sometimes extensive use of chemicals to control plant pests and diseases. In several countries this has initiated sometimes very ambitious national programs to reduce the applications of crop protection chemicals drastically. In the Netherlands, the long-term crop protection program (MJP-G) aimed for the period 1990–2000 a.o. at 50–90% reduction of emissions of plant protection products to the environment. Although ambitious, much progress has been made in reaching these goals, basically through the application of new and more efficient spraying techniques, new compounds and new cultural practices in combination with integrated pest management (IPM). Also a better training and education of farmers, extension officers, and other people directly involved in disease management has contributed significantly. None the less, despite the successes that have been achieved, the general perception of the public toward agriculture has clearly shifted. From an essential role in society and feeding the public after the Second World War toward a more industrial role, the perception of agriculture of the public has become much less positive. The whole debate and in general negative view of the public on genetically modified organisms and crops as well as the concerns on food safety have raised many questions about the environmental impact of agriculture and has clearly shifted attention away from the economic necessity and benefits of agriculture.

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 33–44.

In addition, there have been important changes in the financing of European agricultural research in the last 10–15 years. In many countries long-term government funding has been reduced, sometimes dramatically, while private funding by companies is becoming more and more important. Often this results in a shift toward short-term projects and short-term goals. Direct biological or chemical control of plant viruses is impossible; therefore research on these pathogens does not contribute directly to the political goals set in national programs. At the same time much emphasis is placed on new technologies. Major improvements have been achieved especially at the molecular level but this has been at the expense of epidemiology of plant virus diseases. This has resulted in a significant reduction in the funding for plant virus research, specific infrastructure such as glass houses and electron microscopes and an alarmingly low number of plant virologists.

3.2. Stakeholders

Two groups are especially confronted with the impact of plant viruses in agriculture.

The first group are the growers. Because of the heavy restrictions on pesticide use, growers face increasing losses from insect feeding and virus vectors such as aphids and white flies are particularly difficult to manage/ control. Under an "integrated pest management" strategy, often partially relying on natural enemies, a zero-tolerance policy becomes impossible. Because of these problems growers suffer direct and indirect production losses and economic damage.

The second group are the various national Plant Protection Organizations (NPPO), in charge of phytosanitary control. International trade is still increasing and a growing number of plant pathogens, pests, and viruses are placed on quarantine lists. Phytosanitary regulations and measures necessary to control plant viruses and other plant diseases are no longer national matters and need to be addressed at the European level or even globally. The consequences can be serious and the economic importance is such that these decisions need to be carefully taken on the basis of sound scientific data. Unfortunately, because of the limited funding of plant virus research projects, these scientific data, especially on the host range, ways of transmission, impact on plants and crops of plant viruses, are often either simply not available or seriously outdated.

There are a growing number of plant virus problems. Long recognized viruses, like *Potato virus X* (PVX), *Potato virus Y* (PVY), *Southern bean mosaic virus* (SBMV) and many more, re-occur. In addition, new viruses like the begomoviruses and the criniviruses are introduced with vectors. Every year, viruses with novel properties are "discovered" in association with serious

damage to crops. Vector problems are increasing, durable, and reliable resistance genes are lacking and new strains of virus are selected and overcome resistance traits. An example is the appearance of new isolates of *Tomato spotted wilt virus* (TSWV) that is able to overcome (="break") the SW5 resistance gene in tomato. In addition, there is a lack of appropriate diagnostic technology for fast and large scale applications and all this is set against a background of insufficient epidemiological knowledge and experienced people.

3.3. Emerging New Viruses

Plant virus diseases are significant in view of European crop biosecurity. This especially holds for developing regions or regions that have recently gone through such a phase. In these regions the agricultural industry tends to be very dynamic. The import and export of plant material and produce, the movement of work forces and the introduction of new crops in the local ecosystem often result in the rapid development of new virus diseases. The development of effective phytosanitary control measures takes time; recognition of the problem, gathering epidemiological data, development of detection techniques. Often plant viruses and their vectors move faster and can quickly establish themselves. Eradication becomes virtually impossible and the risk for further spread outside the affected regions is very real.

Almeria is an example of such an agricultural region which has recently undergone a rapid development. Located at the south of Spain's Mediterranean coast it is close to North Africa. This region is nearly completely covered with over 25,000 hectares of plastic houses positioned extremely close together and this acreage is still expanding. There is year-round production of many different, mainly vegetable crops such as tomatoes, cucumbers, melons and French beans. Most of the production is concentrated on small family run farms and in general because of the high density and year-round presence of crops there are serious insect problems especially with whiteflies. In the Almeria region, viruses cause serious economic losses estimated to be in the range of several tens of millions of Euros annually and regularly new virus problems emerge. Over the last 25 years, on average one new virus problem occurred every year (Dirk Janssen, personal communication).

An important driving factor in the growing virus problems in the Almeria region was the introduction of the whitefly species *Bemisa tabaci* which soon there after replaced the pre-existing species (greenhouse whitefly) *Trialeurodes vaporarioum*. The introduction of this new whitefly species led to a number of new virus problems. For instance *Beet pseudo yellows virus* (BPYV), a virus of cucurbit crops and transmitted by *T. vaporariorum*, was very quickly almost completely replaced by *Cucumber yellow stunting disorder*

virus (CYSDV) a new crinivirus transmitted by *Bemisa tabaci*. The occurrence of this new vector also led to the occurrence and rapid spread of new viruses like *Tomato chlorosis virus*, also a crinivirus, and *Cucumber vein yellowing virus* (CVYV), an ipomovirus. One of the latest in these new viruses is *Bean yellow disorder virus* (BnYDV), a new and unknown crinivirus that suddenly emerged in the Almeria region in 2003 (Segundo et al., 2004). Crop losses of up to 100% have been reported for this virus**.**

Another example of a newly emerged virus which became a serious problem in a very short period of time is *Pepino mosaic virus* (PepMV). Early in 1999, an unidentified virus disease appeared in protected tomato crops in the Netherlands. Biological studies, serological comparisons and sequence data soon identified it as the tomato strain of PepMV, a member of the genus *Potexvirus* (Van der Vlugt et al., 2000, 2002). These viruses are easily transmitted mechanically. The only description of this virus (Jones et al., 1980) refers to material collected in 1974 in Peru from Pepino plants (*Solanum muricatum)*. From this study it became apparent that PepMV virus can infect tomato, but also potato and a number of other Solanaceous crops.

Since 2000, numerous findings of the virus have been reported world wide and in some countries the virus has established itself. There are different reports on virus damage but, generally, virus symptoms are few and slight (small yellow spots on leaves) and vary with the cultivar. Some countries however, also reported reduced fruit quality resulting in direct and significant economic losses. At this moment in the EU the virus has a quarantine status on seeds meaning that seed lots have to be 100% free of the virus which despite sensitive diagnostics is something that might be hard to achieve. Unexpectedly severe plant damage (necrosis of stems and leaves of infected plants) has been associated with PepMV. These symptoms obviously directly influence yield. At this moment it is not clear if the more severe symptoms are related to environmental conditions, plant cultivars or possibly other strains of the virus.

To study variability of the virus and to see whether possibly new isolates of PepMV might be involved in the increased problems, different tomato isolates from various sources were analyzed by RT-PCR in using three different primer sets covering a total of 2500 nucleotides from the 5′ and 3′ ends of the PepMV RNA genome. PCR-products were subjected to sequence analysis and comparison to the original tomato isolate from the Netherlands. Different PepMV isolates were included the analysis; the three necrotic isolates (Nec1, 2, and 3), three isolates obtained from Chile (C1, 2, and 3), the Dutch tomato type isolate and the original Pepino strain. Full-length sequences of a French tomato isolate (Cottilon et al., 2002 Acc. no. AJ438767) and a Spanish tomato isolate (Aguilar et al., 2002, Acc. no. AF484251) and two isolates from the USA (US1 and US2; Maroon-Lango et al., 2003, Acc no's AY509926 and

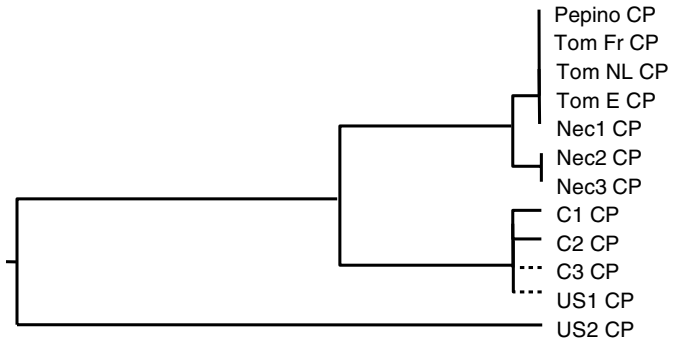


Figure 1. Phylogenetic tree representing the relationships between the coat protein amino acid sequences of isolates of *Pepino mosaic virus*. Pepino: original Pepino strain; Tom NL, Tom Fr, Tom E = Dutch, French and Spanish isolates of the tomato strain respectively; Nec1, Nec2, Nec3 = necrotic isolates; C1, C2, C3 = Chile isolates; US1, US2 = US isolates

AY509927) were included in the sequence comparisons. A phylogenetic tree of the alignment of the full coat protein amino acid sequences is shown in Figure1; a similar tree results from the alignment of the amino acid sequences of ORF3. All tomato isolates are highly homologous with overall sequence similarities of over 96%. From these alignments it is also becomes clear that the necrotic isolates are similar to the tomato isolates while the three isolates from Chile group together with the US1 isolate. Remarkably the US2 isolate clearly differs from all other isolates. This isolate only shares an overall sequence homology with the tomato type strain of around 80%. From these and other analysis (unpublished) it has become clear that a number of different isolates of PepMV exist. At this moment however, it is not clear whether the more severe symptoms reported are caused by new strains of the virus.

The question remains why a virus of which only one scientific reference existed, suddenly "appeared" in Europe and developed into a serious (hard to manage) world wide problem in a period of only 2–3 years. It is very likely that the virus was already present in Europe before 1999 but had remained unnoticed. Infected plants do not necessarily show distinct symptoms (especially not under high light conditions). Fruits from infected plants contain high concentrations of the virus which spreads mechanically very easily and can remain infectious outside the plant for several weeks. Infected tomato fruits are likely to have played a role in the movement of the virus from the natural habitat in South America to Europe and subsequent spread within the European community.

This example simply demonstrates that a virus, when it escapes attention and meets the proper conditions, can quickly become a serious phytosanitary problem.

3.4. Re-occurring Virus Problems

Not just newly emerging viruses cause serious problems but also old and well-known viruses still account for significant economic losses. An example of this is *Potato virus Y*. This virus already known for over 50 years is still a major concern in seed potato production worldwide. The Netherlands is one of the leading seed potato producers in the world with a production area exceeding 39,000 hectares and an annual production of over 900,000 tonnes. Seed potato quality is graded in five classes (S, SE, E, A, and C) with an increasing maximum allowed percentage of virus infection. Constant monitoring of virus infection levels by regular field inspections and laboratory ELISA tests ensures high quality levels of seed potato exports. A key factor in this quality system is an aphid monitoring system. Using air suction traps and yellow water traps the cumulative number of at least 10 different aphid species is determined. When this cumulative number reaches a particular threshold, a mandatory haulm destruction date for the seed potatoes is triggered. Failure to comply with this haulm destruction date automatically leads to degradation of the crop to a lower quality class.

The last few years have shown increasing levels of PVY infections in seed potato lots. At the same time the number of aphids caught shows a gradual decline. The increased levels of PVY mean declassification of seed potato lots, resulting in direct economic damage. What is the explanation of this? Has a shift in the aphid transmission efficiency of PVY occurred or has there been a shift in PVY strains?

Experiments were performed in which four different *Myzus persicae* populations were compared for their transmission efficiency with two PVY strains (PVY-N and PVY-O). Each of the two PVY strains was transmitted by each of the four *M. persicae* clones to 50 *Physalis floridana* plants using one aphid per plant. Ten days after inoculation, plants were assessed for typical symptoms for either PVY-N (mosaic) of PVY-O (necrosis). The results were interesting. Clear differences could be observed between each of the four *M. persicae* clones in their efficiency to transmit PVY. Also clear differences could be observed in the transmission efficiency of PVY-N and PVY-O (seeFigure 2). These results clearly indicate that the transmission efficiency of PVY by *M. persicae* is probably more complicated then generally assumed. PVY is transmitted by a large number of other aphid species and it is probably safe to believe that transmission efficiency by these other aphid species will be also complicated.

Since 1984, a number of new PVY strains (e.g. PVY-NTN and PVYN-Wilga) have been a reported from around the world. Of these, PVY-NTN is of major concern since this isolate is reported to overcome resistance and causes serious necrosis in infected tubers. To investigate whether a shift in PVY field

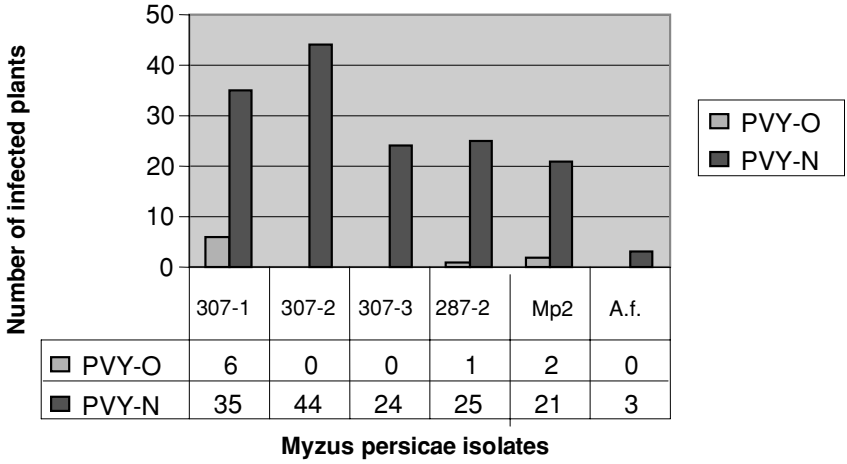


Figure 2. Transmission efficiencies in absolute numbers of infected *Physalis floridana* plants, for four field isolates of *Myzus persicae* (Mp) for PVY-N and PVY-O in comparison to laboratory isolate Mp2 and *Aphis frangulae* (Af)

isolates or strains is responsible for the growing number of problems with *PVY* a total of 108 samples collected in 2003 from fields of the Dutch General Inspection Service (NAK) were investigated.

ELISA analysis using PVY polyclonal and strain-specific monoclonal antibodies showed that seven samples were healthy, 63 plants were infected with PVY-N, 53 plants were infected with PVY-O or PVY-C, and three plants were infected with a mixture of PVY-N and PVY-O/C. These results were compared to similar analyses performed previously in 1978 and in 1994. These results are summarized in Table 1.Interestingly, the percentage of each of the three strains is different in each of the three years. Clearly the relative concentration of each of the three PVY strains in the total population is not stable and can vary considerably over time. For instance PVY-N, first observed in 1959, was long believed to be the predominant strain in the Netherlands. However, Table 1 clearly shows that the percentage of plants infected with

TABLE 1. Differences in % infections of the three different *Potato virus Y* strains as determined in field samples from three different years

	PVY-N	PVY-O	PVY-C
1978	56%	30%	7%
1994	18%	42%	27%
2003	68%	36%	0%

TABLE 2. Strain specific sequence differences between PVY-N and PVY-O at the N-terminus of the CP. Q/G represents the Nib/CP cleavage site. Sequence differences are indicated in bold

PVY-N	Q / G X_9 T X_5 Q X_6 P
PVY-O	Q / **A** X_9 **S** X_5 **P** X_6 **S**

this strain can be as low as 18%, nearly 10% lower than PVY-C, a PVY strain generally considered to be of no importance.

Field isolates collected in 2003 were also subjected to RT-PCR and sequence analysis. Three different PCR-primer sets were used to amplify the 3′ end of the PVY RNA covering the complete coat protein and 3′-nontranslated region (3′-NTR). Alignment of the amino acids of the N-terminus of the viral coat protein confirmed the previous grouping of the isolates as either PVY-N or PVY-O on the basis of the ELISA results. All PVY-N isolates could clearly be distinguished from the PVY-O isolates on the basis of specific amino acids in particular positions in the N-terminus of the coat protein sequence (see Table2).

A similar analysis was performed on PCR fragments obtained from the 3′-NTR. Previously it was shown that base pairing in this region can form very distinct hairpin structures (Van der Vlugt et al., 1993). These hairpin structures can be found in the 3′-region of all PVY potato strains but distinct loop sequences distinguish between PVY-N and PVY-O (see Table3). A total of 19 PVY-N isolates and 17 PVY-O isolates, as determined by ELISA, were compared for the loop sequences in the 3′-nontranslated region and amino acid sequences in the N-terminal region of the coat protein. Of the 19 PVYN isolates two showed sequences in their stem loops typical of PVY-N strains while 17 of these isolates had loop sequences typical of PVY-O. The 17 PVY-O isolates all showed loop sequences typical of PVY-O. This clearly indicates recombination events between PVY-N and PVY-O. These recombination events between different PVY-strains have been reported and appear

TABLE 3. Loop sequences in the four stem loop structures in the 3′-nontranslated region that distinguish between PVY-N and PVY-O strains. Typical sequence differences are indicated in bold

	Loop 1	Loop 2	Loop 3	Loop 4
PVY-N	TTTTGCA	TTCCG	TTCG	TTCTG
PVY-O	**A**T**A**TGCA	TT**T**CG	TTC**T**G	TT**T**CG

to be fairly common. Both PVY-NTN and PVY-N-Wilga are reported to be recombinants between PVY-N and PVY-O strains.

PVY can be considered an old virus still capable of causing new problems. In this work most of the PVY-N field isolates were found to be recombinants between PVY-N and PVY-O. From studies on PVY-NTN it has become clear that recombination events in PVY occur frequently. Most likely viruses use these recombination events to adapt themselves.

3.5. General Conclusions

Plant virus diseases have important economic impacts worldwide and knowledge about their interactions with their hosts, vectors and other viruses is essential if damage is to be minimized. In many regions (i.e. Africa, European states bordering the Mediterranean Europe and in South-America) the problems with white flies and other virus vectors are increasing. Insufficient knowledge of virus uptake and spread by vectors prevents the development of adequate control measures. In Central Africa this has already led to the rapid expansion of a new recombinant isolate of Africa cassava mosaic virus, a virus spread by *Bemisia tabaci* that causes very serious crop losses in cassava, a major food crop in that region.

Durable resistance to plant viruses is not always available in crop plants and there is often the danger that a new virus strain, capable of overcoming resistance traits evolves. Therefore many systems to control plant virus diseases have been developed. Generally, these focus on preventing infections. This means implementation of hygiene protocols and the use of clean seeds and starting planting material. To enable this, adequate detection and identification methods are indispensable. Over the last 50 years very efficient and cost-effective methods were developed that have proven their worth in both small and large-scale testing schemes. The last ten years there is an increasing interest in the development of new molecular-based tests like real-time PCR and multiplex micro array systems, often with the aim of improving sensitivity. Much research efforts and funds are diverted to this type of research. Yet, despite the rapid technical developments, only a few of these tests are implemented and none of them on a large scale. In comparison to more conventional techniques the average price per test is still (too) high but also the problems related with repeatable and reliable sample-extraction and the high-throughput testing of large numbers of samples in a limited time are not yet adequately solved.

There is still a need for reliable and easy-to-use methods virus tests like ELISA. Robust and relatively easily up-scalable serological techniques allow

large-scale indexing and quality control programs at very acceptable prices while maintaining internationally accepted standards regarding sensitivity and specificity. Employment of these techniques requires high-quality serological diagnostics. Production of these antisera however, is becoming more and more problematic, not just because of more strict regulations concerning the use of live animals but mainly because the expertise needed to characterize, and to purify the necessary viruses, is diminishing very fast. Few plant virology groups nowadays are still capable of producing antisera of high enough specificity, in particular to new and uncharacterized viruses or to those viruses which are not so easy to purify. New, often molecular-based techniques to produce specific antisera-like phage display systems have still not yet lived up to their promise and even monoclonal antibodies, despite their proven track record in medical diagnostics are not commonly used in plant virus diagnostics.

For the development of high-quality antisera as well as for the assessments and quality-assurance of new diagnostics, well-defined and characterized plant virus strains and isolates are indispensable. Many established and acknowledged plant virus collections in which this reference material was maintained are nowadays under serious threat, mainly for budgetary reasons. These collections however play a key role in the identification and characterization of new, and old, virus problems. They are also essential for the development and validation of detection and identification methods and the development and availability of "golden standards" required for validated and accredited diagnostic methods. In addition these collections form a condensation of valuable knowledge on biological variability and host range information, including typical symptomatology of virus strains. Especially this type of knowledge and information, critical for the control of virus problems is under threat to disappear. Classical virology is becoming more and more important, but expertise in this area is diminishing.

The erosion of the phytosanitary science base is not restricted to the field of plant virology. This alarming trend prompted the European Plant Protection Organization (EPPO) to publish the following statement.

3.5.1. PLANT HEALTH ENDANGERED—STATE OF EMERGENCY

The work of NPPOs relies on scientific expertise, but the services providing this expertise increasingly lack staff, funds, and training.

The whole scientific basis of the phytosanitary field is quickly eroding. Scientific fields which are vital for sustaining sound public policy are threatened with extinction, because they are no longer in the forefront of science priorities.

The need for phytosanitary expertise, training, and research is substantially and continuously increasing. The number and complexity of plant pest problems increases every year, beyond existing expertise.

Unless urgent action is taken, indispensable expertise and scientific disciplines will irreversibly disappear, and NPPOs will be unable to do their duty.

(http://www.eppo.org/MEETINGS/2004_meetings/council_presentations/state_emergency.htm)

Plant virus diseases are still significant for crop biosecurity in a developing European community. The limited attention for maintaining a decent knowledge infrastructure seriously threatens the European capability to deal adequately with these plant pathogens. In the long run this may be a more serious threat to European agriculture then what is currently politically considered obvious.

Acknowledgments

The author wishes to thank M. Verbeek, A. Dullemans, C. Cuperus, P. Piron, from Plant Research International B.V. and C.C.M.M. Stijger from the Research Station for Floriculture and Glasshouse Vegetables in Naaldwijk, The Netherlands for their valuable contribution to the work described above, Dr Dirk Janssen, CIFA-IFAPA La Mojonera, Almeria, Spain for sharing unpublished data and Dr N. Klijn and Dr. J.W. Roenhorst from the Dutch Plant Protection Service for stimulating discussions.

References

Aguilar, J.M., M.D. Hernández-Gallardo, J.L. Cenis, A. Lacasa, and M.A. Aranda, 2002. Complete sequence of the Pepino mosaic virus RNA genome, Arch. Virol., 147, 2009–2015.

Cottilon, A.-C., M. Girard, and S. Ducouret, 2002. Complete nucleotide sequence of the genomic RNA of a French isolate of *Pepino mosaic virus* (PepMV), Arch. Virol., 147, 2231–2238.

Jones, R.A.C., R. Koenig, and D. Lesemann, 1980. Pepino mosaic virus, a new potexvirus from Pepino (*Solanum muricatum*), Ann. Appl. Biol., 94, 61–68.

Maroon-Lango, C., M.A. Guaragna, R.L. Jordan, M. Bandla, and S. Marquardt, 2003. Detection and characterisation of a US isolate of Pepino mosaic virus, Abstract APS Annual meeting, August 9–13, Charlotte USA.

Segundo, E., G. Martin, I.M. Cuadrado, and D. Janssen, 2004. A new yellowing disease in Phaseolus vulgaris associate with a whitefly-transmitted virus, Plant Pathol., 53, 517.

van der Vlugt, R.A.A., J. Leunissen, and R.W. Goldbach, 1993. Taxonomic relationships between geographically distinct potato virus Y isolates based on detailed comparisons of the viral coat proteins and 3′-nontranslated regions, Arch. Virol., 131, 361–375.

van der Vlugt, R.A.A., C.C.M.M. Stijger, J.Th.J. Verhoeven, and D.-E. Lesemann, 2000. First report of pepino mosaic virus on tomato, Plant Dis., 84, 103.

van der Vlugt, R.A.A., C. Cuperus, J. Vink, C.C.M.M. Stijger, D.-E. Lesemann, J.Th.J. Verhoeven, and J.W. Roenhorst, 2002. Identification and characterization of *Pepino mosaic potexvirus,* EPPO Bull., 32, 503–508.

SIGNIFICANT WAYS TO SPREAD PLANT VIRUS DISEASES IN AGRICULTURAL ECOSYSTEMS: IS AGROTERRORISM POSSIBLE?

Günter Adam

4.1. Introduction

The use of biological weapons is nothing new and has been practiced since centuries to reach war aims and terrorize enemies (Rogers et al., 1999). In most cases the targets were either wariors or their animals which they needed for transport or fight. In these cases either pathogens of humans or animals were set free deliberately or otherwise biotoxins were delivered in form of poisoned food or drinkwater (see also Osborne, this volume). Rarely plant pathogens were used and if, predominantly with the aim to cause shortage of food supply resulting in famine.

However, when the causal connection between microbes and diseases of humans, animals and plants became elucidated by the end of the 19th century, this eventually led to the development of scientific research fields by their own and the planned development of bioweapons started in several countries. In most cases human and animal pathogens were weaponized, i.e. mass propagation, development of ways to deliver them, protection of the own troops and formulations to favor the spread, as well as the virulence under non-favorable conditions after delivery.

Due to the contagious nature of these pathogens also for the producer and deliverer severe security measurements had to be applied, especially during the mass propagation step.

This is where probably the idea, to use plant pathogens as weapons, arose especially in a period where fungicides were more or less unknown and the predictable production of crops for food supply from year to year was pretty uncertain. Also global production of agricultural crops was still unknown and mostly the strategy of states even in Europe was self-supply. Only under such conditions a scenario like the "Irish Famine" during 1845–1850, caused by the potato pathogen *Phytophthora infestans*, was possible. This is the most famous example for the drastic impact a plant pathogen might have. Today, with the available resistant varieties, the possibilities to use highly effective plant protection chemicals and global production of a very efficient agroindustry, it appears highly unlikely that a scenario leading to another "Irish Famine" is in the realm of possibility (see also Morozov and Taliansky, this volume).

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 45–54.

Plant pathogens against which no pesticides are available like plant viruses offer an alternative and this chapter will try to evaluate the possibilities that are visible from the increasing knowledge that has accumulated since the first plant virus was identified a century ago.

4.2. The Scenarios for Agroterrorism Today

Agroterrorism as a new type of terrorism does not try to directly attack humans, but rather their food supply at the most vulnerable site within the farm to fork chain, the farm itself. Agroterrorism, as it is discussed now, may use pathogens of animal or zoonotic diseases which are really threatening agents. Remembering the scenarios of foot and mouth disease where thousands of animals were killed to eradicate the disease, or the delivery of anthrax spores by mail, frightened people, undermined their trust into their governments and caused significant financial damage. Access of terrorists to such pathogens seems to be possible, however, is not easy and the propagation requires a certain level of logistic which may not always be available. The use of plant pathogens against crop plants is much easier. Access to these is rather simple, the mass propagation is possible, even under low level laboratory conditions, since no plant pathogen is infectious for humans. Since in our present agricultural conditions monocultures are characteristic for production, they are vulnerable for epidemic spread which under these conditions is predictable. The establishment of an epidemic infection would almost certainly cause significant damage. In addition the delivery of plant pathogens under these condition appears to be easy and safe against immediate detection (Deen, 2000; Shawn Cupp et al., 2004). This makes the conviction of responsible terrorists difficult if not impossible. In addition the reaction of the population of an attacked country but also that of the world community may be much less influenced by the harmless looking loss of crop plants than by dying animals or even worse humans. So the damnation of the use of such weapons may be milder and the response of the attacked country to the terrorists, if they are identified, might be less aggressive. This is the reason why I avoid the expression weapon in the subsequent sections and use agents instead. It is not biological war that I am discussing here but simply terrorism in its worst.

Plant pathogens may be effective as terror agents in several ways (Scholthof, 2003). The pathogens may damage or destroy the plants on the field directly. They may also damage the yield by reduction of productivity or by produced toxins that make the crops useless as food (Madden and Wheelis, 2003). With storage crops, like most of our cash crops, the damage may not become visible prior to harvest, but developes later during storage like potato tubers infected with PVY_{NTN} (Weidemann, 1993). All above described effects

destroy or damage the yield and may cause food shortage, thus having an impact on the supply with healthy and sufficient agricultural products, however, not immediately.

More important would be the expectable effects of plant pathogens on trade with the agricultural products (Bergsten, 1994). Especially when regulated pathogens are concerned, the importing countries will certainly restrict or even forbid imports, especially when seed or propagation material is involved. But also nonregulated pathogens may cause restrictions in trade and it will certainly be very costly to regain the confidence of trading partners into the safe production of healthy crops, not to speak about eradication costs if this would be possible at all. So the damage caused by an agroterroristic attack may, under optimal conditions, be multifold:

- reduced supply with spoiled food
- financial damage to the farmers
- trade restrictions
- eradication costs and
- increased costs for additional personal and diagnostics necessary to proof eradication

In countries with an infrastructure for efficient monitoring of plant health, and capable advisors that diagnose fast and correctly, the pathogen may be detected early enough and proper countermeasures, for example spraying crops with protection chemicals, may prevent epidemic spread and larger damage. In contrary this means especially for developing countries where food supply is already on a low level, high vulnerability. However, their small scale production as well as growing of diverse crops creates a high biodiversity in an agro-ecosystem that may prevent fast epidemic spread. In our extremely intensive crop production on huge fields of up to hundred's of hectars, leading to reduced biodiversity in such ecosystems, it would rather favor fast spread over large areas, making at least the financial damage significant.

4.3. What are the Agents of Concern and What May be the Possible Damage?

Although it is almost impossible to make a complete list of plant pathogens suitable as terror agents, an adhoc group, set up to develop a package of measures to control the agreement about B-weapons, has published a basic list of pathogens that contains 16 plant pathogens(Table 1).

This list is by far not complete. Other committees have listed in addition more viruses like *plum pox virus*, *sugar beet curly top virus* and even *tobacco mosaic virus* plus some more fungi like *Phytophthora infestans* and

TABLE 1. Plant pathogens with a potential application as weapon

Pathogen	Disease	Crop plant
Colletotrichum coffeanum var. *virulus*	Green berry anthracnose	Coffee
Mycosphaerella pini	Red band needle blight	Pine trees
Erwinia amylovora	Fireblight	Pomaceous fruit trees
Ralstonia solanacearum	Brown rot; bacterial wilt	Potatos, tomatos
Puccinia graminis	Black rust	Cereals
Puccinia striiformis	Striped rust	Cereals
Pyricularia oryzae	Rice blast	Rice
Sugarcane Fiji virus	Fiji disease	Sugarcane
Tilletia indica	Karnal bunt	Wheat
Ustilago maydis	Corn smut	Maize
Xanthomonas albilineans	Sugarcane leaf scald	Sugarcane
Xanthomonas campestris pv. *citri*	Cancer of citrus	Citrus spec.
Xanthomonas campestris pv. *oryzae*	Bacterial leaf blight	Rice
Sclerotinia sclerotiorum	White mold	Salad
Peronospora tabacina	Blue mold	Tobacco
Claviceps purpurea	Ergot	Rey

From (Rogers et al., 1999).

Fusarium graminiforme (Kortepeter and Parker, 1999). The reasons why these pathogens are listed vary, but many of them are known to spread fast in one growing season over large areas and destroy affected crops. Their distribution is either via spores by wind and rain, or via soil, surface-water, seed and also a few insect vectors. Some of them are regulated pathogens for more than one country.

My discussion about risks comes later, but some of the above mentioned pathogens might serve here as examples to show the potential damage involved. A good example is *Ralstonia solanacearum*. The bacterium became introduced to The Netherlands probably by potatoes imported as industrial or food crop. Normally these agricultural products are controlled less than seed potatoes and thus the bacterium got into the abundand creeks and rivers where a solanaceous weed, *Solanum dulcamara*, grows in contact with the surface water. This weed is a latent host for the bacterium and once established there it serves for a continuous supply of new bacteria into the water (Wenneker et al., 1999). The transmission cycle became closed with farmers irrigating their seed potato field with water from such rivers, where an eradication of the bacterium is almost impossible. This desaster had cost the dutch potato industry approximately 20 million dutch guilder per year since 1995, and this bacterium is now excluding one Egyptian production site after the other from the export of early potatoes to Europe.

A vectorially transmitted plant virus has enforced in British Columbia, Canada, the complete rooting out of sweet cherry trees in the Kootenay Valley (Eastwell and Li, 1994). The same might happen when plum pox virus would be deliberatly delivered in the plum, apricot and pear growing areas of the USA. The "Irish Famine" caused by the epidemic spread of *Phytophthora infestans* led in 5 years to the death of about 1 million people in and the emigration of another million from Ireland. Another fungus, *Bipolaris oryzae*, destroying rice yields, caused in only 2 years the death of 2 million Bengali due to starvation.

So the impact of fungi and bacterial plant pathogens is well documented and most attempts to weaponize plant pathogens have used pathogens from these regna (Logan-Henfry, 2000).

But what about plant pathogenic viruses? The few that have been added to various lists appear not very impressive. We live in Europe with *plum pox virus* since centuries without an evident lack of supply with apricots, plums etc. By proper spraying against aphid vectors, providing healthy planting material and use of resistant or at least tolerant varieties the damage is marginal. *Tobacco mosaic virus* is with us, world wide, since centuries and has neither caused a shortage of tobacco nor of tomatoes or other vegetables although it is established in every tobacco growing area in the world. This immediately leads to the question.

4.4. Are Plant Viruses Really that Important?

When looking at the list of regulated pathogens of the European Union (Anonymous, 2000) but also the USA, the number of plant viruses that ought to be absent from imported goods is impressive and outnumbers most other pathogens and pests. This is predominantly due to the quite efficient ways these viruses have for dissemination, the new and modern ways of vegetative propagation of agricultural crops and planting material, and the lack of any useful viruzides in plant protection.

