DIVERSITY OF TOSPOVIRUSES

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DIVERSITY OF TOSPOVIRUSES

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PREPOSITIONS

1. The present emphasis on the role of salicylic acid as a natural inducer of pathogenesis-related proteins and systemic acquired resistance in virus-infected tobacco leaves unjustifiably side-tracks the importance of increased ethylene production in such plants.

   Enyedi, A.J., Yalpani, N., Silverman, P. & Raskin, I. (1992). Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. USA* 89, 2480-2484.

   Malamy, J., Henning, J. & Klessig, D.F. (1992). Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *The Plant Cell* 4, 359-366.

2. The error rate of Taq polymerase used in the PCR, has not been evaluated in the identification of subspecies in the wader bird species.

   Wenink, P.W., Smit, C.J., Tilanus, M.G.J., van Muiswinkel, W.B. & Baker, A.J. (1992). DNA-analyse: achter de grenzen van de biometrie. *Limosa* 65, 109-115.

3. The limited success in the breeding programmes for resistance to TSWV can be explained by the low number of resistance genes to tospoviruses in the plant kingdom.

4. Search for plant species which are immune to tospoviruses will be more fruitful than the continuous identification of new susceptible species.

   Sether, D.M. & de Angelis, J.D. (1992). Tomato spotted wilt virus host list and bibliography. *Agricultural Experiment Station, Oregon State University, Special Report 888.* 16pp.

5. The tospovirus isolate from *Sechium edule* Sw. has improperly been considered to be a tomato spotted wilt virus isolate.

   Silveira, Jr., W.G. & de Ávila, A.C. (1985). Chuchu (*Sechium edule* Sw.): Nova hospedeira do virus de vira-cabeça do tomateiro (TSWV). *Fitopatologia Brasileira* 10, 661-665.

6. In studies to test the efficiency of non-persistently transmitted plant viruses, the aphids should have the opportunity to shuttle between infected and non-infected plants.

   Peters, D., Brooymans, E. & Grondhuis, P.F.M. (1989). Mobility as a factor in the efficiency with which aphids can spread non-persistently transmitted viruses; a laboratory study. *Proc. Exper. & Appl. Entomol.*, N.E.V. Amsterdam, 1, 190-194.

7. Virologists are more concerned with the composition of buffers used to prepare inocula than with the growth conditions of plants to be inoculated.

8. A few countries have greater chances to preserve their natural resources than Brazil.

9. The Dutch postal service (PTT-Post) is more interested in tulips than in seed-potatoes.

10. The often used expression "third world countries" should definitely be banned.

11. The idea that *Homo sapiens* is a vegetarian, is in line with the concept that our ancestors were originally fruit collectors.

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ofereço este trabalho aos meus pais

José Maria e Heloisa

em memória de minha querida irmã

Rosa Maria

e para o bem de todos os sêres vivos

CONTENTS

PREFACE

I am much indebted to EMBRAPA/IICA and all my colleagues of the Department of Virology, particularly to my advisers, Dr. Ir. Dick Peters and Prof. Rob W. Goldbach for their continuous guidance, helpful criticisms and stimulating discussions. I would like to express my thanks to the TSWV group (Peter de Haan, Renato de Oliveira Resende, Richard Kormelink, Elliot Watanabe Kitajima, Claire Huguenot, Ineke Wijkamp, Frank van Poelwijk, Kit Boye, Marcel Prins, Cor Meurs, Wies Smeets), the greenhouse staff and the (ex)students, Aldo Rojas, Marya Korsman, Marie-José Arts and Aat Vogelaar for their cooperation and assistance. I would like to express my appreciation to many friends who always assisted me in good and bad times (Hans van den Heuvel, Miranda Hornes, Jordi Cairó, Jawaid Khan, Renato and Rosane Resende, Ineke and Martinus Beek, Jos and Teresa van den Vooren). Finally, I would like to express my deepest love to my family who supported me unconditionally, specially my loving sister Rosa Maria whose life showed me the real purpose of being here and now.

CHAPTER 1 INTRODUCTION

Tomato spotted wilt virus (TSWV) has been discovered in Australia in 1915 (Brittlebank, 1919) and found, thereafter, in many other countries in (sub)tropical as well as in temperate climate zones. The virus causes severe outbreaks in a considerable number of economic important crops (Illingworth, 1931; Costa, 1944; Cho *et al.*, 1989). The virus vanished after the forties almost completely as the cause of serious diseases in Western Europe. However, the spread of the Western flower thrips, *Frankliniella occidentalis* Perg. over the Northern Hemisphere, revived the occurrence of TSWV in the North American continent and in Europe, causing severe yield losses in many vegetable and greenhouse crops (Zitter *et al.*, 1989; Marchoux, 1990; de Ávila *et al.*, 1991; Stobbs, 1992; Vaira *et al.*, 1992).

TSWV is exclusively transmitted by thrips in a persistent manner. The vector seems to acquire the virus only during its larval stages and becomes infective afterwards for its whole lifespan (Sakimura, 1962; Paliwal, 1974; Cho *et al.*, 1988). The question whether the virus does multiply or not in the vector has not been actualized yet (Ullman *et al.*, 1989). At least eight thrips species have been described as a vector of TSWV (Sakimura, 1962; Paliwal, 1974; Kobatake *et al.*, 1984; Palmer *et al.*, 1990).

The control of spotted wilt virus has been so far extremely difficult for several reasons. This virus has one of the broadest host ranges among plant viruses, infecting at least 550 plant species including mono- and dicots out of approximately 70 botanical families (Peters, 1991, personal communication). Already more than hundred species are recorded within the Solanaceae and Compositae. Control measures like roguing and destruction of the thrips vector by insecticides proved to be very ineffective. Breeding for resistance to TSWV has met very little success (Hartmann, 1991). A limited number of resistance genes has been found which are often difficult to introduce into other breeding lines or species. Recently, de Haan (1991) and Gielen *et al.* (1991) reported genetically engineered resistance to TSWV in several tobacco lines expressing the TSWV N gene. In the future, this strategy may be used and extended to other economically important crops.

TSWV are roughly spherical, enveloped particles ranging from 70-110 nm in diameter

and are covered with knob-like surface projections (Black *et al.*, 1963; Kitajima, 1965; van Kammen *et al.*, 1966; Milne, 1970; Francki & Grivell, 1970; Ie, 1971; Mohamed *et al.*, 1973; Paliwal, 1976). Purified virus preparations contain four to five structural proteins, i.e. a protein of approximately 200 kilodalton (K) which has not been characterized yet, two glycoproteins G1 (78 K) and G2 (58 K), and a 29 K nucleocapsid protein (Mohamed *et al.*, 1973; Tas *et al.*, 1977a).

The virus particles accumulate in the cavities of the endoplasmatic reticulum (ER) and most likely mature by budding of nucleocapsids through the ER membrane (Milne, 1970). Additional viral inclusions have also been detected in the cytoplasm of infected cells (Best & Palk, 1964; Ie, 1964; Kitajima, 1965; Martin, 1964; Francki & Grivell, 1970). Large clusters consisting of moderately dense staining granular material with complexes of dense aggregates, often arranged in chain or string-like structures, are observed. These dense aggregates are formed by non-enveloped nucleocapsids (Ie, 1964, 1982; Verkleij & Peters, 1983; Kitajima *et al.*, 1992). In addition, elongated flexible filaments or paracristalline rods can be found which do not share any antigen associated with virus particles but immunostain with antiserum against a non-structural protein (NSs) encoded by the S RNA (Kormelink *et al.*, 1991; Kitajima *et al.*, 1992).

The genome of TSWV consists of three single-stranded RNA segments, denoted large (L), medium (M) and small (S). These RNA segments are complexed with nucleocapsid (N) protein to form pseudo-circular nucleocapsid structures (van den Hurk *et al.*, 1977; Mohamed, 1981; de Haan *et al.*, 1989; Peters *et al.*, 1991).

Recently, the complete nucleotide sequence of the genome of a Brazilian isolate (BR-01) has been elucidated. Among plant viruses, TSWV appears to have a unique genome organization (de Haan *et al.*, 1990; de Haan, 1991; de Haan *et al.*, 1991; Kormelink *et al.*, 1992a, b and c) (Fig. 1). The L RNA (8897 nucleotides long) has a negative polarity and contains a single open reading frame (ORF) corresponding with a translation product of 331.5 K, which may represent the viral transcriptase (de Haan *et al.*, 1991). Expression of this genome segment occurs via the synthesis of a full-length mRNA (Kormelink *et al.*, 1992a). The M and S RNAs both have an ambisense coding strategy, each containing two ORFs. The M RNA is 4821 nucleotides long, encoding a nonstructural (NSm) protein with a size of 33.6 K in viral sense, and the precursor to the glycoproteins with

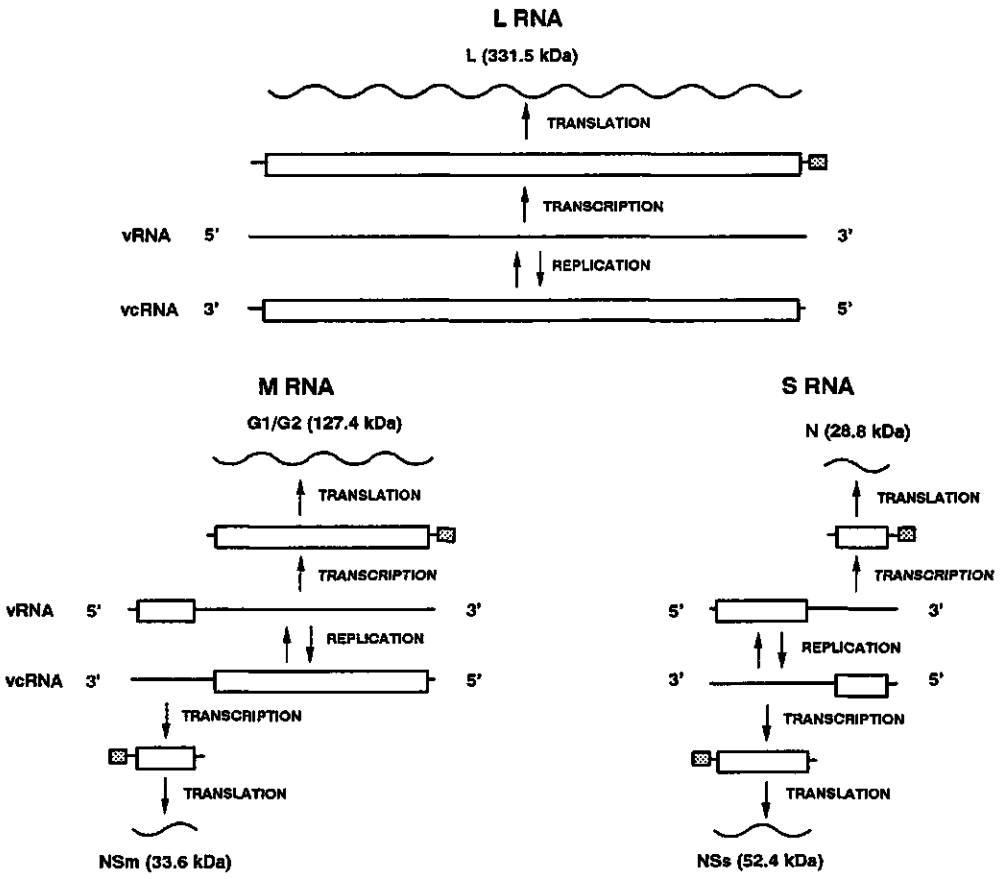

Fig. 1 - Structure and expression of the TSWV BR-01 genome. Data are taken from de Haan et al., 1990, 1992, and Kormelink et al., 1992c. The hatched area refers to non-viral sequences used to initiate transcription of the viral messenger RNAs.

a predicted size of 127.4 K in viral complementary sense (Kormelink *et al.*, 1992c). The precursor of the glycoproteins contains a sequence motif (RGD) which is characteristic for cellular attachment domains (Kormelink *et al.*, 1992c).

The S RNA is 2916 nucleotides long. It encodes a non-structural (NSs) protein of 52.2 K in viral sense and the viral nucleocapsid (N) protein of 28.8 K in viral complementary sense (de Haan *et al.*, 1990). The proteins encoded by M and S RNA are expressed by subgenomic mRNAs, transcribed from complementary strands, and initiated via a process of cap-snatching (Kormelink *et al.*, 1992a, b). The S and M RNA molecules terminate most probably in the central intercistronic region, at a long stable A-U rich hairpin (de Haan *et al.*, 1990; Kormelink *et al.*, 1992c).

All three genomic RNAs have complementary 3' and 5' ends which can be folded in a stable panhandle structure which may be involved in the formation of the pseudo-circular nucleocapsids (Peters *et al.*, 1991, de Haan, 1991; Kormelink *et al.*, 1992b).