4.4.1. WHAT ARE THE PREREQUISITES FOR A PLANT VIRUS TO BECOME A SUCCESSFUL AGENT IN AGROTERRORISM?

Belonging to the regulated pathogens does not necessarily is a must, although it has certain advantages but also disadvantages. The biggest benefit of all would be a broad host range especially in countries with seasonal growth of agricultural crops. Its spread by one way or the other must be fast and if possible difficult to intercept. The virus should also be able to survive outside

a living cell in an infectious state. The impact would be boostered by more than one way to spread to new host plants as well as areas and the presence of natural host plants that tolerate infection and serve as virus reservoir. Among the above criteria the two of highest ranking, according to my judgement, would be: Broad host range and spread without vector, where the later normally implies highly stable virus particles that can survive for long terms outside living cells. Only at third rank comes vectorial transmission but then mainly soilborne which limits the vector species to nematodes or soilborne fungi.

From the terrorists point of view, the ideal virus must be easy to obtain without tracing, easy to propagate to sufficient quantities and simple as well as deliverable unrecognized.

4.4.2. WHAT IS NECESSARY TO ESTABLISH THEM IN AN ECOSYSTEM?

To establish a plant virus in an ecosystem needs more than what has been mentioned above, because here the goal would be to introduce the pathogen only once, like a "fire and forget" weapon. The more stealth character such a pathogen has, the better. Taken the above considerations into account a broad host range again is fine. Second if transmitted by vectors these must be present and visit the original host plant as well as the targeted crops. The climate ought to be favorable or niches must exist like continuous glasshouse crops or weeds. Multiplier sites where the virus remains undetected if not searched for intensively, can be mothergardens where rootstocks are produced or scions for grafting. These sites are ideal for viruses of fruit trees, small fruits and ornamentals, since plants in these places are seldom flowering, fruit bearing or grown normally. Under these circumstances the presence of viral infections might easily remain undetected since hardly any symptoms become visible. Viruses unknown for a given area can therefore easily imported into this production chain, however, it may take centuries until the wanted effects become visible. Establishment, though as desirable as it may be from the terrorist's point of view, is much more difficult to predict and achieve than the damage in one given season with its predictable parameters. An example may be the introduction of new more virulent/aggressive isolates of virus species already present. However, also their behavior as competitors against the indigenous isolates is uncertain (see van der Vlugt in this volume).

4.4.3. WHAT IS NECESSARY TO SPREAD THEM EFFICIENTLY IN AN ECOSYSTEM?

Efficient spread can either be from the first inoculation site after replication and systemic invasion of primary hosts, or it can be delivery from the release site by any other transportation system since plants normally do not move.

Talking about first inoculation sites, a successful attack would mean many of them to gain effects in one season. This would imply plenty of available vectors in an optimal growth stage to acquire the virus and plenty of well suited host plants in the surrounding. Soilborne vector transmission, however, does not belong to the fast spreading ways and the crop rotation performed is usually designed against it. Nevertheless there are examples given by Kühne (this volume) for fungus-transmitted soilborne viruses that successfully spread in twenty years all over the wheat growing areas in Germany. Airborne vectors or pollen transmission may be a way to gain distance faster but the percentage of transmission is either low or easy to prevent.

Therefore vectorless transmission by contaminated irrigation water or hydroponic fluids that are recycled, appear to be the safest and best way to spread efficiently and fast. However, huge amounts of preferably purified virus would be necessary to initiate it. Delivery through irrigation with surface water may be not efficient enough, but when looking at glass or plastic houses with their huge areas for vegetable production in our climates and the increasing use of non-soil substrates like rockwool through which a continuous supply of nutrient solution is circulated, a very vulnerable site becomes visible (see also van der Vlugt, this volume). Huge complexes of such greenhouses are producing almost 80% of our vegetables and small fruits and there an attack with viruses would be easy, almost undiscovered and self-propelling, since some crop plants stay under production more than half a year, i.e. tomatos. Since only very few well educated agro-engineers are running such production sites, the initial attack may remain well be undiscovered before a huge number of plants appear infected and the damage is unavoidable. Again, the highest damage and probability of success can be expected with the stable, vectorless plant viruses that have also medium to large host ranges.

But also vectorial transmission should not be underestimated. The recent increase of *Tomato spotted wilt virus* problems in Europe was only possible with the introduction of the new polyphagus thrips vector *Frankliniella occidentalis* during the 80s of the last century. Here an until then rare and unimportant plant virus became a major problem due to the introduction of a new vector, which is difficult to control. The combination of a plant virus with an extremely large host range and a highly efficient new polyphagus vector had significant effects on greenhouse production and on trade with plant material due to restrictive import regulations worldwide. As outlined by T. Kühne (this volume) especially soilborne fungi acting as vectors for plant viruses can be a serious threat. Their resting spores, when contaminated with virus, are an effective way to disseminate a virus into a new ecosystem. It will be very difficult to eradicate it and provided the proper hosts are grown in such contaminated areas, spread would be predictable and difficult to control.

4.5. Risk Analysis for Plant Virus Usage as Agroterror Agents and Resulting Defensive Strategies

Considering the arguments given above, it appears that plant viruses are suitable agents for terroristic activities against agro-industry. I have tried to list the advantages and disadvantages from a terrorist's point of view. It becomes quite clear that the successful use of plant viruses as a terror agent against agro-industry requires quite a bit of effort, not only financial but also scientific input and it has definitely not the same frightening effects as explosives or bloodshed in a civilian surrounding.

In summary of what has been said about the suitability of plant viruses in Table 2it can be stated that yes, plant viruses are suitable and have many features that might make them attractive especially for low technology groups. However, the scientific knowledge about the target, the compatibility of the agents with the target, besides the capability to obtain, propagate and deliver it appropriately are no trivial factors and might rather lead to the opinion they have no practical terroristic value.

If however, a decision is made for plant viruses to be used as agents, what possible choices are to be expected and what do we need to do in order to avoid success? I have listed in Table 3qualities and features of plant viruses that might favor their choice as agents. I, at least at the moment, do deny any importance for genetically designed plant viruses since this is definitely no low technology and in my opinion wasted money and time, with almost no guarantee for success.

Not necessarily the unknown or absent plant viruses, normally listed as regulated pathogens, are the interesting agents, at least for terrorist usage.

TABLE 2. Advantages and disadvantages of plant viruses to be used as terror agents

Advantages	Disadvantages
Easy access	Establishment in an ecosystem unreliable
Safe propagation to huge quantities	Fast effects not always predictable
Broad host ranges	Too many parameters influence success
Easy dissemination	Resistant host plants are available
Difficult to eradicate	The effects are not terrifying
Several ways of transmission	The effects are slow
Good stability outside of cells	Maybe still to complex as reliable agent
Many regulated pathogens	Chemicals exist to interrupt all transmission pathways
General detection methods not available	
Ways of undetected delivery possible	

TABLE 3. What features make a plant virus attractive as terror agent?

Priority	Features
1.	Stable even under non-living conditions
2.	Broad host range
3.	Non-vector transmission possible by soil or water
4.	Easy to propagate and purify to large amounts
5.	Soilborne and transmitted by fungi
6.	Already known to exist but maybe new isolate with higher virulence or resistance breaking
7.	New, hitherto unknown species
8.	Difficult to diagnose

Rather, the already indigenous ones are dangerous when introduced into modern production conditions, especially if new isolates with altered properties can be selected like resistance breaking isolates or with altered virulence (see also van der Vlugt in this volume).

It will be extremely difficult to guard the production sites of our cash crops, may they be cereals, vegetables or fruits, against deliberate delivery into distribution sites like irrigation or hydroponic systems. It may also be difficult to monitor mothergardens from where the scions for grafting or rootstocks are obtained against deliberate inoculations or even delivery with *in vitro* propagated material that is contaminated with unknown or difficult to diagnose viruses. This means, the risks associated with plant viruses are real. However, I would indicate that the lists do not point into the right direction. It is in most cases not the regulated pathogen, i.e. the unknown, for which we should watch out in the first place, but rather the already present pathogens for which the infection pressure is merely raised by an attack. This would have the additional advantage, that the pathogens are already adapted to the ecosystem and failures due to incompatibilities are excluded. Of course the unknown should not be neglected, but to detect an unusual behavior of already present viruses you need other precautious elements.

A network of plant inspection sites covering the complete country coupled with an efficient and harmonized diagnosis for known endemic viruses/pathogens would allow the detection of unusual behavior or outbreaks in hot spots where a natural explanation for the epidemic is missing. This should be accompanied by an efficient methodology to detect and identify the unknown and might even trace the origin of the pathogen or determine it as not natural. The USA is at present developing such a system on the basis of microarrays made by Affymetrix (Beelosludtsev et al., 2004).

References

Anonymous, 2000. On protection measures against the introduction into the community of organisms harmful to plants or plant products and against their spread within the community, *Counc. Directive* 2000/29/EC.

Beelosludtsev, Y.Y., D. Bowerman, R. Weil, N. Marthandan, R. Balog, K. Luebke, J. Lawson, S.A. Jonston, C. Rick Lyons, H.R. Garner, and T.F. Powdrill, 2004. Organism identification using a genome sequence-independent universal microarray probe set, *BioTechniques* 37, 654–660.

Bergsten, C.F., 1994. APEC and World Trade: A force for worldwide liberalization. *Foreign Aff.,* 73, No 3.

Deen, W.A., 2000. Trends in American agriculture: Their implications for biological warfare against crop and animal resources, *Ann. New York Acad. Sci.*, 164–167.

Eastwell, K.C., and T.S.C. Li, 1994. Status of the little cherry disease eradication program in the Kootenay valley of British Columbia, *Can. Dis. Survey*., 74, 115–116.

Kortepeter, M.G., and G.W. Parker, 1999. Potential biological weapons threats, *Emerg. Infect. Dis.,* 5, 523–527.

Logan-Henfry, L., 2000. Mitigation of bioterrorists threats in the 21st century, *Ann. New York Acad. Sci.*, 121–133.

Madden, L.V., and M. Wheelis, 2003. The threat of plant pathogens as weapons against U.S. crops, *Annu. Rev. Phytopathol.,* 41, 155–176.

Rogers, P., S. Whitby, and M. Dando, 1999. Erntevernichtende Bio-Waffen, *Spektr. Wiss.,* October, 72–77.

Scholthof, K.B., 2003. One foot in the furrow: Linkages between agriculture, plant pathology, and public health, *Annu. Rev. Pub., Health,* 24, 153–174.

Shawn Cupp, O., D.E. Walker, and J. Hillison, 2004. Agroterrorism in the U.S.: Key security challenge for the 21st Century, *Biosecur. Bioterror*., 2, 97–105.

Weidemann, H.-L., 1993. Necrotic ring symptoms on potato tubers caused by a new potato virus Y race, *Kartoffelbau (Germany),* 44, 308–309.

Wenneker, M., M.S.W. Verdel, R.M.W. Groeneveld, C. Kempenaar, A.R. van Beuningen, and J.D. Janse, 1999. *Ralstonia* (*Pseudomonas*) s*olanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (*Urtica dioica*), *Eur. J. Plant Pathol.,* 105, 307–315.

SOIL-BORNE VIRUSES OF CROP PLANTS—POTENTIAL AGENTS FOR BIOTERRORIST ATTACKS?

Thomas Kühne

5.1. Introduction

According to a definition given by Madden and Wheels (2003) bioterrorism is the intentional use, by any human agent other than uniformed military personnel, of organisms (or their products) to cause harm (or death) to humans, animals, or plants.

Since plants are the basis of nutrition, a massive attack on crop plants with pathogens is likely to have severe consequences for humans and animals. We have to consider crops as particularly vulnerable because they are

- susceptible to many pathogens
- grown in large areas (phenomenon of increasing field size in modern plant production)
- impossible to protect (in a military sense)
- poorly monitored, and therefore infections can easily occur unobserved and uncontrolled.

Thus, in a military sense, crop plants are soft targets.

There were numerous scientific discussions on this topic during the last decade of the 20th century. Of course these activities were massively intensified after the disaster of 11 Sept. 2001 and the subsequent anthrax attacks in the USA. As an example, in 2003 the Public Board of the American Phytopathological Society drew the following conclusion in a workshop raising the question "Crop biosecurity—Are we prepared?": "There is a general consensus that the question is not if, but when, a plant disease that can significantly lessen the quality or quantity of our food, feed, or fiber will be purposefully or naturally introduced into the US. If we wish to assure the food security of our nation, the current level of support for pathogen research, diagnostic assay development, and preparation of response tactics will not suffice." In consequence of this statement a number of recommendations were given (http://www.apsnet.org/members/ppb/PDFs/CropBiosecurityWhitePaper5-03.pdf).

On the other hand it is to mention that the threat caused by bioterrorism is interpreted quite differently within the scientific community today. Many specialists even on the human medical sector consider this subject as overestimated (Altman et al., 2005).

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 55–70.

TABLE 1. Soil-borne viruses and their vectors infecting crop plants

Crop plant	Virus	Vector
Barley	*Barley yellow mosaic virus* *Barley mild mosaic virus*	*Polymyxa graminis*
Wheat, triticale, rye	*Soil-borne cereal mosaic virus* *Soil-borne wheat mosaic virus* *Wheat spindle streak mosaic virus*	
Sugar beet	*Beet necrotic yellow vein virus*	*Polymyxa betae*
Potato	*Tobacco necrosis virus* *Potato mop top virus* *Tobacco rattle virus*	*Olpidium brassicae* *Spongospora subterranea* *Trichodorus* spp., *Paratrichodorus* spp.

This paper aims to assess the potential threat by soil-borne virus diseases to assurance of yield and product quality of important agricultural crops, like wheat, barley, triticale, rye, sugar beet, and potato. Although the viruses listed inTable 1 are well known as pathogens of high economic relevance the question is whether these viruses have any potential to be used as agroterrorist agents in Europe.

The listed viruses infecting cereals and sugar beet are exclusively transmitted by *Polymyxa graminis* and *P. betae*. These are obligate soil-borne parasitic micro organisms which can survive in soil as resting spores preserving the incorporated viruses for many years in the absence of the host (Adams et al., 1993; Huth, 2000; Kanyuka et al., 2003). This persistance coupled with the lack of chemical control for these vectors makes the plant diseases caused by those viruses very difficult for growers to control. Further characteristic features of the soil-borne *Polymyxa* spp. are

- ubiquitous occurrence
- mass propagation even in a single plant
- chemical control measures are neither efficient nor acceptable for economic and ecologic reasons
- specific interactions with the viruses in terms of uptake, preservation and transmission
- viruliferous resting spores can easily spread within a partially infested field and to uncontaminated fields in the process of soil cultivation and due to soil adhering to farm machinery, respectively. Further, the resting spores are easily transported by wind and surface water.

As experiments revealed crop rotation measures or continuous growing of virus resistant varieties for up to 3 years in infested fields do not produce a measurable decline in soil virus populations (Adams et al., 1993).

What is the current situation for the indicated pathosystems?

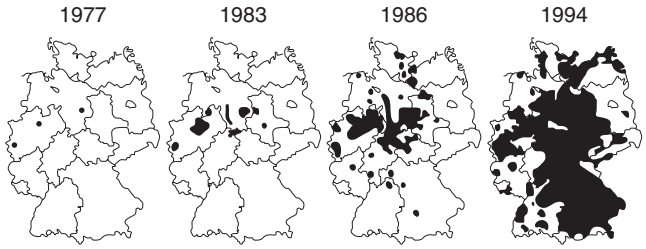


Figure 1. Occurrence of the yellow mosaic viruses (BaMMV and BaYMV) of barley in Germany (courtesy of W. Huth)

5.2. Cereal Crops

5.2.1. WINTER BARLEY—BAYMV, BAMMV

The first report on the occurrence of the yellow mosaic disease of winter barley in Europe was given for Germany (Huth and Lesemann, 1978). In the following years the disease was detected in many other European regions, too (Lapierre, 1980; Hill and Evans, 1980; Maroquin et al., 1982, Proeseler et al., 1984; Langenberg and Van der Wal, 1986; Fantakhun et al., 1987; Rubies-Autonell et al., 1995; Katis et al., 1997). It has been also described in several Asian countries (Kashiwazaki et al., 1989; Chen et al., 1996; Lee et al., 1996). The infectious agents are *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV) that both cause identical symptoms in barley plants regardless of a single or mixed infection. In Germany they have spread very rapidly over large areas after the first detection in 1978(Figure 1; Spaar et al., 2002).

Yield losses of >50% may occur when susceptible barley crops are grown on severely infected soils (Plumb et al., 1986; Adams and Hill, 1992). Moreover infected plants are significantly more sensitive to extended periods of black frost, which can lead even to almost total crop loss (Huth, 1988). Therefore, the only opportunity to control the disease and to impede further spread of viruliferous resting spores was breeding of resistant barley varieties. For more than 20 years one recessive gene, designated *rym4*, that confers complete immunity against BaMMV and BaYMV has been extensively used in breeding for resistance against these viruses in Europe, where the majority of resistant commercial barley cultivars carry this gene (Graner and Bauer, 1993; Stein et al., 2005). However, in the late 1980s another pathotype of BaYMV, BaYMV-2, which is able to overcome *rym4*-controlled resistance, was detected in Germany and the United Kingdom and later in several other European countries (Adams, 1991; Hariri et al., 1990; Huth, 1989; Steyer et al., 1995). The reason for the qualitative change in pathogenicity has not yet been finally resolved, but it was shown recently that the ability to overcome

TABLE 2. Mapped resistance genes against barley yellow mosaic virus disease (from Werner et al., 2003, mod.)

Resistance gene	Chromosome	Resistance to		
		BaYMV		
		1	2	BaMMV
rym1	4HL	X	X	X
rym2	7HL	X	X	X
rym3	5HS	X	X	
rym4	3HL	X		X
rym5	3HL	X	X	X
rym6	3HL	X*		
rym7	1HS			X
rym8	4HL	X		X
rym9	4HL			X
rym10	3HL	X	X	
rym11	4HL	X	X	X
rym12	4HL	X	X	X
rym13	4HL	X	X	X
$Rym14^{Hb}$	6HS	X	X	X
rym15	6HS	X	X	X
	5HS	X	X	X
$Rym16^{Hb}$	2HL	X	X	X

* Japanese Strains.

the *rym4*-mediated resistance correlates with a single substitution at nucleotide position 4094 (VPg coding region) of RNA1 leading to exchange of amino acid lysine in BaYMV-1 to arginine in BaYMV-2 at position 1307 of the polyprotein (Kühne et al., 2003). The spontaneous appearance of the new pathotype shortly after introducing barley varieties harboring the *rym4* gene into practice once more reflects the fundamental disadvantage of monogenic resistances. By evaluating barley germplasm up to now eight independent genetic loci distributed over the barley genome have been identified that confer mostly recessive resistance to either one or several strains of this virus complex (Table2). They are available to be taken over into breeding programs.

As a reaction to spontaneous formation of BaYMV-2 several new varieties carrying the *rym5* gene instead of *rym4* have been developed and registered over the last years. But again, shortly after their introduction into practice first reports appeared, this time on BaMMV isolates breaking this type of resistance (Hariri et al., 2003; Habekuss et al., 2005). As genome analyses revealed substitutions of single amino acid residues in the RNA1 encoded VPg protein seem to be responsible for the altered biological behavior of the new pathotype (Kanyuka et al., 2004; Habekuss et al., 2005).

Does the ability of both viruses to quickly overcome monogenic resistances of barley in combination with the extreme persistance of infectious BaMMV and BaYMV in resting spores of the ubiquitous vector *P. graminis* make them suitable objects for agroterrorist attacks?

Under natural conditions the two viruses are exclusively transmitted by their fungal vector. Zoospores of *P. graminis* are motile but can actively move in soil only over very short distances. This keeps the areas of virus-infected plants always restricted to those parts of a field which are infested with viruliferous populations of *P. graminis.* Primary infestation of so far non-affected fields would be possible only by massive contamination of soil with viruliferous resting spores. To achieve a massive contamination a large scale production of root powder (dried grinded roots) from previously infected barley plants is needed, which would be extremely laborious and time consuming and therefore cannot be regarded as a realistic strategy. But even under the assumption that this logistic problem could be solved to a certain extent one had to pass several cycles of growing the host plant in freshly contaminated soil to gain a high infection pressure. Therefore, significant negative effects on production of winter barley—as a possible aim of an agroterrorist action—could be achieved only with a tremendous input in work and time. Nevertheless, using such a strategy it would be at least theoretically possible to spread the viruses unnoticed over large areas. This approach would be not specific for the yellow mosaic viruses of barley but valid for any soil-borne virus that is kept infectious inside resting spores of its vector.

As already mentioned above numerous sources of resistance to BaMMV and/or BaYMV have been identified in barley germplasm over the last 25 years (Table 2). In principle, all these resistance genes are available for breeders today. Up to now only two genes, the allelic *rym4* and *rym5*, have been utilized in approved cultivars and both resistances have been already overcome by new pathotypes of BaYMV and BaMMV. With the aim to solve this well known problem of low durability of monogenic resistance, breeding programs to pyramidize different resistance genes, i.e. to combine them in a single genotype, have been started. The aim is to generate a broader base of resistance to a single virus which will significantly reduce the selection pressure on the pathogen by decreasing the probability that just one spontaneous mutation in the genome of the attacking agent may be already enable it to overcome plant defense.

A second aspect has to be noted in this context. The first detection of pathotype BaYMV-2 which overcomes the *rym4* mediated resistance was already in 1987 (Huth, 1989). Despite the fact that until the year 2000 this gene was the only source of resistance in the assortments of barley cultivars in Europe a rapid spread of this pathotype into other areas has not been observed, neither in Germany nor in France and the UK. The main reason for this phenomenon is probably the significantly lower fitness of BaYMV-2 in comparison to the

original isolate BaYMV-1. After mechanical inoculation of barley seedlings under controlled conditions the infection rate of the resistance breaking pathotype is 2–3 times lower than that of BaYMV-1. Symptom expression of plants infected with BaYMV-2 is clearly delayed. After co-inoculation of susceptible barley plants with both BaYMV-1 and BaYMV-2, the standard isolate replicates normally while RNA1 of pathotype BaYMV-2 can be hardly detected (Kühne et al., 2003).

Results of investigations on relative fitness of the resistance (*rym5*) breaking BaMMV isolates from France and Germany are awaited, but it is now possible to state that the two yellow mosaic viruses of winter barley are not ideal agents for any bioterrorist.

5.2.2. WHEAT, TRITICALE, RYE—SBCMW, SBWMV, WSSMV

In Europe these crop species may become infected by the following economically important soil-borne viruses:

- *Soil-borne cereal mosaic virus*
- *Soil-borne wheat mosaic virus*
- *Wheat spindle streak mosaic virus*

Recently another virus named Aubian wheat mosaic virus has been detected in wheat plants in France. It is assumed to be transmitted by *P. graminis* but this has not been experimentally proved (Hariri et al., 2001).

SBCMV and SBWMV are serologically very closely related to furoviruses which induce identical disease symptoms in their host plants. A few years ago when their unambiguous differentiation became possible on base of nucleotide sequence data of the bipartite genomes (Koenig et al., 1999) it turned out that the virus disease of winter wheat (*T. aestivum*, *T. durum*) which has been known in Italy and France already for many years and in UK since 1999 is actually caused by SBCMV but not by SBWMV as assumed in the past. Under natural conditions both SBCMV and WSSMV frequently occur together in infected plants. While SBCMV can easily be detected by ELISA from early spring through the whole vegetation period the bymovirus WSSMV can be reliably detected only in the short period when plants just start to grow after the winter period (Kastirr et al., 2005a).

In contrast to the situation in Italy, France and England with wheat as the predominantly affected crop the three soil-borne viruses cause most damage to rye and triticale in countries like Germany, Denmark, and Poland (Huth, 2002). Even total yield losses have been observed under unfavorable conditions.

SBCMV was first detected in Europe more than 20 years ago (Proeseler et al., 1982) but at that time still regarded as SBWMV. In Germany it has not

been an object of intensive research until the late 1990s when it became clear that the virus is already widely distributed in areas characterized by intensive production of rye and triticale. This picture very much resembles the process of rapid spreading of BaMMV and BaYMV in the barley growing areas about two decades ago.

In 2002 a first report on occurrence of the closely related SBWMV in Southwest Germany was given. This isolate is characterized by a high virulence and an almost identical genome sequence to the Nebraska strain previously described in the US (Koenig and Huth, 2003). The latter was not to expect because different isolates of SBCMV and SBWMV display considerable genetic diversity, respectively, while in contrast, the genome of bymoviruses is much less variable. Despite this pronounced genetic diversity significant differences in the biological properties of the furovirus isolates have not been observed so far (Koenig et al., 1999; Koenig and Huth, 2000; Koenig et al., 2002).

Can SBCMV, SBWMV and WSSMV seriously threaten the production of wheat, triticale, and rye?

In wheat, sources of resistance against the furoviruses have been known for several years. They have been successfully introduced into breeding programs and an increasing number of resistant cultivars has been developed and registered over the last 10 years. Fortunately, in many instances this resistance is tightly linked to a remarkably reduced susceptibility against WSSMV (Hariri et al., 1987; Huth, 2002). The genetic background of the observed resistance of wheat to the two furoviruses and the bymovirus has been attributed to various mechanisms including

- Reduced virus accumulation and spread within roots (Hariri et al., 1987; Driskel et al., 2002)
- Reduced movement of virus from roots to shoots and leaves, the tissues for massive virus multiplication (Myers et al., 1993; Carroll et al., 2002)
- Reduced virus accumulation in leaves (Himmel et al., 1991).

Inheritance of SBWMV resistance has been studied for several commercial wheat varieties with contradictory results. A single dominant gene (Modawi et al., 1982), two genes (Barbosa et al., 2001) or even three genes (Nakagawa et al., 1959) have been proposed to control this resistance. According to Kanyuka et al. (2003) the resistance to SBCMV in one UK wheat variety is controlled by a single gene.

In case of WSSMV many sources of resistance have been identified in commercial wheat varieties and wild *Triticum* species. The resistance is generally described as qualitative in nature and controlled by more than one gene (Khan et al., 2000). Plants are not immune to the virus (Cunfer et al., 1988; Carroll et al., 2002). WSSMV resistance probably acts by limiting distribution

of the virus in the root system and/or subsequent virus transport from roots into the tillers.

In case of rye and triticale all current varieties are susceptible to the virus complex. However extensive evaluation programs are running particularly in Germany and Poland to screen genetic resources for resistance to the above mentioned soil-borne viruses. First promising results have been obtained recently (Kastirr et al., 2005b).

Considering the previously outlined specific aspects of virus transmission by *P. graminis* and the fact that in case of wheat, as the most important cereal crop, sources of resistance to SBCMW, SBWMV, and WSSMV are available and have been already transferred into modern cultivars which have confirmed their efficiency in large scale production one can clearly conclude that none of the three viruses is useful as an effective agent for agroterrorist activities.

5.3. Sugar Beet—BNYVV

The so-called Rhizomania-Virus (*Beet necrotic yellow vein virus*, BNYVV) is the economically most important among a number of different soil-borne viruses that can infect sugar beet. It is transmitted by the parasitic organism *Polymyxa betae* which is morphologically indistinguishable from *P. graminis*. The virus can infect all types of beet, dramatically reducing root and sugar yields (up to 80%) in susceptible varieties (Giltrap et al., 2002). The disease was first officially recorded in Italy in 1952. Since then it has spread to most areas in the Northern hemisphere where sugar beet is grown and it is very widespread in Continental Europe. A survey in 2000 indicated that in Western Europe alone, 700,000 hectares of sugar-beet crop were infected. The Netherlands, France, and Germany are particularly badly affected by rhizomania, with estimated 70%, 46%, and 35% respectively of their land in beet production in that year (Giltrap et al., 2002). Characteristic symptoms are expressed mainly on roots and depend on the severity of the infection. Where infection is severe, roots remain very small, growth of the main tap root ceases and proliferation of laterals (root "bearding") occurs. The most specific symptoms associated with rhizomania are seen when the root is cut vertically. Root vascular elements are thickened and discolored and tumerous outgrowths occur at the site of lateral root proliferation.

Up to now three different types of BNYVV have been detected (Koenig et al., 1997; Koenig and Lennefors, 2000). The so-called A type is widespread in Europe as well as in the US, China, and Japan. Type B is geographically more restricted and occurs frequently in Germany, France and the UK. The

P type which seems to be more aggressive than the A and the B types has so far been found in Europe only in a small area around the French town of Pithiviers. It resembles the A type but contains a fifth RNA species whereas other isolates are characterized by four genomic components. A characteristic feature of BNYVV is the pronounced sequence stability of the various parts of the genome. They are highly conserved and exceptionally stable among isolates of A, B, and P type.

Despite yield losses of up to 80% in susceptible beet cultivars BNYVV cannot be considered a potential agroterrorist agent because

- viruliferous vector populations are already present and widespread in almost all European sugar beet growing areas.
- similar to soil-borne cereal viruses, large scale effective contamination of so far "healthy" areas with virus containing resting spores of *P. betae* is not a realistic scenario.
- the latent period between a potential purposeful initial introduction and the symptom development is long. Experience has shown that at least two or three crops of beet have to be grown from the time infection is introduced into field for the soil inoculum levels to build up sufficiently to cause significant economic harm, i.e. obvious symptoms in beet crops (Giltrap et al., 2002).
- during the last 10 years breeders have developed a whole spectrum of varieties (including transgenic genotypes) showing resistance (tolerance) to rhizomania (Scholten and Lange, 2000). The conventional resistance is a single major gene resistance controlled by the gene *Rz* originating from the American cultivar "Holly." Since 1995 this gene has been solely the basis of resistance in sugar beet cultivars throughout Europe and the US. It confers tolerance to the virus, i.e. leads to reduction in multiplication of BNYVV in lateral roots and reduced movement of the virus into the main tap root where infection is most damaging. As a consequence a significantly lower proportion of viruliferous resting spores are produced in the root of partially resistant than in susceptible cultivars.
- the problem with the P type of BNYVV, presently known in France but liable to be more widespread for the reason given above, that is highly aggressive and able to cause significant damage to sugar beet cultivars with "Holly" resistance is going to be solved. Recently in France a new cultivar was registered which possesses a second resistance gene different to *Rz*.
- resistance to the vector that is expressed in wild beet species *Beta procumbens* and *B. patellaris* has been difficult to incorporate into agronomically acceptable cultivars, but efforts are continuing in this area (Scholten and Lange, 2000).

5.4. Potato

In 2003 only 0.9% of the total agricultural area in the EU countries was used for production of potatoes, which is even less than for cultivation of sugar beet (1.5 %) (http://europa.eu.int/comm/agriculture). Because less than 50% of harvested tubers were directly used for human consumption, potato cannot longer be regarded as a main agricultural crop. Nevertheless, it has kept its traditional, very specific role and its high value as a staple food for many people, particularly in central and east Europe.

Therefore, a massive terrorist attack leading to extreme yield losses and/or tremendous decline in tuber quality could have significant psychological consequences that might be expressed as a general concern and a feeling of uncertainty within large groups of consumers. An example of dramatic social effects resulting from the epidemic occurrence of a single pathogen in potato fields is well known from history. In 1845 many potato fields in Ireland suddenly became severely infected. Many of the potatoes were found to have gone black and rotten and their leaves had withered. About half of the potato crop was destroyed. In the subsequent years almost the entire crop had been wiped out. With the massive rotting of harvested tubers people almost totally lost the basis of nutrition ("Irish Famine"). As a result about 1 million people died from hunger and a significant part of the population had to leave the country to survive (http://www.wesleyjohnston.com/users/Ireland). At that time potato was grown in Ireland almost in monoculture, which is completely different to the current situation in any European country and the infectious agent was not a virus but *Phytophthora infestans*, which is still regarded as the most important pathogen for potato. The majority of the numerous viruses infecting potato plants are naturally transmitted by aphids; only the three following species have soil-borne vectors:

- *Tobacco necrosis virus* (TNV) /*Olpidium brassicae*
- *Potato mop top virus* (PMTV) /*Spongospora subterranea*
- *Tobacco rattle virus* (TRV) / trichodorid nematodes.

What are the characteristic features of these viruses?

5.4.1. TNV

The virus particles are very stable in soil after release from infected plants. They are not incorporated in resting spores of *O. brassicae* but are becoming adsorbed onto the surface membrane of motile zoospores being on their way to host plant roots (Campbell, 1996). Virus gains entry into root cell following encystment of fungal zoospores. The virus induces typical symptoms on tubers

but not on leaves. It is not transmitted to plants grown from infected tubers. As the virus has not gained any economic relevance yet it clearly cannot serve as an effective agroterrorist agent.

5.4.2. PMTV

Potato mop top virus is transmitted by the plasmodiophorid vector *S. subterranea*, which in contrast to the *Polymyxa* species itself is much more pathogenic to its host plant and responsible for the powdery scab disease in potato tubers. The virus PMTV, a cause of spraing, is a persistent problem in potato crops in many potato production systems worldwide, including northern and central Europe (Jones, 1988). It causes rust colored rings, arcs and flecks in tubers of susceptible varieties infected during the growing season, and yellow chevrons and shortening of internodes of stems of plants grown from infected tubers. Symptoms can significantly vary depending on the cultivar and the environmental conditions (Kurppa, 1989). The reported effects of a PMTV infection on potato tuber yield range from no influence on total yield and dry matter content (Nielsen and Molgaard, 1997) to losses of up to 20 % (Van de Graaf et al., 2003). As tuber quality is generally affected by the virus and sources of resistance have still not been incorporated into modern potato cultivars, more effective control methods for both powdery scab and PMTV are needed in future. But despite the still lacking resistance the economic relevance of the two diseases is much too low to regard the corresponding pathogens as potential agroterrorist agents.

5.4.3. TRV

The virus which is transmitted by several species of *Paratrichodorus* and *Trichodorus* nematodes is the second of the two viruses that can cause spraing symptoms in potato. They appear as arcs and lines of cork necrotic tissue in the tubers and may render entire crops unmarketable at relatively low levels of symptom expression (Brown and Sykes, 1973). Depending on environmental and other conditions symptom expression can be highly variable. In addition to tubers, also leaves and shoots of infected plants may be affected up to a virtually total failure to produce daughter tubers (Robinson et al., 2004). TRV has a notably wide host range, infecting over 100 plant species in nature, including potato and a number of other important crop plants, like tobacco, sweet pepper, gladiolus, or tulip. Once established at a site, TRV and its vector nematodes are very difficult to control or eradicate without use of fumigant soil treatments that are currently environmentally unacceptable.

Because the bipartite virus genome is highly variable a large number of strains and serotypes is known. For many years it was thought that the primary

effects of TRV were visible spraing symptoms and that the virus was generally self-eliminating from seed potato stocks. By the procedure of consequent visual control of seed potato lots for typical spraing symptoms the uncontrolled distribution of the virus could be successfully prevented in the past. But as recent results demonstrated, some potato cultivars can become persistently systemically infected with TRV without developing spraing symptoms and such plants can serve as sources for virus acquisition by vector nematodes. Stocks of these cultivars can become chronically infected and show negative effects on yield and quality. The most evident phenotypic effects are a significant decrease in tuber size, accompanied by a large increase in tuber number and also a notable degree of secondary growth resulting in misshapen tubers (Dale et al., 2004). From the phytopathological point of view these new findings are very important, because they make obvious that the visual control of seed material for presence of TRV does not suffice anymore. However, a large scale production and distribution of TRV-infected but symptomless tubers to farmers in order to purposefully carry the virus into virus free regions, would be definitely a non-realistic scenario for an agroterrorist activity.

Therefore, I conclude that none of the above-mentioned soil-borne viruses has much, if any, potential to be used as a bioweapon—even with expensive and time-consuming genetic modification for enhanced pathogenicity.

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 Pathol., 40, 53–58.

Adams, M.J., and S.A. Hill, 1992. Soil-borne mosaic viruses of cereals: The UK situation, Home-Grown Cereals Authority, 8 pp.

Adams, M.J., D.R. Jones, T.M. O'Neill, and S.A. Hill, 1993. The effect of cereal break crops on barley mild mosaic virus, Ann. Appl. Biol., 123, 37–45.

Altman, S., B.L. Bassler, J. Beckwith, M. Belfort, H.C. Berg, B. Bloom, J.E. Brenchley, A. Campbell, R.J. Collier, N. Connell, N.R. Cozzarelli, N.L. Craig, S. Darst, R.H. Ebright, S.J. Elledge, S. Falkow, J.E. Galan, M. Gottesman, R. Gourse, N.D.F. Grindley, C.A. Gross, A. Grossman, A. Hochschild, M. Howe, J. Hurwitz, R.R. Isberg, S. Kaplan, A. Kornberg, S.G. Kustu, R.C. Landick, A. Landy, S.B. Levy, R. Losick, S.R. Long, S.R. Maloy, J.J. Mekalanos, F.C. Neidhardt, N.R. Pace, M. Ptashne, J.W. Roberts, J.R. Roth, L.B. Rothman-Denes, A. Salyers, M. Schaechter, L. Shapiro, T.J. Silhavy, M.I. Simon, G. Walker, 2005. An open letter to Elias Zerhouni, Science, 307, 1409–1410.

Barbosa, M.M., L.R. Goulart, A.M. Prestes, and F.C. Juliatti, 2001. Genetic control of resistance to soil-borne wheat mosaic virus in Brazilian cultivars of *Triticum aestivum* L. Thell, Euphytica, 122, 417–422.

Brown, E.B., and G.B. Sykes, 1973. Control of tobacco rattle virus (spraing) in potatoes, Ann. Appl. Biol., 75, 462–464.

Campbell, R.N., 1996. Fungal transmission of plant viruses, Ann. Rev. Phytopathol., 34, 87–108.

Carroll, J.E., G.C. Bergstrom, and S.M. Gray, 2002. Assessing the resistance of winter wheat to wheat spindle streak mosaic bymovirus, Can. J. Plant Pathol., 24, 465–470.

Chen, J.P., M.J. Adams, F.T. Zhu, Z.Q. Wang, J. Chen, S.Z. Huang, and Z.C. Zhang, 1996. Response of foreign barley cultivars to barley yellow mosaic virus at different sites in China, Plant Pathol., 45, 1117–1125.

Cunfer, B.M., J.W. Demski, and D.C. Bays, 1988. Reduction in plant development, yield, and rain quality associated with wheat spindle streak mosaic virus, Phytopathology, 78, 198–204.

Dale, M.F.B., D.J. Robinsson, and D. Todd, 2004. Effects of systemic infections with *Tobacco rattle virus* on agronomic and quality traits of a range of potato cultivars, Plant Pathol., 53, 788–793.

Driskel, B.A., R.M. Hunger, M.E. Payton, and J. Verchot-Lubicz, 2002. Response of hard red winter wheat to *Soilborne wheat mosaic virus* using novel inoculation methods, Phytopathology, 92, 347–354.

Fantakhun, A.T., L.A. Pavlenko, and A.D. Bobyr, 1987. Barley yellow mosaic agent in the Ukraine, Mikrobiol. Zhurnal, 49, 76–80.

Giltrap, N., R. Baker, and C. Henry, 2002. Sugar Beet Rhizomania—a technical overview. http://www.defra.gov.uk/planth/Rhizback.pdf, 26 pp.

Graner, A., and E. Bauer, 1993. RFLP mapping of the ym4 virus resistance gene in barley, Theor. Appl. Genet., 86, 689–693.

Habekuss, A., T. Kühne, F. Rabenstein, I. Krämer, F. Ehrig, B. Ruge-Wehling, W. Huth, and F. Ordon, 2005. Detection of an rym5 resistance breaking virus strain in Germany. Abstr. VIth Int. Symp. Working Group on Plant Viruses with Fungal Vectors, Bologna, p. 60.

Hariri, D., M. Courtillot, P. Zaoui, and H. Lapierre, 1987. Multiplication of soil-borne wheat mosaic virus in wheat roots infected by soil carrying SBWMV and wheat yellow mosaic virus, Agronomie, 7, 789–796.

Hariri, D., M. Fouchard, and H. Lapierre, 1990. Resistance to barley yellow mosaic virus and to barley mild mosaic virus in barley, in: *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors (Schriftenreihe der Deutschen Phytomedizinischen Gesellschaft),* edited by R. Koenig, Verlag Eugen Ulmer, Stuttgart, pp. 109–112.

Hariri, D., M. Meyer, and H. Prud'homme, 2003. Characterization of a new barley mild mosaic virus pathotype in France, Eur. J. Plant Pathol., 109, 921–928.

Hariri, D., H. Prud'homme, M. Fouchard, G. Boury, P. Signoret, and H. Lapierre, 2001. Aubian wheat mosaic virus, a new soil-borne wheat virus emerging in France, Eur. J. Plant Pathol., 107, 775–785.

Hill, S.A., and E.J. Evans, 1980. Barley yellow mosaic virus, Plant Pathol., 29, 197–199.

Himmel, P.T., A.D. Hewing, and D.A. Glawe, 1991. Incidence of soil-borne mosaic virus and its reported vector *Polymyxa gaminis*, in field grown soft red winter wheat, Plant Dis., 75, 1008–1012.

Huth, W., and D.E. Lesemann, 1978. Eine für die Bundesrepublik Deutschland neue Virose an Wintergerste, Nachrichtenbl. Dt. Pflanzenschutzd., 30, 184–185.

Huth, W., 1988. Ein Jahrzehnt Barley Yellow Mosaic Virus in der Bundesrepublik Deutschland, Nachrichtenbl. Dt. Pflanzenschutzd., 40, 49–55.

Huth, W., 1989. Ein weiterer Stamm des barley yellow mosaic virus aufgefunden, Nachrichtenbl. Dt. Pflanzenschutzd., 41, 6–7.

Huth, W., 2000. Im Getreidebau in Deutschland und in Europa wird eines der größten Probleme erwartet: die bodenbürtigen Viren des Weizens und Roggens, Nachrichtenbl. Dt. Pflanzenschutzd., 52, 196–198.

Huth, W., 2002. Die bodenbürtigen Viren von Weizen und Roggen in Europa—ein zunehmendes aber durch ackerbauliche Maßnahmen und Anbau resistenter Sorten lösbares Problem, Ges. Pflanze, 54, 51–57.

Jones, R.A.C., 1988. Epidemiology and control of potato mop-top virus, in Developments in Applied Biology, II. Viruses with fungal vectors, edited by I. Cooper and M.J.C. Asher, AAB Wellesbourne, pp. 255–270.

Kanyuka, K., G. McGrann, K. Alhudaib, D. Hariri, and M.J. Adams, 2004. Biological and sequence analysis of a novel European isolate of *Barley mild mosaic virus* that overcomes the barley *rym5* resistance gene, Arch. Virol., 149, 1469–1480.

Kanyuka, K., E. Ward, and M.J. Adams, 2003. *Polymyxa graminis* and the cereal viruses it transmits: a research challenge, Mol. Plant Pathol., 4, 393–406.

Katis, N., K. Tzavella-Klonari, and M.J. Adams, 1997. Occurrence of barley mild mosaic and barley yellow mosaic bymoviruses in Greece, Eur. J. Plant Pathol., 103, 281–284.

Kashiwazaki, S., K. Ogawa, T. Usugi, T. Omura, and T. Tsuchizaki, 1989. Characterization of several strains of barley yellow mosaic virus, Ann. Phytopathol. Soc. Japan, 55, 16–25.

Kastirr, U., F. Rabenstein, and T. Kühne, 2005a. Epidemiolgical aspects of soil-borne viruses of wheat, triticale and rye in Germany, Abstr. VIth Int. Symp. Working Group on Plant Viruses with Fungal Vectors, Bologna, p. 40.

Kastirr, U., R. Schachschneider, T. Hammann, and H. Wortmann, 2005b. Investigation of resistance to soil-borne viruses in wheat, triticale and rye, Abstr. Xth Conf. Viral Dis. of Graminae in Europe, Louvain-la-Neuve, p. 56.

Khan, A.A., G.C. Bergstrom, J.C. Nelson, and M.E. Sorrells, 2000. Identification of RLP markers for resistance to wheat spindle streak mosaic bymovirus (WSSMV) disease, Genome, 43, 477–482.

Koenig, R., G.C. Bergstrom, S.M. Gray, and S. Loss, 2002. A New York isolate of *Soil-borne wheat mosaic virus* differs considerably from the Nebraska type strain in the nucleotide sequences of various coding regions but not in the deduced amino acid sequences, Arch. Virol., 1477, 617–625.

Koenig, R., A.M. Haeberlé, and U. Commandeur, 1997. Detection and characterization of a distinct type of beet necrotic yellow vein virus RNA 5 in a sugar beet growing area in Europe, Arch. Virol., 142, 1499–1504.

Koenig, R., and W. Huth, 2000. *Soil-borne rye mosaic* and *European wheat mosaic virus*: two names for a furovirus with variable genome properties which is widely distributed in several crops in Europe, Arch. Virol., 145, 689–697.

Koenig, R., and W. Huth, 2003. Natural infection of wheat by the type strain of *Soil-borne wheat mosaic virus* in a field in Southern Germany, Eur. J. Plant Pathol., 109, 191–193.

Koenig, R., C.W.A. Pleij, and W. Huth, 1999. Molecular characterization of a new furovirus mainly infecting rye, Arch. Virol., 144, 2125–2140.

Koenig, R., and B.L. Lennefors, 2000. Molecular analyses of European A, B and P type sources of Beet necrotic yellow vein virus and detection of a rare P type in Kazakhstan, Arch. Virol., 145, 1561–1570.

Kühne, T., N. Shi, G. Proeseler, M.J. Adams, and K. Kanyuka, 2003. The ability of a bymovirus to overcome the *rym4*-mediated resistance in barley correlates with a codon change in the VPg coding region on RNA1, J. Gen. Virol., 84, 2853–2859.

Kurppa, A., 1989. Reaction of potato cultivars to primary and secondary infection by potato mop-top furovirus and strategies for virus detection, EPPPO Bull., 19, 593–598.

Langenberg, W. G., and D. Van der Wal, 1986. Identification of barley yellow mosaic virus by immuno-electron microscopy in barley but not in *Polymyxa graminis* or *Lagena radicicola,* Neth. J. Plant Pathol., 92, 133–136.

Lapierre, H., 1980. Nouvelles maladies à virus sur ceréales d'hiver, Le Producteur Agricole Français, 270, 11.

Lee, K.-J., S. Kashiwazaki, T. Hibi, and I.-Y. So, 1996. Properties and capsid protein gene sequence of a Korean isolate of barley mild mosaic virus, Ann. Phytopathol. Soc. Japan, 62, 397–401.

Madden, L.V., and M. Wheels, 2003. The threat of plant pathogens as weapons against U.S. crops, Ann. Rev. Phytopathol., 41, 155–176.

Maroquin, C.M., M. Cevalier, and A. Rassel, 1982. Premières observations sur le virus de la mosaìcque jaune de l'orge en Belgique, Bull. Rech. Agron. Gembloux, 17, 157–172.

Modawi, R.S., E.G. Hyne, D. Brunetta, and W.G. Willis, 1982. Genetic studies of field reaction to wheat soil-borne mosaic virus, Plant Dis., 66, 1183–1184.

Myers, L., J.L. Sherwood, W.C. Siegerist, and R.M. Hunger, 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.

Nakagawa, M., Y. Soga, S. Watanabe, H. Gocho, and K. Nishio, 1959. Genetical studies on the wheat mosaic virus. II. Genes affecting the inheritance of susceptibility to strains of yellow mosaic virus in varietal crosses of wheat, Jap. J. Breed., 9, 118–120.

Nielsen, S.L., and J.P. Molgaard, 1997. Incidence, appearance and development of potato mop-top furovirus-induced spraing in potato cultivars and the influence on yield, distribution in Denmark and detection of the virus in tubers by ELISA, Potato Res., 40, 101–110.

Plumb, R.T., E.A. Lennon, and R.A. Gutteridge, 1986. The effects of infection by barley yellow mosaic virus on yield and components of yield of barley, Plant Pathol., 35, 314–318.

Proeseler, G., A. Stanarius, and K. Eisbein, 1982. Nachweis weiterer Viren an Getreide in der DDR, Arch. Phytopathol. Pflanzenschutz, 18, 397–403.

Proeseler, G., A. Stanarius, and T. Kühne, 1984. Vorkommen des Gerstengelbmosaik-Virus in der DDR, Nachrichtenbl. Pflanzenschutz DDR, 38, 89–91.

Robinson, D.J., M.F.B. Dale, and D. Todd, 2004. Factors affecting the development of disease symptoms in potatoes infected by *Tobcco rattle virus*, Eur. J. Plant Pathol., 110, 921–928.

Rubies-Autonell, C., G. Toderi, A. Marenghi, and V. Vallega, 1995. Effects of soil village and crop rotation on BaYMV and BaMMV mixed infection, Agronomie, 15, 511–512.

Scholten, O.E., and W. Lange, 2000. Breeding for resistance to rhizomania in sugar beet: A review, Euphytica, 112, 219–231.

Spaar, D., W. Huth, and F. Rabentein, 2002. Problem of virus diseases of cereal crops in Europe, Plant Prot. News (Institute of Plant Protection, St. Petersburg, Russia), 1, 8–14.

Stein, N., D. Perovic, J. Kumlehn, B. Pellio, S. Stracke, S. Streng, F. Ordon, and A. Graner, 2005. The eukaryotic initiation factor of translation 4E confers multiallelic recessive bymovirus resistance in *Hordeum vulgare* (L.), Plant J., 42, 912–922.

Steyer, S., J. Kummert, and F. Froidmont, 1995. Characterisation of a resistance-breaking BaYMV isolate from Belgium, Agronomie, 15, 433–438.

Van de Graaf, P., A. Lees, and J. Duncan, 2003. Effect of environmental factors n infection of potato by *Spongospora subterranea* f. sp. *subterranea*, the vector of PMTV. Proc. 5th Symp. Int. Working Group on Plant Viruses with Fungal Vectors, Zurich, July 22–25, 2002, 88–91.

Werner, K., W. Friedt, E. Laubach, R. Waugh, and F. Ordon, 2003. Dissection of resistance to soil-borne yellow-mosaic-inducing viruses of barley (BaMMV, BaYMV, BaYMV-2) in a complex breeders cross by means of SSRs and simultaneous mapping of BaYMV/BaYMV-2 resistance of var. 'Chirukin Ibaraki 1', Theor. Appl. Genet., 106, 1425–1432.

GENOMIC APPROACHES IN VIRUS DIAGNOSTICS A PERSONAL ASSESSMENT OF REALITIES WHEN FACED WITH VIRUSES IN A PLANT BIOSECURITY CONTEXT

Ian Cooper

6.1. Introduction

During the past 30 years, most plant pathogenic viruses and viroids have been characterized in terms of their base sequence. A few, such as viruses in the family *Luteoviridae*, were harder to crack than others but recently yielded to this approach (e.g. Huang et al., 2005). With the primary base sequences of these viruses determined, it was possible to infer relationships but also to identify the positions of functional units. Additionally, it was often possible to unravel the complex interactions in time and space involved with genome expression. Thus, because of their relatively small genome sizes, viruses were in the vanguard of what has come to be grouped under the generic title "omic" technologies; the word "transcriptomics" was not used to describe these early technological thrusts but might be now.

Genome level characterization of a tiny number of cellular organisms that are virus hosts is now approaching completeness (if there can really be such a thing given the biodiversity among individuals in biological systems). Only a few green vascular plants (e.g. rice, poplars, brassicas, wheat, tomatoes, arabidopsis) are currently under investigation in this way. Nevertheless, drawing upon experience gained in vertebrate or microbial systems, it is beginning to be possible to develop new insights into the activities of plant proteins, including enzymes, produced when genes are active under particular conditions (metabolomics/proteomics). Predictably, viruses elicit in infected plants the expression of special genes. Interestingly, different viruses trigger the expression of common genes (e.g. Whitham et al., 2003). The very expensive robotics that lie at the heart of "omic" technologies could be applied to the simultaneous testing of many samples against a diverse range of targets. However, this would be something of a sledgehammer to crack a nut and I will now consider a number of detection approaches that seem to be more likely.

Genomic knowledge about humans is valued because it enables more focused treatment than hitherto. However, there are concerns that such information enables the targeting of pathogens into particular genotypes (ethnic groups). I am presently of the opinion that knowledge of plant genomics (gene location, function and control) is much too fragmentary to be similarly

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 71–80.

exploited in the near future. There has undoubtedly been a very substantial investment in a range of plant "omic" technologies and it is conceivable that the knowledge obtained may expose new ways to increase the economic impact of specific pathogens on plants. However, I do not intend to speculate on how this might be achieved.

Building on genomic information, gene delivery systems derived from viruses that infect vertebrates have been made to expand therapeutic options through the provision of essential genes and products that the host lacks (e.g. Nathwani et al., 2004; Edelstein et al., 2004). Such gene vector systems may lend themselves to accidental or deliberate use for the enhancement of pathogenicity in vertebrates (Jackson et al., 2001) and other contributors at this meeting are likely to indicate parallel opportunities that exist with genetically modified plant infecting viruses.

If faced with exotic or hitherto uncommon viruses (whether natural or manufactured), it seems to me that the most likely tools for routine use for diagnosis or detection will target genomes. The rise of genome-based detection technologies has been accompanied by a decline in usage of other methods; some that were popular have been totally eclipsed.

6.2. Electron Microscopy and Bioassay Systems

In 1986 (Jones and Torrance), a notable electron microscopist (Ian Roberts), wrote "electron microscopy continues to play a vital role in plant virology." At least in a diagnostic context, this is no longer true. It is undoubtedly very satisfying for a virus pathologist to "see" the size and shape of a virus in suspect samples and this information may give taxonomic clues that inform management opportunities. However, knowledge about the external appearance of virions has only limited value. Electron microscopes are expensive to buy and maintain and are no longer normally operated even in the best-equipped virus laboratories. Their place as pieces of sophisticated and expensive "must have" equipment for detection/ diagnosis is now occupied by robots for standardized nucleic acid characterization and discrimination; automated fluorescence sequencing, micro array handling and "real time" polymerase chain amplification (e.g. Mackay et al., 2002). Although unquestionably useful, biological systems for virus recognition are being sidelined because of time and cost. Bioassays that depend upon grafting of materials under test into (particularly woody) indicator plants have almost completely "had their day." However, other biological assay systems, such as the manual inoculation of plants with extracts from other plants or through the use of vectors, remain valuable—despite the often hard to justify cost in terms of labor, time, maintenance and management of appropriate glasshouses.

6.3. Current Practice-A Case Study

I picked the following from current literature to show what was recently done in a diagnostic context. Electron microscopy was not mentioned in the work which concerned the detection of *Turnip mosaic virus* in rhubarb (*Rheum hybridum*) in Alaska. In itself, this record is exceptional (and I hope pertinent) only in so far as Alaska has few crops and I suspect very few specialist "virologists." When faced with a virus-like disease in rhubarb, Robertson and Ianson (2005) described serological tests as the primary diagnostic screen but also the use of manual inoculation of plants with sap extracts to show pathogen transmissibility. The clinching tests were made using extracted nucleic acid from the plants under test. Thus, suspicious leaf tissue was first tested using a "shop bought" indirect enzyme-amplified immuno assay kit from Agdia Inc. and a panel of reagents detecting a range of potyviruses. Confirmatory tests used a kit of sera from the same company to reveal strong evidence for a specific potyvirus—turnip mosaic virus. Then, total RNA was extracted from the foliage, purified using a kit from Qiagen Sciences and assayed using reverse transcription and polymerase chain amplification (Mullis, 1990) with primers specific for the suspected virus. Amplified sequences were characterized and checked against databases to reaffirm the suspicion.

In the following, I have not attempted to cover in equal depth all of the diagnostic methods that have been reported to be useful for plant viruses. I consider that a sufficient number of the plant virus detection systems were described in the book edited by Jones and Torrance (1986) for that compilation to be an appropriate and adequate introduction to the prior art. As a consequence, I have deliberately not described some methods (e.g. diagnosis through recognition of ds RNA) even though they have specific utility (e.g. Gibbs et al., 1996). I have concentrated on the pros and cons of "genomic" approaches to detection/diagnosis.

6.4. Mainly Enzyme Amplified Immunoassays

A diverse range of serological methods is in use for detection/diagnosis but enzyme-amplified immunoassays are routine and seem unlikely to be eclipsed in the near future. Cooper and Edwards (1986) reviewed much of the relevant literature but numerous other reports of modifications (often very trivial) in this technology burden library shelves.

Despite the technical simplicity of radioimmune assays and their potential for measurement, increased awareness of the health hazards associated with radionuclides contributed to a decline in the popularity of this approach. Their place is occupied by a family of methods that exploit amplification

systems in which enzymes act on chromogenic, chemiluminescent, or fluorogenic substrates. None of these is constrained to any important extent by signal generation or detection and many have been reliably automated. As a consequence, the methods have readily lent themselves to use in large scale testing programs. Although antigenic detection is based on only a small part of the total genomic coding capacity of a target virus and the process may be confounded by reversible antibody-antigen interactions, such assays have become routine except when the target pathogen lacks protein (as with viroids). Nevertheless, their future is not secure because only a narrow spectrum of sera is available and the range does not seem to be increasing in step with the diversity of potential hazard.

I think it is worth emphasizing that not all of the economically important viruses have had sera prepared against them. For some, there are only minute amounts "for research purposes only." Given the extremely limited quantities of appropriate antisera and the increasing cost of regulation in animal rearing facilities, I consider that the future of serological detection systems is somewhat threatened. Hybridoma lines that secrete potentially unlimited amounts of monoclonal antibodies have very substantial "set up" costs and are only available for a few dozen of the plant pathogenic viruses that are subjects in crop improvement programs and thereby have guaranteed markets. Whereas human health justifies substantial (speculative) investment, plant diagnostics are often judged to serve only a minuscule boutique market and this is reflected in the range of tools available "of the shelf."

6.5. Detection Systems that Target Specific Nucleic Acid Sequences

Detection systems that target specific nucleic acid sequences currently hold prime of place in the repertoire of the diagnostician because of their versatility, throughput, and efficiency. However, sensitivity and sophistication comes at a cost that is hard to bear generally and effective co-operation with a central laboratory or even a research facility in another country may be crucial if there is to be a rapid and appropriate response to an unexpected phytopathological event.

Using protocols developed from systems first used for the detection of viruses in animal cells (e.g. Brandsma and Miller, 1980), nucleic acid hybridization has often been used separately for the detection of viruses or viroids in plants. Whether on a solid surface or in solution, detection requires hybridization of target with a labeled probe of complementary base sequence (Gould and Symons, 1983). When radioisotopic labels are used, there is ample published evidence that shows methods based on nucleic acid hybridization are acceptably sensitive for the detection of single-stranded RNA.

6.6. Polymerase Chain Reactions (PCR)

The PCR is a synthesis that uses two oligonucleotide primer sequences hybridized to target strands of opposite polarity and flanking a region of interest in the target genome. A repeating series of cycles involving template denaturation, primer annealing, and extension facilitated by DNA polymerase results in the exponential increase of segments defined by the 5′ terminal bases in the primers. The method that was invented by Mullis and colleagues (e.g. Mullis, 1990) initially used the Klenow fragment of DNA polymerase I from *Escherichia coli* but the availability of thermostable enzymes from thermophilic organisms has transformed the protocols and facilitated automation (Saiki et al., 1988). Whereas products of PCR were once analyzed by gel electrophoresis, many modifications in detection technology now exist; products may be used for nucleic acid hybridization with a standard, for direct sequencing, for cloning or as a diagnostic probe in hybridization. Whatever the end use, these approaches requires either new work or that the genomic nucleotide sequences of the viruses, viroids, etc. to have been lodged in public databases. Internet accessible resources enable PCR optimization and even group discussions (McCann, 1999) but the design of oligomeric primers is absolutely limited by the sequence data available and it is naive to assume that all viruses that have been characterized are described in the public literature. Analogously, not all genomic data obtained from organisms is in the public domain.

Polymerase chain amplification that has hybridization at its heart now leads the field in virus detection—whether for amplifying DNA or RNA (when base sequences are copied [= reverse transcribed] as a prelude to the amplification step; RT-PCR). The technologies are popular and draw upon developments in genetics of plants, people, and microbes but often need to be tailored to particular use. Furthermore, viruses that have extensive sequence diversity among isolates or which integrate in their host genomes (e.g. Harper et al., 1999) create problems for PCR amplification/detection that may be hard to surmount.

6.7. Variations on the PCR Theme

RT-PCR, in various forms, has been used successfully to detect and to differentiate between viruses at the family, genus, species or isolate levels and has often been reported to enable virus detection when serological or biological methods failed. The general approach has been claimed to be several-fold more sensitive than enzyme amplified immunoassays or nucleic acid hybridization for virus detection (Olmos et al., 1997) and there are a bewildering array

of opportunities for future refinement. However, specific PCR protocols are not a universal technofix and need to be optimized for each new problem. Thus, commercial RNA extraction systems may, in some instances, facilitate sample preparation (e.g. Stevens et al., 1997; MacKenzie et al., 1997) but should not be assumed to be satisfactory in all. Furthermore, cells differ in the readiness with which viral nucleic acids are released and also in the abundance of inhibitory chemicals (e.g. Thomson and Dietzgen, 1995). Phenolics or polysaccharides in woody tissue, tubers, or old and tough foliage may be a nuisance (Newbury and Possingham (1979) that may or may not be eliminated by sample dilution or through the use of commercial products such as GeneReleaser (BioVentures Inc.). Alternatively, generally reliable but expensive commercial column systems (e.g. Rneasy from Qiagen) have become routine where "time is money." Inhibitors of reverse transcription or polymerase enzymes can be avoided by capturing virions non-specifically on plastic or more specifically with antisera before attempting the amplification. Indeed, when sera are freely available, immunocapture PCR (e.g. Wetzel et al., 1992) may be the detection approach of choice. Primer oligonucleotides can be rationally identified when numerous genome sequences have been determined in the target and when specific regions (of greatest variability and least homology or target specificity or vector specific coding or antigenicity) can be identified. Selection of a presumed stable sequence of bases such as form the polymerase coding region may be a good first objective when seeking to design a primer oligomer but even that coding sequence may be prone to vary and turn out to be unusable. There is sometimes merit in using mixtures of primer sequences in which the nucleotides at one or a few positions differ deliberately to represent a conserved sequence of bases in the target. Unfortunately, these do not always work because of unintended amplifications of host components and the inevitable diminution in sensitivity. Combinations of primers can be useful for simultaneous detection/differentiation of different viruses (e.g. Bariani et al., 1994) or the simultaneous amplification of different target regions in a common template. Gibbs and MacKenzie (1997) designed primer pairs that amplified 17 virus species in the Potyviridae and analogous broad reactivity has been attained for use when seeking other viruses such as closteroviruses (Tian et al., 1996), geminiviruses (Rybicki and Hughes, 1990) or luteoviruses (Robertson et al., 1991). These approaches undoubtedly have value for screening purposes but viral genome characteristics can also be investigated after PCR; either through use of many primers each targeting different parts of a genome or as a result of repeated amplification of cDNA ends. Entire genomic sequences can be determined in specific pathological contexts (e.g. Revers et al., 1997) and unexpected insertions can clearly be revealed by direct sequencing of PCR products.

6.8. Being Prepared; Back to the Future

In 2002, when signing the Homeland Security Act into federal law, President G.W. Bush said "we can neither predict nor prevent every conceivable attack." In that speech he was mainly concerned with direct attacks on human health but his opinion would have been no less appropriate in the context of indirect/economic impacts. Biological attacks on food supplies, forests, or natural vegetation have been envisaged for many years and must be considered "likely." That potential hazard is given much less publicity (and research funding) than issues of human or animal health probably because, on past experience, farm animals and humans are more likely direct targets than crop plants—especially if engineered pathogens with supernatural pathogenicity attributes provide the means of damage.

The issues raised by the possibility that plant pathogenic viruses will be used as weapons calls for enhanced scrutiny of plant populations, recognition of abnormal incidence or severity of disease and training of people in aspects of "green" plant pathology-an area that has been of declining interest in recent years. There is a need for sensitive detection, characterization and diagnosis if the most appropriate countermeasures are to be operated. Furthermore, if harm is to be minimized, rapid response teams are desirable for mobilization in the hope that they can effectively assess the situation and assemble information about uncommon/unusual/wholly new pathogens/diseases.

A few early warning systems are in place but past experience suggests that national phytosanitary services may not be well prepared to react quickly to uncommon (even though "expected" events). Co-ordinated activity from staff in a national body should provide the continuity and expertise to address the actual cause of a possible new disease. Given appropriate support from the field they should assess whether the cause of an increase in incidence is linked to enhanced dispersal of a previously recognized pathogen or intensified cropping or weather patterns- or to something substantially new. In addition, they should determine whether the causal agent (virus or other) is infectious and whether it falls within the established range of natural variation. Nucleic acid analysis that reveals a novel juxtaposition of genetic elements may indicate something more sinister but this is likely to be uncovered only after time-consuming and costly effort—a potentially harmful economic impact in itself. In the first instance, absolute quality assurance and cost-effectiveness is not crucial but if containment or ideally eradication is to be attempted, detection methods should be available that are specific, sensitive and rapid. Furthermore, there is a need to know the pattern of disease spread in the field-giving hints about possible vectors and their management. Thus, there is a need (perhaps even an urgent need) to monitor growing plants

including crops and to establish reporting systems that respond to unusual observations having regard to the history of past virus infections/vectors. At one level there is a need to alert forestry workers and environmentalists to keep their eyes open to changes in their landscape but grower education is key with the aim of encouraging regular inspections and reporting. Clearly the farmers and horticulturalists must be given knowledge of all aspects of normal crop production; varietal variation and management. This information was absorbed passively (by immersion and diffusion) by their forebears who walked and personally weeded in fields rather than drove and sprayed mechanically (and pre-emergence). I think that sensitization and training of field workers is crucial but that effort must be complemented by strengthened "political" willingness to respond effectively and rapidly to unusual events if they are not to be preludes to damaging epiphytotics. In the past, issues of "trade" and other political considerations have undoubtedly been responsible for delaying effective action. When urgent drastic action would have been appropriate, months or years of debate were allowed before stable doors were closed—by which time horses had bolted. Rates of pathogen spread vary and the insidious "slow burn" epiphytotic may have more long-term economic impact than an eyecatchingly explosive pattern of spread. As a consequence, there is a need for diligence "to nip the problem in the bud"—if possible.

6.9. Conclusion

Reagents for use in PCR technologies are costly, unstable and some must be made "to design and for order." Furthermore, there are substantial costs attributable to laboratory supply and management to eliminate contamination if PCR assays are to be delivered at the highest standards of reliability and reproducibility. Almost inevitably, because of the hardware and personnel, the technologies that use bioinformatics and genomics to support virus detection are more likely to be centralized than dispersed in areas where crops are grown and where suspicious "new" diseases may be introduced. Battery powered portable thermocycling instruments (e.g. RAZOR from Idaho Technology) are starting to be offered for sale and may find a use but, for the well funded central laboratory, ultrafast "real time" PCR instrumentation is increasingly the "norm". One supplier (Applied Biosystems) claims to deliver in approximately 25 minutes results of PCR tests after fully automated robotic loading of 96 well or larger format plates. Thus, the tests and data handling systems are available but proponents of new technological opportunities such as the "molecular beacons" which fluoresce when bound to a target sequence (e.g. Tyagi et al., 1998) do not always give appropriate prominence to the

realities of development time, sample preparation time and also the substantial "licence" costs attributable to proprietary tools. What may be tolerated in human medicine normally cannot be considered in the context of plants. Nevertheless, hospitals may have underused "real time" PCR facilities and may be prepared to share them but there will be an inevitable learning curve while the staff in the hospital lab adapts their technology.