TSWV has several biological properties by which it can be distinguished from other viruses. It exhibits a wide range of virulence patterns in different plant species, which makes identification of TSWV solely by symptomatology possible, although sometimes difficult. The virus can induce a wide variety of symptoms like mosaic, mottling, ring spots, vein clearing, stem necrosis, leaf-distortion, flower breaking, wilting and even latent infections. The symptoms are influenced by the host variety, time of infection, age of the host, temperature and isolates involved. Mechanical transmission of the virus to a selected host range provides a reliable identification of TSWV. *Petunia hybrida* Vilm. has been widely used as a test plant. The virus causes within two days small brown or black local lesions on the inoculated leaves of this species (Francki & Hatta, 1981). Alternatively, the virus may be identified by electron microscopy, due to its unique morphology and characteristic inclusions in the cytoplasm. Extension of such studies with immunogold-labelled virus-specific antibodies can precisely confirme the identity of the virus particles in extracts and thin sections (Peters *et al.*, 1991; Kitajima *et al.*, 1992). The use of electron microscopy for detection and diagnosis is, however, restricted as this approach is time consuming and only a limited number of samples can be examined.

Transmission by thrips, although a unique property, has not routinely been applied to identify TSWV due to difficulties in handling these tiny and fragile insects and the

elaborate rearing techniques.

In the past, TSWV could poorly be detected serologically due to the conspicuous lack of simple and sensitive serological techniques and to the difficulties in obtaining sufficient amounts of pure antigen (Best & Hariharasubramanian, 1967; Feldman & Boninsega, 1968; Tsakirides & Gooding, 1972; Joubert *et al.*, 1974; Paliwal, 1976; Tas *et al.*, 1977b; Francki & Hatta, 1981). However, the possibilities to detect the virus reliably in plants and thrips have dramatically been increased with the production of sensitive antisera and the development of ELISA techniques (Gonsalves & Trujillo, 1986; Cho *et al.*, 1988; Huguenot *et al.*, 1990; Wang & Gonsalves, 1990; Resende *et al.*, 1991). The use of other techniques as tissue blotting (Hsu & Lawson, 1991) hybridization with either cDNA or riboprobes (Ronco *et al.*, 1989; Huguenot *et al.*, 1990; German & Hu, 1990; Rice *et al.*, 1990) and the polymerase chain reaction (de Haan, 1991) has been advocated but are not yet widely applied in the identification and diagnosis of TSWV.

The first attempts to classify TSWV isolates were based on host responses (Norris, 1946). Five distinct "strains" were placed in three groups, while Best and Gallus (1953) distinguished six "strains" by symptom expression on three indicator hosts. These studies have not been followed up as other descriptors were lacking. Hence, new isolates of TSWV have poorly been described and clear proposals to classify TSWV in terms of species and/or strains have not been made. Due to the lack of any useful defined descriptor in the past, TSWV was first classified as the sole member of a monotypic plant virus group (Ie, 1970; Matthews, 1982), a position which was almost unique in the taxonomy of viruses. However, as detailed studies have revealed, the virus shares many similarities with the arthropod-borne Bunyaviridae, such as the mode of transmission, particle morphology and genome structure. As a result of these similarities, TSWV has recently been placed into this family and classified therein as the sole member of the newly created genus, *Tospovirus* (Francki *et al.*, 1991).

In view of the worldwide spread of the virus, its ability to infect a high number of plant species and its occurrence in different biological niches, taxonomic differences may exist between the various isolates. Elucidation of the taxonomic relations between the tospoviruses is not only of taxonomical interest but also essential for resistance breeding and understanding their epidemiology.

The present study was aimed to analyze a number of parameters which could be used to distinguish isolates by stable characteristics. For this purpose, a large number of isolates from different geographical areas and crops were compared as to their biological, serological and molecular properties. Chapter 2 describes the differentiation of twenty different isolates using polyclonal and monoclonal antibodies raised against the nucleocapsid (N) protein of the Brazilian isolate BR-01. A comparative study on the cytopathology of these isolates by electron microscopy is described in Chapter 3. Further detailed analyses made on the serological properties of these isolates, revealed that three serogroups could be distinguished on the basis of the results presented in Chapter 4 and 5. Finally, based on comparisons of sequence data, the phylogenetic relations between a selected set of isolates have been elucidated while a proposal for classification of tospoviruses is discussed (Chapter 6).

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CHAPTER 2 SEROLOGICAL DIFFERENTIATION OF TWENTY ISOLATES OF TOMATO SPOTTED WILT VIRUS

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SUMMARY

Twenty tomato spotted wilt virus (TSWV) isolates were serologically compared in ELISA employing five different procedures using a rabbit polyclonal antiserum against nucleocapsid proteins ($NuAb^R$), and mouse monoclonal antibodies (MAbs), two directed to nucleocapsid proteins (N1 and N2) and four directed to glycoproteins (G1 to G4). All antisera were raised against the TSWV - isolate TSWV-$CNPH_1$. The 20 isolates were differentiated into two distinct serogroups. Serogroup I consisting of 16 isolates, strongly reacted with $NuAb^R$. The other four isolates were poorly recognized by $NuAb^R$ and were placed in another serogroup, designated II. The panel of MAbs differentiated the TSWV isolates into three serotypes. The 16 isolates forming serogroup I reacted strongly with the MAbs generated and were identified as serotype I isolates. The four isolates which made up serogroup II were split in two serotypes II and III. The serotype II isolates did not respond or responded poorly with the MAbs N1, N2, and G3. The two other isolates placed in serotype III were recognized by N1 but not by N2 and G3. Two isolates became defective after several mechanical passages and failed to respond or responded very poorly with MAbs directed to glycoproteins. Our results show that ELISA employing polyclonal and monoclonal antisera is a useful tool to differentiate TSWV isolates and to detect defective forms. The results also strongly suggest that TSWV nucleocapsid proteins are less conserved than glycoproteins.

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INTRODUCTION

The spread of *Frankliniella occidentalis* (Pergande) from the Western part to the rest of the USA and into Canada and Europe has caused numerous outbreaks of tomato spotted wilt virus (TSWV) in different crops in the field and in glasshouses (Peters *et al.*, 1991). Although TSWV can easily be distinguished from other plant viruses, methods by which different isolates can readily be characterized are not available. Identification of the various isolates using symptoms in different indicator plants, as advocated by Norris (1946) and by Best & Gallus (1955), has not widely been used. Also, serology has not been explored extensively in the diagnosis and identification of TSWV and differentiation of the various isolates since antisera produced against TSWV often lack the required reliability or sensitivity (Paliwal, 1974; Reddy & Wightman, 1988; Tas *et al.*, 1977). Peters *et al.* (1991) have argued that serological techniques, like agar gel diffusion or ring tests applied in the past, are not suited for a sensitive detection of TSWV.

ELISA techniques seem well suited to detect TSWV as has been shown recently. A polyclonal antiserum produced against a TSWV isolate from papaya (Gonsalvez & Trujillo, 1986) detected TSWV in sap from infected plants. Cho *et al.* (1988) found TSWV in individual infected thrips with ELISA using an antiserum against a TSWV isolate from lettuce. Wang & Gonsalves (1990) compared 30 TSWV isolates from several countries and concluded that some non-USA isolates may be categorized into distinct serogroups. Recently, Sherwood *et al.* (1989) reported the use of a monoclonal antibody (MAb) directed to the nucleocapsid protein in the detection of five TSWV isolates from different hosts and geographically different areas. Polyclonal antisera against complete virus, purified nucleocapsid protein and six MAbs directed to the nucleocapsid protein or envelope glycoproteins have been produced by Huguenot *et al.* (1990). These polyclonal antisera are very useful in the detection of TSWV (Resende, R. de O., unpublished results). Huguenot *et al.* (1990) compared the usefulness of different ELISA procedures using MAbs with a molecular hybridization technique using riboprobes for the detection of TSWV. The use of riboprobes and also of cDNA (Ronco *et al.*, 1989) has not yet widely been applied.

Here we report the results of a study on the serological identification and

differentiation of 20 TSWV isolates employing five different ELISA procedures using polyclonal and monoclonal antibodies.

METHODS

TSWV identification and maintenance

Twenty TSWV isolates from different hosts, climatic zones and geographical regions (Table 1) were identified on differential hosts and by electron microscopy as being TSWV. All the isolates were maintained in *Nicotiana rustica* L. plants by mechanical inoculation. The original isolates were stored in leaf tissue in liquid nitrogen.

Table 1. Geographical areas and hosts from which the TSWV isolates originated.

Isolate designation		Country	Crop
Original*	Present#		
$CNPH_1$	BR-01	Brazil	Tomato
B2	BR-02	Brazil	Tomato
B3	BR-03	Brazil	Tomato
B1	BR-04	Brazil	Pea
B5	BR-05	Brazil	Tomato
B6	BR-06	Brazil	Tomato
B8	BR-08	Brazil	Tomato
B13	BR-13	Brazil	Tomato
B15	BR-15	Brazil	Tomato
B16	BR-16	Brazil	Tomato
Fi	SF-01	Finland	Tomato
A1	D-01	Germany	Ranunculus
S1	E-01	Spain	Sweet-pepper
A4	ZA-04	South Africa	Pea
A5	SA-05	South Africa	Peanut
H1	NL-01	The Netherlands	Impatiens
H2	NL-02	The Netherlands	Tomato
H3	NL-03	The Netherlands	Tomato
H4	NL-04	The Netherlands	Chrysanthemum
A7	YU-07	Yugoslavia	Tobacco

(*) Used in this chapter, (#) used in the following chapters

Purification of TSWV complete virus and nucleocapsid proteins

The virus was purified by the method described by Tas *et al.* (1977) with one modification. Prior to homogenization of the leaves, PMSF (Sigma) was added from a 0.2 M stock solution in isopropanol to the extraction buffer to a final concentration of 1 Mm.

The nucleocapsid purification protocol used was according to (D. Peters, unpublished results). The method consisted of grinding 50 g of infected *N. rustica* leaves in the extraction buffer (0.01 M Tris-HCl, 0.1 M sodium sulphite and 0.01 M EDTA pH 8.0) 1:4 (w/v). After a low speed centrifugation (1000 *g*), the supernatant was submitted to a high speed centrifugation at 50,000 *g* for 30 min. The pellets were resuspended in resuspension buffer (0.01 M Tris-HCl, 0.01 M sodium sulphite and 0.01 M EDTA, pH 7.9) containing 1% Nonidet P40. After another low-speed centrifugation at 8000 *g* for 10 min, the supernatant was centrifuged on a 30% sucrose cushion for 1 h at 125,000 *g*. The pellet was resuspended in 0.01 M citrate buffer, pH 6.0, and subsequently centrifuged in a 20-40 % sucrose gradient for 2 h at 190,000 *g*. The nucleocapsid bands were collected using a Uvicord III 2089 LKB fraction collector. The complete virus and nucleocapsid protein concentration was estimated as described by Lowry *et al.* (1951).

Polyclonal and monoclonal antisera

Polyclonal antisera against complete virus, the purified nucleocapsid fraction and six monoclonal antibodies prepared against the isolate $CNPH_1$ as described by Huguenot *et al.* (1990) were used in the serological tests. The monoclonal antibodies N1 (originally designated (6.12.15), and N2 (2.9) are directed to nucleocapsid proteins, while the other four, G1 to G4 (3.22.6, 7.22.6, 6.7, and 7.22.1) are directed to the envelope glycoproteins (Huguenot *et al.*, 1990).

Biotinylation of MAb and preparation of enzyme-labelled streptavidin

Biotinyl N-hydroxysuccinimide (E.Y. Laboratories; 5 mg/ml in 0.01 M $NaHCO_3$) was mixed at a 1:25 (v/v) ratio with N1 gammaglobulin containing ascitic fluid diluted 1:10 (v/v) in 0.01 M $NaCO_3$. The mixture was incubated for 4 h at 25°C and the reaction was stopped by adding 10 µl 1M NH_4Cl per ml (Zrein *et al.*, 1986). Subsequently, the

mixture was dialysed extensively in phosphate buffered saline (0.14 M NaCl, 1 Mm KH_2PO_4, 8 Mm Na_2HPO_4, 2.5 Mm KCl)(PBS) at 4°C.

ELISA procedures

Twenty TSWV isolates were serologically compared using five different ELISA procedures which are summarized in Table 2. In all the procedures, the microtiter plates (Nunc) were coated with antibodies in carbonate buffer, pH 9.6, overnight (200 µl/well). The dilutions of the antibody stock solutions used in the several steps of the ELISA procedures according to Huguenot *et al.* (1990) are indicated in Table 3. After coating, the plates were incubated with 200 µl PBS, pH 7.4, containing 0.05% Tween 20 (PBS-T) and 1% bovine serum albumin (BSA) per well for 30 min to block non-specific binding sites.