References

Bariani, H.S., A.L. Shannon, P.G.W. Chu, and P.M. Waterhouse, 1994. Detection of five seedborne legume viruses in one sensitive multiplex polymerase chain reaction test, Phytopathology, 84, 1201–1205.

Brandsma, J., and G. Miller, 1980. Nucleic acid spot hybridization: Rapid quantitative screening of lymphoid cell lines for Epstein-Barr viral DNA, Proc. Natl. Acad. Sci. USA, 77, 6851–6855.

Cooper, J.I., and M.L. Edwards, 1986. Variations and limitations of enzyme amplified immunoassays, in Developments and applications in Virus Testing, edited by R.A.C. Jones and L. Torrance, Association of Applied Biologists, Wellesbourne, U.K., pp. 139–154.

Edelstein, M.L., M.R. Abedi, J. Wixon, and R.M. Edelstein, 2004. Gene therapy clinical trials worldwide 1989–2004—an overview, J. Gene Medic., 6, 597–602.

Gibbs, M.J., I. Cooper, and P.M. Waterhouse, 1996. The genome organization and affinities of an Australian isolate of carrot mottle umbravirus, Virology, 224, 310–313.

Gibbs, A.J., and A. MacKenzie, 1997. A primer pair for amplifying part of the genome of all potyvirids by RT-PCR, J. Virol. Meth., 63, 9–16.

Gould, A.R., and R.H. Symons, 1983. A molecular biological approach to relationships among viruses, Ann. Rev. Phytopathol., 21, 179–199.

Harper, G., J.O. Osuji, J.P.S. Heslop Harrison, and R. Hull, 1999. Integration of banana streak badnavirus into the Musa genome: Molecular and cytological evidence, Virology, 255, 207–213.

Huang, L.F., M. Naylor, D.W. Pallett, J. Reeves, J.I. Cooper, and H. Wang, 2005. The complete genome sequence, organisation and affinities of carrot red leaf virus, Arch. Virol., 150, 1845–1855.

Jackson, R.J., A.J. Ramsay, C.D. Christensen, S. Beaton, D.F. Hall, and I.A. Ramshaw, 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox, J. Virol., 75, 1205–1210.

Jones, R.A.C., and L. Torrance, 1986. Developments in Applied Biology 1. Developments and Applications in Virus Testing, Association of Applied Biologists, Wellesbourne, U.K., 312 p.

Mackay, I.M., K.E. Arden, and A. Nitsche, 2002. Real–time PCR in virology, Nucl. Acids Res., 30, 1292–1305.

McCann, S., 1999. Web PCR, Nat Biotechnol., 17, 304.

MacKenzie, D.J., M.A. McLean, S. Mukerji, and M. Green, 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription–polymerase chain reaction, Plant Dis., 81, 222–226.

Mullis, K.B., 1990. The unusual origins of the polymerase chain reaction, Sci. Am., 263, 56–65.

Nathwani, A.C., A.M. Davidoff, and D.C. Leach, 2004. A review of gene therapy for haematological disorders, Br. J. Haematol., 128, 3–17.

Newbury, H.J., and J.V. Possingham, 1979. Factors affecting the extraction of intact ribonucleic acid from plant tissues containing interfering phenolic compounds, Plant Physiol., 60, 543–547.

Olmos, A., M. Cambra, M.A. Dasi, T. Candresse, O. Esteban, M.T. Gorris, and M. Asensio, 1997. Simultaneous detection and typing of plum pox virus (PPV) isolates by Semi-nested PCR and PCR-ELISA, J. Virol. Meth., 68, 127–137.

Revers, F., H. Lot, S. Souche, O. Le Gall, T. Candresse, and J. Dunez, 1997. Biological and molecular variability of lettuce mosaic virus isolates, *Phytopathology*, 87, 397–403.

Robertson, N.L., and D.C. Ianson, 2005. First Report of Turnip mosaic virus in rhubarb in Alaska, Plant Dis., 89, 430.

Robertson, N.L., R. French, and S.M. Gray, 1991. Use of group specific primers and the detection and identification of luteoviruses, J. Gen. Virol., 72, 1473–1477.

Rybicki, E.P., and F.L. Hughes, 1990. Detection and typing of maize streak virus and other distantly related geminiviruses of grasses by polymerase chain reaction amplification of a conserved viral sequence, J. Gen. Virol., 71, 2519–2526.

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

Stevens, M., R. Hull, and H.G. Smith, 1997. Comparison of ELISA and RT-PCR for the detection of beet yellows closterovirus in plants and aphids, J. Virol. Meth., 68, 9–16.

Tian, T., V.A. Klaasen, J. Soong, G. Wisler, J.E. Duffus, and B.W. Falk, 1996. Generation of cDNAs specific to lettuce infectious yellows closterovirus and other whitefly-transmitted viruses by RT-PCR and degenerate oligonucleotide primers corresponding to the closterovirus gene coding the heat shock protein, Phytopathology, 86, 1167–1173.

Thomson, D., and R.G. Dietzgen, 1995. Detection of DNA and RNA plant viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenization, J. Virol. Meth., 54, 85–95.

Tyagi, S., D.P. Bratu, and F.R. Kramer, 1998. Multicolor molecular beacons for allele discrimination, Nat. Biotechnol., 16, 49–53.

Wetzel, T., T. Candresse, G. Macquaire, M. Ravelonandro, and J. Dunez, 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection, J. Virol. Meth., 39, 27–37.

Whitham, S.A., S. Quan, H.S. Chang, B. Cooper, B. Estes, T. Zhu, X. Wang, and Y.M. Hou, 2003. Diverse RNA viruses elicit the expression of common sets of genes in susceptible Arabidopsis thaliana plants, Plant J. 33, 271–283.

MOLECULAR METHODS FOR DETECTION AND QUANTITATION OF VIRUS IN APHIDS

Mariano Cambra, Edson Bertolini, Antonio Olmos, and Nieves Capote

7.1. Introduction

Following the introduction of a hitherto uncommon or wholly novel virus into a crop it is necessary to do three things: to identify the target, to manage spread, and (ideally) to eliminate sources of inoculum. Here, we have contrasted a number of approaches that have value for these purposes. In any geographic region (whether "developing" or "developed") it is crucial to be prepared for the unexpected and to have developed a range of technologies that enable these objectives to be addressed. Aphids provide the means of spread for most of the viruses that threaten crops in Europe (indeed worldwide) and it is clearly prudent to have well-proven means for detecting viruses in these vectors (Ng and Perry, 2004). The benefits of detection and quantitative determination of the number of virions carried by a single aphid concern multiple topics ranging from basic studies on virus replication process to epidemiological studies (Fabre et al., 2003). An accurate evaluation of the number of virions used to challenge (by graft, mechanical, or vector inoculation) the resistance of plants obtained from conventional breeding programs or after genetic manipulation, will be essential in a near future (Olmos et al., 2005). Provided that reliable quantitative means can be devised and focused on RNA or DNA targets in single insects, a number of key questions can be answered. In the particular case of aphids, these questions include the following: (a) is the efficiency of transmission of "nonpersistent" viruses by a given insect related with the amount of acquired virus (b) what proportion of the viruses are able to infect plants after different vector feeding periods? (c) what is the relationship between the number of viruses detected in a single aphid and the percentage of virus transmission? (d) what is the number of aphids carrying viral-targets that land and feed on a single plant? (e) is the number of viruliferous aphids captured in a given ecological area directly related with the number of infected hosts (inoculum) present in the same area? (f) are all biotypes of aphids actually vectors? etc. For the control of viruses transmitted by aphids, these and other issues are expected to key.

A number of features contribute to the success of aphids as vectors of plant viruses. These include (a) their polyphagous nature (migratory habits) that allows them to visit and feed on a wide range of plant species, facilitating

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 81–88.

the spread of viruses that infect a large number of plant hosts, (b) the ability to undergo parthenogenesis (rapid generation of large numbers of individuals), and (c) the possession of a stylet capable of piercing plant cell walls and delivering viruses into a host cell (Ng and Perry, 2004). In addition, basically three factors determine the viral incidence in field: (a) the number of immigrant winged aphids and the timing of colonization, (b) their viruliferous state, and (c) the dynamics of the secondary epidemic spread (e.g. Plumb, 1990). Thus, the assessment of infectivity of aphids appears to be one of the most important factors to evaluate the spread and epidemics of aphid-borne viruses and for development of any decision-making system to improve viral control strategies (Fabre et al., 2003; Marroquín et al., 2004).

The viruliferous state of aphid species was, at first, indirectly assessed by observation of symptoms in plants and by experimental assays of transmission (Pirone and Harris, 1977). The development of the serological ELISA technique (Clark and Adams, 1977) revolutionized diagnostics in plant virology and, from the beginning, was successfully applied to detect circulative (propagative and nonpropagative) and noncirculative (semipersistent and nonpersistent) aphid-transmitted plant viruses (Gera et al., 1978; Denèchère et al., 1979; Clarke et al., 1980; Du Plessis and Von Wechmar, 1981; Cambra et al., 1981). However, when not propagative, the low concentration of viruses in single aphids was close to the normal limit of reliable detection by ELISA. In the last decade, due to its high sensitivity, conventional PCR and reverse transcription coupled to PCR in a single step involving reverse transcription (RT-PCR) is a molecular method frequently used for the detection of plant viruses. Different PCR or RT-PCR variants including immunocapture (with plant extracts) (Wetzel et al., 1992; Nolasco et al., 1993) or print/squash-capture PCR (with immobilized targets on paper) (Olmos et al., 1996, 1997) enable the detection of minute quantities of DNA or RNA targets. Nevertheless, when targets are at very low concentration and/or the samples (plant material or insects) contain PCR inhibitors, more sensitive detection can be achieved by seminested or nested-PCR. These techniques enhance sensitivity but greatly favor the risk of contamination with amplicons when two different tubes are used in the subsequent reactions. To avoid this problem, nested-PCR in a single closed compartmentalized Eppendorf tube was proposed (Olmos et al., 1999). Usually the PCR products are detected by gel electrophoresis but colorimetric detection of PCR products, in membrane or on plastic microtiter plates, has been successfully employed to increase sensitivity and to facilitate the interpretation of results (McManus and Jones, 1995; Bertolini et al., 2001).

Detection of nonpersistently transmitted viruses is the most problematic due to the very low titer of these viruses [formerly named stylet-borne viruses (Pirone, 1964)] in aphid vectors. There are few reports demonstrating the feasibility of detecting nonpersistently transmitted viruses in individual aphid

vectors using PCR-based assays (Singh et al., 1996, 2004; Olmos et al, 1997; Singh, 1998; Nie and Singh, 2001; Cambra et al., 2004), but the information provided to date was only qualitative. Real-time quantitative RT-PCR is gaining acceptance as a sensitive method for nucleic acids detection because it allows a large dynamic range of target quantitation (*see also Cooper, this volume*). The method has been applied to quantify RNA viruses in aphid species (Fabre et al., 2003; Schneider et al., 2004; Olmos et al., 2004, 2005) opening new possibilities in plant virology and epidemiology.

7.2. Aphid Monitoring

In addition to the classical direct sampling of established aphid colonies on the young leaves or shoots, several trapping methods have been used in surveys to determine or to evaluate the aphid species present or visiting an orchard or a plantation. These methods included the conventional suction traps (Taylor, 1955), water traps (Moericke, 1951), sticky fishing-line traps (Labone et al., 1983), and the sticky tree or shoot method using glue-covered bait leaves or shoots/budsticks (Avinent et al., 1993; Cambra et al., 2000). The latter one is the most efficient way for estimating and predicting the numbers of aphids (winged adults) landing on the plants or visiting young shoots and leaves, according to Hermoso de Mendoza et al. (1998) and Derron and Goy (1998).

The sticky shoot method has been extensively used for monitoring aphid species in adult trees (Marroquín et al., 2004; Cambra et al., 2004). Young shoots (15–20 cm long) are sprayed with an adhesive glue (Souverode aerosol, Scotts France SAS), detached after 7 or 10 days, and new sticky shoots prepared in trees to complete the scheduled period of survey. The removed sticky shoots with aphids stuck on the surface of the leaves are placed in turpentine to dissolve the glue and then the aphids washed in soapy water to remove the solvent. The collected aphid species are kept in 70% alcohol for later identification and counting.

7.3. Sample Preparation (Viral Nucleic Acids Extraction from Aphids)

The identified aphid species can be analyzed for detection of viral targets as above recommended. The first step is the extraction of the viral targets from individual aphids or for multiple aphid species together. The extraction of targets can be performed by a detergent solution (Singh, 1999), by conventional methods of nucleic acids extraction after extracts preparation (Mehta et al., 1997; Naidu et al., 1998; Fabre et al., 2003) or after immobilization of viral targets on paper (Olmos et al., 1996, 1997) or in nitrocellulose membranes (Singh et al., 2004).

The squashing of aphid species onto paper avoids the preparation of extracts and any consequential release of plant or insect inhibitors as well as potential contamination problems. In addition, the immobilization of targets on paper is simpler and much faster than extractions and can be used with quarantine viruses without risks. The paper harboring printed or squashed aphid species can be stored for a long time before being used or mailed, thus allowing their preparation directly in the field if needed (Olmos et al., 1996).

The presence of viral targets in individual aphid species can be assessed from fresh as well as from previously captured individuals stored in alcohol and/or squashed on paper (Marroquín et al., 2004).

7.4. Recommended Protocols for Amplification of Viral RNA Targets of Nonpersistent and Semipersistent Viruses from Aphids

7.4.1. RT-NESTED-PCR IN A SINGLE CLOSED TUBE

Detailed description of RT-nested-PCR in a single closed tube protocol can be obtained from Olmos et al. (2003). Briefly, the method is based in the use of a 0.5 ml Eppendorf tube compartmentalized with the end of a 200 μl plastic tip (Olmos et al., 1999). The cocktail for reverse transcription and external amplification is dispensed in the bottom of the Eppendorf tube. The internal cocktail is dispensed into the plastic tip where it remained due to capillarity. After the first amplification the tube is vortexed and centrifuged to mix the cocktail contained in the cone tip with the products of the RT-PCR.

The method has been successfully applied for *Plum pox virus* (PPV) and *Citrus tristeza virus* (CTV) detection (Olmos et al., 1999).

7.4.2. REAL-TIME RT-PCR

Real-time quantitative RT-PCR assay based on Taqman chemistry (Fabre et al., 2003; Olmos et al., 2005) seems to be more sensitive than intercalating dye SYBR Green I for detection and quantitation of RNA targets from the nonpersistently transmitted PPV (Olmos et al., 2004).

Samples quantitation can be reported as copies of amplified virus targets per cell, per gram of tissue, per copy of some other target, etc. When only relative changes are important (for instance, comparison of gene expression level), the quantitation is made relative to a housekeeping gene, because the absolute number of copies is unnecessary. However, for systems in which absolute changes in copy numbers are important, as in viral load determination, careful use of controls is critical. Quantitation by external standards is denominated absolute quantitation and results in an actual number of DNA

molecules. Standard curves may be derived from DNA or transcript targets. To evaluate the suitability of external standards for quantitation it is necessary to verify the amplification efficiencies of standards and unknown samples to minimize under/overestimation of input template copy numbers (Rasmussen, 2001; Tichopad et al., 2003).

The sensitivity afforded by real-time RT-PCR was 100 times higher than RT-nested-PCR and 1,000 times higher than DAS-ELISA and conventional RT-PCR. The quantities of PPV-RNA targets detected (by real-time RT-PCR) in a single aphid ranged from 40 to more than 2×10^3 units (Olmos et al., 2005). Other authors (e.g. Fabre et al., 2003) investigating *Barley yellow dwarf virus* in its vector *Rhopalosiphum padi* have described obtaining a sensitivity of real-time PCR which was assessed to be between 10 and 1,000 times greater than RT-PCR and ELISA assays, respectively.

7.5. Conclusion

The detection of virus-associated targets in aphids that are capable of transmitting viruses is crucial not only for the study of viral replication but also for the optimization of control strategies. Molecular methods targeting nucleic acids are required for a sensitive detection of the viruliferous state of aphid species. RT-PCR methods has been applied to a wide variety of virus families (Naidu et al., 1998; Vercruysse et al., 2000; Singh et al., 2004), nevertheless more sensitive methods are required for accurate detection of semipersistently and nonpersistenly transmitted viruses. Variants of RT-nested-PCR have been successfully applied for the detection of minute quantities of viral targets (Olmos et al., 1999; Marroquín et al., 2004).

However, the information available to date has been only qualitative. Real-time quantitative RT-PCR is now known to be particularly sensitive for the detection of viral nucleic acid in aphid species (Fabre et al., 2003; Olmos et al., 2005). It has been demonstrated the possibility of quantitation from fresh individual aphids as well as from aphids previously captured on traps and squashed on paper, without the need of previous RNA extraction. These combined technologies (direct squash capture and real-time target amplification) open possibilities for a better understanding of the role of vectors in spreading nonpersistently transmitted viruses.

Acknowledgments

The reported activity has been basically supported by INIA RTA 03-099/1306 and Ministerio de Educación y Ciencia AGL2005-01546, projects.

E. Bertolini was in receipt of a fellowship grant (CTBPDC/2004/034) from Generalidad Valenciana.

References

Avinent, L., A. Hermoso de Mendoza, and G. Llácer, 1993. Comparison of sampling methods to evaluate aphid populations (Homoptera, Aphidinea) alighting on apricot trees, Agronomie, 13, 609–613.

Bertolini, E., A. Olmos, M.C. Martínez, M.T. Gorris, and M. Cambra, 2001. Single-step multiplex RT-PCR for simultaneous and colorimetric detection of six RNA viruses in olive trees, J. Virol. Methods, 96, 33–41.

Cambra, M., M.T. Gorris, N. Capote, M. Asensio, M.C. Martínez, E. Bertolini, C. Collado, A. Hermoso de Mendoza, E. Mataix, and A. López, 2004. Epidemiology of *Plum pox virus* in Japanese plums in Spain, Acta Hort., 657, 195–200.

Cambra, M., M.T. Gorris, C. Marroquín, M. Román, A. Olmos, M.C. Martínez, A. Hermoso de Mendoza, A. López, and L. Navarro, 2000. Incidence and epidemiology of *Citrus tristeza virus* in the Valencian Community of Spain, Virus Res., 71, 75–85.

Cambra, M., A. Hermoso de Mendoza, P. Moreno, and L. Navarro, 1981. Use of enzyme-linked immunosorbent assay (ELISA) for detection of citrus tristeza virus (CTV) in different aphid species, Proc. Int. Soc. Citricult., 1, 444–448.

Clark, M.F., and A.M. Adams, 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses, J. Gen. Virol., 34, 475–483.

Clarke, R.G., R.H. Converse, and M. Kojima, 1980. Enzyme-linked immunosorbent assay to detect potato leafroll virus in potato tubers and viruliferous aphids, Plant Dis., 64, 43–45.

Denèchère, M., F. Cante, and H. Lapierre, 1979. Détection immunoenzymatique du virus de la jaunisse nanisante de l'orge dans son vecteur *Rhopalosiphum padi* (L.), Ann. Phytopathol., 11, 507–514.

Derron, J.O., and G. Goy, 1998. Aptitude de diferentes techniques de piégeage des pucerons vecteurs à prévoir les épidémies de jaunisse nanisante d l'orge (BYDV), Rev. Suisse Agric., 30, 125–129.

Du Plessis, D.H., and M. Von Wechmar, 1981. Detection of cauliflower mosaic virus in leaf extracts, protoplasts and aphids by enzyme-linked immunosorbent assay (ELISA), *Phytopath. Z.*, 100, 270–278.

Fabre, F., C. Kervarrec, L. Mieuzet, G. Riault, A. Vialatte, and E. Jacquot, 2003. Improvement of Barley yellow dwarf virus—PAV detection in single aphids using a fluorescent real time RT-PCR, J. Virol. Methods, 110, 51–60.

Gera, A., G. Loebenstein, and B. Raccah, 1978. Detection of cucumber mosaic virus in viruliferous aphids by enzyme linked immunosorbent assay, Virology, 86, 542–545.

Hermoso de Mendoza, A., E. Péres, E.A. Carbonell, and V. Real, 1998. Sampling methods to establish percentages of species and population patterns in citrus aphids, in Aphids in Natural and Managed Ecosystems, edited by J.M. Nieto Nafría and A.F.G. Dixon, Universidad de León, León, pp. 561–568.

Labone, G., G. Fauvel, F. Leclant, and J.B. Quiot, 1983. Intérêt des pièges à fils dans l'étude des populations de pucerons ailés, Agronomie, 3, 315–325.

Marroquín, C., A. Olmos, M.T. Gorris, E. Bertolini, M.C. Martínez, E. Carbonell, A. Hermoso de Mendoza, and M. Cambra, 2004. Estimation of the number of aphids carrying *Citrus tristeza virus* that visit adult citrus trees, Virus Res., 100, 101–108.

McManus, P.S., and A.L. Jones, 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridization, Phytopathology, 85, 618–623.

Mehta, P., R.H. Brlansky, S. Gowda, and R.K. Yokomi, 1997. Reverse transcription polymerase chain reaction detection of Citrus tristeza virus in aphids, Plant Dis., 81, 1066–1069.

Moericke, V., 1951. Eine Farbfalle zur Kontrolle des Fluges von Blattläusen insbesondere der Pfirsichblattlaus, Myzodes persicae (Sulz.), Nachrichtenbl. Deut. Pflanzenschutzd., 3, 23–24.

Naidu, R.A., D.J. Robinson, and F.M. Kimmins, 1998. Detection of each of the causal agents of groundnut rosette disease in plants and vector aphids by RT-PCR, J. Virol. Methods, 76, 9–18.

Ng, J.C.K., and K.L. Perry, 2004. Transmission of plant viruses by aphid vectors, Mol. Plant Pathol., 5, 505–511.

Nie, X., and R.P. Singh, 2001. A novel usage of random primers for multiplex RT-PCR detection of virus and viroid in aphids, leaves, and tubers, J. Virol. Methods, 91, 37–49.

Nolasco, G., C. de Blas, V. Torres, and F. Ponz, 1993. A method combining immunocapture and PCR amplification in a microtiter plate for the routine diagnosis of plant viruses and subviral pathogens, J. Virol. Methods, 45, 201–218.

Olmos, A., E. Bertolini, M. Gil, and M. Cambra, 2004. Real-time RT-PCR for quantitative detection of *Plum pox virus*, Acta Hortic., 657, 149–153.

Olmos, A., E. Bertolini, M. Gil, and M. Cambra, 2005. Real-time assay for quantitative detection of nonpersistently transmitted *Plum pox virus* RNA targets in single aphids, J. Virol. Methods, 128, 151–155.

Olmos, A., M. Cambra, M.A. Dasí, T. Candresse, O. Esteban, M.T. Gorris, and M. Asensio, 1997. Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by heminested-PCR and PCR-ELISA, J. Virol. Methods, 68, 127–137.

Olmos, A., M. Cambra, O. Esteban, M.T. Gorris, and E. Terrada, 1999. New device and method for capture, reverse transcription and nested PCR in a single closed tube, Nucleic Acids Res., 27, 1564–1565.

Olmos, A., M.A. Dasi, T. Candresse, and M. Cambra, 1996. Print-capture PCR: a simple and highly sensitive method for the detection of plum pox virus (PPV) in plant tissues, Nucleic Acids Res., 24, 2192–2193.

Olmos, A., O. Esteban, E. Bertolini, and M. Cambra, 2003. Nested-PCR in a single closed tube, in Methods in Molecular Biology, Vol. 226: PCR Protocols: Methods and Applications, edited by J. Bartlett and D. Stirling, 2nd edition. Humana Press, Totowa, pp. 151–159, 545.

Pirone, T.P., 1964. Discussion and preliminary reports. Aphid transmission of a purified Stylet-Borne virus acquired through a membrane, Virology, 23, 107–108.

Pirone, T.P., and K.F. Harris, 1977. Nonpersistent transmission of plant viruses by aphids, Annu. Rev. Phytopathol., 15, 55–73.

Plumb, R.T., 1990. The epidemiology of barley yellow dwarf in Europe, in Word Perspectives on Barley Yellow Dwarf, edited by P.A. Burnett, CIMMYT, México, pp. 215–227.

Rasmussen, R., 2001. Quantitation on the LightCycler instrument, in Rapid Cycle Real-Time PCR: Methods and Applications, edited by S. Meuer, C. Wittwer, and K. Nakagawara, Springer, Heidelberg, pp. 21–34, 408.

Schneider, W.L., D.J. Sherman, A.L. Stone, V.D. Damsteegt, and R.D. Frederick, 2004. Detection and quantitation of plum pox virus in aphid vectors by real-time fluorescent reverse transcription-PCR, Acta Hortic., 657, 135–139.

Singh, R.P., 1998. Reverse-transcription polymerase chain reaction for the detection of viruses from plants and aphids, J. Virol. Methods, 74, 125–138.

Singh, R.P., 1999. A solvent-free, rapid and simple virus RNA-release method for potato leafroll virus detection in aphids and plants by reverse transcription polymerase chain reaction, J. Virol. Methods, 83, 27–33.

Singh, R.P., A.D. Dilworth, M. Singh, and D.L. McLaren, 2004. Evaluation of a simple membrane-based nucleic acid preparation protocol for RT-PCR detection of potato viruses from aphid and plant tissues, J. Virol. Methods, 121, 163–170.

Singh, R.P., J. Kurz, and G. Boiteau, 1996. Detection of stylet-borne and circulative potato viruses in aphids by duplex reverse transcription polymerase chain reaction, J. Virol. Methods, 59, 189–196.

Taylor, L.R., 1955. The standardization of air-flow in insect suction traps, with an appendix by W.S. Coleman, Ann. Appl. Biol., 43, 390–408.

Tichopad, A., M. Dilger, G. Schwarz, and M.W. Pfaffl, 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up, Nucleic Acids Res., 31, e122.

Vercruysse, P., M. Gibbs, L. Tirry, and M. Höfte, 2000. RT-PCR using redundant primers to detect the three viruses associated with carrot motley dwarf disease, J. Virol. Methods, 88, 153–161.

Wetzel, T., T. Candresse, G. Macquaire, M. Ravelonandro, and J. Dunez, 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection, J. Virol. Methods, 39, 27–37.

THE USE OF MONOCLONAL ANTIBODIES IN PLANT PROTECTION AND FOR STUDYING VIRUS-INDUCED PATHOGENIC PROCESSES

Tatyana N. Erokhina

8.1. Introduction

Since the development of hybridoma technology (Köhler and Milstein, 1975), monoclonal antibodies (mAbs) produced by the technique of somatic cell hybridization have been widely used in plant virology for diagnostics (e.g. Halk et al., 1982; Hsu et al., 1983; Jordan and Hammond, 1991; D'Arcy et al., 1989; Massalski and Harrison, 1987; Erokhina et al., 1993; Erokhina, 1995), antigenic analysis, and epitope mapping (e.g. Andreeva et al., 1994; Commandeur et al., 1992; Koenig et al., 1990; van den Heuvel et al., 1990; Erokhina et al., 2001; Gorshkova et al., 2003) or subcellular localization of viruses and viral proteins (e.g. Lesemann et al., 1990; Bandla et al., 1994; MacKenzie and Tremaine , 1988; Liu et al., 1999; Erokhina et al., 2001; Yelina et al., 2005).

mAbs against plant viruses were first produced in the early 1980s to members of all the major groups of plant viruses (van Regenmortel,1984). The main advantages of mAbs in comparing with polyclonal antisera are as follows:

1. Large quantities of standard antibody preparations can be produced for reference purposes but such large scale production is not possible with polyclonal antisera.
2. Specificity and uniformity: the molecular homogeneity of mAbs ensures that only one antigenic determinant (epitope) of the virus is analyzing at one time; mAbs can distinguish epitopes unique to virus strain or common to a virus group. If two strains of virus differ in only one epitope, the corresponding mAb will possess a superior discriminatory capacity compared to a polyclonal antiserum. Thus, provided that appropriate resources are applied, it is possible to make very fine distinctions between closely related virus isolates/strains in a reproducible manner.
3. Efficiency of material use in immunization: the very small quantities for immunization of mice and producing mAbs.

There are disadvantages: the production of mAbs is both expensive and time-consuming. Furthermore, some mAbs do not work in certain assays formats. Importantly, mAbs raised against viral proteins expressed in bacteria often fail to recognize protein *in planta*.

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 89–100.

8.2. Experience with Barley Yellow Dwarf Virus

There are several groups of viruses where individual members or strains have common epitopes. In this case using mAbs for strain and/or virus diagnosis can be hampered. For example, in the luteovirus group, tests with *Barley yellow dwarf virus* (BYDV) have shown high cross-reactivity between different type/strains (e.g. D'Arcy et al., 1989; Erokhina et al., 2002). Multiple sequence alignments of 10 luteovirus coat proteins showed that they contained similar or identical amino acids in many areas, suggesting a reason why there are so many cross reactions among anti-luteovirus mAbs (Mayo and Ziegler-Graff, 1996).

8.3. Some Uses for mAbs

mAbs are useful tools to study the antigenic structure of viral structural and non-structural proteins. Virus epitopes are usually classified into "continuous" and "discontinuous." "Continuous" epitopes are composed of linear sequence of a polypeptide, whereas "discontinuous" epitopes are formed by amino acids that assembled together by folding of polypeptide chains (van Regenmortel, 1990). Some information about the location of epitopes can be obtained by testing mAbs reactivity with synthetic peptides, fusion proteins, or mutant strains, and by visualizing binding in the electron microscope. Using the Pepscan technique (Geysen et al., 1987), continuous epitopes have been identified by the reactions of mAbs with short (4–8 amino acids) peptides in the linear sequence of viral protein. In this way in the *Beet yellows virus* (BYV) replicase 1a protein mAbs obtained against methyltransferase (MT) domain of 1a protein, reacted with three different octapeptides. mAbs 4A2 and 4A5 reacted with sequence SRLLENET (amino acids, aa 686–692 amino acids), mAbs 2A4 with SREQLVEA (aa 750–757), and 3C5, 4B4, 4C5 recognized TMVTPGEL (aa 806–813) octapeptide (Erokhina et al., 2001). In addition, mAb 1C4 raised against helicase (HEL) domain of 1a protein reacted with three overlapping peptides, KFQEDDPF, QEDDPFRS, and DDPFRSEN, whereas mAb 1D1 reacted with only the latter two. Obviously, the sequences DDPF and DDPFRS (aa 2493–2496 and 2493–2498) are thus the likely respective epitopes. It should be noted, however, that the actual size of an epitope may be larger than revealed by Pepscan analysis, and that the amino acids surrounding the key epitope in the native protein may influence its reactivity (van Regenmortel, 1992; Commandeur et al., 1994). Using the data from peptide scanning and computer-assisted predictions of secondary structure in the BYV MT and HEL proteins, we showed that the amino acid string SRLLENET is located within a amphipathic alpha-helix, which appears to

be largely buried in the protein globule. By contrast, the epitope TMV**TPGEL** overlaps with a predicted hydrophobic beta-strand and is probably buried in the protein globule. Furthermore, the SREQLVEA epitope appears to be located in a hydrophilic loop, and is predicted to be exposed. DDPF epitope is located between conserved subdomains HEL V and VI; but is exposed on the BYV HEL protein molecule. Failure of 1C4 mAb to detect on immunoblots any proteins of *Citrus tristeza virus* (CTV) (Erokhina et al., 2000) may be due to the change of DDPF to DTPF in the CTV HEL domain.

Two mAbs, specific to TGBp3-GFP fusion protein, presented an interesting case of mAb specificity when 2D5 mAb epitope, revealed by Pepscan analysis, has been showed consists from six C-terminal residues of GFP (shown in bold) and two N-terminal residues of TGPp3 protein: **ELYKGS**MA (Gorshkova et al., 2003).

Rat mAbs to *Beet necrotic yellow vein virus* (BNYVV) were used to identify three different epitopes on the surface of virus particles (Lesemann et al., 1990). One group of mAbs reacted with antigenic determinants along the entire length of the particles, whereas a second and third group of mAbs reacted with determinants on the opposite extremities of the particles.

Knowledge of intracellular localization of viral proteins provides important clues as to the functions of these proteins and mechanisms of their interaction with cell components. There are a few reports only of the use of mAbs to detect and localize viral proteins in plant cells (Wieczorek and Sanfacon, 1993; Erokhina et al., 2001; Zinovkin et al., 2003; Erhardt et al., 2005). Wieczorek and Sanfacon located putative movement protein of *Tomato ringspot virus* (TRSV) in *Nicotiana clevelandii.* This protein was associated with tubular structures in/or near cell wall. It was done using immunogold labeling (IGL) technique with mAbs raised against putative movement protein of TRSV. The authors presumed that this protein is involved in the cell-to-cell movement of the virus and that this movement might take place via the formation of tubular structures.

The new data concerning subcellular localization of BYV replicative proteins were obtained using mAbs against MT, HEL, and papain-like proteinase (PCP) domains of BYV ORF 1a polyprotein. Firstly, these proteins were detected on immunoblots of the infected *Tetragonia expansa* plants as 63, 100, and 66 kDa products, respectively (Erokhina et al., 2000; Zinovkin et al., 2003). But little was known about the subcellular localization of BYV replicative-associated proteins. Closteroviruses caused of formation "BYV-type" vesicles formed by membranes of unknown origin. These structures are either discrete vesicles of ca. 100 nm, each delimited by double membrane, or clusters of single-membrane vesicles surrounded by a common outer membrane (Esau and Hoefert, 1971; Lesemann, 1988). The vesicles contain interior networks of fine fibrils interpreted as double-stranded nucleic acid.

The gold particles were mostly found associated with the outer surface of BYV-type vesicle membranes and the cytoplasmic matrix between but not within the vesicle lumens (Erokhina et al., 2001). This fact, and observation of the virions and ribosomes in the cytoplasmic matrix between the aggregates of vesicles, prompted Esau and Hoefert (1971) to propose that these may be the sites of BYV multiplication. Erokhina et al. (2001) and Zinovkin et al. (2003) provided electron microscopic evidence that the closterovirus PCP and replicative-associated proteins are associated with the BYV-type vesicle membranes. It is known that replication of positive-strand RNA viruses of animals and plants is connected with vesicles or spherules derived from various membranous organelles of the cell (Buck, 1996). Co-localization of PCP with closterovirus replication-associated proteins agrees with its involvement in RNA accumulation (Peng and Dolja, 2000). However, the possibility that the BYV leader protein is also involved in fleeting interactions with other cell compartments and/or virus products to perform activities such as long-distance transport (Peng et al., 2003) cannot be excluded.