Table 2. ELISA procedures used to differentiate TSWV isolates.

Procedure	Coating	Summary of successive steps		
		Antigen	Detecting Ab	Conjugate
1	DAS*: $CvAb^R$	Ag		$CvAb^R$-PAL+
2	DAS: $NuAb^R$	Ag		$NuAb^R$-PAL
3	TAS#: $CvAb^R$	Ag	MAb	GAM/PAL
4	DAS: MAb	Ag		$CvAb^R$-PAL
5	TAS^B: MAb	Ag	MAb^BN1	SAv-PAL

* *Double antibody sandwich,*
\+ *See material and methods for the meaning of acronyms*
\# *Triple antibody sandwich*

In the following steps of the five procedures, the antigen and antibody solutions were diluted in PBS-T and 100 µl samples were added per well. The plates were incubated in each step at 37°C for 2 h and between each incubation step rinsed three times with PBS-T. The antigens bound were detected by adding 100 µl of *p*-nitrophenyl phosphate at a concentration of 1 mg/ml dissolved in 0.01 M diethanolamine buffer, pH 9.6. An EL 312 ELISA reader (BIO-TEK Instruments) was used to measure the absorbance at 405 nm. Samples of sap from infected *N. rustica* leaves, purified virus and nucleocapsid

preparations (100 µl/well) were tested in duplicate in the ELISA plates and average values are presented.

The reactivity of the different antisera with the TSWV isolates tested was studied in five ELISA procedures in order to eliminate the possibility that procedure-specific results were obtained. In procedures 1 and 2, sap from infected plants was diluted 300-fold and in the procedure 3, 4 and 5, 30-fold (Table 2). In each plate, samples of purified TSWV-CNPH$_1$ isolate (150 ng/100 µl), or sap from a healthy *N. rustica* plant as a negative control. The experiments described were repeated at least twice for all the isolates, and six times for an isolate, representing each proposed serogroup.

In the ELISA procedures 1 and 2, rabbit polyclonal antibodies (Ab^R) against complete virus (Cv), or against nucleocapsid proteins (Nu) were used at a concentration of 1 µg/ml to coat the wells. The same antibodies conjugated to alkaline phosphatase ($CvAb^R$-PAL and $NuAb^R$-PAL) were used in a concentration of 1 µg/ml. In procedure 3, the wells were coated with $CvAb^R$ at 2 µg/ml.

The dilutions at which the six MAbs were used are indicated in Table 3. The alkaline phosphatase-labelled goat anti-mouse globulin (GAM-PAL) solution (Sigma) was used at a 1/2000 dilution. In procedures 4 and 5, the six MAbs were used for coating the wells at dilutions indicated in Table 3. After adding the antigens, alkaline phosphatase-labelled

Table 3. Dilutions of the MAbs used in experiments described.

MAb	Subclass type	Ascitic fluid dilution		
		Coated	Detected	Biotinylated
N1	IgG1	1/1000	1/10000	1/1000
N2	IgG2a	1/1000	1/10000	-
G1	IgG2a	1/1000	1/1000	-
G2	IgG1	1/1000	1/1000	-
G3	IgG1	1/100	1/1000	-
G4	?	1/1000	1/1000	-

polyclonal antibody was used at 1 μg/ml in procedure 4, and biotinylated MAb N1 ($MAb^{B}N1$) in procedure 5 was used to detect viral antigen. The biotin labelled antibodies immobilized to the solid phase were detected with 5000-fold diluted streptavidin-alkaline phosphatase conjugate (SAv-PAL).

Immunogold labelling of sections

To detect TSWV *in situ* using the available antisera we applied the immunogold procedure described by van Lent *et al.* (1990). Leaf tissue from host plants systemically infected with TSWV, was fixed with aldehyde and embedded in LR Gold medium 2 to 3 weeks after inoculation. Thin sections were treated with 1% BSA in PBS before incubation in the specific antiserum, then gold-labelled with protein-A gold (pAg) and examined after staining with uranyl acetate and lead citrate. Tissues from healthy plants were used as control.

RESULTS

General characteristics of the monoclonal and polyclonal antibodies

TSWV is a plant virus which has many properties in common with viruses of the family of arthropod-borne Bunyaviridae (de Haan *et al.*, 1989a). To differentiate and identify the isolates of TSWV, the same criteria which are used for the serological differentiation of bunyaviruses (Bishop & Shope, 1979) may be applied to TSWV. Serogroups and serotypes are defined by the reactions between the nucleocapsid proteins and antibodies in ELISA. A serogroup refers to antigenic relationships between polyclonal antisera and the nucleocapsid protein of the different viruses. The designation serotype is based on serological differences found with a panel of MAbs and the nucleocapsid protein.

From the panel of six MAbs used in this study, N1 and N2 are directed to nucleocapsid protein and react in ELISA with both, intact virus particles and purified nucleocapsid protein. The other four MAbs, G1, G2, G3 and G4 are most probably directed to the envelope glycoproteins. They do not react in ELISA with purified nucleocapsid protein but only with intact virus particles (data not shown). These MAbs

label virus particles *in situ* with gold, but do not react with structures consisting of nucleocapsids in dip preparations or nucleocapsid aggregates *in situ* (Kitajima *et al.*, 1992), the latter being accumulations of nucleocapsids, which do not become enveloped into virus particles (Ie, 1982; Chapter 3).

Serological differentiation of 20 TSWV isolates using sap from infected plants

Twenty isolates of TSWV were studied using five different ELISA procedures with a polyclonal antiserum against the $CNPH_1$ isolate, a polyclonal antiserum against the nucleocapsid (N) protein of $CNPH_1$ and six MAbs against the N and G proteins of this isolate. All isolates tested reacted in the five procedures but responded in a quantitatively different way as shown in Fig. 1 for the results using procedure 1.

Using procedures 1 and 2, the anti-$CNPH_1$ serum (data not shown) and anti-N serum (Fig. 1) differentiated the isolates into two serogroups. Sixteen isolates, B1, S1, A7, B15, A1, H1, H2, H3, B2, A4, Fi, B5, B16, H4, B13 and $CNPH_1$, reacted strongly with both

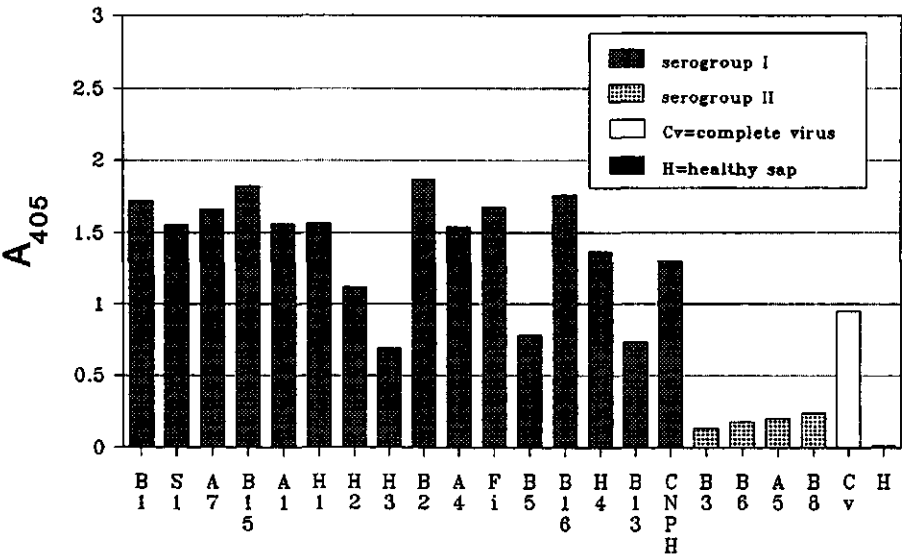

Fig. 1 - Reaction of 20 TSWV isolates using sap from infected plants and a polyclonal antiserum against $CNPH_1$ nucleocapsid protein and the division of these isolates in two serogroups. The A_{405} was measured 15 min after substrate addition. Cv: complete virus; H: healthy sap.

antisera and may belong to one serogroup denoted I. The isolates B3, B6, A5, B8 were poorly recognized by both antisera. Since these isolates reacted strongly with antisera prepared to B3 and A5 nucleocapsid proteins (data not shown), they were placed in serogroup II.

Clear differentiation of the TSWV isolates was obtained with procedure 3. Three reaction patterns were discernible with the MAbs N1 and N2 by which three different serotypes were distinguished (Fig. 2). The 16 TSWV isolates, forming serogroup I reacted with MAb N1 as well as with MAb N2; thus the group I viruses form one serotype, denoted I. The isolates A5 and B8 reacted strongly with MAb N1 but not with MAb N2 (Fig. 2). The isolates B3 and B6 showed only a weak reaction with MAb N1 and did not react with MAb N2. This serological differentiation did not change in procedure 3, when the MAbs N1 and N2 were tested in a 1000-fold dilution instead of 10,000 fold. It is evident from these results that these MAbs are directed to different epitopes on the nucleocapsid protein. Therefore, the four viruses have been placed into two different serotypes; the isolates B3 and B6 are denoted serotype II viruses, and A5 and B8 as serotype III viruses.

The 16 isolates (serogroup I) reacted strongly with the MAbs G1 and G3 (Fig. 2). The reaction profiles obtained with MAbs G2 and G4 were identical to those with MAb G1 (data not shown). The division of the isolates B3, B6, A5 and B8 into two serotypes is not supported by the reaction with the four MAbs reacting with the G proteins. These isolates reacted strongly with the three MAbs G1, G2 and G4, whereas no reaction was obtained with MAb G3 (Fig. 2).

The results obtained with the procedures 4 and 5 were similar to those with procedure 3, but the discriminative power of the latter was higher than that of the former two.

Serological differentiation of the TSWV isolates using purified antigens

To eliminate the possibility that the serological differences were due to the use of sap as antigen source, the proposed serogrouping and -typing of CNPH$_1$, A5, B3, B6 and B8 was studied using purified antigens. Attempts to purify the virus particles of isolates A5, B3 and B6 using the procedure described by Tas *et al.* (1977) were unsuccessful.

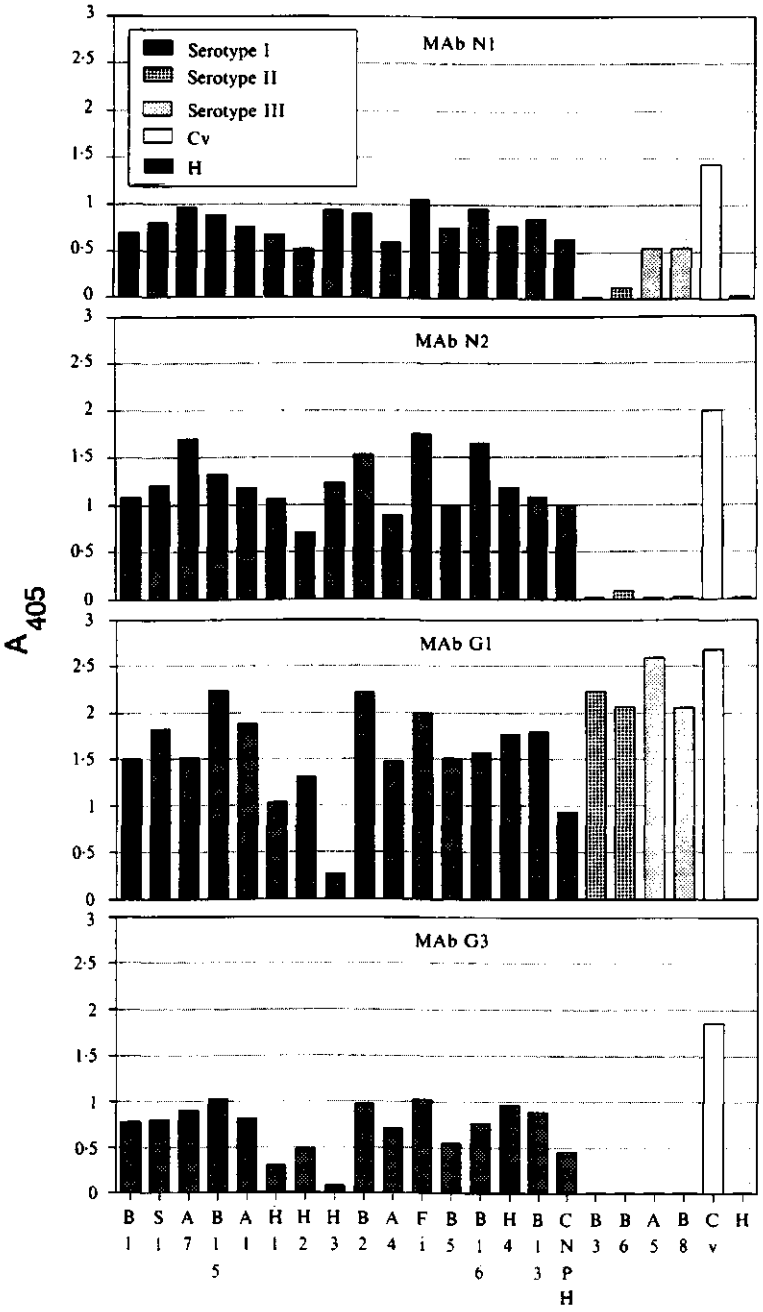

Fig. 2 - Allocation of 20 TSWV isolates into three serotypes using sap from infected plants and four MAbs, two directed to nucleocapsid proteins (N1 and N2) and two to glycoproteins (G1 and G3). The A_{405} was measured 1 h after substrate addition. Cv: complete virus; H: healthy sap.

The nucleocapsid proteins from these isolates were purified, tested in ELISA and used to prepare antisera. Fig. 3 shows that the nucleocapsid proteins of B3, B6, A5 and B8 (serogroup II) reacted poorly in ELISA with nucleocapsid polyclonal antiserum against $CNPH_1$. Similar results as with sap from infected plants were obtained with the MAbs N1 and N2 (Fig. 4). As expected, almost no reaction was observed with the four MAbs directed to the glycoproteins when the purified nucleocapsid protein preparations were used as antigens. The weak reaction of the nucleocapsid protein of isolate A5 with MAbs directed to glycoproteins was due to a slight contamination with glycoproteins as shown by protein gel electrophoresis (data not shown). The serological results did not change (data not shown) when MAbs were used at a 10 times higher concentration.

Detection of TSWV defective forms using a panel of MAbs

The serological results are very stable because changes were not observed after several passages of most of the isolates in *N. rustica* and other host plants. However, the reaction of the isolates H4, B13 and H3 changed considerably during our studies. After a number of passages, samples of *Nicotiana benthamiana*, *N. rustica* and tomato plants infected with the isolates H4 and B13 reacted with MAbs N1 and N2, but did not react or reacted only very slightly with the four MAbs directed to glycoproteins (Fig. 5). Moreover, mechanical transmission of these isolates became slightly more difficult and attenuation of symptoms on *N. rustica* was observed. Electron microscopy studies revealed that complete virus particles were virtually absent in leaf-dip preparations. In ultrathin sections, only dense masses characteristic of defective forms (Ie, 1982) were observed. The dense masses were recognized as aggregates of nucleocapsids using immunogold labelling techniques with a polyclonal nucleocapsid antiserum (Fig. 6). The H3 isolate also showed a decreased reactivity with MAbs directed to glycoproteins after several mechanical inoculations. In leaf-dip preparations a few complete virus particles were still observed. Since the ratio of antigen that could be detected changed in favor of a reaction with the antibodies directed to the nucleocapsid protein, we suppose that this isolate is in the process of becoming defective.

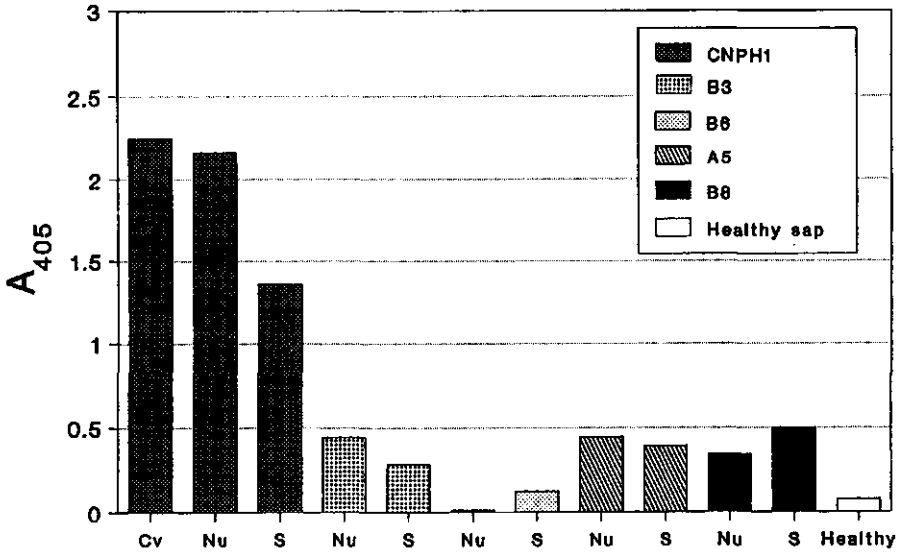

Fig. 3 - Reaction of complete virus (Cv), nucleocapsid protein preparations (Nu) and sap from infected plants (S), with TSWV-CNPH$_1$ nucleocapsid polyclonal antiserum. Cv-CNPH$_1$ (150 ng), Nu-CNPH$_1$ (100 ng), Nu-B3 (100 ng), Nu-B6 (100 ng), Nu-A5 (100 ng) and Nu-B8 (25 ng). The sap from the infected and healthy plants was diluted 300-fold. The A_{405} was measured 45 min after substrate addition. H: healthy sap.

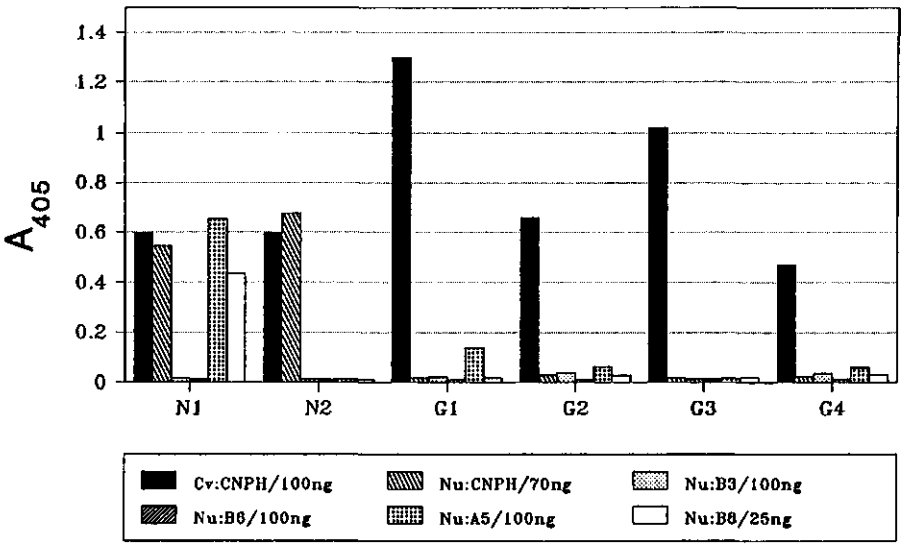

Fig. 4 - Reaction of purified complete virus (Cv), and nucleocapsid preparations (Nu) of CNPH$_1$, B3, B6, A5 and B8 with MAbs N1, N2, G1, G2, G3 and G4. The A_{405} was measured 1 h after substrate addition.

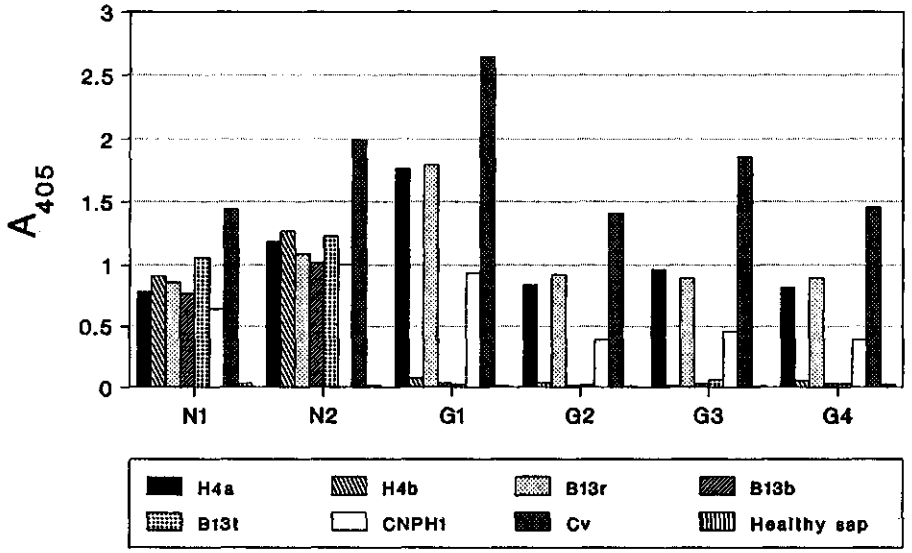

Fig. 5 - Detection of defective isolates of TSWV in leaf sap with a panel of MAbs. The NL-04a isolate was obtained after a few mechanical passages of H4 in Nicotiana rustica. H4b was obtained after 10 mechanical transmissions of H4 in N. rustica. B13a, B13b and B13t were obtained after transmitting B13 several times through N. benthamiana, N. rustica and tomato plants respectively. $CNPH_1$ was used as a positive control. Cv: complete virus preparation; H: healthy sap. The A_{405} was measured 1 h after substrate addition.

DISCUSSION

Owing to a lack of distinguishing methods, TSWV isolates have so far not been differentiated by stable characteristics. In the past, TSWV has been distinguished by symptoms on tomatoes (Norris, 1946) or on a few differential host plants (Best & Gallus, 1955). Antisera have rarely been used to study the serological relationships of different isolates of TSWV.

Different ELISA procedures are often used to clarify and analyze the virus serologically (Dekker *et al.*, 1989). To study the serological relationships of the TSWV isolates, five different ELISA procedures were used. The results obtained varied quantitatively but not fundamentally. The sharpest discrimination was obtained with procedure 3. In the present study, 20 TSWV isolates were differentiated into two serogroups and three serotypes. Sixteen isolates, which were also not differentiated by MAbs N1 and N2, were placed in serogroup I (Fig. 1). Four isolates which were poorly

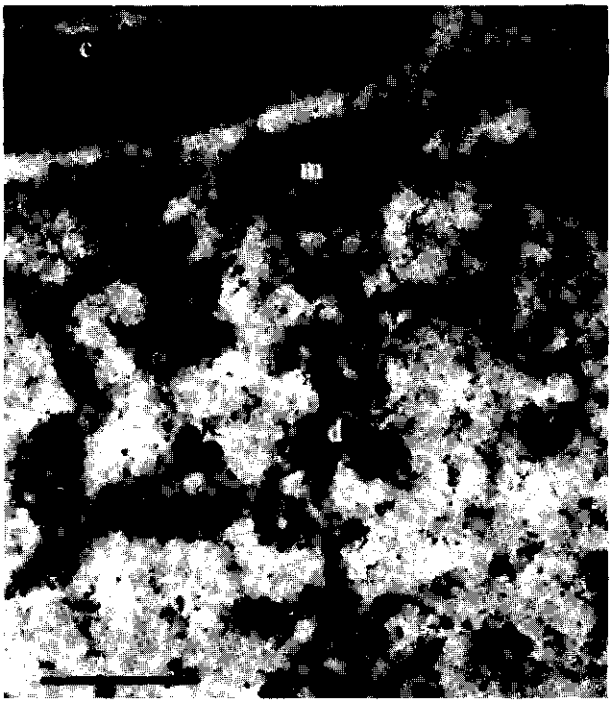

Fig. 6 - Mesophyl cell of N. benthamiana infected with H4b-TSWV defective form showing only a virus-induced inclusion of nucleocapsid aggregates (d) being gold-labelled with anti-nucleocapsid protein antibodies. A chloroplast (c) and mitochondria (m) are shown. Bar: 0.2 nm.

recognized by the polyclonal antiserum against the N protein were placed in serogroup II (Fig. 1). These isolates reacted differently with MAbs N1 and N2 and were considered to be members of two distinct serotypes, II and III (Fig. 2).

Wang & Gonsalves (1990) compared 30 TSWV isolates (most of which are from the USA, a few from other countries) in various ELISA procedures using specific antisera to the whole virion of their BL isolate, its nucleoprotein (26 K) and membrane protein (78 K). These authors, although finding variable results with 11 isolates, did not establish either serogroups or -types. However, isolates with distinct nucleocapsid proteins were found by Kameya-Iwaki *et al.* (1988) in a virus from watermelon, and by Law & Moyer (1990) in a virus from *Impatiens*. The latter authors provided circumstantial evidence that their isolates had to be placed in a different serogroup using the distant serological relationship of the N protein as a criterion.