8.4. Antibody-Based Resistance

Antibody-based resistance is a novel strategy for generating resistance to viruses. Decades ago it was shown that mAbs can neutralize the infectivity of viruses. This approach has been improved by the development of recombinant antibodies (rAbs) (Hiatt et al., 1989; Düring et al., 1990). Antibody-based resistance seems uniquely flexible as a tool to protect crop plants because theoretically inhibitory antibodies can be generated to any virus or viral protein involved in pathogenesis. This strategy has been advanced by the progress in understanding the mechanism of plant diseases and the identification of many proteins critical to virus infection, replication, and spread. The first steps toward the generation of virus-resistance plants require mAbs cloning, efficient mAbs expression, antibody stabilization, and targeting to appropriate cellular compartments. Cloning of antibody-encoding genes from hybridoma cell lines requires of mRNA isolation, cDNA generation, and polymerase chain reactions (PCR). Cloned recombinant full-size antibodies can be converted into Fab or $F(ab)_2$ fragments. Furthermore, monovalent single-chain antibodies (scFv) that have the variable domains linked by a short polypeptide linker, can be constructed from the original antibody (Bird et al., 1988). In addition, fusion proteins can be generated, for example by genetic coupling of a scFv gene to a toxin or enzyme.

During last 15 years, secretory antibodies (Ma et al., 1995), full-size antibodies (Van Engelen et al., 1994; Voss et al., 1995; Baum et al., 1996), Fab-fragments (De Neve et al., 1993), scFv fragments (Owen et al., 1992;

Tavlodoraki et al., 1993; Boonrod et al., 2004), bispecific scFvs (Fisher et al., 1999), and single domain antibodies (Benvenuto et al., 1991) have been expressed in leaves, roots, and seeds of tobacco, potato, rice, wheat, or Arabidopsis plants. Furthermore, targeting of rAbs has been shown for different compartments of plants including the cytosol, the endoplasmic reticulum (ER), and the intercellular space (Schillberg et al., 2001; Boonrod et al., 2004) (Table 1).

It has been shown that signal peptides (SP) are necessary for directing the light and heavy chains into the ER of plant cells (Hiatt et al., 1989), where they are folded and assembled. Signal sequences from mouse, plants, and yeast (De Neve et al., 1993; Hein et al., 1991; Hiatt et al., 1989; Ma et al., 1995; Van Engelen et al., 1994; Voss et al., 1995) have been used successfully to target rAbs to the required cell compartment. Correct cleavage of the signal sequences has been shown by N-terminal sequencing of the immunoglobulin chains (Hein et al., 1991) and assembled antibodies have been detected in the ER by IGL (Düring et al., 1990).

Although the maximum level of antibody accumulation depends on the plant species, on the properties of the antibody itself, and on the tissue, it may conclude that full-size antibodies and Fab fragments are accumulated at high levels (more than 0.1% of total soluble protein (TSP) upon secretion by adding an N-terminal ER signal sequence. For the scFv fragments the highest levels (1–5% of TSP) are obtained when they are retained in the ER by adding an N-terminal signal sequence and a C-terminal KDEL retention signal (Schouten et al., 1996). The low accumulation level of secreted scFv fragments could be improved by fusing a camel long-hinge region with a murine IgM CH4 domain (Schouten et al., 1997) or by making bispecific single-chain antibodies (Fisher et al., 1999). In conclusion, for immunomodulation of target molecules present in the apoplast, IgGs or Fabs are the best option. For immunomodulation in the ER, scFv fragments are the best choice; however IgGs and Fabs can also be used.

A novel approach of plantibodies is an immunomodulation (Jaeger et al., 2000). Antigen-antibody interaction in vivo could result in modulation of the antigen activity. Binding of the antibody on the substrate or ligand-binding site of an enzyme or receptor blocks interactions between enzyme and substrate or between receptor and ligand by competitive inhibition. Pathogen-specific rAbs can be targeted to the cellular compartment where the pathogen inactivates by binding to its surface or to proteins necessary for its spread or replication. The potential of rAbs to interfere with the infection of a plant virus was demonstrated in 1993 (Tavladoraki et al., 1993). In this case, the expression of a cytosolic scFv against coat protein of *Artichoke mottled crinkle virus* (AMCV) in transgenic tobacco caused a reduction in viral infection and a delay in symptom development. This result supported the hypothesis that

TABLE 1. Recombinant antibodies produced in transgenic plants

Antigen	Plant	Signal sequence	Antibody form	Cell compartment	References
Not mentioned	*N. tabacum*	Barley alpha amylase peptide	Full-size Abs	ER	Düring et al. (1990)
Fungal cutinase	*Tobacco* (roots)	Murine IgG signal peptide	Full-size Abs/F(ab')$_2$	Apoplast	Van Engelen et al., 1994
TMV	*N. tabacum*	Murine IgG signal peptide	Full-size Abs IgG/F(ab)	Apoplast	Voss et al. (1995)
Not mentioned	*Nicotiana, Arabidopsis*	2S storage protein	scFv	Primary callus tissue	De Neve et al. (1993)
Artichoke mottled crinkle virus coat protein	*Transgenic plants*	Murine IgG signal peptide	Full-size Abs, scFv	Cytosol	Tavlodoraki et al. (1993)
TMV	*N. tabacum*	Murine antibody leader sequence	scFV	Cytosol	Schillberg et al. (1999)
Human carcinoembrionic antigen	*N. tabacum (leaves)*	ER-targeting sequence KDEL	scFv, mouse/human full-size chimeric Ab	ER	Vaquero et al., 1999
TMV	*N. tabacum*	Murine IgG signal peptide	scFv	Cytosol, apoplast	Zimmermann et al. (1998)
BNYVV	*N. benthamiana*	N-terminal signal sequence	scFv	Apoplast, ER	Fecker et al. (1997)

transgenically expressed antibodies or antibody fragments recognizing critical epitopes on structural or non-structural proteins of invading viruses may interfere with viral infection and confer viral resistance. Most plant viruses are RNA viruses that replicate in the cytosol. As such, the highest resistance is expected by targeting the antibody to this cell compartment. Zimmermann et al. (1998) have evaluated and compared protection of tobacco plants against *Tobacco Mosaic Virus* (TMV) infection by the expression of a TMV virion-binding scFv fragment in the cytosol and apoplast. Though much higher accumulation levels were obtained for the scFv targeted to the apoplast, the most dramatic reduction of necrotic local lesion numbers was observed in plants accumulating scFv fragments in the cytosol. Infectivity could be reduced by more than 90%. Moreover, several plant lines showed inhibition of systemic virus spread.

In another study, a scFv fragment specific to the coat protein of BNYVV was produced in transgenic *N. benthamiana* (Fecker et al., 1997). The scFv was produced in the cytosol or targeted to the apoplast by an N-terminal signal sequence. No scFv proteins could be detected in the cytosol. The apoplast-targeted scFv seemed, by unknown reasons, to be retained in the ER. Upon infection of these plants, the average time needed for infection symptoms to appear was longer in the scFv-producing plants than in non-producing control plants. In addition, the scFv-producing plants were partially protected against the pathogenic effects exerted by the virus in the late stages of infection. It is surprising that the scFv proteins targeted to the secretory pathway were able to interfere with cytosolic replication of the virus. Possibly higher degrees of resistance might have been obtained when the scFv would have been able to accumulate in the cytosol.

8.5. Conclusion

Plantibody-based approaches are one of the most recent innovations in the field of molecular techniques for the analysis and manipulation of virus infection and plant cellular pathways. The resistance against plant-pathogenic viruses can be obtained, and activity of anti-virus agents can be targeted although it should be said that issues of intellectual property (= patent protection) must be addressed. There are indications that this approach can also be applied to more complex pathogens. Specific epitopes, instead of the whole proteins, could be targeted. Applying antibodies with the same target specificity but with different binding affinities might permit different levels of epitope blocking. It is possible that blocking a single epitope may leave other protein interactions intact, resulting in less pleiotropic effects than a total gene knock-out. In this

way, the functional study of epitopes instead of complete proteins becomes feasible.

As indicated by Morozov (this volume), the opportunity to express rAbs specifically in different organs, cells, and compartments of transgenic plants offers possibilities to study the cellular mode of action of regulatory factors but also to change disease severity for good or ill.

References

Andreeva, L., L. Jarvekülg, F. Rabenstein, L. Torrance, B.D. Harrison, and M. Saarma, 1994. Antigenic analysis of potato virus A particles and coat protein, Ann. Appl. Biol., 125, 337–348.

Bandla, M.D., D.M. Westcot, K.D. Chenault, D.E. Ulmann, T.L. German, and J.L. Sherwood, 1994. Use of monoclonal antibody to the nonstructural protein encoded by the small RNA of the tomato spotted wilt tospovirus to identify viruliferous thrips, Phytopathology, 84, 1427–1431.

Baum, T.J., A. Hiatt, W.A. Parrott, L.H. Pratt, and R.S. Hussey, 1996. Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of the root-knot nematode, Mol. Plant Microbe Interact, 9, 382–387.

Benvenuto, E., R.J. Ordas, R. Tavazza, G. Ancora, S. Biocca, A. Cattaneo, and P. Galeffi, 1991. Phytoantibodies: A general vector for expression of immunoglobulin domains in transgenic plants, Plant Mol. Biol., 17, 865–874.

Bird, R.E., K.D. Hardman, J.W. Jacobson, S. Johnson, B.M. Kaufman, S.M. Lee, T. Lee, S.H. Pope, G.S. Riordan, and M. Whitlow, 1988. Single-chain antigen-binding proteins, Science, 242, 423–426.

Boonrod, K., D. Galetzka, P.D. Nagy, U. Conrad, and G. Krczal, 2004. Single-chain antibodies against a plant viral RNA-dependent RNA polymerase confer virus resistance, Nature Biotechnol., 22, 856–862.

Buck, K., 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals, Adv.Virus Res., 47, 159–251.

Commandeur, U., R. Koenig, D.-E. Lesemann, L. Torrance, W. Burgermeister, Y. Liu, A. Schots, M. Arlic, and G. Grassi, 1992. Epitope mapping on fragments of beet necrotic yellow vein virus coat protein, J. Gen. Virol., 73, 695–700.

Commandeur, U., R. Koenig, R. Manteuffel, L. Torrance, P. Luedecke, and R. Frank, 1994. Location, size, and complexity of epitopes on the coat protein of beet necrotic yellow vein virus studied by means of synthetic overlapping peptides, Virology, 198, 282–287.

Conrad, U., and U. Fiedler, 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: An essential tool for antibody production and immunomodulation of physiological functions and pathogen activity, Plant Mol. Biol., 38, 101–109.

D'Arcy, C.J., L. Torrance, and R.R. Matrin, 1989. Discrimination among luteoviruses and their strains by monoclonal antibodies and identification of common epitopes, Phytopathology, 79, 869–873.

De Jaeger, G., C. De Wide, D. Eeckhout, E. Fiers, and A. Depicker, 2000. The plantibody approach: Expression of antibody genes in plants to modulate plant metabolism or to obtain pathogen resistance, Plant Mol. Biol., 43, 419–428.

De Neve, M., M. De Loose, A. Jacobs, H. Vanhoudt, B. Kaluza, U. Weidle, M. Vanmontagu, and A. Depicker, 1993. Assembly of an antibody and its derived antibody fragment in Nicotiana and Arabidopsis, Transgenic Res., 2, 227–237.

Düring, K., S. Hippe, F. Kreuzaler, and J. Schell, 1990. Synthesis and selfassembly of a functional monoclonal antibody in transgenic Nicitiana tabacum, Plant Mol. Biol., 15, 281–293.

Erhardt, M., G. Vetter, D. Gilmer, K. Bouzoubaa, G. Richards, G. Jonard, and H. Guilley, 2005. Subcellular localization of the triple gene block movement proteins of Beet necrotic yellow vein virus by electron microscopy, Virology, 340, 155–166.

Erokhina, T.N., 1995. Monoclonal antibodies to barley yellow dwarf virus: An immuno-enzyme test system for virus diagnostics, Bioorg. Khim. (in Russian), 21, 256–260.

Erokhina, T.N., S.M. Ambrosova, Yu.A. Varitsev, Yu.S. Malofeeva, V.P. Knyazeva, and A.V. Kulyavtsev, 1993. A hybrid immuno-enzyme test system for the potato virus A detection, Bioorg. Khim. (in Russian), 19, 941–949.

Erokhina, T.N., T.B. Kastalieva, and K.A. Mozhaeva, 2002. Preparing of monoclonal antibodies against BYDV using viruses purified from naturally infected plants, Proceedings of International Symposium "Barley Yellow Dwarf Disease: Recent Advances and Future Strategies," 1–5 Sept., El Batan, Texcoco, Mexico, pp. 101–103.

Erokhina, T.N., R.A. Zinovkin, M.V. Vitushkina, W. Jelkmann, and A.A. Agranovsky, 2000. Detection of beet yellows closterovirus methyltransferase-like and helicase-like proteins in vivo using monoclonal antibodies, J. Gen. Virol., 81, 597–603.

Erokhina, T.N., M.V. Vitushkina, R.A. Zinovkin, D.-E. Lesemann, W. Jelkmann, E.V. Koonin, and A.A. Agranovsky, 2001. Ultrastructural localization and epitope mapping of beet yellows closterovirus methyltransferase-like and helicase-like proteins, J. Gen. Virol., 82, 983–1994.

Esau, K., and L.L. Hoefert, 1971. Cytology of beet yellows virus infection in Tetragonia. I. Parenchyma cells in infected leaf, Protoplasma, 72, 255–273.

Fecker, L.F., R. Koenig, and C. Obermeier, 1997. Nicotiana benthamiana plants expressing beet necrotic yellow vein virus (BNYVV) coat protein-specific scFv are partially protected against the establishment of the virus in the early stages of infection and its pathogenic effects in the late stages of infection, Arch. Virol., 142, 1857–1863.

Fisher, R., D. Schumann, S. Zimmermann, J. Drossard, M. Sack, and S. Schillberg, 1999. Expression and characterization of bispecific single chain Fv fragments produced in transgenic plants, Eur. J. Biochem, 262, 810–816.

Geysen, H.M., S.J. Rodda, T.J. Mason, G. Tribbik, and P.G. Schoofs, 1987. Strategies for epitope analysis using peptide synthesis, J. Immunol. Methods., 102, 259–274.

Gorshkova, E.N, T.N. Erokhina, T.A. Stroganova, N.E. Yelina, A.A. Zamyatnin, Jr., N.O. Kalinina, J. Schiemann, A.G. Solovyev, and S.Yu. Morozov, 2003. Immunodetection and fluorescent microscopy of transgenically expressed hordeivirus TGBp3 movement protein reveals its association with endoplasmic reticulum elements in close proximity to plasmodesmata, J. Gen. Virol., 84, 985–994.

Halk, E.L., H.T. Hsu, and J. Aebig, 1982. Properties of virus-specific monoclonal antibodies to Prunus necrotic ringspot, apple mosaic, tobacco streak and alfalfa mosaic viruses, Phytopathology, 72, 953–959.

Hein, M.B., Y. Tang, D.A. McLeod, K.D. Janda, and A. Hiatt, 1991. Evaluation of immunoglobulins from plant cells, Biotechnol. Prog., 7, 455–461.

Hiatt, A., R. Cafferkey, and K. Bowdish, 1989. Production of antibodies in transgenic plants, Nature, 342, 76–78.

Hsu, H.T., J. Aebig, W.F. Rochow, and R.H. Lawson, 1983. Isolations of hybridomas secreting antibodies reactive to RPV and MAV isolates of Barley yellow dwarf virus and Carnation etched ring virus, Phytopathology, 73, 790.

Jordan, R., and J. Hammond, 1991. Comparison and differentiation of potyvirus isolates and identification of strain-, virus-, subgroup-specific and potyvirus group-common epitopes using monoclonal antibodies, J. Gen. Virol., 72, 25–36.

Koenig, R., U. Commandeur, D.-E. Lesemann, W. Burgermeister, L. Torrance, G. Grassi, M. Arlic, J. Kallerhoff, and A. Schots, 1990. Antigenic analysis of the coat protein of beet necrotic yellow vein virus by means of monoclonal antibodies, J. Gen. Virol., 71, 2229–2232.

Köhler, G., and C. Milstein, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity, Nature, 256, 495–497.

Lesemann, D.-E., 1988. Cytopathology, in *The Plant Viruses*, edited by R.G. Milne, Plenum, New York, pp. 179–235.

Lesemann, D.-E., R. Koenig, L. Torrance, G. Buxton, P.M. Boonekamp, D. Peters, and A. Schots, 1990. Electron microscopical demonstration of different binding sites for monoclonal antibodies on particles of beet necrotic yellow vein virus, J. Gen. Virol., 71, 731–733.

Liu, F., E. Sukhacheva, T. Erokhina, and J. Schubert, 1999. Detection of potyviral nuclear inclusion b proteins by monoclonal antibodies raised to synthetic peptides, Eur. J. Plant. Pathol, 105, 389–395.

Ma, J.K.-C., A. Hiatt, M. Hein, N.D. Vine, F. Wang, P. Stabila, C. Vandolleweerd, K. Mostov, and T. Lehner, 1995. Generation and assembly of secretory antibodies in plants, Science, 268, 716–719.

MacKenzie, D.J., and J.H. Tremaine, 1988. Ultrastructural location of non-structural protein 3A of cucumber mosaic virus in infected tissue using monoclonal antibodies to a cloned chimeric fusion protein, J. Gen. Virol., 69, 2387–2395.

Massalski, P.R., and B.D. Harrison, 1987. Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolates differing in aphid transmissibility, J. Gen. Virol., 68, 1813–1821.

Mayo, M.A., and V. Ziegler-Graff, 1996. Molecular biology of luteoviruses, Adv. Virus Res., 46, 413–460.

Owen, M., A. Gandecha, B. Cockburn, and G. Whitelam, 1992. Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco, Biotechnology, 10, 790–794.

Peng, C.W., and V.V. Dolja, 2000. Leader proteinase of the beet yellows closterovirus: Mutation analysis of the function in genome amplification, J. Virol., 74, 9766–9770.

Peng, C.W., A.J. Napuli, and V.V. Dolja, 2003. Leader proteinase of beet yellows virus functions in long-distance transport, J. Virol., 77, 2843–2849.

Schillberg, S., S. Zimmermann, A. Voss, and R. Fisher, 1999. Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in Nicotiana tabacum, Transgenic Res., 8, 255–263.

Schillberg, S., S. Zimmermann, M.-Y. Zhang, and R. Fisher, 2001. Antibody-based resistance to plant pathogens, Transgenic. Res., 10, 1–12.

Schouten, A., J. Roosien, F.A. van Engelen, G.A.M.I. de Jong, A.W.M. BorstVrenssen, J.F. Zilverentant, D. Bosch, W.J. Stiekema, F.J. Gommers, A. Schots, and J. Bakker, 1996. The C-terminal KDEL sequence increases the expression level of a single chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco, Plant Mol. Biol., 30, 781–793.

Schouten, A., J. Roosien, J.M. de Boer, A. Wilmink, M.N. Rosso, D. Bosch, W.J. Stiekema, F.J. Gommers, J. Bakker, and A. Schots, 1997. Improving scFv antibody expression levels in the plant cytosol, FEBS Lett., 415, 235–241.

Tavladoraki, P., E. Benvenuto, S. Trinca, D. De Martinis, A. Cattaneo, and P. Galeffi, 1993. Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack, Nature, 366, 469–472.

Vaquero, C., M. Sack, J. Chandler, J. Drossard, F. Schuster, M. Monecke, S. Schillberg, and R. Fischer, 1999. Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves, Proc. Natl. Acad. Sci. USA, 96, 11128–11133.

van den Heuvel, J.F.J.M., C.M. de Blank, R.W. Goldbach, and D. Peters, 1990. A characterization of epitopes on potato leaf roll virus coat protein, Arch. Virol., 115, 185–197.

Van Engelen, F.A., A. Schouten, J.W. Molthoff, J. Roosien, J. Salinas, W.G. Dirkse, A. Schots, J. Bakker, F.J. Gommers., M.A. Jongsma, D. Bosch, and W.J. Stiekema, 1994. Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco, Plant Mol. Biol., 26, 1701–1710.

van Regenmortel, M.H.V., 1984. Monoclonal antibodies in plant virology, Microbiol. Sci., 1 (3), 73–78.

van Regenmortel, M.H.V., 1990. Plant viruses, in Immunochemistry of Viruses, II. The Basis for Serodiagnosis and Vaccines, edited by M.H.V. van Regenmortel and A.R. Neurath, Elsevier, Amsterdam, pp. 505–515.

van Regenmortel, M.H.V., 1992. Molecular dissection of protein antigens, in Structure of Antigens, edited by M.V.H. van Regenmortel., CRC Press, Boca Baton, pp. 1–29.

Voss, A., M. Niersbach, R. Hain, H.J. Hirsch, Y.C. Liao, F. Kreuzaler, and R. Fischer, 1995. Reduced virus infectivity in N. tabacum secreting a TMV-specific full-size antibody, Mol. Breed., 1, 39–50.

Yelina, N.E., T.N. Erokhina, N.I. Lukhovitskaya, E.A. Minina, M.V. Schepetinnikov, D.-E. Lesemann, J. Schiemann, A.G. Solovyev, S.Yu. Morozov, 2005. Localization of *Poa semilatent virus* cysteine-rich protein in peroxisomes is dispensable for the ability to suppress RNA silencing, J. Gen. Virol., 86, 479–489.

Zimmermann, S., S. Schillberg, Y.-C. Liao, and R. Fisher, 1998. Intracellular expression of TMV-specific single-chain Fv fragments to improved virus resistance in Nicotiana tabacum, Mol. Breed., 4, 369–379.

Zinovkin, R.A., T.N. Erokhina, D.-E. Lesemann, W. Jelkmann, and A.A. Agranovsky, 2003. Processing and subcellular localization of the leader papain-like proteinase of *Beet yellows closterovirus*, J. Gen. Virol., 84, 2265–2270.

IDENTIFICATION OF PLANT HOST FACTORS INTERACTING WITH VIRUSES: NOVEL TARGETS FOR VIRUS CONTROL

Michael E. Taliansky

9.1. Introduction

Plant viruses and their vectors cause serious economic losses, limit crop production, and have negative effects on the quality and security of food supplies. The disease induced by a particular virus may be significantly exacerbated by the presence of a second unrelated virus or a subviral agent (viroids, satellite RNAs) or by infection with other cellular parasites (fungi, bacteria). Current approaches to the protection of plants from viruses are primarily based on poorly understood mechanisms and it is likely that more detailed knowledge will lead to improved virus management. Plant virus genomes are relatively small and therefore are physically unable to encode all the products needed for development of virus infection. When establishing infection, viruses recruit natural host factors to replicate and spread. These factors are candidate targets for novel virus resistance approaches. However, knowledge of such factors may also lead to misuse of plant viruses (by potential bioterrorists) for the development of methods inactivating these factors by recombinant (modified) viruses and hence destroying plant (crop) functions. To protect plants from consequences of such misuse we need to know more about host factors interacting with plant viruses. Here, I present information on one such factor.

The genus Umbravirus comprises seven distinct virus species: Carrot mottle virus, *Carrot mottle mimic virus*, *Groundnut rosette virus*, *Lettuce speckles mottle virus*, *Pea enation mosaic virus-2*, *Tobacco mottle virus*, and Tobacco bushy top virus. The past few years have brought remarkable progress in our understanding of the genome organization and expression of umbraviruses. At the same time, the recent findings, particularly the involvement of the nucleolus in umbravirus infection, have raised some new and fascinating questions related to basic molecular processes in plants.

The genomes of umbraviruses differ from those of most other viruses in that they do not encode a coat protein, and thus no virus particles are formed in infected plants. Besides an RNA-dependent RNA polymerase (encoded by ORF1 and ORF2; Figure 1),umbravirus genomes encode two other proteins from almost completely overlapping open reading frames. One of these (encoded by ORF4; Figure 1) is a cell-to-cell movement protein that can

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 101–106.

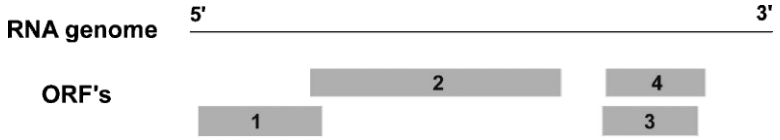


Figure 1. The genome of GRV. Line represents RNA molecule and boxes represent open reading frames (ORFs)

mediate the transport of homologous and heterologous viral RNAs through plasmodesmata without the participation of a coat protein (Ryabov et al., 1998; Taliansky et al., 1996).

The other umbravirus-encoded protein, the ORF3 protein is a multifunctional RNA-binding protein involved in phloem-associated long-distance RNA movement (Nelson and Bel, 1998), and protection from RNase attack, including possibly siRNA-guided RNA degradation (RNA silencing) (Ryabov et al., 1999; Ryabov et al., 2001a; Taliansky et al., 2003). Localization studies showed that the ORF3 protein encoded by GRV accumulated in cytoplasmic granules (Taliansky et al., 2003). These granules consisted of filamentous ribonucleoprotein (RNP) particles, contained viral RNA and the ORF3 protein. The granules were detected in all types of cells and were abundant in phloem-associated cells. It is suggested that these RNP particles serve to protect viral RNA, and may be the form in which it moves through the phloem. Formation of the cytoplasmic RNP complexes may also be involved in the protection of viral RNA from the plant's defensive RNA silencing response, although it is not a suppressor of silencing (Ryabov et al., 2001b).

9.2. The Nucleolus is a Gateway to Umbravirus Systemic Infection

The studies of localization of the GRV ORF3 protein also provided another quite unexpected finding: in addition to the cytoplasmic granules containing RNP particles described above (see also Taliansky et al., 2003), the ORF3 protein labeled with green fluorescent protein (GFP) was also found in nuclei, preferentially but not exclusively targeting nucleoli (Figure2) (Ryabov et al., 1998).

The nucleolus is a prominent subnuclear domain and is classically regarded as the site of transcription of rRNA, processing of the pre-rRNAs and biogenesis of pre-ribosomal particles. However, in addition to these traditionally recognized nucleolar activities, the nucleolus also participates in many other aspects of cell function as well. Thus, because it is a site of transient sequestration and maturation of several factors and regulatory complexes, the nucleolus may be involved in the regulation of signal recognition particle biogenesis, small nuclear RNA processing, mRNA nuclear export, telomerase

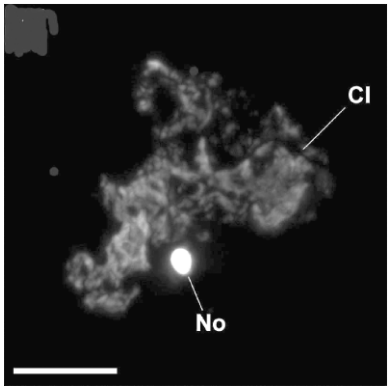


Figure 2. Confocal image a of *Nicotiana benthamiana* epidermal cell infected with a TMV derivative expressing GFP-tagged ORF3 protein of GRV. No, nucleolus; CI, cytoplasmic inclusions containing filamentous RNP particles. Bar, 10 μm

activity, the cell cycle, cell growth and aging (see for recent reviews, Carmo-Fonseca et al., 2000; Lamond and Earnshaw, 1998; Olson et al., 2000). A number of animal viruses interact with the nucleolus and its proteins. Certain viral proteins co-localize with, reorganize, and redistribute some nucleolar antigens such as nucleolin, B23, and fibrillarin (see for review Hiscox, 2002). There have been several reports of plant virus-encoded proteins targeting the nucleolus but the specific role of the nucleolus remains obscure.

Database searches with the sequence of the umbraviral 26–29 kDa ORF3 proteins revealed no significant similarity with any other viral or non-viral proteins, except the corresponding proteins encoded by different umbraviruses (Taliansky et al., 1996) suggesting that there are no analogous proteins encoded by other viruses. Further analysis revealed that the most conserved central region of these proteins consists of a rather basic and highly hydrophilic domain (amino acids 108–130), which seems to be exposed on the protein surface and includes a highly basic arginine (R)-rich sequence (amino acids 109–123) (Figure 3)that resembles a nuclear localization signal and moreover, is not unlike some of the nucleolar localization signals listed in (Taliansky et al., 2003). Another conserved region (amino acids 151–180) of the umbravirus ORF3 protein is hydrophobic and contains invariant leucine (L) residues in a motif **LXXLL** (Figure 3) that resembles a nuclear export signal (NES). Mutagenesis studies confirmed that the arginine-rich domain is a nucleolar localization signal and the leucine-rich domain functions as a NES. Thus, the presence of these sequences may explain the accumulation of ORF3 protein in the nucleolus. The putative NES may be conserved among the ORF3 proteins to ensure that they can be exported back to the cytoplasm and prevent their being trapped in the nucleus.

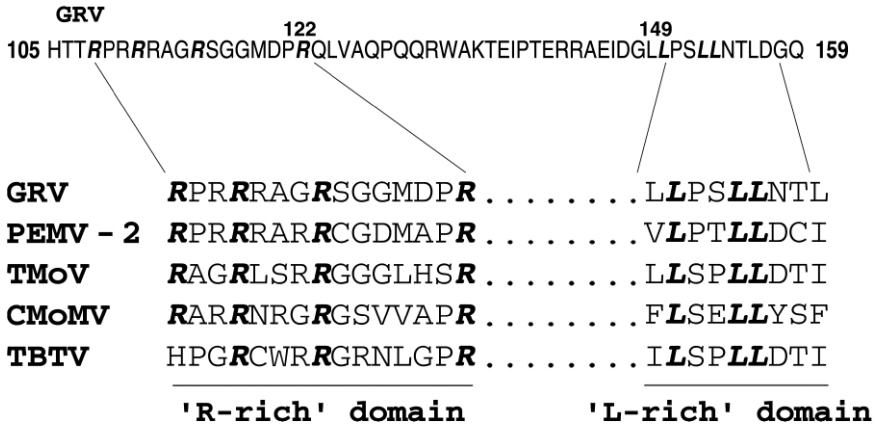


Figure 3. Two conserved domains of umbravirus-encoded ORF3 proteins. Amino acid sequence of the GRV-encoded ORF3 protein (residues 105–159) and partial alignments of arginine (R)-rich and leucine (L)-rich domains for the five 1umbraviruses that have been sequenced are shown. R- and L- residues are in bold. *Groundnut rosette virus*,GRV; *Pea enation mosaic virus-2*, PEMV-2; *Tobacco mottle virus*,TMoV; *Carrot mottle mimic virus*, CMoMV; and *Tobacco bushy top virus*, TBTV

Functional analysis of the ORF3 mutants revealed a correlation between nucleolar localization of the ORF3 protein and its ability to transport viral RNA long distances via the phloem. The likely pathway taken by ORF3 protein in an infected plant cell is illustrated in Figure 4.

The distribution of GFP-labeled GRV ORF3 protein within the nucleolus is not uniform (Ryabov et al., 1998) but resembles that of the granular

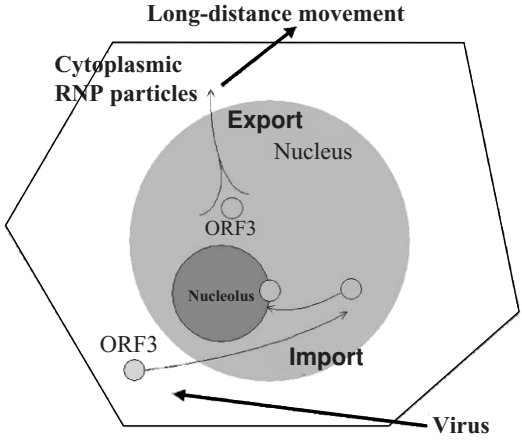


Figure 4. Schematic diagram of the pathway taken by umbraviral ORF3 protein in an infected cell

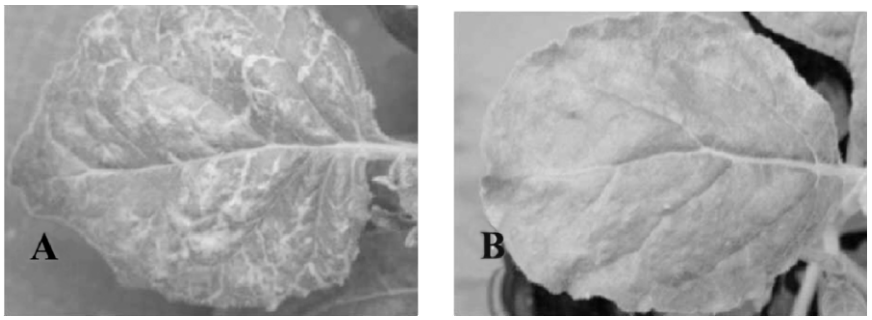


Figure 5. Fibrillarin gene knock down experiments. To partially silence (knock down) fibrillarin gene *N. benthamiana* plants were inoculated with a TRV vector expressing antisense nucleotide sequence corresponding to fibrillarin gene. The silenced (B) and non-treated (control) plants were challenged with GRV YB strain. The silenced plants (B) were resistant to GRV systemic infection whereas control (non-silenced; (A) plants showed bright yellow blotch symptoms characteristic of GRV infection

component that is the site for later stages of ribosome biogenesis (Beven et al., 1996). However, the ORF3 protein-associated RNP structures identified in the cytoplasm (see above) were not found in ultrathin sections of nuclei (Taliansky et al., 2003). Thus the ORF3 protein in nuclei is apparently in a different form from that in the cytoplasm.