Recently, TSWV has been placed in a new genus of the family Bunyaviridae (Francki *et al.*, 1991). The genome organization of the TSWV S RNA is identical to that of phleboviruses and uukuviruses (Ihara *et al.*, 1984; Simons *et al.*, 1990; de Haan *et al.*, 1990). Several serological techniques have been used to differentiate the bunyaviruses (Bishop & Beaty, 1988). The study of group-specific antigenic determinants on nucleocapsid proteins has been successfully used in this family (Shope, 1985). Group-specific antigenic determinants also occur in the nucleocapsid protein of TSWV as shown by the use of MAbs and polyclonal antiserum produced against the N protein. The 20 isolates were split into three serotypes by two MAbs against the N protein. These results suggest that the nucleocapsid protein is not a highly conserved protein of TSWV. Shope (1985) concluded that the nucleocapsid protein of the phleboviruses, which form another genus of the Bunyaviridae, is less conserved than G1 and G2 glycoproteins. Serological relationships have not yet been found between TSWV and the bunyaviruses (Wang *et al.*, 1988).

This study has shown that changes or differences in serological reactions can be explained not only by serological differences, but also by misinterpretation due to the generation of defective isolates (Figs. 5 and 6) which may arise during the subsequent mechanical virus transfers. The panel of MAbs available appears to be a useful tool to identify the defective isolates.

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CHAPTER 3 COMPARATIVE CYTOLOGICAL AND IMMUNOGOLD LABELLING STUDIES ON DIFFERENT ISOLATES OF TOMATO SPOTTED WILT VIRUS

E.W. Kitajima, A.C. de Ávila, R. de O. Resende, R.W. Goldbach and D. Peters

SUMMARY

Ultrastructural changes in plant cells induced by infection with 32 different isolates of tomato spotted wilt virus (TSWV) were studied. Most of the isolates studied showed different macroscopical symptoms but caused similar cytopathological reactions. Observed cytopathic effects included formerly described reactions as the accumulation of virus particles in the endoplasmic reticulum, and the formation of viroplasm and aggregates, consisting of non-enveloped viral nucleocapsids in the cytoplasm. For two isolates virus particles were consistently absent in tissue of infected plants. In almost half of the isolates examined, inclusions were found which consisted of fibrous material, either arranged in loose, irregular aggregates or forming paracrystalline arrays. Immunogold analysis using antibodies to purified virus preparations or purified nucleocapsid fractions of the isolates BR-01 and NL-04, demonstrated the presence of nucleocapsid protein in virus particles, viroplasm and electron dense aggregates. Antiserum to viral glycoprotein labelled only virus particles. The fibrous structures found were not immunostained with antisera against either TSWV structural protein, but did so with antiserum raised against a non-structural protein (NSs) encoded by S RNA. In a few occasions, structures were observed which could be interpreted as images of a budding process of TSWV particles on possible Golgi complex derived membranes.

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INTRODUCTION

Tomato spotted wilt virus (TSWV), an important crop damaging pathogen, recently regained much interest not only because of its revived spread in the Northern Hemisphere, but also for its peculiar replication strategy and taxonomic relationship with the Bunyaviridae, a family of animal viruses (Milne & Francki, 1984; de Haan *et al.*, 1989, 1990; Peters *et al.*, 1991).

TSWV has a very wide host range and is spread in nature by several thrips species; it has a lipid membrane bound, spheroidally shaped particle with a diameter of 70-110 nm. Protein analysis reveals that at least 4 major polypeptides, a nucleocapsid protein N (29 K), two glycoproteins, G1 (78 K) and G2 (54 K), and a possible polymerase L (120-200 K) can be found in the virus particles (Mohamed *et al.*, 1973; Tas *et al.*, 1977). The genome consists of three different single stranded RNA segments with sizes of 8.9 (L RNA), 5.0 (M RNA), and 2.9 (S RNA) kilobases. The S RNA has an ambisense character, containing the genes for the nucleocapsid (N) and a non-structural (NSs) protein (de Haan *et al.*, 1990; Kormelink *et al.*, 1991). M RNA probably encodes the viral membrane glycoproteins, while the L RNA, the sequence of which has recently been elucidated, encodes a putative protein of 331.5 K which may have viral polymerase activity (de Haan *et al.*, 1989, 1991). Serological studies, using both mono- and polyclonal antibodies, resolved distinct serogroups and serotypes among twenty isolates analyzed sofar (de Ávila *et al.*, 1990). Repeated mechanical transmission generates defective isolates, which have lost the capacity to form complete virus particles, possibly as a consequence of a malfunctional M RNA (Ie, 1982; Resende *et al.*, 1991).

Concerning the cytopathology of TSWV infections, it has well been demonstrated that virus particles accumulate in the cisternae of the endoplasmic reticulum (Kitajima, 1965; Francki & Grivell, 1970; Milne, 1970; Ie, 1971). In addition, formations of amorphous matrix material in which aggregates of more electron dense material with a periodicity of 5 nm are embedded, as well as aggregates of fibrous or thin rodlike appearance are found in the cytoplasm (Francki *et al.*, 1985; Law & Moyer, 1990). For some isolates only dense masses and no virus particles at all were detected in infected plant tissue (Ie, 1982).

So far, cytopathological studies have been limited to a single or a few isolates which originated from distinct geographical regions, and occurred on a few plant species. Since different isolates can cause distinct types of symptoms upon infection, a comparative study may elucidate possible relations between the cytopathology and symptomatology. In this paper we describe the results of an extensive study on the cytopathic effects of a large number of isolates of TSWV found on different plant species and originating from different geographical regions. This study was complemented by immunogold analysis using different antisera to detect and locate the corresponding viral antigens within the infected cell and the various virus-induced structures.

METHODS

Virus isolates

A collection of TSWV isolates was maintained in leaf tissues in liquid nitrogen, and inoculated mechanically onto *Nicotiana rustica* L. cv America (Table 1) when required. In some cases, the plants in which the isolate was found, were kept under greenhouse conditions.

Antisera

Polyclonal antisera to the Brazilian isolate BR-01, originally designated $CNPH_1$, (Chapter 2), were prepared by injecting rabbits with purified virus preparations (BR-01 antiserum), with purified nucleocapsid preparations (anti-N serum), with the G1 glycoprotein eluted from a SDS-polyacrylamide gel (anti-G serum) or the nonstructural protein encoded by the S RNA (anti-NSs serum) (de Ávila *et al.*, 1990; Resende *et al.*, 1991; Kormelink *et al.*, 1991). Antisera prepared to purified virus (NL-04 antiserum) and of its purified nucleocapsids (anti-N NL-04 serum) of the TSWV isolate NL-04, belonging to the same serogroup as BR-01 (de Ávila *et al.*, 1990) were also used. The monoclonal antibodies (MAb G1, G2, G3 and G4) used were directed to the glycoproteins and the MAb N1 and N2 to the nucleocapsid protein of BR-01 (de Ávila *et al.*, 1990; Huguenot *et al.*, 1990).

Table 1. The cytopathological effects in cells of plants infected with different TSWV isolates.*

Isolates	Cytopathology#				Isolates	Cytopathology#			
	V	VP	NCA	F		V	VP	NCA	F
D-01	+	+	+	-	DK-01	+	+	+	+
BG-02	+	+	+	-	SF-01	+	+	+	-
SA-05	+	+	+	+	SF-03	+	+	+	-
BR-01	+	+	+	-	NL-01	+	+	+	-
BR-02	+	+	+	-	NL-02	+	+	+	+
BR-03	+	+	+	-	NL-03	+	+	+	-
BR-05	+	+	+	-	NL-04	+	+	+	+
BR-06	+	+	+	-	NL-04(*env-*)	-	+	+	+
BR-08	+	+	+	+	NL-05	+	+	+	+
BR-13	+	+	+	+	NL-06	+	+	+	+
BR-13(*env-*)	-	+	+	+	NL-07	+	+	+	+
BR-15	+	+	+	-	NL-08	+	+	+	+
BR-19	+	+	+	-	NL-09	+	+	+	-
BG-02	+	+	+	-	NL-10	+	+	+	-
BG-03	+	+	+	-	NL-11	+	+	+	-
BG-04	+	+	+	-	E-01	+	+	+	+
BG-05	+	+	+	-	NL-12	+	+	+	+

** The country from which these isolates originate is indicated by the international coding used for automobiles. The number refers to the isolate studied. # V: virus particle, VP: viroplasm NCA: nucleocapsid aggregate, F: inclusion of fibrous material, -: absence and +: presence of the cytopathic effects, env-: morphologically defective particle.*

Electron microscopy

Small samples of leaf tissues from *N. rustica*, tomato (*Lycopersicon esculentum* Mill.), pepper (*Capsicum annuum* L.), *Datura stramonium* L. plants, mechanically infected with different TSWV isolates, or from *Impatiens* sp. and pepper plants, each of which was naturally infected with one of the studied isolates, were fixed in a modified Karnovsky fixative (2% paraformaldehyde and 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2) for 2-3 h, postfixed in 1% OsO_4 in the same buffer, and embedded in Spurr's low viscosity medium. Sections were cut in a LKB Ultratome V with a Diatome diamond knife, stained with uranyl acetate and Reynold's lead citrate.

Immunogold labelling

For immuno-electron microscopy, tissues were fixed only with aldehyde and embedded in LRGold as previously described (van Lent *et al.*, 1990). Sections mounted on gold or nickel grids, were preincubated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min, and then treated with specific antiserum for two hours. The polyclonal antibody solutions (1 mg/ml) were usually diluted 500 or 1000 times, while the monoclonal antibody suspensions (1 mg/ml) were used at dilutions of 1:100. The sections were then exposed to gold particles of 7 or 15 nm in diameter, conjugated with protein A when polyclonal antibodies were used, and with gold-labelled (15 nm in diameter) goat anti-mouse immunoglobulin (from Johanssen), diluted to an OD_{520} of 0.1 - 0.2, when monoclonal antibodies were used (van Lent *et al.*, 1990).

Sections from uninfected leaves of *N. rustica* as well as from pelleted purified nucleocapsid or intact virus particles of the BR-01 isolate were used as additional controls in the immunolabelling experiments. Examination of the sections were made in a Philips CM12 or Zeiss EM109 electron microscope.

RESULTS

Comparative cytopathology of different TSWV isolates

Roughly spherical, enveloped virus particles are always observed within membrane bound cavities of the endoplasmic reticulum system in ultrathin sections of cells from infected plants. Usually, several to many particles occur in a single cavity (Fig. 1A), but a few isolates often disperse their virus particles individually in a vesicle with distinct membranes (Fig. 1B). This may be a stable characteristic for a given isolate, but is not so for all members of the same serogroup. Numerous particles of SA-05 and BR-03, serogroup II isolates (Table 2), are often arranged in a crystalline array within a single cavity (not shown), whereas the particles of BR-06 and BR-08, also isolates of the same serogroup, occur singly in a vesicle (Fig. 1B).

Practically, virus particles were found in all leaf cell types, e.g. trichoma, epidermis, mesophyll and vascular parenchyma cells. They were also seen in xylem vessels undergoing differentiation (Fig. 2A) but seldom in sieve tubes (Fig. 2B). Virus particles

were not found near or in the plasmodesmata, though the desmotubules were continuously connected with endoplasmic reticulum containing virus particles (Fig. 2C).

Basically, all isolates studied exhibited similar cytopathic effects. Besides the occurrence of mature virus particles, a common feature of infected cells is the presence of amorphous, moderately stained dense material to which will be referred to as viroplasm (Fig. 3A, B). They occur next to the regions where virus particles accumulate. The viroplasms usually contain small complexes of electron dense material, often arranged in apparent chains or strings (Fig. 3A, B). To these complexes has been referred to in the past as dense masses (Ie, 1982). They are usually between 30 and 120 nm in diameter and consist of rough cubic, circular or epiliptic profiles. They are bordered by a small zone in which the density fades away into the viroplasm. Some cross sections of these complexes revealed a 5-6 nm periodicity, an observation which confirms earlier results (Ie, 1982). The amount of these complexes, which are considered to be aggregates of nucleocapsids (Ie, 1982; Verkley & Peters, 1983) occurred in varying

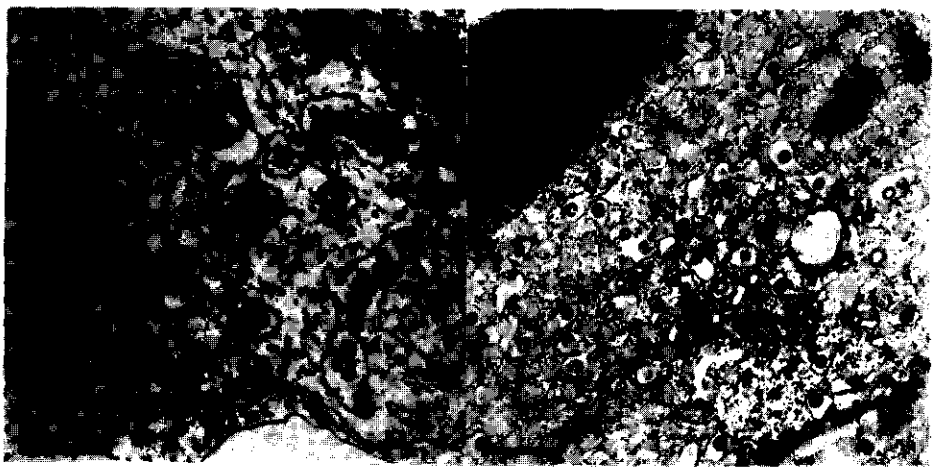

Fig. 1 - Accumulation of virus particles in the cisternae of the endoplasmic reticulum of cells infected with TSWV. (A) Accumulation of particles between membranes of a cell infected with isolate SA-04. (B) Single particles in apparent vesicles in a cell infected with BR-06. Bars: 500 nm.

amounts in the sectioned cells. They are rarely found in field isolates, but abundantly when the same isolates have been maintained in the laboratory for longer periods and being mechanically transmitted for several times.

In almost half of the isolates examined, inclusions with filamentous material were found (Fig. 4). The filaments, about 10 nm thick with a flexous nature, form inclusions in which they are packed either in an unarranged way (Fig. 4A) or in parallel arrays (Fig. 4B). In two of the isolates examined (NL-07 and NL-08) the filaments were rigid, rodlike, and formed criss-cross paracrystalline arrays (Fig. 4B, C); depending on the plane of the section they could be seen as a series of dots, interspersed with lines (Fig. 4D). Similarly arranged filaments have also been described by de Ávila *et al.* (1991) for isolate E-01, designated C-TSWV by these authors, and by Law & Moyer (1990) for an isolate from *Impatiens*. The inclusions formed by these filaments differ in amount, when related to that of the other structures. For some isolates very large numbers of fibrous inclusions were found in infected cells, while for other isolates only small numbers were occasionally seen in a minority of infected cells. The development of the filamentous material might also be time and host dependent, because sometimes they could not be found in samples of the same host, collected at different moments, and were consistently absent in plants of other host species.

Immuno-labelling of thin sections

Immuno-gold antibodies were used to analyze the antigenic composition of the various virus induced structures and to reveal their localization in the infected cells. These studies were made on thin sections of cells infected with five virus isolates of serogroup I and four viruses of serogroup II as characterized by de Ávila *et al.* (1990) (Table 2). Most of the analyses described here below, were performed with BR-01 (serogroup I). The specificity and reactivity of the antibodies were tested on purified virus and nucleocapsid preparations (Fig. 5; Table 2). Polyclonal anti-N serum strongly labelled sections of sedimented purified nucleocapsid fractions and purified virus particles (Fig. 5A, B), showing that these antibodies reacted with free nucleocapsid protein as well as that assembled in virus particles. However, labelled antibodies against glycoprotein, either mono- or polyclonal, reacted only with sedimented purified particles (Fig. 5C),

Table 2. Results of immunolabelling of thin sections using polyclonal antibodies and monoclonals to the tomato spotted wilt virus TSWV serogroup I isolates BR-01 and NL-04.

TSWV	@Induced cytopath. structures	Antibodies											
		Polyclonal						Monoclonal					
		BR-01				NL-04		BR-01					
		V*	NC	G	NSs	V	NC	G1	G2	G3	G4	N1	N2
Serogroup I#													
BR-01	V	++	++	+	-	++	+	+	+	+	+	-	-
	VP	++	++	-	-	++	++	-	-	-	-	-	-
	NCA	++	++	-	-	++	++	-	-	-	-	-	-
NL-04	V	++	++	++	-	++	++	+	+	+	+	-	-
	VP	++	++	-	-	++	++	-	-	-	-	-	-
	NCA	++	++	-	-	++	++	-	-	-	-	-	-
	F	-	-	-	+	-	-	-	-	-	-	-	-
NL-04 *(env.)*	VP	++	++	-	-	++	++	+	+	+	+	-	-
	NCA	++	++	-	-	++	++	-	-	-	-	-	-
	F	-	-	-	+	-	-	-	-	-	-	-	-
E-01	V	++	++	+	-	++	++	+	+	+	+	-	-
	VP	++	++	-	-	++	++	-	-	-	-	-	-
	NCA	++	++	-	-	++	++	-	-	-	-	-	-
	F	-	-	-	+	-	-	-	-	-	-	-	-
DK-01	V	++	++	-	-	o	o	o	o	o	o	o	o
	VP	++	++	-	-	o	o	o	o	o	o	o	o
	NCA	++	++	-	-	o	o	o	o	o	o	o	o
	F	-	-	-	+	o	o	o	o	o	o	o	o
Serogroup II, type I#													
BR-03	V	+	+	+	-	+	+	+	+	+	-	-	-
	VP	+	+	-	-	+	+	-	-	-	-	-	-
	NCA	+	+	-	-	+	+	-	-	-	-	-	-
BR-06	V	+	+	+	-	+	+	+	+	+	-	-	-
	VP	+	+	-	-	+	+	-	-	-	-	-	-
	NCA	+	+	-	-	+	+	-	-	-	-	-	-
Serogroup II, type II#													
SA-05	V	+	+	+	-	+	+	+	+	+	-	-	-
	VP	+	+	-	-	+	+	-	-	-	-	-	-
	NCA	+	+	-	-	+	+	-	-	-	-	-	-
	F	-	-	-	+	-	-	-	-	-	-	-	-
BR-08	V	+	+	+	-	+	+	+	+	+	-	-	-
	VP	+	+	-	-	+	+	-	-	-	-	-	-
	NCA	+	+	-	-	+	+	-	-	-	-	-	-
	F	-	-	-	+	-	-	-	-	-	-	-	-

**V: purified virus, NC: nucleocapsid fraction, G: glycoprotein, NSs: non-structural. @V: virus particles, VP: viroplasm, NCA: nucleocapsid aggregates, F: inclusion of fibrous dense material, env.: morphologically defective particle, -: no immunolabelling, +: weak to moderate positive and ++: strong labelling by gold particles, o: not done. #Serogroup division to the proposal by de Ávila et al. (1990).*

Fig. 2 - Occurrence of TSWV particles in various leaf cells. (A) Low magnification micrograph of a N. rustica plant infected with isolate SA-05 showing a xylem vessel (X) in the final stage of differentiation. Its content is almost completely lysed, but groups of virus particles (arrows) are still identifiable. Most of the surrounding parenchyma cells are infected. (B) Group of virus particles (V) in the lumen of a sieve tube (ST) of a N. rustica plant infected with isolate NL-13. (C) Detail of a plasmodesmata connecting 2 parenchyma cells in a N. rustica leaf infected with isolate BR-03. Note a desmotubule (arrow) in contact with an ER element containing TSWV particle.

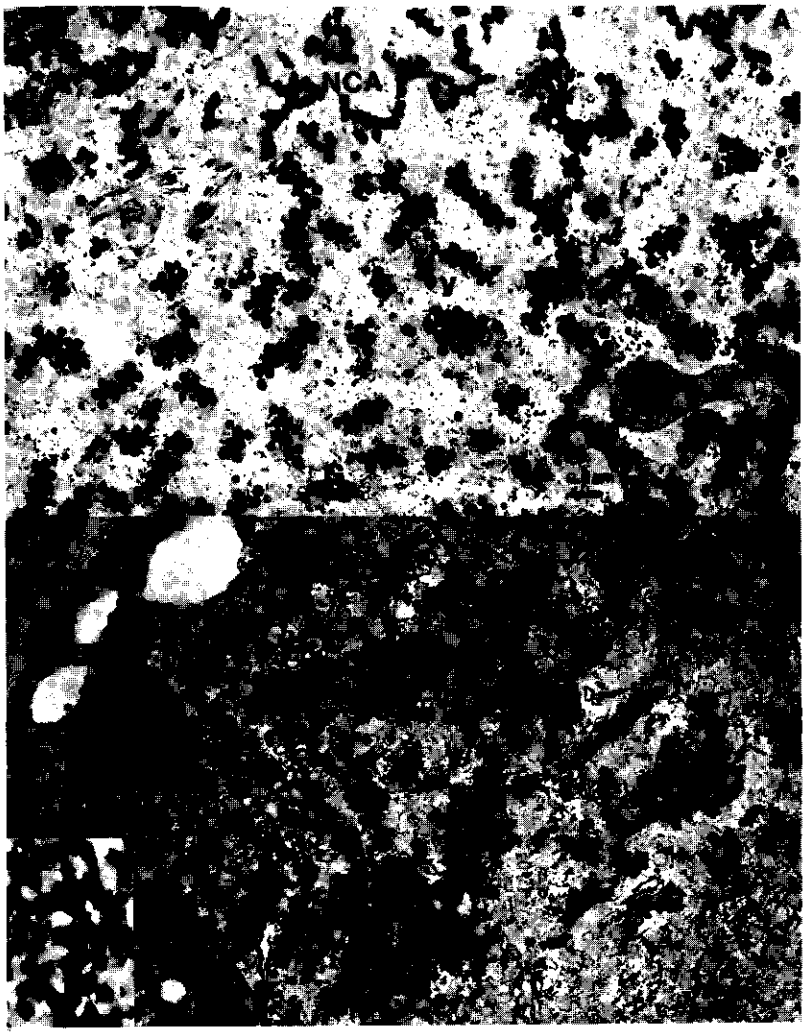

Fig. 3 - Cytopathology of TSWV leaf mesophyll infected cells showing the quantitative differences in the accumulation of virus particles, viroplasm interspersed with strings of nucleocapsid aggregates and inclusions of fibrous material as seen in ultrathin sections. (A) N. rustica infected with isolate NL-12. Accumulation of virus particles (V) in the endoplasmic reticulum cisternae with some nucleocapsid aggregates (NCA), believed to be formed by non-enveloped nucleocapsids, and a small aggregate of filamentous material (F). (B) Accumulation of a large amount of nucleocapsid aggregates (NCA) embedded in an amorphous material of moderate density, the viroplasm (VP), in a D. stramonium leaf cell infected with isolate E-01. Aggregates of fibrous material (F) occur in the lower part of the micrograph. Inset shows details of cross sections of these fibrils, revealing their tubular form. Only a few virus particles (arrowheads) are present. G: Golgi complex; L: lysosome; M: mitochondrion; P: chloroplast.

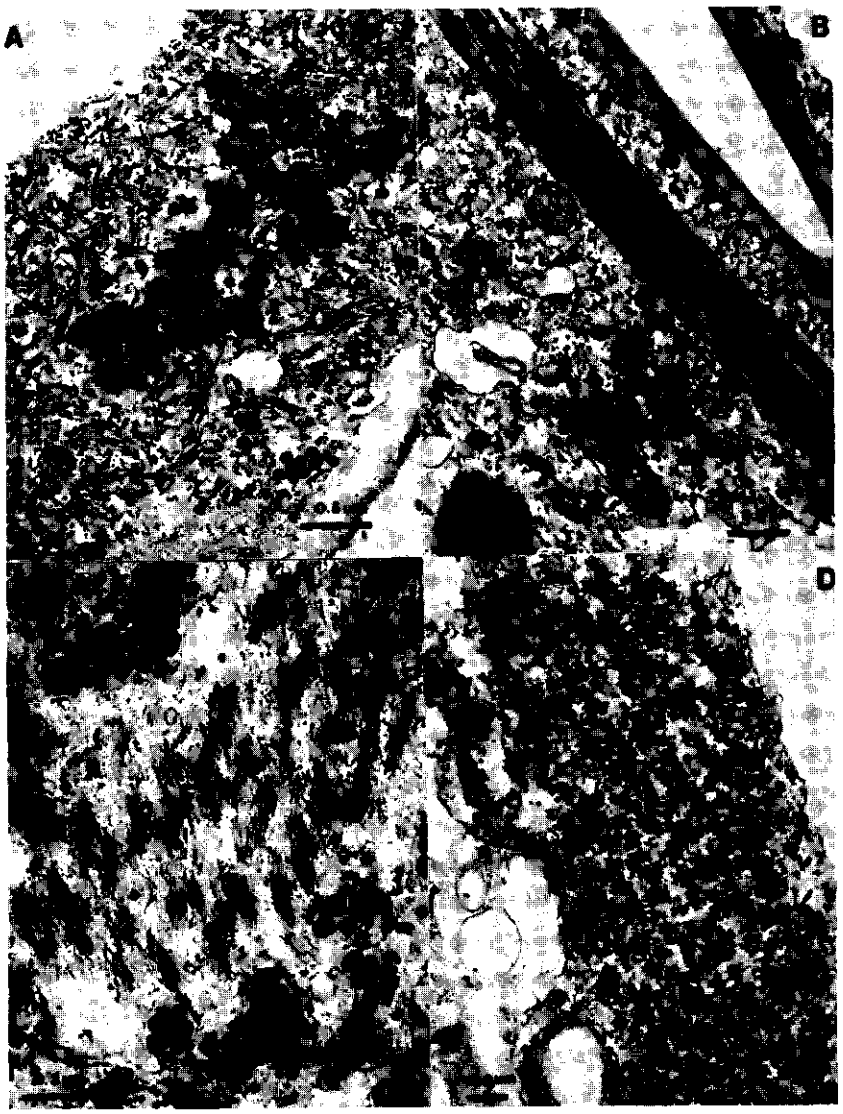

Fig. 4 - Different aspects of aggregates with fibrous material induced by some isolates of TSWV. (A) Large complex of filaments (F) interspersed with nucleocapsid aggregates (NCA) in a N. rustica cell infected with isolate DK-01. (B) A large bundle of parallelly oriented fibers (F) in a pallisade parenchyma cell of N. rustica infected with isolate SA-05. (C) Rigid, rodlike fibers (F), organized in layers of criss-cross, paracrystalline arrays, in a parenchyma cell of N. clevelandii infected with isolate NL-07. Note that virus particles (V) are often somewhat oval shaped. (D) A large aggregate of rigid rods in paracrystalline array (F) in a mesophyl parenchyma cell of a local lesion induced by isolate NL-07 in N. rustica. Rows of alternating dots and lines are observable. M: mitochondrion; V: virus particle.

indicating that the glycoproteins are virtually absent in the purified nucleocapsid protein extracts. It is also evident that a high number of labelled antibodies to the N protein was associated with the core of the virus particles (Fig. 5B) while the antibodies to the G-proteins were often associated with the outer regions of the particles (Fig. 5C).