To investigate a functional role of nucleolar localization of the ORF3 protein we analyzed its interactions with host nucleolar proteins. It was found the virus-encoded ORF3 protein binds to nucleolar protein, fibrillarin, in vitro. It was also shown that fibrillarin is re-distributed from the nucleolus to cytoplasm during GRV infection (unpublished results).

To determine if the interaction of the ORF3 protein with fibrillarin is essential for virus invasion, the expression was silenced of the host fibrillarin gene in *Nicotiana benthamiana* plants using virus induced gene silencing. A *Tobacco rattle virus* (TRV)-based vector was used to deliver antisense nucleotide sequence (approximately 200 nts) corresponding to a *N. benthamiana* fibrillarin gene. Ten days post-inoculation with GRV YB strain, bright yellow blotch symptoms formed in control plants (in which the fibrillin gene had not been silenced; Figure 5A).By contrast, plants in which the fibrillarin had been silenced did not display any symptoms (Figure 5B). Furthermore, although infected locally, GRV did not systemically invade the silenced plants (data not shown). These results suggest that the fibrillarin plays an essential role in the establishment of umbraviral infection and further research will be carried out to further elucidate the role of fibrillarin on the assembly of movement–competent umbravirus RNP and/or trafficking via the phloem.

Acknowledgments

This work was supported by a grant-in-aid from the Scottish Executive Environment and Rural Affairs Department.

References

Beven, A.F., R. Lee, M. Razaz, D.J. Leader, J.V.S. Brown, and P.J. Shaw, 1996. The organization of ribosomal RNA processing correlates with the distribution of nucleolar snRNAs, J. Cell Sci., 109, 1241–1251.

Carmo-Fonseca, M., L. Mendes-Soares, and I. Campos, 2000. To be or not to be in the nucleolus, Nat. Cell Biol., 2, E107–E112.

Hiscox, J.A., 2002. The nucleolus—a gateway to viral infection? Arch. Virol., 147, 1077–1089.

Lamond, A.I. and W.C. Earnshaw, 1998. Structure and function in the nucleus, Science 280, 547–553.

Nelson, R.S., and A.J.E. van Bel, 1998. The mystery of virus trafficking into, through and out of the vascular tissue, Progr. Botany, 59, 476–533.

Olson, M.O.J., M. Dundr, and A. Szebeni, 2000. The nucleolus: an old factory with unexpected capabilities, Trends Cell Biol., 10, 189–196.

Ryabov, E.V., K.J. Oparka, S. Santa Cruz, D.J. Robinson, and M.E. Taliansky, 1998. Intracellular location of two groundnut rosette umbravirus proteins delivered by PVX and TMV vectors, Virology, 242, 303–313.

Ryabov, E.V., D.J. Robinson, and M.E. Taliansky, 1999. A plant virus-encoded protein facilitates long-distance movement of heterologous viral RNA, Proc. Natl. Acad. Sci. USA, 96, 1212–1217.

Ryabov, E.V., D.J. Robinson, and M.E. Taliansky, 2001a. Umbravirus-encoded proteins both stabilize heterologous viral RNA and mediate its systemic movement in some plant species, Virology, 288, 391–400.

Ryabov, E.V., G. Fraser, M.A. Mayo, H. Barker, and M. Taliansky, 2001b. Umbravirus gene expression helps Potato leafroll virus to invade mesophyll tissues and to be transmitted mechanically between plants, Virology, 286, 363–372.

Taliansky, M.E., D.J. Robinson, and A.F. Murant, 1996. Complete nucleotide sequence and organization of the RNA genome of groundnut rosette umbravirus, J. Gen. Virol., 77, 2335–2345.

Taliansky, M., I.M. Roberts, N. Kalinina, E.V. Ryabov, S.K. Raj, D.J. Robinson, and K.J. Oparka, 2003. An umbraviral protein, involved in long-distance RNA movement, binds viral RNA and forms unique, protective ribonucleoprotein complexes, J. Virol., 77, 3031–3040.

CURRENT VIEWS ON HOST COMPONENTS INVOLVED IN PLANT VIRUS INTERCELLULAR TRAFFICKING

Sergey Yu. Morozov

10.1. Introduction

Plant virus diseases are one of the major threats to the world food supply and mitigation of crop losses caused by viral pathogens will be necessary if the stability and abundance of the food supply is to be sustained. In the 21st century, new threats of agroterrorism with the use of plant viruses as potential biological weapon are expected to boost existing problems and to demand improved remedies. In the past, disease due to viral pathogens was controlled using breeding to introduce natural resistance genes into crop plants or protective immunization (cross protection) when prior infection with one virus affords protection against closely related and more damaging ones (Pennazio et al., 2001; Campbell et al., 2002). Proven strategies for combating viruses include also chemicals to kill vectors or to stimulate systemic acquired resistance responses (Oostendorp et al., 2001; Campbell et al., 2002).

Breeding and selection of crops plants is slow and inappropriate when urgency is needed. Therefore, several novel "engineered" forms of virus resistance in transgenic plants have been developed over the past 20 years. These strategies include expression in transgenics of non-viral genes and pathogen-derived sequences including both coding and non-coding parts of viral genomes (Goldbach et al., 2003). Among these, antibody-based resistance is one of the more promising approaches. Decades ago it was shown that polyclonal and monoclonal antibodies can neutralize viruses, and this approach has been recently improved by the development of *in planta* expression of recombinant antibodies (rAbs). Crop resistance has been engineered by the expression of pathogen-specific antibodies, antibody fragments or antibody fusion proteins ("plantibodies") (Wilson, 1993; Benvenuto and Taviadoraki, 1995; Conrad and Fiedler, 1998; Schillberg et al., 2001; Stoger et al., 2002; Goldbach et al., 2003). The advantage of this approach is that rAbs can be selected and rapidly synthesized against any virus-specific target molecule (e.g. coat protein, replicase etc) as described, for example, by Boonrod et al., 2004). Moreover, future development of "plantibody" strategies may result in generation of new resistance approaches aimed to alter the plant phenotype by immunomodulation with rAbs against host proteins (Conrad and Fiedler, 1998; Stoger et al., 2002). In addition to "plantibodies", further strategies for

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 107–120.

engineered virus resistance with non-viral sequences have been explored, including the use of plant genes controlling systemic acquired resistance (Campbell et al., 2002), pokeweed (*Phytolacca americana*)-derived antiviral protein and mammalian 2′,5′-oligoadenylate system (Wilson, 1993; Truve et al., 1994; Goldbach et al., 2003; Honda et al., 2003).

As described by Fitchen and Beachy (1993), the first report of a successful use of pathogen-derived resistance to construct transgenic plants with viral genes or sequences, expression of which blocks a specific step during virus multiplication, was published in 1986 and specifically concerned coat protein-"mediated" protection against *Tobacco mosaic virus* (TMV). Further development of pathogen-derived resistance approach focused also on the non-virion proteins (such as replication proteins) and defective-interfering RNAs (Fitchen and Beachy, 1993; Wilson, 1993; Goldbach et al., 2003). However, the most successful way to engineer virus resistance is currently through the use of post-transcriptional gene silencing (PTGS) which is generally believed to mimic a natural defense system in plants and is mediated by the expression of both coding and non-coding sequences derived from viral genomes (Johansen and Carrington, 2001; Baulcombe, 2004; Vanitharani et al., 2005).

10.2. Virus Spread and Subcellular Localization of Movement Proteins

In general, cell-to-cell movement of viral RNAs and their long-distance transport through sieve elements is a crucial prerequisite for host colonization by plant viruses that may result in severe plant diseases or massive production of substances hazardous for human and animals. It is generally accepted that plant infecting viruses use plasmodesmata (PD) and endogenous transport machineries to transfer their genomes throughout the plant (Oparka, 2004; Heinlein and Epel, 2004; Waigmann et al., 2004; Boevink and Oparka, 2005; Nelson and Citovsky, 2005; Scholthof, 2005; Lucas, 2006). In green vascular plants, PD are plasma membrane-lined cell wall-crossing cytoplasmic channels containing an appressed tubule derived from endoplasmic reticulum (ER) (Heinlein and Epel, 2004). Plant virus-encoded "movement" proteins (MPs) were the first such faciltators to be recognized. MPs are able to increase the Size Exclusion Limit (SEL) of PDs (Haywood et al., 2002; Heinlein and Epel, 2004; Waigmann et al., 2004; Lucas, 2006). MPs exhibit nucleic acid-binding properties, and it is obvious that many plant-virus genomes are transported through PDs as ribonucleoprotein complexes with MPs (Tzfira et al., 2000; Waigmann et al., 2004). In many viruses genomes encode only one MP, and the TMV 30 kDa protein is one of the best studied in this category (Waigmann et al., 2004; Heinlein and Epel, 2004).

Transgenic expression of partially functional MPs was expected to act as a defective molecular decoy to compete against the virus-coded wild-type

MP and it was shown that expression of defective MPs reduces substantially the spread (and hence yield) of plant viruses (Wilson, 1993; Seppanen et al., 1997). A development of this strategy (by immunomodulation of cell physiology with rAbs against host proteins: Conrad and Fiedler, 1998; Stoger et al., 2002) successfully inhibited interactions between MPs and crucial host components. A number of host proteins that specifically interact with virus coded MPs have been identified. However, although subsequent molecular analyses of the respective interactions confirmed specificity of the protein-protein interferences, their functionality during cell-to-cell trafficking of MPs and viral genomes is still obscure in most cases (Heinlein, 2002). The current model of MP transport through PD (Lucas and Lee, 2004; Lucas, 2006) involves at least two processes. First, MPs are recognized by specific PD pathway receptors to move to the cell peripheral compartments in the vicinity of PD. Second, at the PD orifices, MP is recognized by a hypothetical "docking complex." This interaction induces structural PD modification resulting in transient increase of SEL, as well as (partial) unfolding of transported molecules, that governs protein translocation through PD (Haywood et al., 2002; Lucas and Lee, 2004; Lucas, 2006). Thus, the multiple examples of localization of MPs to PD within infected cells have a clear biological sense and are realistically predicted (Oparka, 2004; Heinlein and Epel, 2004; Waigmann et al., 2004; Lucas and Lee, 2004; Tremblay *et al*., 2005). Targeting of MPs to the ER is also understood to some degree (e.g. Blackman and Overall, 2001; Morozov and Solovyev, 2003; Heinlein and Epel, 2004; Waigmann *et al*., 2004; Scholthof, 2005). However, finding of MPs in other subcellular compartments raises specific concerns related to the intracellular dynamic of MPs and real amounts of MP molecules required for cell-to-cell movement of viral genomes (Waigmann et al., 2004). Thus, more knowledge will be needed of molecular virus-plant interactions to reveal host protein targets of MPs and to address the fundamental questions of whether and in which ways a particular virus MP-host protein interaction is physiologically relevant for movement of a respective virus *in planta*. It appears that dozens, or even hundreds, of host proteins interact with viral MPs and positively or negatively influence virus transport towards and through PD. Table 1contains a list of known plant proteins interacting with MPs and potentially involved in virus cell-to-cell and long-distance movement.

10.3. MP Interactions with Protein Kinases

The most detailed picture of sites and functions of protein phosphorylation is available for MP of tobamoviruses (Waigmann et al., 2004). MP phosphorylation may occur sequentially: on one hand, MP phosphorylation might ensure effective viral movement; on another hand, further phosphorylation

TABLE 1. Host proteins that interact with viral MPs

	Function of host protein	Viral genus—source of MP	References
Host protein			
1.3. Tobacco RIO protein	Kinase	*Tobamovirus* *Cucumovirus*	Yoshioka et al. (2004)
2.3. LRR-containing protein	Kinase	*Begomovirus*	Fontes et al. (2004)
1.4. Tomato KELP protein	Transcription factor	*Tobamovirus*	Matsushita et al. (2001)
2.4. Tobacco MBF1 protein	Transcription factor	*Tobamovirus*	Matsushita et al. (2002)
3.4. Homeodomain protein Hfi22	Transcription factor	*Tombusvirus*	Desvoyes et al. (2002)
1.5. Nuclear protein AtNSI	Nuclear acetyltransferase	*Begomovirus*	Carvalho and Lazarowitz (2004) Carvalho et al. (2006)
2.5. Fibrillarin	Nucleolar protein	*Umbravirus*	Ryabov et al. (2004) Taliansky et al., this issue
1.6. Initiation factor eIF4E	Translation initiation factor	*Potyvirus*	Leonard et al. (2004) Gao et al. (2005) Miyoshi et al. (2005)
1.7. Arabidopsis DnaJ-like protein	Protein chaperone	*Tospovirus*	Soellick et al. (2000)
2.7. Tobacco RME-8 protein	DnaJ-like chaperone	*Pomovirus*	Haupt et al. (2005)
3.7. Tobacco TLP1 protein	Pathogenesis- related protein	*Cucumovirus*	Kim et al. (2005)
1.8. Actin protein	Cytoskeleton	*Tobamovirus*	McLean and Zambryski (2000)
2.8. Tubulin	Cytoskeleton protein	*Tobamovirus*	Heinlein (2002) Gillespie et al. (2002)
3.8. MPB2C protein	Microtubule-associated protein	*Tobamovirus*	Kragler et al. (2003)
1.9. Calreticulin	Calcium-sequestering protein	*Tobamovirus*	Chen et al. (2005)
2.9. KNOLLE protein	t-SNARE protein	*Nepovirus*	Laporte et al. (2003)
3.9. MPI7 protein	Rab protein receptor	*Caulimovirus*	Huang et al. (2001)
4.9. At4/1 protein	Unknown	*Tospovirus*	Von Bargen et al. (2001) Paape et al. (2006)
1.10. Pectin methylesterase	Cell-wall modification	*Tobamovirus*	Chen et al. (2000) Dorokhov et al. (1999)
2.10. TIP protein	Regulation of ß-1,3-glucanase	*Potexvirus*	Fridborg et al. (2003)
3.10. Atp8 protein	RGD motif cell wall protein	*Turnip crinkle virus*	Lin and Heaton (2001)

events may lead to inactivation of TMV MP transport function. However, phosphorylation of TMV MP negatively regulates TMV-MP gating function and viral spread in *Nicotiana tabacum* but not in *Nicotiana benthamiana*, indicating a host-dependent inactivation strategy (Trutnyeva *et al*., 2005). In general agreement with these findings, direct interaction of tobamovirus and cucumovirus MPs with tobacco RIO kinase was demonstrated (Yoshioka *et al*., 2004). Moreover, leucine rich-repeat receptor-like kinases were found to interact specifically with the nuclear shuttle protein (NSP) required for cell-to-cell movement of geminivirus DNA genomes (Fontes *et al*., 2004).

10.4. MP Interactions with the Proteins Involved in Transcriptional Control

Recent studies have revealed that some MPs bind to transcription factors normally localized to nuclei. Particularly, tobamovirus MP interacts with transcriptional co-activators, KELP and MBF1 (Matsushita *et al*., 2001, 2002), whereas tombusvirus MPs bind to homeodomain leucine-zipper protein Hfi22 and transcriptional co-activator REF (Desvoyes et al., 2002; Uhrig et al., unpublished, cited in Oparka, 2004). Such interactions may be involved in transport function of MPs indirectly by modulating host gene expression (Waigmann et al., 2004). Alternatively, by analogy with non-cell-autonomous plant transcription factors, some MP-interacting transcription control proteins may move between cells and, thus, help MP-RNA complexes to shuttle through PD (Desvoyes et al., 2002; Scholthof, 2005).

10.5. MP Interactions with Nuclear Proteins

Transcription control proteins are not sole representatives of the nuclear proteins known to bind plant viral MPs. Functionally important interactions between geminivirus nuclear-shuttle movement protein NSP and expressed in vascular tissue AtNSI have been recently revealed (Carvalho and Lazarowitz, 2004; Carvalho et al., 2006). Moreover, the umbraviral ORF3 long-distance movement protein locates in the nucleolus (Kim et al., 2004; Ryabov et al., 2004) and binds to fibrillarin (M. Taliansky this volume; also cited in Oparka, 2004). Interestingly, *Barley yellow dwarf virus* MP is able to interact with the nuclear membrane and seems to assist the transport of viral genome into the nuclear compartment of host plant cells (Liu et al., 2005).

10.6. Interactions of Viral Proteins Involved in Cell-to-Cell Movement with the Proteins of Plant Translational Apparatus

Recent genetic studies identified an essential role of translation initiation factor eIF4E in the infection cycle of potyviruses, particularly, in cell-to-cell trafficking (Leonard et al., 2004; Gao et al., 2005; Sato et al., 2005). In line with this, it was shown that potyviral VPg specifically binds to isoform eIF(iso)4E of this translational factor (Wittmann et al., 1997; Leonard et al., 2000, 2004; Miyoshi et al., 2005). It was suggested that recruitment of eIF4E to potyviral virion complex results in trafficking this complex to and through PD and rapid initiation of cap-independent translation in newly infected cells (Gao et al., 2005). Indeed, recent data showed that potyviruses contain specialized, morphologically distinct structure at one end of virion. This structure contained an exposed VPg protein bound to the 5′ end of viral RNA and was implicated with cell-to-cell and long-distance movement of virus particles (Torrance et al., 2006).

10.7. MP Interactions with Protein Chaperones and Pathogenesis-related Proteins

It is strongly suspected that, cellular chaperones play an important role in the translocation of MPs and viral nucleic acids through the PD pore (Roberts and Oparka, 2003; Lucas and Lee, 2004). Particularly, Aoki et al. (2002) identified a new sub-family of cell Hsp70 proteins which show the ability to interact with PD and to modify the PD SEL. In line with this, *Tomato spotted wilt virus* MP was also shown to bind DNAJ-like proteins known to regulate Hsp70 chaperone activity (Soellick et al., 2000; Von Bargen et al., 2001). The specific binding of *Potato mop-top virus* MP to an endocytic DNAJ-like chaperone from *"the"* RME-8 family (Haupt et al., 2005) further argues that recruiting components of cell chaperone system by viral intercellular transport machine has obvious biological sense. *Cucumber mosaic virus* MP was found recently to bind thaumatin-like PR protein, TLP1 (Kim et al., 2005). Similar proteins are synthesized by plants in response to fungal and viral infections. However, their exact functions are poorly understood.

10.8. MP Interactions with Components of Cytoskeleton

Although, the cytoskeleton may provide the principle route for trafficking of MPs to PD (Heinlein, 2002), the available data suggest that such an association of TMV MP with tubulin and microtubules might transport this protein

for degradation by proteasomes (Reichel and Beachy, 2000; Gillespie et al., 2002). Furthermore, binding of TMV MP to microtubule-associated protein MPB2C interferes with its cell-to-cell movement (Kragler et al., 2003). On the other hand, interactions of TMV MP with microfilaments and actin (McLean et al., 1995; McLean and Zambryski, 2000) may be required for intra- and intercellular trafficking of this MP assuming the presence of acto-myosin complex in PD (Oparka, 2004).

10.9. MP Interactions with the ER-associated Proteins and Vesicle Trafficking Proteins

Targeting of some MPs to the ER (Blackman and Overall, 2001; Morozov and Solovyev, 2003; Heinlein and Epel, 2004; Waigmann et al., 2004; Zamyatnin et al., 2006) strongly suggest an association of MPs with membrane-bound ER proteins and ER-associated plant proteins. Indeed, recent findings showed that movement of TMV MP is dependent on NCAPP1 which represents cell membrane protein associated with cortical ER in the vicinity of PD (Lee et al., 2003). Similarly, protein At-4/1 from *Arabidopsis thaliana* is capable of interacting with *Tomato spotted wilt virus* MP (Von Bargen et al., 2001) and localizes mostly in the similar ER compartment and, importantly, is able to be transported through PD. Using polyclonal At-4/1 antibodies obtained after heterologous expression in *E. coli*, western blot analysis of protein extracts isolated from different plant species as well as genome database screenings, it was showed that homologues of At-4/1 seemed to be present in a number of vascular plants (Paape et al., 2006).

In general, a functional endomembrane system appears to be essential for the cell-to-cell trafficking of viral genomes by different MPs (Reichel and Beachy, 1999; Pouwels et al., 2002; Laporte et al., 2003; Haupt et al., 2005). A common feature of membrane-dependent trafficking of some MPs is also their movement to the cell surface via secretory pathway (Heinlein et al., 1998; Huang et al., 2001; Pouwels et al., 2002; Laporte et al., 2003). In line with this, it was found that TMV MP interacts with plant calreticulin (calcium-sequestering protein) both *in vivo* and *in vitro*. Calreticulin is ER-associated protein and may participate in the endomembrane-mediated transport of TMV MP to and through PD (Chen et al.,2005). Remarkably, the oligomeric membrane-embedded MP of TMV directs to and through plasmodesmata vesicular membrane structures containing whole viral replication complexes (Kawakami et al., 2004). This ER-derived trafficking involves vesicular membrane containers and actin cytoskeleton (Morozov and Solovyev, 2003; Haupt et al., 2005).

In plants and animals, vesicular transport requires a family of Rab GTPases and specific receptors (v-SNAREs) exposed on the surface of the transport membrane vesicles interacting with their cognate target receptors (t-SNAREs) on the acceptor membrane. The vesicle fusion event involves three major steps: an initial tethering mediated by specific classes of peripheral membrane proteins; docking dependent on numerous specific pairs of v-SNARE and t-SNARE proteins holding the vesicle in close proximity to the target membrane, and fusion of lipid bilayers (Neumann et al., 2003; van Vliet et al., 2003). Involvement of this vesicular trafficking system in plant virus movement is supported by recent data indicating that t-SNARE protein KNOLLE (Laporte et al., 2003) and Rab receptor MPI7 (Huang et al., 2001) interact with MPs of *Grapevine fanleaf virus* and *Cauliflower mosaic virus*, respectively.

10.10. Interactions of MP with the Cell Wall and Putative PD Proteins

Many examples of localization of MPs to PD within infected cells suggest that some of PD proteins may directly interact with plant viral MPs and may be involved in increasing the PD permeability (Roberts and Oparka, 2003). In line with this hypothesis, potexvirus MP TGBp2 has recently been shown to interact with TIP, a host protein regulator of β-1,3-glucanase which is a key enzyme of callose turnover (Fridborg et al., 2003). One can speculate that keeping the PD neck region open by callose degradation (or prevention of callose accumulation) is a possible function of TGBp2 at the early stage of infection (Fridborg et al., 2003; Roberts and Oparka, 2003). Another cell wall enzyme with important role in cell wall status, pectin methylesterase, was also found to interact with MP of TMV (Dorokhov et al., 1999; Chen et al., 2000; Chen and Citovsky, 2003). This interaction could be also involved in PD SEL increase by changing the structural state of cell wall pectins that are enriched in a wall compartment surrounding PD microchannels (Heinlein and Epel, 2004). The role of binding of *Turnip crinkle virus* MP with putative cell wall protein Atp8 is less understood (Lin and Heaton, 2001). Atp8 contains the so-called RGD motifs implicated in regulation of attachment of extracellular proteins to cell surfaces.

10.11. Host Proteins Involved in Spread of Viroid RNAs

Systemic spread of viroids and viruses occurs via the vasculature. Viroid translocation through the phloem is facilitated by host proteins. Particularly, phloem proteins PP2 and VirP1 from several plants form translocatable RNPs with RNAs of several viroids (Owens et al., 2001; Gomez and Palas, 2001,

2004; Maniataki et al., 2003; Gomez et al., 2005). Some properties of PP2 such as the RNA-binding activity, phloem translocatability as well as the capacity to increase PD SEL strongly suggest their activity as viroid-transporting protein (Balachandran et al., 1997; Gomez et al., 2005). Interestingly, phloem sap proteins also include strong interactors for tobamoviral and cucumoviral MPs (Shalitin and Wolf, 2000).

References

Aoki, K., F. Kragler, B. Xoconostle-Cázares, and W.J. Lucas, 2002. A subclass of plant heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata, Proc. Natl. Acad. Sci., USA, 99, 16342–16347.

Balachandran, S., Y. Xiang, C. Schobert, G.A. Thompson, and W.J. Lucas, 1997. Phloem sap proteins from Cucurbita maxima and Ricinus communis have the capacity to traffic cell to cell through plasmodesmata, Proc. Natl. Acad. Sci., USA, 94, 14150–14155.

Baulcombe, D., 2004. RNA silencing in plants, Nature, 431, 356–363.

Benvenuto, E. and P. Tavladoraki, 1995. Immunotherapy of plant viral diseases, Trends Microbiol., 7, 272–275.

Blackman, L.M. and R.L. Overall, 2001. Structure and function of plasmodesmata, Austr. J. Plant Physiol., 28, 709–727.

Boevink, P. and K. Oparka, 2005. Virus-host interactions during movement process, Plant Physiol., 138, 4–6.

Boonrod, K., D. Galetzka, P.D. Nagy, U. Conrad, and G. Krczal, 2004. Single-chain antobodies against a plant viral RNA-dependent RNA polymerase confer virus resistance, Nat. Biotechnol., 22, 856–862.

Campbell, M.A., H.A. Fitzgerald, and P.C. Ronald, 2002. Engineering pathogen resistance in crop plants, Transgenic Res., 11, 599–613.

Carvalho, M.F. and S.G. Lazarowitz, 2004. Interaction of the movement protein NSP and the Arabidopsis acetyltransferase AtNSI is necessary for cabbage leaf curl geminivirus infection and pathogenicity, J. Virol., 78, 11161–11171.

Carvalho, M.F., R. Turgeon, and S.G. Lazarowitz, 2006. The geminivirus nuclear shuttle protein NSP inhibits the activity of AtNSI, a vascular-expressed Arabidopsis acetyltransferase regulated with the sink-to-source transition, Plant Physiol., 140, 1317–1330.

Chen, M.H., J. Sheng, J. Hind, A.K. Handa, and V. Citovsky, 2000. Interaction between the tobacco mosaic virus movement protein and host cell pectin methylesterase is required for viral cell-to-cell movement, EMBO J., 19, 813–820.

Chen, M.H., G.-W. Tian, Y. Gafni, and V. Citovsky, 2005. Effects of calreticulin on viral cell-to-cell movement, Plant Physiol., 138, 1866–1876.

Chen, M.H. and V. Citovsky, 2003. Systemic movement of a tobamovirus requires host cell pectin methylesterase, Plant J., 35, 386–392.

Conrad, U. and U. Fiedler, 1998. Compartment-specific accumulation of recombinant immunoglobulis in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity, Plant Mol. Biol., 38, 101–109.

Desvoyes, B., S. Faure-Rabasse, M.H. Chen, J.W. Park, and H.B. Scholthof, 2002. A novel plant homeodomain protein interacts in a functionally relevant manner with a virus movement protein, Plant Physiol., 129, 1521–1532.

Dorokhov, Y.L., K. Makinen, O.Y. Frolova, A. Merits, J. Saarinen, N. Kalkkinen, J.G. Atabekov, and M. Saarma, 1999. A novel function for a ubiquitous plant enzyme pectin methylesterase: The host-cell receptor for the tobacco mosaic virus movement protein, FEBS Lett., 461, 223–228.

Fitchen, J.M. and R.N. Beachy, 1993. Genetically engineered protection against viruses in transgenic plants, Ann. Rev. Microbiol., 47, 739–753.

Fontes, E.P.B., A.A. Santos, D.F. Luz, A.J. Waclawovsky, and J. Chory, 2004. The geminivirus nuclear shuttle protein is a virulence factor that suppresses transmembrane receptor kinase activity, Genes Develop., 18, 2545–2556.

Fridborg, I., J. Grainger, A. Page, M. Coleman, K. Findlay, and S. Angell, 2003. TIP, a novel host factor linking callose degradation with the cell-to-cell movement of Potato virus X., Mol. Plant Microbe Interact., 16, 132–140

Gao, Z., E. Johansen, S. Eyers, C.L. Thumas, T.H.N. Ellis, and A.J. Maule, 2005. The potyvirus recessive resistance gene, sbm1, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking, Plant J., 40, 376–385.

Gillespie, T., P. Boevink, S. Haupt, A.G. Roberts, R. Toth, T. Valentine, S. Chapman, and K.J. Oparka, 2002. Movement protein reveals that microtubules are dispensable for cell-to-cell movement of tobacco mosaic virus, Plant Cell, 14, 1207–1222.

Goldbach, R., E. Bucher, and M. Prins, 2003. Resistance mechanisms to plant viruses: An overview, Virus Res., 92, 207–212.

Gomez, G. and V. Pallas, 2001. Identification of a ribonucleoprotein complex between a viroid RNA and a phloem protein from cucumber, Mol. Plant Microbe Interact., 14, 910–913.

Gómez, G. and V. Pallás, 2004. A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex in vivo with hop stunt viroid RNA, J. Virol., 78, 10104–10110.

Gomez, G., H. Torres, and V. Pallas, 2005. Identification of a translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system, Plant J., 41, 319–331.

Haupt, S., G.H. Cowan, A. Ziegler, A.G. Roberts, K.J. Oparka, and L. Torrance, 2005. Two plant-viral movement proteins traffic in the endocytic recycling pathway, Plant Cell, 17, 164–181.

Haywood, V., F. Kragler, and W.J. Lucas, 2002. Plasmodesmata: Pathways for protein and ribonucleoprotein signaling, Plant Cell, 14, Suppl., S303–S325.

Heinlein, M., 2002. The spread of tobacco mosaic virus infection: Insights into the cellular mechanism of RNA transport, Cell. Mol. Life Sci., 59, 58–82.

Heinlein, M. and B.L. Epel, 2004. Macromolecular transport and signalling through plasmodesmata, Int. Rev. Cytol., 235, 93–164.

Heinlein, M., H.S. Padgett, J.S. Gens, B.G. Pickard, S.J. Casper, B.L. Epel, and R.N. Beachy, 1998. Changing patterns of localization of the tobacco mosaic virus movement protein and replicase to the endoplasmic reticulum and microtubules during infection, Plant Cell, 10, 1107–1120.

Honda, A., H. Takahashi, T. Toguri, T. Ogawa, S. Hase, M. Ikegami, and Y. Ehara, 2003. Activation of defense-related gene expression and systemic acquired resistance in cucumber mosaic virus-infected tobacco plants expressing the mammalian 2'5' oligoadenylate system, Arch. Virol., 148, 1017–1026.

Huang M., L. Jongejan, H. Zheng, L. Zhang, and J. Bol, 2001. Intracellular localization and movement phenotypes of alfalfa mosaic virus movement protein mutants, Mol. Plant-Microbe Interact., 14, 1063–1074.

Johansen, L.K. and J.C. Carrington, 2001. Silencing on the spot. Induction and suppression of RNA silencing in the agrobacterium-mediated transient expression system, Plant Physiol., 126, 930–938.

Kawakami, S., Y. Watanabe, and R.N. Beachy, 2004. Tobacco mosaic virus infection spreads cell to cell as intact replication complex, Proc. Natl. Acad. Sci., USA, 101, 6291–6296.

Kim, S.H., E.V. Ryabov, J.W.S. Brown, and M. Taliansky, 2004. Involvement of the nucleolus in plant virus systemic infection, Bioch. Soc. Trans., 32, 557–560.

Kim, M.J., B.-K. Ham, H.R. Kim, I.-J. Lee, Y.J. Kim, K.H. Ryu, Y.I. Park, and K.-H. Paek, 2005. In vitro and in planta interaction evidence between Nicotiana tabacum thaumatin-like protein 1 (TLP1) and *Cucumber mosaic virus* proteins, Plant Mol Biol, 59, 981–994.

Kragler, F., M. Curin, K. Tritnyeva, A. Gansch, and E. Waigmann, 2003. MPB2C, a microtubule associated plant protein binds to and interferes with cell-to-cell transport of tobacco-mosaic-virus movement protein, Plant Physiol., 132, 1870–1883.

Laporte, C., G. Vetter, A.-M. Loudes, D.G. Robinson, S. Hillmer, C. Stussi-Garaud, and C. Ritzenthaler, 2003. Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of grapevine fanleaf virus movement protein in tobacco BY-2 cells, Plant Cell, 15, 2058–2075.

Lee, J.-Y., B.-C. Yoo, M.R. Rojas, N. Gomez-Ospina, L.A. Staehelin, and W.J. Lucas, 2003. Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1, Science, 299, 392–396.

Leonard, S., D. Plante, S. Wittmann, N. Daigneault, M.G. Fortin, and J.F. Laliberte, 2000. Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity, J. Virol., 74, 7730–7737.

Leonard, S., C. Viel, C. Beauchemin, N. Daigneault, M.G. Fortin, and J.F. Laliberte, 2004. Interaction of VPg-Pro of turnip mosaic virus with the translation initiation factor 4E and the poly(A)-binding protein in planta, J. Gen. Virol., 85, 1055–1063.

Lin, B. and L. Heaton, 2001. An *Arabidopsis thaliana* protein interacts with a movement protein of turnip crinkle virus in yeast cells and in vitro, J. Gen. Virol., 82, 1245–1251.

Liu, K., Z. Xia, Y. Zhang, Y. Wen, D. Wang, K. Brandenburg, F. Harris, and D.A. Phoenix, 2005. Interaction between the movement protein of barley yellow dwarf virus and the cell nuclear envelope: role of a putative amphiphilic alpha-helix at the N-terminus of the movement protein, Biopolymers, 79, 86–96.

Lucas, W.J., 2006. Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes, Virology, 344, 169–184.

Lucas, W.J. and J.-W. Lee, 2004. Plasmodesmata as a supracellular control network in plants, Nat. Rev. Mol. Cell. Biol., 5, 712–726.

Maniataki, E., A.E. Martinez de Alba, R. Sagesser, M. Tabler, and M. Tsagris, 2003. Viroid RNA systemic spread may depend on the interaction of a 71-nucleotide bulged hairpin with host protein VirP1, RNA, 9, 346–354.

Matsushita, Y., M. Deguchi, M. Youda, M. Nishiguchi, and H. Nyunoya, 2001. The tomato mosaic tobamovirus movement protein interacts with a putative transcriptional coactivator KELP, Mol. Cells, 12, 57–66.

Matsushita, Y., O. Miyakawa, M. Deguchi, M. Nishiguchi, and H. Nyunoya, 2002. Cloning of a tobacco cDNA coding for a putative transcriptional coactivator MBF1 that interacts with the tomato mosaic virus movement protein, J. Exp. Botany, 53, 1531–1532.

McLean, B.G., J. Zupan, and P. Zambryski, 1995. TMV P30 movement protein associates with the cytoskeleton in tobacco cells, Plant Cell, 7, 2101–2114.

McLean, B.G. and P. Zambryski, 2000. Interactions between viral movement proteins and the cytoskeleton, in Actin: A dynamic framework for multiple plant cell functions, edited

by C.J. Staiger, F. Baluska, D. Volkmann, and P.W. Barlow, Kluwer Academic Publishers, Dordrecht.

Miyoshi, H., N. Suehiro, K. Tomoo, S. Muto, T. Takahashi, T. Tsukamoto, T. Ohmori, and T. Natsuaki, 2006. Binding analyses for the interaction between plant virus genome-linked protein (VPg) and plant translational initiation factors, Biochimie, 88, 329–340.

Morozov, S.Yu. and A.G. Solovyev, 2003. Triple gene block: modular design of a multifunctional machine for plant virus movement, J. Gen. Virol., 84, 1351–1366.

Nelson, R.S. and V. Citovsky, 2005. Plant viruses. Invaders of cells and pirates of cellular pathways, Plant Physiol., 138, 1809–1814.

Neumann, U., F. Brandizzi, and C. Hawes, 2003. Protein transport in plant cells: In and out of the Golgi, Ann. Botany, 92, 167–180.

Oostendorp, M., W. Kunz, B. Dietrich, and T. Staub, 2001. Induced disease resistance in plants by chemicals, Eur. J. Plant Pathol., 107, 19–28.

Oparka, K.J., 2004. Getting the message across: How do plant cells exchange macromolecular complexes?, Trends Plant Sci., 9, 33–41.

Owens, R.A., M. Blackburn, and B. Ding, 2001. Possible involvement of a phloem lectin in long distance viroid movement, Mol. Plant Microbe Interact., 14, 905–909.

Paape, M., A.G. Solovyev, T.N. Erokhina, E.A. Minina, M.V. Schepetilnikov, D.E. Lesemann, J. Schiemann, S.Yu. Morozov, and J.-W. Kellmann, 2006. At-4/1, an interactor of the Tomato spotted wilt virus movement protein, belongs to a new family of plant proteins capable of directed intra- and intercellular trafficking, Mol. Plant-Microbe Interact., 19, 874–883.

Pennazio, S., P. Roggero, and M. Conti, 2001. A history of plant virology. Cross protection, New Microbiol., 24, 99–114.

Pouwels, J., G. van der Krogt, J. van Lent, T. Bisseling, and J. Wellink, 2002. The cytoskeleton and the secretory pathway are not involved in targeting the cowpea mosaic virus movement protein to cell periphery, Virology, 297, 48–56.

Reichel, C. and R.N. Beachy, 1999. The role of the ER and cytoskeleton in plant viral trafficking, Trends Plant Sci., 4, 458–462.

Reichel, C. and R.N. Beachy, 2000. Degradation of tobacco mosaic virus movement protein by the 26S proteasome, J. Virol., 74, 3330–3337.

Roberts, A.G. and K.J. Oparka, 2003. Plasmodesmata and the control of symplastic transport, Plant, Cell Environ., 26, 103–124.

Ryabov, E.V., S.H. Kim, and M. Taliansky, 2004. Identification of a nuclear localization signal and nuclear export signal of the umbraviral long-distance RNA movement protein, J. Gen. Virol., 85, 1329–1333.

Sato, M., K. Nakahara, M. Yoshii, M. Ishikawa, and I. Uyeda, 2005. Selective involvement of members of the eukaryotic initiation factor 4E family in the infection of Arabidopsis thaliana by potyviruses, FEBS Lett., 579, 1167–1171.

Schillberg, S., S. Zimmermann, M.Y. Zhang, and R. Fischer, 2001. Antibody-based resistance to plant pathogens, Transgen. Res., 10, 1–12.

Scholthof, H.B., 2005. Plant virus transport: Motions of functional equivalence, Trends Plant Sci., 10, 376–382.

Seppanen, P., R. Puska, J. Honkanen, L.G. Tyulkina, O. Fedorkin, S.Yu. Morozov, and J.G. Atabekov, 1997. Movement protein-derived resistance to triple gene block-containing plant viruses, J. Gen. Virol., 78, 1241–1246.

Shalitin, D. and S. Wolf, 2000. Interaction between phloem proteins and viral movement proteins, Aust. J. Plant Physiol., 27, 801–806.

Soellick, T., J.F. Uhrig, G.L. Bucher, J.W. Kellmann, and P.H. Schreier, 2000. The movement protein NSm of tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the

TSWV N protein, and identification of interacting plant proteins, Proc. Natl. Acad. Sci., USA, 94, 14150–14155.

Stoger, E., M. Sack, R. Fischer, and P. Christou, 2002. Plantibodies: Applications, advantages and bottlenecks, Curr. Opin. Biotechnol., 13, 161–166.

Torrance, L., I.A. Andreev, R. Gabrenaite-Verhovskaya, G. Cowan, and M.E. Taliansky, 2006. An unusual structure at one end of potato potyvirus particles, J. Mol. Biol., 357, 1–8.

Tremblay, D., A.A. Vaewhongs, K.A. Turner, T.L. Sit, and S.A. Lommel, 2005. Cell wall localization of red clover necrotic mosaic virus movement protein is required for cell-to-cell movement, Virology, 333, 10–21.

Trutnyeva, K., R. Bachmaier, and E. Waigmann, 2005. Mimicking carboxyterminal phosphorylation differently effects subcellular distribution and cell-to-cell movement of tobacco mosaic virus movement protein, Virology, 332, 563–577.

Truve, E., M. Kelve, A. Aaspollu, A. Kuuksalu, P. Seppanen, and M. Saarma, 1994. Principles and background for the construction of transgenic plants displaying multiple virus resistance, Arch. Virol., 9, Suppl., 41–50.

Tzfira, T., Y. Rhee, M.H. Chen, T. Kunik, and V. Citovsky, 2000. Nucleic acid transport in plant-microbe interactions: The molecules that walk through the walls, Ann. Rev. Microbiol., 54, 187–219.

Vanitharani, R., P. Chellappan, and C.M. Fauquet, 2005. Geminiviruses and RNA silencing, Trends Plant Sci., 10, 144–161.

van Vliet, C., E.C. Thomas, A. Merino-Trigo, R.D. Teasdale, and P.A. Gleeson, 2003. Intracellular sorting and transport of proteins, Progress Biophys. Mol. Biol., 83, 1–45.

Von Bargen, S., K. Salchert, M. Paape, B. Piechulla, and J. Kellmann, 2001. Interaction between the tomato spotted wilt virus movement protein and plant proteins showing homologies to myosin, kinesis, and DnaJ-like chaperons, Plant Physiol. Biochem., 39, 1083–1093.

Waigmann, E., S. Ueki, K. Trutnyeva, and V. Citovsky, 2004**.** The Ins and Outs of nondestructive cell-to-cell and systemic movement of plant viruses, Crit. Rev. Plant Sci., 23, 195–250.

Wilson, T.M.A., 1993. Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms, Proc. Natl. Acad. Sci., USA, 90, 16342–16347.

Wittmann, S., H. Chatel, M.G. Fortin, and J.-F. Laliberte, 1997. Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso)4E of Arabidopsis thaliana using the yeast two-hybrid system, Virology, 234, 84–92.

Yoshioka, K., Y. Matsushita, M. Kasahara, K.-I. Konagaya, and H. Nyunoya, 2004. Interaction of tomato mosaic virus movement protein with tobacco RIO kinase, Mol. Cells, 17, 223–229.

Zamyatnin, A.A., Jr., A.G. Solovyev, P.V. Bozhkov, J.P.T. Valkonen, S.Yu. Morozov, and E.I. Savenkov, 2006. Assessment of the integral membrane protein topology in living cells, Plant J., 46, 145–154.

ABIOTIC ENVIRONMENTAL FACTORS: EFFECTS ON EPIDEMIOLOGY OF PLANT VIRUS INFECTIONS

Valery P. Polishchuk, Oleksiy V. Shevchenko, Irene G. Budzanivska, and Tetyana P. Shevchenko

11.1. Introduction

Virus infections are a serious threat for plant growth and development in agroecosystems because of the absence of reliable controlling measures for most of them. Recently it has been shown that due to the non-compliance with generally approved agricultural practices, significant spreading of plant virus infections occurred on the territory of Ukraine (Polishchuk et al., 2001).

There are many environmental factors, biotic and not, that affect virus infection development in plants. Some Ukrainian regions are characterized by serious heavy metal contamination of land used for agricultural purposes. The main reason for such chemical pollution of the soil is industrial activity (Shevchenko et al., 2003). In Ukraine, seven regions are particularly seriously contaminated by heavy metal compounds (Donetsk, Zaporizhya, Lugansk, Dnipropetrivsk, Kharkiv, Mykolayiv, Odessa), and, in total, more than 4.5 million hectares of agricultural lands are polluted by heavy metals and radionuclides (Shevchenko et al., 2002). Exploration of soils in Chernobyl area also supposes growing of agricultural crops in the region. However, plants in this area are affected by the residual radioactivity (Boyko, 1990). These stress factors may severely inhibit development of plants in natural inhabitance as well as in agroecosystems leading to mostly non-specific physiological and biochemical changes, affecting enzyme and transport activities, photosynthetic apparatus, resistance mechanisms, and growth, resulting in yield losses (Barcelo and Poschenrieder, 1990; Foy et al., 1978).

It is not known whether or how these environmental conditions affect the development of plant virus infections, as possible increase of virus content due to an abiotic stress factor might potentiate further easier/faster spreading of the virus raising thus questions of biosafety and epidemiology.

As we demonstrated in numerous experimental works, ionizing radiation and heavy metal contamination of soil may lead to significant changes in symptoms induced by virus infection, elevation of virus content in plants, and mutations in plant virus genome. We suspected that at the population level there might be an increase in the prevalence and diversity of

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 121–132.

viruses—raising questions about biosafety. Hereafter, these questions and possible consequences for the environment and plant production are discussed.

11.2. Alterations in the Development of Virus Infection

Following the monitoring of viruses spreading in various regions in Ukraine, we isolated *Tobacco mosaic virus* (TMV) in association with a range of different symptoms in the same host species; *Plantago major*. Two TMV isolates were compared with a reference isolate (TMV strain U1); one from the Chernobyl area (designated TMV_{ch}) and one from the Shatsk National Park in Volyn region (TMV_{sh}). Gamma-radiation dose rates in these regions were in the range 300–520 μR/h for the Chernobyl Nuclear Power Plant (NPP) area, and 5–10 μR/h for the ecologically "clean" territory of Shatsk National Park. We expected the TMV isolates to differ because of the constant radioactive pressure driving mutation and possibly evolution in the virus genome. Indeed, the TMV_{ch} differed from U1 and TMV_{sh} in bioassay tests (Tyvonchuk et al., 1998).

We have also analyzed the impact of soil chemical contamination with heavy metals on the development of plant viral infection. As we have seen from natural and agricultural ecosystems, heavy metal pollution of soil may result in the extensive relative abundance and diversity of plant viruses. This gave us the idea to simulate such conditions in the laboratory and in the small-scale field experiments in order to trace the development of virus-specific visual symptoms of the infection, and accumulation of virus antigens in the plants.

In the first set of experiments, we used TMV and *Lycopersicon esculentum* (tomato), which is systemically invaded. To simulate contamination we added heavy metals copper, zinc, and lead in the form of water-soluble salts added to soil separately (monometal contamination) at 10 times maximum permissible concentration (MPC) (Kabbata-Pendias and Pendias, 1986). We observed no difference in the type of symptoms induced by TMV on tomato plants, whether heavy metals were applied or not. All infected plants developed mild leaf mosaic with following deformation of upper leaves. However, it is worth mentioning that among the metals tested, lead caused a delay in the time of appearance of virus-induced symptoms on the plants. Whereas, on all the remaining TMV-infected tomatoes (either grown in sterile or Cu-/Zn-contaminated soil), the symptoms developed by 19 day post infection (dpi), those in the virus-infected *L. esculentum* plants grown in Pb-treated soil developed similar visual signs of infection only by 26 dpi.

In these experiments, we have also investigated whether there were any changes in the virus content in tomatoes undergoing additional heavy metal

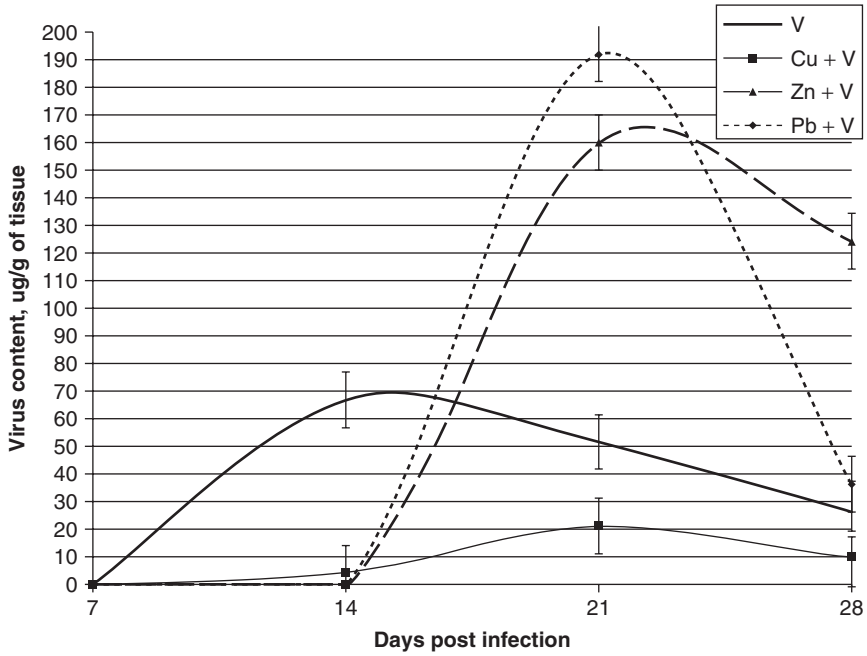


Figure 1. Temporal dynamics of TMV content in fresh leaf tissue of tomato plants as affected by heavy metals lead (Pb), zinc (Zn), and copper (Cu). *V—virus infection*

stress (Figure 1). It was evident that TMV-infected plants grown in non-amended soil have been accumulating virus for up to 14–16 dpi with following gradual decrease up to 28 dpi—as is common in many plants (Matthews, 1992). Maximum TMV concentration in infected tomatoes not being treated with heavy metals constituted approximately 70 μg/g of fresh leaf tissue. Conversely, virus content in Cu-treated plants reached its maximum (21 μg/g of fresh tissue) on 22 dpi; in Pb-treated (192 μg/g of fresh tissue), and in Zn-treated (160 μg/g of fresh tissue) plants—on 22–24 dpi (Figure 1) (Shevchenko et al., 2004). From this result it is clear that zinc and lead did actually induce significant (more that 2-fold) increment of TMV content in tomato plants. The metals did not have any effects on the type or severity of the virus-specific symptoms but a 7-day delay in onset was noted for lead-treated plants.

We used another model system: *Potato virus X* (PVX)—*Solanum tuberosum* cv. "Povin" (potato) plants. In this case, we applied zinc, lead, and copper at a range of concentrations (5×, 10×, and 50× MPC) to attain low, medium, and high level of soil contamination. PVX-infected potato plants grown in soil not amended with heavy metals have developed visual symptoms of virus infection by 14 dpi; the symptoms were typical—mild leaf mosaic (Figure 2(*A*)). Input of heavy metal to soil at any concentration tested substantially

Figure 2. Symptoms induced by PVX on potato plants. *A*—plants grown in non-contaminated soil; mild mosaic of leaves by 14 dpi. *B*—plants grown in soil amended with Zn in 10 × MPC; delayed development of severe leaf mosaic by 21 dpi, followed by deformation of younger leaves by 28 dpi

delayed the appearance of the symptoms up to 21 dpi. Moreover, Zn and Cu at 10× MPC induced more severe symptoms on the later stage of virus infection (28 dpi), namely strong leaf mosaic followed by deformation of the upper leaves (Figure 2(*B*)) (Kamzel et al., 2005).

Our study of PVX in potatoes showed that untreated virus-infected plants accumulated up to 25 μg/g of fresh leaf tissue by 14–21 dpi. All the heavy metals tested in 5× MPC induced statistically significant elevation in PVX concentration. Zn caused the greatest changes in virus content (1.6-fold increase on 42 dpi; 42 μg/g of tissue) and also in temporal dynamics (Figure 3(*A*)). Amendment of Zn and Cu in 10× MPC led to exceptional differences in time dynamics of PVX content. Virus concentration in these plants climbed to 63 μg/g on 42 dpi (2.5-fold elevation). Moreover, even by 42 dpi, we have not detected any decrease in PVX content (which normally follows a maximum of concentration) in plants grown in Cu-/Zn-contaminated soil. Pb in 10× MPC did not induce any differences comparing to 5× MPC values (Figure 3(*B*)). Contrary to these data, all heavy metals in 50× MPC did not induce any statistically significant changes in PVX concentration when comparing to infected plants grown in non-amended soil (Figure 3(*C*)) (Kamzel et al., 2005).

Taking this altogether, we believe that abiotic stresses, either heavy metals or radioactivity, altered the expression of virus-induced symptoms on plants. These alterations can affect type, severity, and time of appearance of the symptoms invoked by a virus. More significantly, we explicitly showed that heavy metals may provoke an enormous increment in virus content in the host tissues. Sometimes this effect seems to be dependent on the nature of the metal, as in the 'TMV-tomato' model system. Conversely, in the "PVX-potato" system, practically all metals tested (zinc, copper, and lead) induced

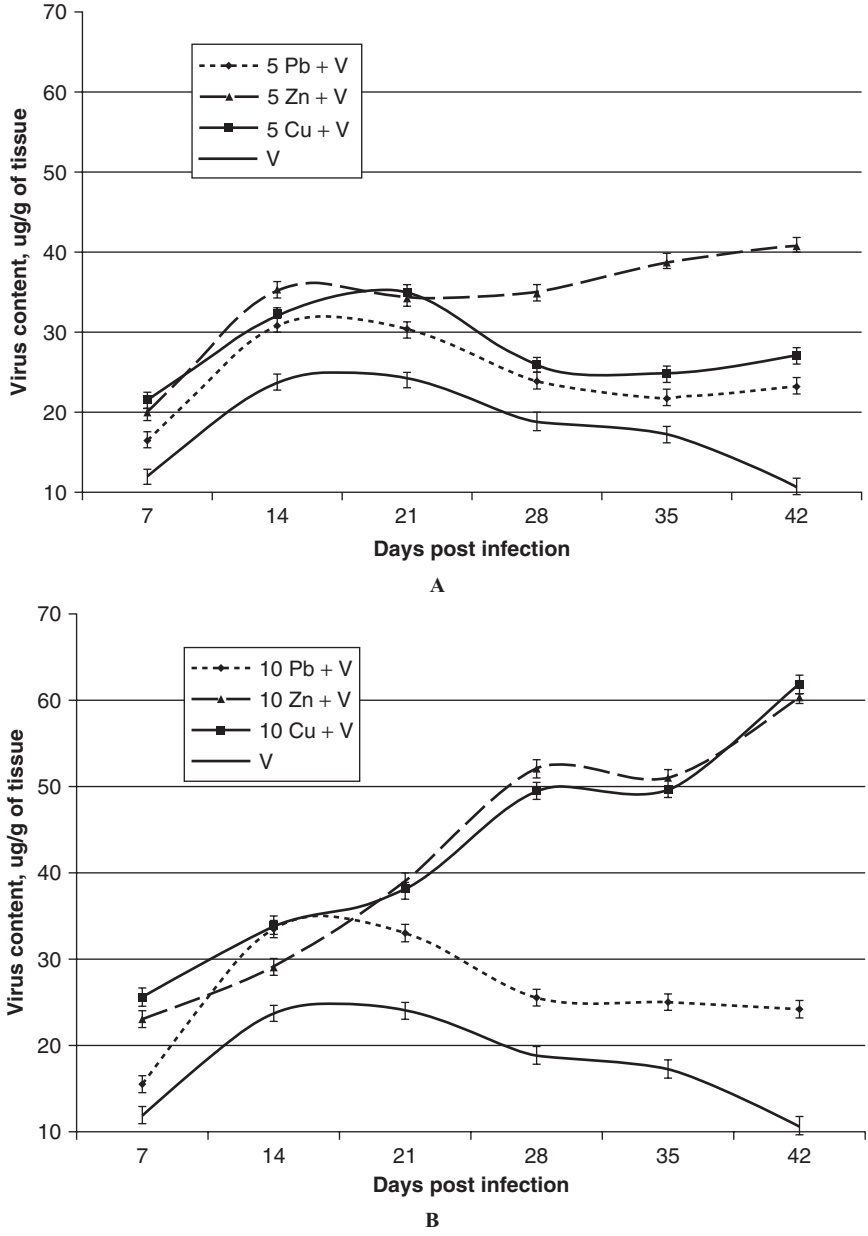
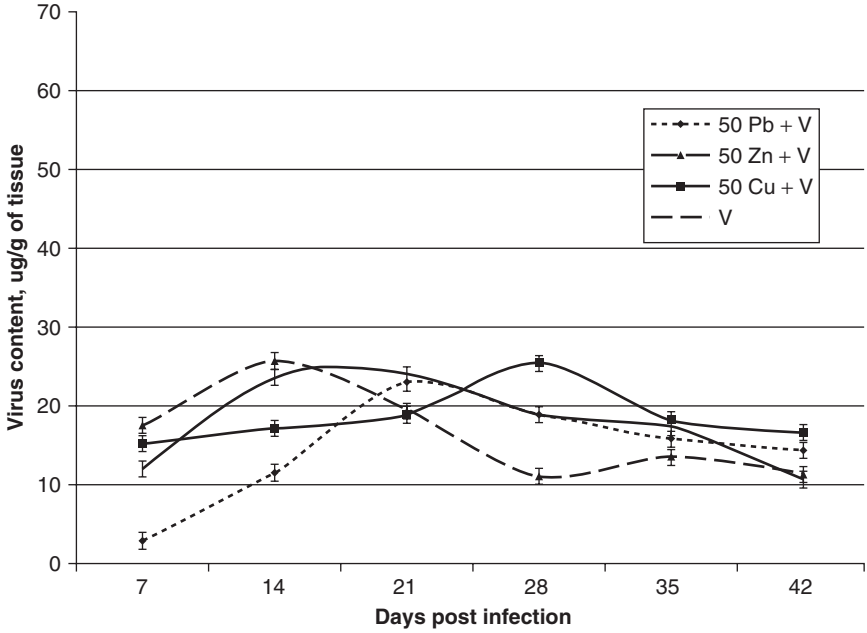


Figure 3. Temporal dynamics of PVX content in fresh leaf tissue of potato plants as affected by heavy metals lead (Pb), zinc (Zn), and copper (Cu) applied in a range of concentrations. *A*—5 × MPC of heavy metals. *B*—10 × MPC of heavy metals. *C*—50 × MPC of heavy metals. *V—virus infection*



C

Figure 3. (*Continued*)

an elevation in virus concentration. However, for zinc and copper, the most peculiar feature of virus content temporal dynamics has been the absence of the "plateau" stage and the absence of any decrease in virus concentration on the late stage of infection process. Constantly high concentration of the virus in the host may further increase the prospects of virus transmission to other plants. Furthermore, the duration of infectivity when plants were under abiotic stress seemed longer comparing to the virus-infected untreated plants.

11.3. Effects on Virus Distribution in Cenoses

In our work we sought links between heavy metal or radioactive contamination and the relative abundance of plant viruses in the region. From one point of view, abiotic stresses might have caused an inhibition of natural plant defense responses, leading to their higher susceptibility to viruses. On the other hand, being applied constantly, these environmental factors could have induced the narrowing of living conditions for plants and their respective pathogens, which could provoke a situation when not all plants would be able to survive in such

Figure 4. Map of Ukraine with sampling points indicated. Red dots, from left to right, represent Shatsk National Park (Volyn region), Kyiv region, and Zmiyiv Power Plant area (Kharkiv region), respectively. Black dot represents sampling sites' area in Chernobyl region

conditions, and possibly not every pathogen would still be able to exert its "power" on the hosts.

Keeping this in mind, we selected three regions in Ukraine differing from the ecological point of view: Chernobyl region (Chernobyl NPP area) with a durable high rate of radioactivity, Kharkiv region (Zmiyiv Power Plant area) with confirmed serious heavy metal contamination of soil, and the Volyn region (Shatsk National Park). In some experiments, we used samples from the Kyiv region as "clean" controls—even though somewhat polluted (Figure 4). For radioactive contamination, gamma-radiation dose rates for compared regions were 300–520 μR/h for Chernobyl NPP area, and 5–10 μR/h for the territory of Shatsk National Park.

For radioactive contamination assay, we sampled several different areas within the Chernobyl region: Kopachy, Novo- and Staro-Shepelychi, Chernobyl surroundings, Opatchychi, arid areas in Shepelychi and Buryakivka villages. For the purpose of chemical pollution analysis, we collected plant and soil samples from Kyiv region, and area of Zmiyiv Power Plant in Kharkiv region for following heavy metal content investigation using atomic absorbance spectroscopy. As reference points we also collected samples of plants and soil from the area of Shatsk National Park (evidently, ecologically safe region) as referent samples. Further on, all plant samples were tested in ELISA for the

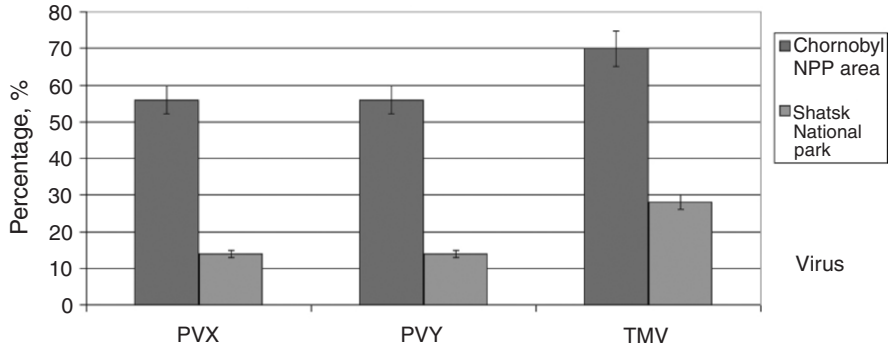


Figure 5. Comparison of encountering frequency for PVX, PVY, and TMV in plant samples from regions differing in the level of radioactivity pollution: Chernobyl NPP area and Shatsk National Park

presence of *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Beet mosaic virus* (BMV), *Barley yellow dwarf virus* (BYDV), and *Cucumber mosaic virus* (CMV), which are widespread in Ukraine (Polishchuk et al., 2001). The investigations have been focused on the assessment of plant viruses' distribution and their frequency of occurrence in wild and cultural flora of contaminated region.

Comparison of the Chernobyl region with that in Shatsk National Park revealed much less abundant plant species diversity in radioactively contaminated area, probably reflecting pressure exerted by the stress. Furthermore, analysis of some specimen of *Elytrigia repens*, *Taraxacum officinale*, *Plantago major,* and *Cirsium arvense* demonstrated substantial differences in virus amounts and representation between Chernobyl samples and other areas. Investigations in two fields near the Opatchychi settlement showed that plant virus frequency distribution was much greater in the Chernobyl samples than in samples from Shatsk National Park. PVX, TMV, and PVY were the most abundant (Figure 5). Similarly to the Opatchychi area, plant virus frequency distribution was greater in the samples from the Chernobyl NPP area compared to control plants. This could be a reflection of the impoverished species diversity. It should be stressed that this research supports the tendency toward polluted ecosystems being hotbeds of virus capable of fuelling epidemics (Polishchuk et al., 2001).

Analyzing possible effects of chemical contamination of soil on the spreading of plant virus infections, we used atomic absorbance spectroscopy to measure the concentration of several heavy metals in soil samples from the area of Zmiyiv Power Plant in Kharkiv region. We have used soil samples from Kyiv region (medium pollution level) and Shatsk National Park as well. As we expected, remarkably high values of metals' content in soil were characteristic

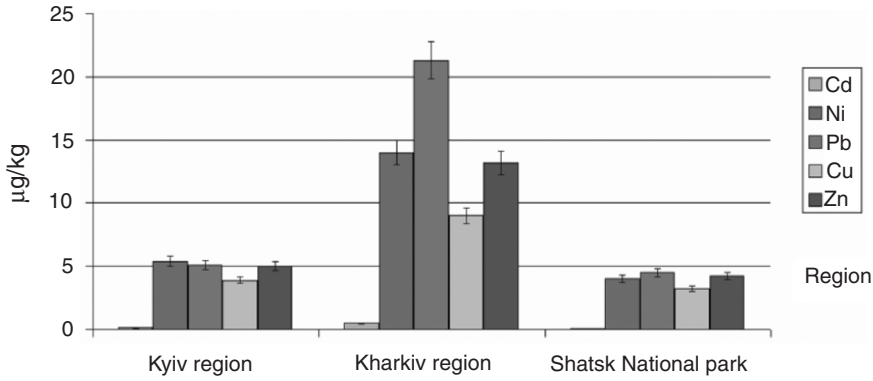


Figure 6. Content of heavy metal ions in soil sampled in Kyiv region, Kharkiv region (Zmiyiv Power Plant area), and Shatsk National Park

for Kharkiv region. Surprisingly, comparison of metals' concentrations in Kyiv region and the area of Shatsk National Park did not show any statistically significant differences for the majority of metal ions tested (Figure 6). It is worth mentioning that the soil samples from these geographical regions analyzed in the study had similar absorbing capacity (Peterson, 1983). Obviously, increased heavy metal concentration in the area of Zmiyiv Power Plant mainly is due to the activity of the power plant.

Afterward, we studied plant samples from these sites on the presence of virus antigens in ELISA. Our results showed drastically frequent occurrence of virtually all viruses tested—TMV, BMV, CMV, BYDV, and PVY—in plant samples from chemically polluted area (Zmiyiv PP), contrary to plants from wild flora from Shatsk National Park. Samples from Kyiv region demonstrated an intermediate position (Figure 7). Indeed, the Kyiv region was quite similar to the Shatsk area from the point of view of heavy metal content in soil (Figure 6). We suggest that increased abundance of plant viruses was due to the agricultural practice conducted there. Thus, another reason for low frequency of virus occurrence in the samples from Shatsk National Park might as well be the absence of any agricultural production. This is not, however, the case for Kharkiv region (Zmiyiv Power Plant area), as plant samples from this region were collected from uncultivated lands (Polishchuk et al., 1998).

Based on the results discussed above, we believe that abiotic environmental stresses, namely radioactive pollution of the cenoses and heavy metal soil contamination, have significant impact on virus distribution. The first reason may be the narrowing of the diversity of plant species, growing on contaminated soil. The second reason is the possible non-specific decrease in the ability of plants to defend themselves. Whatever the reasons, the consequence might be, again, more efficient further spreading of plant viruses, as

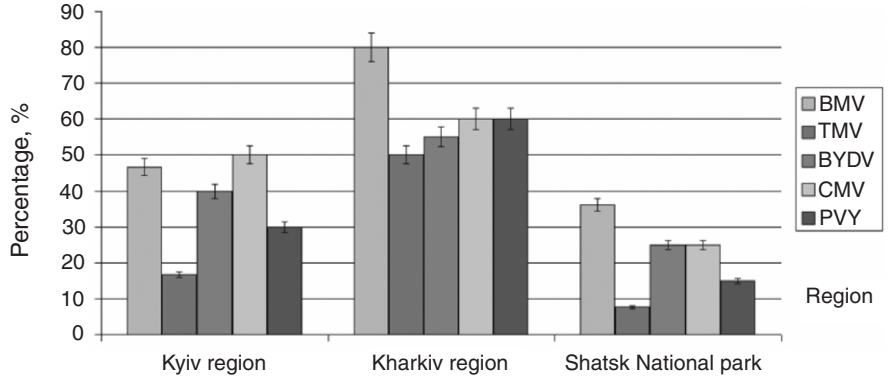


Figure 7. Comparison of virus frequency distribution in plant samples from regions differing in the level of heavy metal' soil contamination: Kyiv region, Kharkiv region (Zmiyiv Power Plant area), and Shatsk National Park

the plants growing in environmentally polluted areas represent potent foci of infection.

11.4. Conclusion

In this work we analyzed possible "crosstalk" between negative effect of abiotic environmental stress factors, radioactivity and heavy metals, on plants, and development of virus infection in these hosts. We have been particularly concerned with two main aspects: (i) virus infection progress in a single plant and (ii) distribution of virus infections in plants at the population level.

Here we have demonstrated that abiotic stressors can induce changes in the appearance of virus-specific symptoms on the plants. Following the results of a set of laboratory and small-scale field experiments with two model systems it is clear that chemical contamination of soil may and do favor an enhanced accumulation of viruses in host plants. Sometimes heavy metals invoked more than a 2.5-fold increment in virus content, comparing to virus-infected plants grown in non-polluted soil. The principal moment is that this elevation of virus content has not been temporary; it remained high for long time.