Analysis of ultrathin sections with immunogold labelled antisera against nucleocapsid protein demonstrates the presence of nucleocapsid protein in virus particles, viroplasms (VP) and the nucleocapsid aggregates (NCA; Fig. 6). Staining of high intensity was obtained using antisera prepared to viruses of the same serogroup (Fig. 6A), but not when antiserum to viruses of other serogroups was used (Fig. 6B; Table 2). This observation indicates that viruses belonging to different serogroups could also be discriminated using gold labelled antibodies.

The inclusions consisting of fibrous material did not give any positive response using either antiserum to intact virus particles or anti-N sera (Fig. 6C, D), but they were specifically tagged (Fig. 6E) by antibodies against the 52 K NSs protein encoded by the S RNA (Kormelink *et al.*, 1991). Gold labelled NSs antiserum did not immunostain virus particles, viroplasm or nucleocapsid aggregates in ultrathin sections.

Antiserum, either polyclonal or monoclonal, to the glycoproteins only probed virus particles in thin sections (Fig. 7). The MAbs (G1-G4) stained the virus particles less intensive than anti-G serum (Fig. 7A, B; Table 2).

Morphogenesis

In approximately 5% of the sectioned cells, structures were found that could be interpreted as intermediate stages of the morphogenetic process of TSWV particle envelopment. Flattened and curved vesicles, with dense material in the concave side were found in cytoplasmatic areas within or near viroplasm and the Golgi bodies (Fig. 8). When these structures were found, the Golgi bodies always contained several single virus particles surrounded by a closely posed membrane (double enveloped particles), while also smooth-surfaced vesicles were present in the neighborhood, forming a distinct and differentiated area in the cytoplasm. Eventually, TSWV-containing endoplasmic reticulum elements were also present in the surrounding area, and some of the small, virus-containing vesicles appeared to be continuous with them (Fig. 8B). Despite the low

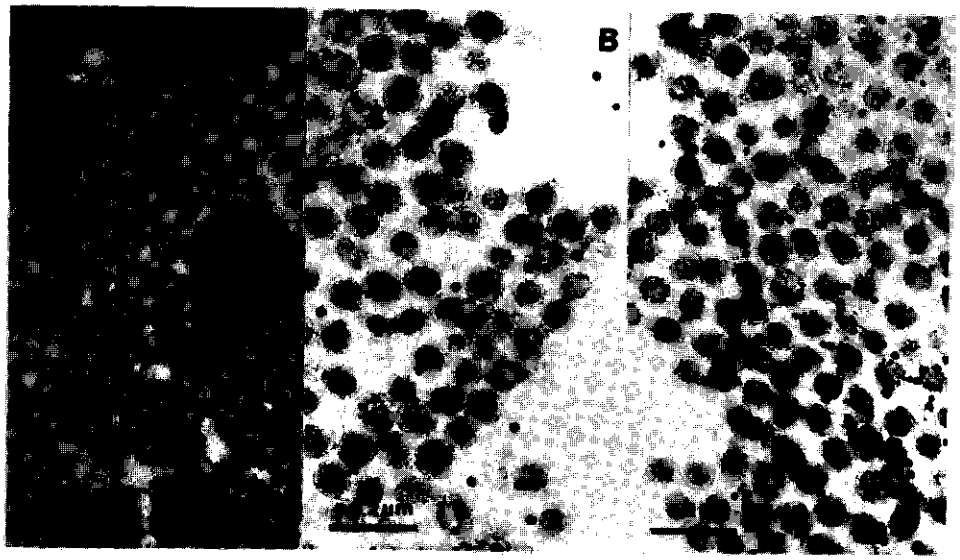

Fig. 5 - Immunogold-labelling of pellets of either purified nucleocapsids (A) or purified virus (B, C) preparations from isolate BR-01 infected plants using antibodies to nucleocapsid protein or glycoprotein of BR-01. (A) Gold particles are mainly associated with nucleocapsid aggregates using anti-N protein serum. (B) Section of a pellet of purified BR-01 virus particles tagged with immunogold labelled with anti-N serum. (C) Section of the same pellet immunostained with polyclonal anti-G serum. Labels occur only on the virus particles.

frequency of these structures, they occurred in most of the cells studied whenever they were observed in a given tissue sample. The possible involvement of the structures found in the morphogenesis of TSWV particles has schematically been pictured in Fig. 9.

DISCUSSION

Examination of ultrathin sections of tissues from plants infected with different isolates of TSWV revealed that essentially all isolates, despite great differences in their macroscopical effects on plants, exhibit similar cytopathic effects. Virus particles accumulate in the cavities of the endoplasmic reticulum, while several types of inclusions occur in the cytoplasm. Concerning a possible discrimation of the isolates studied, it may be concluded that it is difficult to identify a given isolate by the way the virus particles

accumulate, or by the form and amount of intracellular inclusions induced, and to relate these characteristics to symptom severity or the plant species infected. Only isolates which are morphological defect can be discerned by the complete absence of intact virus particles (Ie, 1982; Resende *et al.*, 1991).

Virus particles usually had a circular profile, but sometimes particles with an elliptical profile were observed, especially in samples from plants infected with the NL-07 isolate. The particle size varies between 70 and 110 nm. These dimensions, which have also been measured in leaf dip as well as in purified preparations, may reflect the number of nucleocapsids enveloped. Some particles might contain more nucleocapsids and others less than the three forming a complete genome.

Some confusion exists in previous reports on the description and nomenclature of the inclusions which appear in the cytoplasm during TSWV infection. Terms like viroplasm, dense strands, diffuse masses or material, amorphous masses, densely staining granular material, etc. are used (Kitajima, 1965; Milne, 1970; Francki & Grivell, 1970; Ie, 1971, 1982). The following picture emerges from the studies by these authors and the one reported here. Large clusters consisting of moderately dense staining material are usually found in the cytoplasm. They are and will be coined in this report as viroplasms. Immersed in the viroplasms, aggregates of more densely stained material, often arranged in chain or string-like structures, occur. These aggregates have been designated in the past with different names as mentioned above and which have also been described for morphologically defective isolates (Ie, 1982). These aggregates mainly consist of nucleocapsid material (Ie, 1982; Verkley & Peters, 1983; de Ávila *et al.*, 1991).

In addition, fibrous material, accumulating in different ways, occurs in at least half of the isolates studied. These inclusions can readily be distinguished from the chain-like accumulations of the nucleocapsid aggregates in the viroplasm by their fibrous nature.

The viroplasms immunostain with anti-N, but not with anti-G immunoglobulins, and might represent focal accumulations of nucleocapsid material and replicating RNA complexes.

The nucleocapsid aggregates react intensively with anti-N protein sera and not at all with anti-G protein sera. They represent aggregates of nucleocapsid material which are not enveloped or fail to acquire an envelope during the replication of the virus. This may

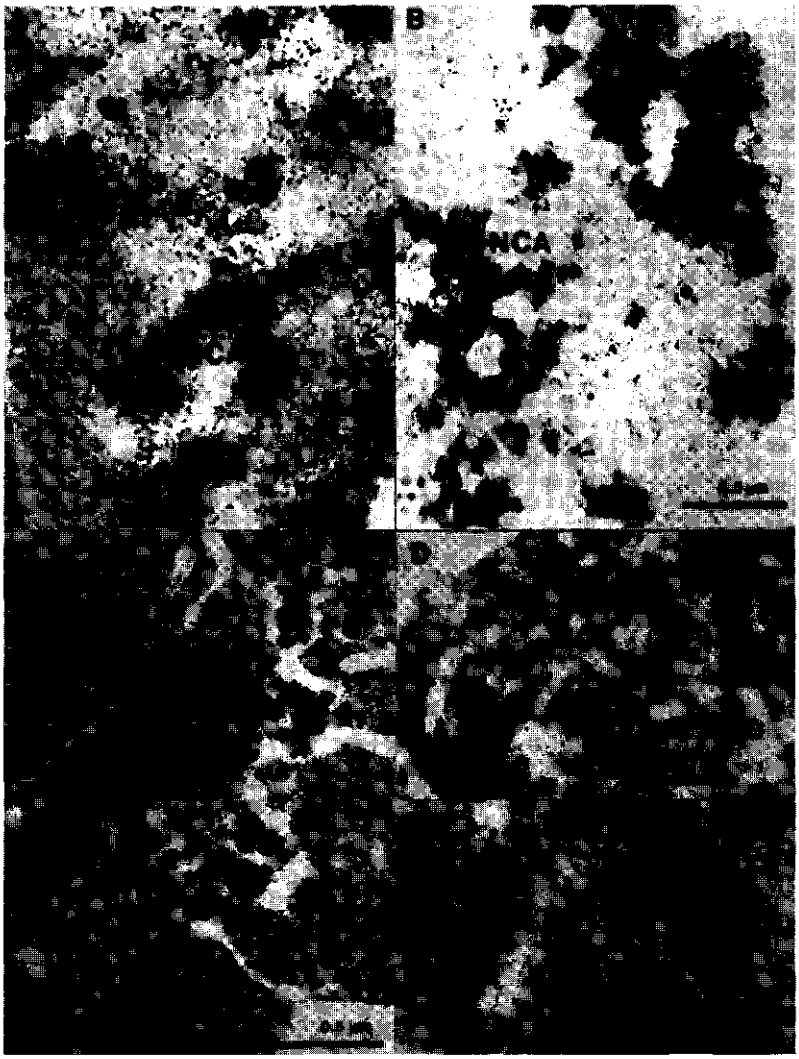

Fig. 6 - Immunostaining of viral material in cells infected with different isolates of TSWV using anti-N and anti-NSs serum. Intense gold labelling of the nucleocapsid aggregates (NCA) in cells infected with BR-01 (A) and the almost absence of any label on BR-03 nucleocapsid aggregates (B) using anti-N serum of isolate BR-01. (C) Labelling of viroplasm (VP) and virus particles (V) in a N. rustica cell infected with E-01 after incubation with anti-N serum of BR-01. Note that the fibrous inclusions (F) are not labelled. (D) Cell infected with a defective form of isolate NL-04 (NL-01(env-)) and immunostained with anti-N serum of BR-01. Gold particles are found on the nucleocapsid aggregates (NCA), but not on the fibrous inclusion (F). (E) Immunostaining of a paracrystalline array of fibers (F) but not of virus particles (V) in cells infected with isolate NL-07 with anti NSs serum.