Another side of the story was revealed at the population level. We showed that both the abiotic stressors we studied potentially inflicted broader harm from viruses on a given territory. Viruses have been detected in their respective hosts more frequently. As this has been shown for five different viruses isolated from different plant species, and for two different stress factors separately, we suggest it is indeed the case. Our results and comments are summarized in Figure 8. However, it still remains elusive what exactly is

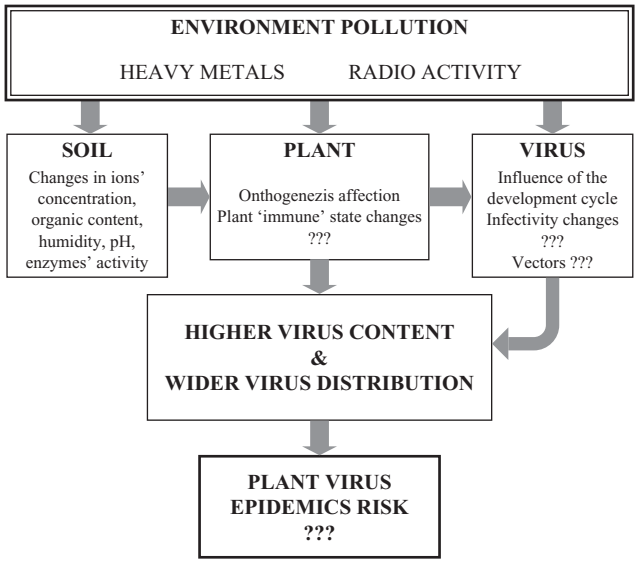


Figure 8. Schematic representation of possible connections and consequences concerning the development and distribution of virus infections in plants undergoing additional abiotic stress

happening to the plants (undergoing stresses of abiotic nature), making possible easier/faster/more efficient development, and further spreading of virus infection. Furthermore, more work is needed to determine if there is any influence of radioactivity/chemical pollution on virus vectors which would, in turn, affect plant virus epidemics.

Generally, we propose that virus infections behave quite differently when their hosts undergoing additional stresses of abiotic nature. Viruses tend to accumulate to higher levels in plant tissues; virus infections tend to spread more successfully. Speculating, we believe this may pose a significant risk in the context of uncontrollable distribution of these pathogens, proving a need for careful monitoring of virus circulation in (radioactively/chemically) contaminated environments to avoid their spreading to the neighboring agrocenoses.

References

Barcelo, J. and Ch. Poschenrieder, 1990. Plant water relations as affected by heavy metal stress: A review, J. Plant Nutr., 13 (1), 1–37.

Boyko, A.L., 1990. Ecology of Plant Viruses, Vischa Shcola, Kyiv, 167 pp. (in Russian).

Foy, C.D., R.L. Chaney, and M.C. White, 1978. The physiology of metal toxicity in plants, Ann. Rev. Plant Physiol., 29, 511–566.

Kabbata-Pendias, A., and H. Pendias, 1986. Trace Elements in Soils and Plants, CRC press, Inc., Boca Raton, Florida, 349 pp.

Kamzel, O.A., O.V. Shevchenko, and I.G. Budzanivska, 2005. Elevation of *Potato virus X* in *Solanum tuberosum* plants when growing in soil contaminated by heavy metals, Bulletin of Taras Shevchenko' Kyiv National University. Biology, 44, 15–16 (in Ukrainian).

Matthews, R.E.F., 1992. Fundamentals of Plant Virology, Academic Press, San Diego, California, 403 pp.

Peterson, P.J., 1983. Adaptation to toxic metals, in Metals and Micronutrients: Uptake and Utilization by Plants, edited by D.A. Robb and W.S. Pierpoint, Academic Press, New York, pp. 51–69.

Polishchuk, V.P., I.G. Budzanivska, S.M. Ryzhuk, V.P. Patyka, and A.L. Boyko, 2001. Monitoring of plant virus infections in biocenoses of Ukraine, Phytosociocentre, Kyiv, 220 pp. (in Ukrainian).

Polishchuk, V.P., N.A. Senchugova, I.G. Budzanivska, O.L. Holovenko, and A.L. Boyko, 1998. Monitoring and strategy in virus diseases' prognosing for agricultural plants in various agrocenoses of Ukraine, *DAN*, 2, 184–187 (in Ukrainian).

Shevchenko, O.V., I.G. Budzanivska, V.P. Patyka, A.L. Boyko,. and V.P. Polishchuk, 2003. Influence of Heavy Metals on the Development of Plant Virus Infections, Phytosociocentre, Kyiv, 224 pp. (in Ukrainian).

Shevchenko, A.V., I.G. Budzanivska, T.P. Shevchenko, and V.P. Polishchuk, 2002. Stress caused by plant virus infection in presence of heavy metals, Plant Prot. Sci., 38, 455–457.

Shevchenko, A.V., I.G. Budzanivska, T.P. Shevchenko, V.P. Polishchuk, and D. Spaar, 2004. Plant virus infection development as affected by heavy metal stress, Arch. Phytopathol. Plant Prot., 37, 139–146.

Tyvonchuk, T.P., V.P. Polishchuk, and A.L. Boyko, 1998. Studying *Tobacco mosaic virus* strain diversity on the territory of Ukraine, Agroecol. Biotechnol., 2, 209–213 (in Ukrainian).

SOMACLONAL VARIATION AS A SOURCE OF TOMATO SPOTTED WILT VIRUS-RESISTANCE IN PLANTS

Ivan S. Shcherbatenko, Lubov T. Oleshchenko

12.1. Introduction

Tomato spotted wilt virus (TSWV) is the type species of the plant-infecting *Tospovirus* genus within the arthropod-borne *Bunyaviridae* family (van Regenmortel et al., 2000; Fauquet et al., 2005).

Like other bunyaviruses, TSWV has a tripartite genome consisting of three single-stranded RNA species called L, M, and S. The fully negative-sense L RNA encodes the viral RNA-dependent RNA polymerase (Adkins et al., 1995). The ambisense M RNA codes for the precursor to the membrane glycoproteins G1 and G2 and the viral movement protein NSm (Soellick et al., 2000). The ambisense S RNA encodes the N protein, the main constituent of the TSWV nucleocapsid, and nonstructural protein NSs involved in gene silencing suppression (Kormelink et al., 1994; Bucher et al., 2003; Voinnet et al., 1999).

The virions consist of a core of nucleocapsids in which the genomic RNA molecules are tightly associated with nucleocapsid (N) proteins and a few copies of the L protein. These nucleocapsids are surrounded by a lipid membrane envelope that contains two virally encoded glycoproteins. Virions are isometric, 85 nm in diameter, rounded in profile and contain 5% nucleic acid, 70% protein, 20% lipid and 5% carbohydrate (Brunt et al., 1996; Sherwood et al., 2001).

TSWV is transmitted by mechanical inoculation, by grafting and by eight thrips species of the genera *Thrips* and *Frankliniella* in a propagative–circulative way (Nagata et al., 2002; Ullman et al., 2002; Medeiros et al., 2004). This virus is characterized by high genetic variability and worldwide distribution, has an extremely broad host range, and causes substantial economic losses in many important crops (Prins and Goldbach, 1998; Reddy and Wightman, 1988). Tospoviruses are among the most threatening for crop biosecurity—particularly in developing countries. There is ample evidence showing that the introduction of tospoviral pathotypes into regions where vectors occur or the introduction of more efficient vectors into regions with well-developed phytosanitary services such as Western Australia have resulted in substantial economic harm.

I. Cooper et al. (eds.), Virus Diseases and Crop Biosecurity, 133–144.

To a large extent, TSWV management in field crops is based on vector control with some effective antiviral chemicals that have undesirable environmental impacts (Reddy and Wightman, 1988; Riley and Pappu, 2004; Caner et al., 1984; De Fazio and Kudamatsu, 1983; De Fazio et al., 1980). The important management measures are reliable diagnostic systems (*e.g.* Rice et al., 1990; Mason et al., 2003; Jones, 2004) and production of resistant cultivars by traditional breeding using appropriate parental lines (Brommenschenkel and Tanksley, 1997; Czech et al., 2003; Jahn et al., 2000; Moury et al., 2000). Alternatively, genetic engineering to obtain resistance may have utility (Gielen et al., 1991; MacKenzie and Ellis, 1992; Pang et al., 1992; Rudolph et al., 2003). However, taking into consideration the social resistance in some localities to transgenic plants (Rudolph et al., 2003), a more realistically promising source of virus-resistant varieties may be somaclonal variants (Van den Bulk, 1991; Liu and Zheng, 2002).

Our investigations on TSWV resistance in tobacco somaclones (Kovalenko et al., 1989; Shcherbatenko et al., 1989, 1991a; Shcherbatenko and Oleshchenko, 1993, 1995, 1999) showed significant variability both in the yield of resistant variants and in the inheritance of resistance as well as in the morphological traits, and fertility of regenerants. The results suggest that resistance to TSWV in tobacco somaclones is due to spontaneous genetic and epigenetic variability, with many genes involved. Therefore, virus-resistant somaclones tend frequently to lose resistance or self-fertility in progeny. Our attempts to decrease these undesirable features are summarized below.

12.2. Somaclone Regeneration and Testing for TSWV-Resistance

Experiments were performed using *Nicotiana* species, hybrids, and cultivars and a severe isolate of TSWV obtained from the Crimean Experimental Station for tobacco research in Tabachnoe, Ukraine. Interspecific hybrids between tobacco cultivars and wild species of *Nicotiana sanderae, N. glauca* or *N. alata* as well as intraspecific hybrids were produced by mechanical pollination of emasculated immature flower buds.

All plants were grown in glasshouses. Somaclones were regenerated from leaf callus (somaclones SC_0) or mesophyll protoplasts (protoclones P_0). For in vitro culture we used: protoplast culture medium K_3NM of Nagy and Maliga (1976); anther culture medium LSU (Shcherbatenko and Oleshchenko, 1993); callus culture medium (MSK), shoot induction medium (MSP), shoot elongation medium (MS42) and root induction medium (MSR) described by Shcherbatenko et al., 1991b.

The regenerated somaclones (SC_0), protoclones (P_0), and F_1 hybrids were inoculated mechanically with TSWV using as inoculum crude extracts of

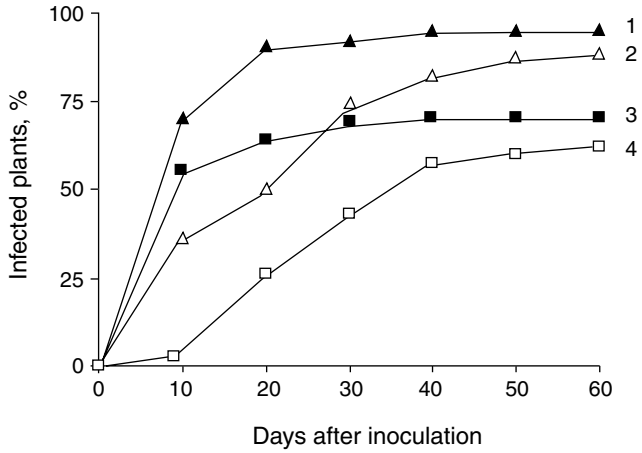


Figure 1. Dynamics of TSWV-infection in tobacco somaclones. 1—clone 21 (American 3j SC_0); 2—clone 10 (Krymsky stepovy SC_3); 3—clone 47 (American 3 SC_1 × *N. alata*) F_2; 4—clone 40 (American 3 SC_1 × *N. alata*) F_2.

infected tobacco leaf tissue. The responses of plants were recorded over a 10-day period up to 60 days post inoculation. Plants that were not infected after inoculation were inoculated once more and those of them that were not infected after four challenge inoculations were tested for TSWV resistance in successive selfed generations: SC_1—SC_3 or F_2 and F_3.

12.3. Differences Between Somaclones and Source Plants in Their Response to TSWV

Plants of all tobacco varieties tested showed severe disease symptoms after 8–10 days postinoculation. In contrast to this, the somaclones became infected after two to four successive inoculations till 60 days post first inoculation, and virus was not detected in some of them (Figure 1).

Figure 1 illustrates two types of invasion dynamics. One type (represented by curves 1 and 3) indicates that the majority of plants become infected until 20 days post inoculation with no obvious subsequent increase in the numbers of infected individuals. In this instance, the somaclone populations fell into two distinct classes (resistant and susceptible) and the reaction to challenge was maintained in progeny. So, clone 21 (American 3j SC_0) produced 5% of resistant plants, its SC_1 progeny—60%; clone 47 (American 3 SC_1 × *N. alata*) F_2)—30%, and its progeny F_3—65% (Table 1). In the second case (curves 2 and 4) the somaclone populations show significant variability in the time of plant infecting. In spite of downright differences in the percentage of resistant

TABLE 1. Inheritance of TSWV resistance in tobacco somaclones and somaclone-derived hybrids

Clone number	Plant	Infection dynamics[1]	Percentage of resistant plants	
			Initially	In their progeny
21	American 3j SC_0	TSLC	5	60
47	American 3 SC_1 × *N. alata*	TSLC	30	65
40	American 3 SC_1 × *N. alata*	MSLC	38	0
10	Krymsky stepovy SC_3	MSLC	12	0

[1] TCLC—two-step-like curve of plant infecting dynamics (Figure 1, curves 1, 3); MSLC—multi-step-like curve of plant infecting dynamics (Figure 1, curves 2, 4).

plants between clone 10 (12%) and clone 40 (38%), no resistant samples was revealed in theirs progenies.

The results of this study indicate that the yield of resistant variants in populations of somaclones strongly depends on both the tobacco variety and plant clone, but the inheritance of resistance—on dynamics of plant damaging of parental clones. The clones with two-step curves (Figure 1, curves 1, 3) had the heritable resistance, but those with multi-step curves (Figure 1, curves 2, 4) did not. Thus, the rate of plant damaging is the more useful criterion for selection of virus-resistant variants than the yield of resistant specimens in populations of somaclones.

12.4. The Yield of TSWV-Resistant Plants in Tobacco Somaclones and Protoclones

12.4.1. INTERDEPENDENCE BETWEEN RESISTANCE AND SELF-FERTILITY OF PLANTS

In essence, results from our previous investigations showed the negative correlation between TSWV-resistance and plant self-fertility in tobacco somaclones and protoclones (Table 2). The somaclones of TSWV-infected tobacco cultivars have the least yield of resistant plants (9.2%), but the most of selected resistant samples (82.4%) are able to set viable seeds. The greater proportion of somaclones of a virus-free interspecific hybrids (98.3%) were resistant to TSWV, but none of these plants was self-fertile.

A similar negative correlation between the yield of resistant plants (77.0%, 57.9%) and self-fertility of their progenies (12.6%, 16.1%) was observed in the protoclones of tobacco cultivars in which virus was not detected after challenge and the numbers of somaclones that gave rise to intraspecific hybrids.

TABLE 2. Resistance to TSWV and plant fertility in tobacco somaclones and protoclones

Source of plant	No. of plants tested	% of plants	
		Resistant	Resistant and self-fertile
Somaclones of TSWV-infected cultivars	184	9.2	82.4
Protoclones of virus-free cultivars	257	77.0	12.6
Somaclones of virus-free intercultivar hybrids	216	57.9	16.1
Somaclones of virus-free interspecific hybrids	174	98.3	0

Assuming that some of the self-sterile TSWV-resistant plants may have had partial (male or female) fertility, we made some crosses between resistant somaclones with the aim to overcome the self-sterility.

12.4.2. CROSSES BETWEEN TWO RESISTANT SOMACLONES

The somaclones used in sexual crosses (Table 3) were regenerated from leaf explants of tobacco cultivars (American 361, Immunny 580), wild species *Nicotiana sanderae*, *N. tabacum* × *N. sanderae* hybrids (Asan, KS, Asansan) and *N. tabacum* × *N. glauca* hybrid (TG). The regenerated somaclones of *N. sanderae* and of *N. tabacum* × *N. sanderae* hybrids produced from 66.7 (KS) to 100% of resistant plants (*N. sanderae*, Asansan). A small percentage of resistant plants was observed in somaclones of American 361 (5.6%), Immunny 580 (6.2%), and TG (7.1%).

All somaclones of interspecific hybrids used in the experiment were self-sterile, but some crosses resulted in fertile and resistant hybrid progeny. The

TABLE 3. Resistance to TSWV and plant fertility in tobacco hybrids obtained by resistant somaclones crossing

Somaclones crossed[1]	Resistant plants (%)			Self-fertile plants (%)
	Parents		Hybrids	
Asan × *N. sanderae*	86.5	100	100	0
KS × Asan	66.7	86.5	97.1	2.9
Asansan × KS	100	66.7	99.8	0
American 361 × Asan	5.6	86.5	0	100
TG × Asan	7.1	86.5	13.7	58.8
TG × Immunny 580	7.1	6.2	3.2	32.3

[1]Asan—American 361 × *N. sanderae*; Asansan—Asan × *N. sanderae*; KS—Khurchavy 73 × *N. sanderae*; TG—Trapesond 19 × *N. glauca*.

TABLE 4. Resistance to TSWV in tobacco hybrids obtained by crossing resistant somaclones with *Nicotiana alata*

Somaclones SC_0 crossed with *Nicotiana alata*	Generation	No. plants tested	Resistant plants	
			No.	%
American 5j	F_1	0	0	0
American 307	F_1	1	0	0
Krupnolistny B3	F_1	2	0	0
American 19 × American 5	F_1	4	1	0.25
	F_2	0[1]	0	0
American 3	F_1	189	8	4.2
	F_2	73	1	1.4
	F_2	89	3	3.4
	F_2	123	5	4.1
	F_3	25	0	0
	F_3	28	0	0
	F_3	29	0	0
American 3 × *N. Alata*	F_1	4	2	50.0
	F_2	43	21	48.8
	F_3	38	28	73.7
	F_3	48	42	87.5
	F_3	32	28	87.5

[1] The resistant hybrid plant obtained in F_1 had sterile seeds, and produced no progeny.

crosses of KS × Asan, TG × Asan, TG × Immunny 580 show that Asan have female fertility and TG—male fertility. Thus, some of the self-infertile TSWV-resistant somaclones could be used as male or female parents in the production of hybrids and subsequent selection of tobacco for resistance to the virus.

12.4.3. CROSSES OF RESISTANT TOBACCO SOMACLONES WITH *N. ALATA*

Since numerous crosses between tobacco cultivars and TSWV-resistant *Nicotiana alata* resulted in only a few self-sterile plants, we used tobacco somaclones to overcome the crossability barriers (Table 4).

In the three crosses between resistant tobacco somaclones SC_0 and *N. alata,* only two (Krupnolistny B3), one (American 307), or none (American 5j) hybrids were obtained, and no hybrid plants were observed to have a TSWV-resistant phenotype. The resistant hybrids were produced by crossing resistant somaclones of American 19 × American 5, American 3 and American 3 × *N. alata* with *N. alata*. We observed the resistance to TSWV in F_1(American 19 × American 5), in F_1 and F_2 (American 3), and also in F_1,

TABLE 5. Plants regeneration from sterile seeds of TSWV-resistant interspecific tobacco hybrids

Hybrids	Nutrition medium	Yield per 100 seeds		No. of plants obtained		
		Seedlings	Callus	All	Resistant	Resistant and self-fertile
Khurchavy 73 × *N. sanderae*	MS42	1	0	0	0	0
American 361 × *N. sanderae*	MSP	2	0	0	0	0
Trapesond 19 × *N. Glauca*	MS42	0	0	0	0	0
Khurchavy 140 × *N. glauca*	MSP	4	0	4	4	0
(American 3 × *N. alata*) × *N. alata*	MS42	0	1	0	0	0
American 3 × *N. alata*	MSP	6	1	6	5	2

F_2, and F_3 (American 3 × *N. alata*). Furthermore, we observed an increase in resistant plants in subsequent selfed generations F_2 and F_3. It was observed in crosses between somaclones of American 3 × *N. alata* and *N. alata*.

The results of this study indicate that it is possible to produce of *N. tabacum* × *N. alata* hybrids with inheritable resistance to TSWV.

12.4.4. REGENERATION OF PLANTS FROM STERILE SEEDS

Although some hybrids between tobacco cultivars and TSWV-resistant species of *N. sanderae*, *N. glauca*, or *N. alata* reached maturity, flowered, formed pollen, berry, and seeds, but none produced progeny. Therefore we attempted to overcome the sterility by regenerating plants from the sterile seeds.

Harvested seeds were surface sterilized with sodium hypochlorite and plated on MSP or MS42 nutrient agar medium in Petri dishes at 16–22°C with 14 h illumination. In this experiment very few seeds produced seedlings or callus (Table 5). However, the seedlings from two hybrids (Khurchavy 140 × *N. glauca* and American 3 × *N. alata)* were available for the assessment of regeneration.

All but one out of ten regenerants were resistant to TSWV, and two out of five resistant plants from American 3 × *N. alata* set viable seeds.

The results of this study indicate that it is possible to overcome the sterility of TSWV-resistant interspecific tobacco hybrids by regeneration of plants from sterile seeds but the yield of plants is very low and depends both on plant genotype and nutrient medium (growth regulator requirements). Possibly, in vitro manipulations may provide a way to increase the yield of fertile regenerants.

12.5. Conclusion

Our investigations show that somaclonal variation may be used in tobacco breeding for resistance to TSWV and to provide sources of TSWV-resistant somaclones, protoclones and androclones (Shcherbatenko and Oleshchenko, 1995). Additionally, somaclonal variation provides opportunities to increase the fertility of TSWV-resistant tobacco hybrids and to overcome the cross-ability barriers between tobacco cultivars and TSWV-resistant *Nicotiana* species. Fertile virus-resistant hybrids were produced by regeneration of somaclones from leaf-derived callus of sterile hybrids; by hybridization of selected virus-resistant somaclones of susceptible tobacco varieties with resistant wild species; by intercrossing between semifertile somaclones of interspecific hybrids and for the in vitro production of plants from nongermitable seed.

Unfortunately, the approach is both time-consuming and somewhat uncertain in terms of durability. Somaclones from tobacco cultivars and hybrids showed a negative correlation between TSWV-resistance and plant self-fertility as well as significant variability in response to TSWV, from delay in symptom development to complete resistance. The yield of resistant variants varied widely and was strongly influenced by tobacco variety whereas the inheritance of resistance seemed more to depend on the dynamics of plant damage in parental clones. The dynamics of pathogenicity is the more suitable criterion for selection of virus-resistant variants than the yield of resistant specimens in populations of somaclones.

The resistant variants segregated in selfed progenies and tended to loose resistance in subsequent generations. However, some clones showed heritable resistance, which leads to an increase in the proportion of resistant plants in successive generations. These somaclones seemed likely to be potentially useful breeding material and have indeed been used in breeding of tobacco for TSWV resistance. The American 63 tobacco selected (Rud et al., 2000) combines a high productivity, high production quality and moderate TSWV resistance in the field.

Somaclonal variation has been used successfully for the selection of Fijivirus-resistant sugarcane (Krishnamurthi and Thaskal, 1974), PVX- and TMV-resistant tobacco (Shepard, 1975; Murakishi and Carlson, 1976, 1982; Saha and Gupta, 1989; Toyoda et al., 1989), TMV-resistant tomato (Barden et al., 1986), PVX-, PVY-, and PLRV-resistant potato (Jellis et al., 1984; Wenzel and Uhrig, 1981), BaYMV-resistant barley and wheat (Foroughi-Wehr and Friedt, 1984; Comeau and Plourde, 1987), virus-resistant melon (Lotfi et al., 2003) or in the breeding of plants for resistance to other pathogens (e.g. Liu and Zheng, 2002; Van den Bulk, 1991).

Thus, somaclonal variation offers a promising source of virus resistance in breeding programs to enhance crop biosecurity but, as with most breeding approaches, there is a very long lead time.

References

Adkins, S., R. Quadt, T.J. Choi, P. Ahlquist, and T. German, 1995. An RNA-dependent RNA polymerase activity associated with virions of tomato spotted wilt virus, a plant- and insect-infecting bunyavirus, Virology, 207, 308–311.

Barden, K.A., S. Schiller, and N.N. Murakishi, 1986. Regeneration and screening of tomato somaclones for resistance to tobacco mosaic virus, Plant Sci., 45, 209–213.

Brommenschenkel, S.H. and S.D. Tanksley, 1997. Map-based cloning of the tomato genomic region that spans the Sw-5 tospovirus resistance gene in tomato, Mol. Gen. Genet., 256, 121–126.

Brunt, A.A., K. Crabtree, M.J. Dallwitz, A.J. Gibbs, L. Watson, and E.J. Zurcher, 1996. Plant Viruses Online: Descriptions and Lists from the VIDE Database, Version: 20 Aug. 1996.

Bucher, E., T. Sijen, P. De Haan, R. Goldbach, and M. Prins, 2003. Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions, J. Virol., **77**, 1329–1336.

Caner, J., M. Amelia, V. Alexandre, and M. Vicente, 1984. Effect of tiazofurin on tomato plants infected with tomato spotted wilt virus, Antiviral Res., 4, 325–331.

Comeau, A., and A. Plourde, 1987. Cell, tissue culture and intergeneric hybridization for barley yellow dwarf virus resistance in wheat, Can. J. Plant Pathol., 9, 188–192.

Czech, A.S., M. Szklarczyk, Z. Gajewski, E. Zukowska, B. Michalik, T. Kobylko and K. Strzalka, 2003. Selection of tomato plants resistant to a local Polish isolate of tomato spotted wilt virus (TSWV), J. Appl. Genet., 44, 473–480.

De Fazio, G., J. Caner, and M. Vicente, 1980. Effect of virazole (ribavirin) on tomato spotted wilt virus in two systemic hosts, tomato and tobacco, Arch. Virol., 63, 305–309.

De Fazio, G., and M. Kudamatsu, 1983. Inhibitory effect of Distamycin-A and a pyrazino-pyrazine derivative on tomato spotted wilt virus, Antiviral Res., 3, 109–113.

Fauquet, C.M., M.A. Mayo, J. Maniloff, U. Desselberger,. and L.A. Ball, 2005. Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, Amsterdam, 1259 pp.

Foroughi-Wehr, B., and W. Friedt, 1984. Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture, Theor. Appl. Genet., 67, 377–382.

Gielen, J.J.C., P. de Haan, A.J. Kool, D. Peters, M.Q.V.N. Van Grinsven, and R.W. Goldbach, 1991. Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus, Biotechnology, 9, 1363–1367.

Jahn. M., I. Paran, K. Hoffmann, E.R. Radwanski, K.D. Livingstone, R.C. Grube, E. Aftergoot, M. Lapidot, and J. Moyer, 2000. Genetic mapping of the Tsw locus for resistance to the *Tospovirus* Tomato spotted wilt virus in *Capsicum* spp. and its relationship to the Sw-5 gene for resistance to the same pathogen in tomato, Mol. Plant Microbe Interact., 13, 673–682.

Jellis, G.J., R.E. Gunn, and R.E. Boulton, 1984. Variation in disease resistance among potato somaclones, edited by F.A. Winiger and A. Stockly, Abstr. Conf. Pap. Triennal Conf EAPR, Interlaken, EAPR, Wageningen, pp. 380–381.

Jones, R.A.C., 2004. Using epidemiological information to develop effective integrated virus disease management strategies, Virus Res. 1, 5–30.

Kormelink. R., E.W. Kitajima, P. de Haan, D. Zuidema, D. Peters, and R. Goldbach, 1994. The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells, Virology, 181, 459–468.

Kovalenko, A.G., I.S. Shcherbatenko, L.T. Oleshchenko, E.A. Rud, and N.I. Strelaeva, 1989. The production of fertile somaclones of interspecific tobacco hybrids with high resistance to tomato spotted wilt virus, Cytol Genet, 24, 59–65.

Krishnamurthi, M., and J. Thaskal, 1974. Fiji disease resistant Saccharum officinarum var. Pindar subclones from tissue cultures, Proc. Int. Soc. Sugarcane Technol., 15, 130–137.

Liu, J.P., and C.M. Zheng, 2002. Application of in vitro selection and somaclonal variation in improvement of disease resistance, Yi Chuan., 24, 617–630.

Lotfi M., A.R. Alan, M.J. Henning, M.M. Jahn, and E.D. Earle, 2003. Production of haploid and doubled haploid plants of melon (*Cucumis melo* L) for use in breeding for multiple virus resistance, Plant Cell Rep., 21, 1121–1128.

MacKenzie, D.J., and P.J. Ellis, 1992. Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene, Mol. Plant Microbe Interact., 5, 34–40.

Mason, G., P. Roggero, and L. Tavella, 2003. Detection of tomato spotted wilt virus in its vector *Frankliniella occidentalis* by reverse transcription-polymerase chain reaction, J. Virol. Methods, 109, 69–73.

Medeiros, R.B., R.O. Resende, and A.C. de Avila, 2004. The plant virus tomato spotted wilt tospovirus activates the immune system of its main insect vector, *Frankliniella occidentalis*, J. Virol., 78, 4976–4982.

Moury, B., S. Pflieger, A. Blattes, V. Lefebvre, and A. Palloix, 2000. A CAPS marker to assist selection of tomato spotted wilt virus (TSWV) resistance in pepper, Genome, 43, 137–142.

Murakishi, H.H., and P.S. Carlson, 1976. Regeneration of virus-free plants from dark-green islands of tobacco mosaic virus-infected tobacco leaves, Phytopathology, 66, 931–932.

Murakishi, H.H., and P.S. Carlson, 1982. In vitro selection of *Nicotiana sylvestris* variants with limited resistance to TMV, Plant Cell Rep., 1, 94–97.

Nagata, T., A.K. Inoue-Nagata, J. van Lent, R. Goldbach, and D. Peters, 2002. Factors determining vector competence and specificity for transmission of Tomato spotted wilt virus, J. Gen. Virol., 83, 663–671.

Nagy, J.I., and P. Maliga, 1976. Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*, Z. Pflanzenphysiol., 78, 453–455.

Pang, S-Z., P. Nagpala, , M. Wang, J.L. Slighton, and D. Gonsalves, 1992. Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene, Phytopathology, 82, 1223–1229.

Prins, M., and R. Goldbach, 1998. The emerging problem of tospovirus infection and nonconventional methods of control, Trends Microbiol., 6, 31–35.

Reddy, D.V.R., and J.A. Wightman, 1988. Tomato spotted wilt virus: thrips transmission and control, Adv. Dis. Vector Res., 5, 203–220.

Rice, D.J., T.L. German, F.R.L. Mau, and F.M. Fujimoto, 1990. Dot blot detection of tomato spotted wilt virus RNA in plant and thrips tissues by cDNA clones, Plant Dis., 74, 274–276.

Riley, D.G., and H.R. Pappu, 2004. Tactics for management of thrips (*Thysanoptera*: *Thripidae*) and tomato spotted wilt virus in tomato, J. Econ. Entomol., 97, 1648–1658.

Rud, E.A., A.G. Kovalenko, I.S. Shcherbatenko, L.T. Oleshchenko, N.I. Strelaeva, and L.M. Kargina, 2000. The Author's witness N 1215. Ukrainian State Commission on testing and protecting of plant cultivars.

Rudolph, C., P.H. Schreier, and J.F. Uhrig, 2003. Peptide-mediated broad-spectrum plant resistance to tospoviruses, Proc. Natl. Acad. Sci. USA, 100, 4429–4434.

Saha, S., and S. Gupta, 1989. Isolation of disease free plants from tissue cultures of the TMV-infected leaf of tobacco var, *Jayasri.* Phytomorphology, 38, 241–248.

Shcherbatenko, I.S., A.G. Kovalenko, L.T. Oleshchenko, Z.M. Olevinskaya, E.A. Rud, and N.I. Strelyaeva, 1991a. Resistance of tobacco somaclones to tomato spotted wilt virus, Mikrobiol. Zh., 53, 75–80.

Shcherbatenko, I.S., A.G. Kovalenko, L.T. Oleshchenko, E.A. Rud, and N.I. Strelyaeva, 1989. The production of somatic clones of tobacco resistant to tomato spotted wilt virus, Biol. Nauk., 6, 24–27.

Shcherbatenko, I.S., and L.T. Oleshchenko, 1999. Selection of androgenetic tobacco somaclones resistant to tomato spotted wilt virus, Mikrobiol. Zh., 61, 9–14.

Shcherbatenko, I.S., and L.T. Oleshchenko, 1993. The production of tobacco androgenetic plants resistant to tomato spotted wilt virus, Cytol. Genet., 27, 48–52.

Shcherbatenko, I.S. and L.T. Oleshchenko, 1995. The display of high resistance to tomato spotted wilt virus in cellular clones and hybrids of tobacco, Mikrobiol. Zh., 57, 65–71.

Shcherbatenko, I.S, L.T. Oleshchenko, and Z.M. Olevinskaya, 1991b. Display of hypersensitivity and acquired resistance to TMV in tobacco regenerants, Mikrobiol. Zh., 53, 69–75.

Shepard, J.F., 1975. Regeneration of plants from protoplasts of potato virus X-infected tobacco leaves, Virology, 66, 492–501.

Sherwood, J.L., T.L. German, A.E. Whitfield, J.W. Moyer, and D.E. Ullman, 2001. "Tospoviruses", in Encyclopedia of Plant Pathology, edited by O.C. Maloy and T.D. Murray, John Wiley and Sons Inc., New York, pp. 1034–1040.

Soellick, T., J.F. Uhrig, G.L. Bucher, J.W. Kellmann, and P.H. Schreier, 2000. The movement protein NSm of tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the TSWV N protein, and identification of interacting plant proteins, Proc. Natl Acad. Sci. USA, 97, 2373–2378.

Toyoda, H., K. Khatani, Y. Matsuda, and S. Ouchi, 1989. Multiplication of tobacco mosaic virus in tobacco callus tissues and in vitro selection for viral disease resistance, Plant Cell Rep., 8, 433–436.

Ullman, D.E., R.B. Medeiros, L.R. Campbell, A.E. Whitfield, J.L. Sherwood, and T.L. German, 2002, Thrips as vectors of tospoviruses, Adv. Bot. Res., 36, 113–140.

Van den Bulk R.F., 1991. Application of cell and tissue culture and in vitro selection for disease resistance breeding—a review, Euphytica, 65, 269–285.

van Regenmortel, M.H.V., C.M. Fauquet, D.H.L. Bishop, E.B. Carstens, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, and R.B. Wickner, 2000. Virus Taxonomy: Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses, Academic Press, San Diego, 1162 pp.

Voinnet, O., Y.M. Pinto, and D.C. Baulcombe, 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants, Proc. Natl. Acad. Sci. USA, 96, 14147–14152.

Wenzel, G., and H. Uhrig, 1981. Breeding for nematode and virus-resistance in potato via anther culture, Theor. Appl. Genet., 59, 333–340.

INDEX