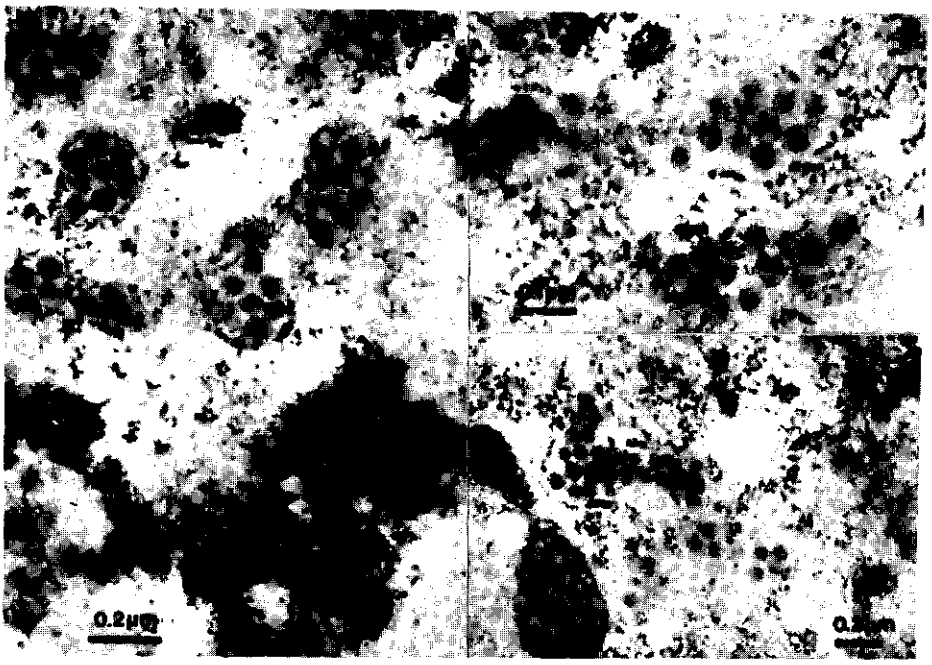

Fig. 7 - Immunolabelling studies of thin sections using either anti-G serum or monoclonals to the BR-01 G protein. (A) Labelling of BR-01 virus particles (V) of isolate BR-01 but not of nucleocapsids aggregates (NCA) with anti-G serum. (B, C) Immunostaining of virus particles (V) of isolate E-01, using the monoclonal antibodies, G1 (B) or G4 (C) directed against G protein, respectively. F: fibrous inclusion; M: mitochondrion.

be caused by an unbalance between nucleocapsid and glycoprotein production, the synthesis of defect glycoproteins, or even the absence of glycoproteins, caused by mutations or deletions in the M RNA (Verkleij & Peters, 1983; Resende *et al.*, 1991). The sizes of these aggregates are often almost equal to those of the complete virus. This might suggest that they are precursors of the nucleocapsids in mature virus particles. However, this will probably not be the case as these free aggregates stain much more intense than the core of the virus particles.

The structures formed by the fibrous material do not share any antigenic determinant associated with the virus particle. They only reacted with serum against the non-structural protein encoded by S RNA (Kormelink *et al.*, 1991; Kitajima *et al.*, 1992).

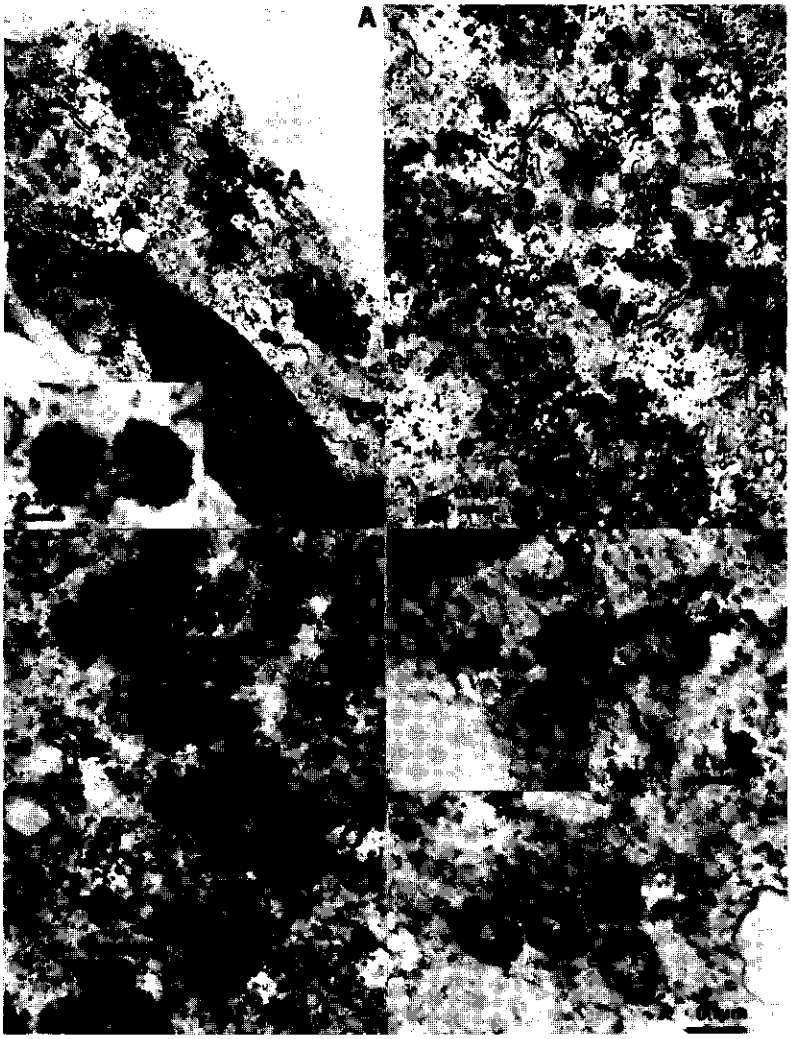

Fig. 8 - Details of TSWV particles in a presumptive process of morphogenesis. (A) A general view of a cytoplasmic area in a leaf mesophyl parenchyma cell of N. rustica infected with isolate BU-03. Groups of double enveloped (DE) particles appear associated with viroplasm (VP). Arrow points to a pair of budding particles, detail of which can be seen in the inset. They seem to be small flattened vesicles arching due to the association of dense material, presumably nucleocapsids, with the membrane in the concave side. NCA: nucleocapsid aggregates; M: mitochondrion; P: chloroplast. (B) Viroplasm (VP) interspersed with double enveloped (DE) particles and some few budding particles (arrows) in a parenchyma cell of N. rustica infected with isolate BU-03. Some DE appear to be fusing with endoplasmic reticulum (arrowhead). (C) Group of budding particles (arrows) in a leaf parenchyma cell infected with isolate BR-08. Note the density of the membranes (arrowheads) embedded in the viroplasm as well as that of arching vesicles, (D, E) Details of the presumptive budding of TSWV particles, in N. rustica leaf cells, infected with isolates NL-07 and BR-08 respectively.

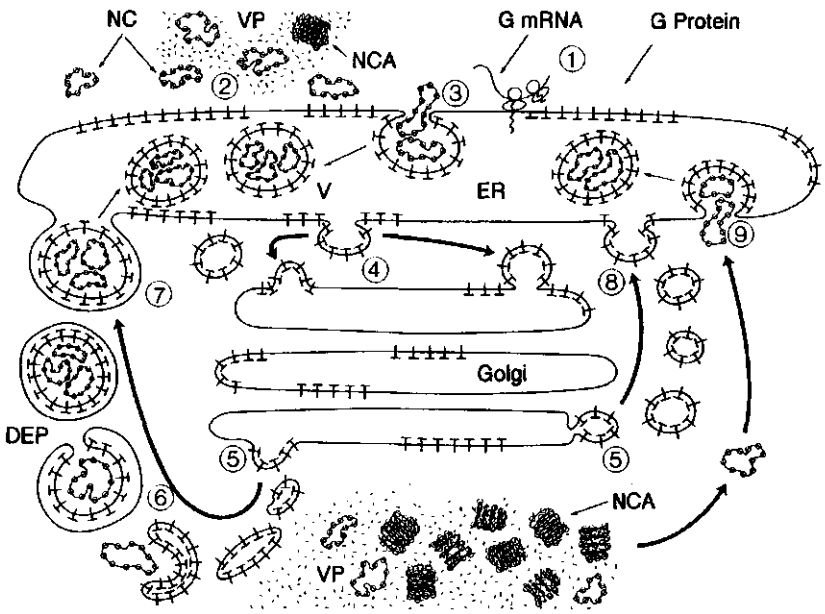

Fig. 9 - Schematic representation of three possible pathways of TSWV-morphogenesis as described in the Discussion. NC: nucleocapsids; NCA: nucleocapsid aggregate; V: virus particles; VP: viroplasm; ER: endoplasmic reticulum; DEP: double enveloped particle.

The function of this protein and the need of its accumulation in fibers has remained unknown.

Mature virus particles were never seen in the plasmodesmata, confirming previous observations, although desmotubules were connected with the ER cisternae of adjacent cells, containing virus particles. Thus cell-to-cell spread of the TSWV infection probably not involves enveloped particles but presumably free nucleocapsids. In this regard, the enveloped particles occasionally seen in sieve tubes, and more frequently in xylem vessels, are probably not relevant for the spread of infection throughout the plant as such, as they could have no means to leave the vascular system.

Two serogroups have been distinguished using polyclonal antisera and a panel of monoclonal antibodies against nucleocapsid protein or glycoprotein of isolate BR-01 (de Ávila *et al.*, 1990). Although not as clear as in ELISA tests, immunogold labelling experiments lent convincing support for this proposed distinction. Monoclonal antibody

G4, which did not react with particles of the isolates of serogroup II, also failed to react in immunolabelling experiments (Fig. 7C). A similar trend was noticed using BR-01 and NL-04 anti-N sera. Viroplasm and nucleocapsid aggregates in cells infected by serogroup I isolates were more strongly immunostained than those produced by serogroup II viruses. This observation qualitatively confirms the separation of the different TSWV isolates in serogroups as obtained by ELISA (de Ávila *et al.*, 1990). The serum prepared against glycoprotein G1 did not discriminate between isolates of serogroup I and II. This suggests that the glycoproteins are more conserved than the nucleocapsid proteins, a fact previously noted in ELISA studies by de Ávila *et al.* (1990). Since this serum failed to label virus particles in suspension (data not shown), it is likely that it might be directed against some internal epitopes.

The way complete TSWV particles acquire their membrane remains an enigma. Finding structures which can be interpreted as maturing particles is a rarity in TSWV infection. Francki *et al.* (1985) argued that this might be due to the fact that the envelopment is very short lived. Since the replication of the virus proceeds in various stages in a cell and certainly in different cells it might be expected that maturing particles will occasionally be found in some cells. In a few occasions during this study, structures were observed which could be interpreted as having a function in the process of maturation.

Figure 9 depicts diagrammatically three possible maturation pathways occurring either at the endoplasmic reticulum (ER) or Golgi derived membranes, and excludes *de novo* formation of viral envelopes as inferred by Ie, (1971). In the first proposed pathway nucleocapsids formed by a condensation of viral RNA with N protein bud through the endoplasmic membrane and become enveloped with a glycoprotein-studded membrane (Fig. 9, step 1 - 3). Alternatively, the glycoproteins may migrate to the Golgi complex for additional changes in the polysaccharides before the nucleocapsids are enveloped (Fig. 9, step 4). In one pathway, supported by the structures shown in Fig. 8 and depicted by step 5 to 7 in Fig. 9, the envelopment could take place on the Golgi derived vesicles. The budding of Bunyaviruses, of which TSWV is a member which will thus have evolutionary relationships and many molecular properties and structures in common with this large family of animal infecting viruses (Milne & Francki, 1984; de Haan *et al.*, 1989), is known

to occur at the Golgi complex (Dubois-Dalcq *et al.*, 1984; Schmaljohn & Patterson, 1990). Some of our micrographs seem to support this view, especially those depicting small arches formed by flattened and curved vesicles with dense material in the concave part (Fig. 8D, E). These flattened vesicles may form a double membraned vesicle during the incorporation of nucleocapsids, resulting in a mature virus particle within a very tight vesicle (DEP). These vesicles would be equivalent to the so called double enveloped TSWV and Punta Toro virus particles, respectively, described by Milne, (1970) and Smith & Pifat (1982). Eventually, these virus containing vesicles could fuse together or with the ER membrane, producing larger cavities with many particles. Finally, in the third pathway the glycoproteins could be reinserted into the ER membrane after maturation of the polysaccharides in the Golgi system. Particle formation may then occur at ER membranes (Fig. 9, steps 8 and 9). Identification of the G1 glycoprotein on the endoplasmatic reticulum or Golgi-complexes may be indicative for the maturation pathway of TSWV. However, experiments aimed to demonstrate the presence of glycoprotein antigens on the membranes of the endoplasmic reticulum and Golgi apparatus derived membranes, were unsuccessful. It was noticed that, when these double enveloped structures were found, they occurred in most of the cells studied. This suggests that there might be some sort of synchrony in the maturation of the virus in a number of cells. How such a synchrony can be achieved by the replicating virus is still an open question.

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CHAPTER 4 CHARACTERIZATION OF A DISTINCT ISOLATE OF TOMATO SPOTTED WILT VIRUS (TSWV) FROM *Impatiens* sp. IN THE NETHERLANDS

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SUMMARY

This paper describes the characterization of a distinct tomato spotted wilt virus (TSWV) isolate from New Guinea impatiens (*Impatiens* sp.) in The Netherlands. Several plant species responded in a hypersensitive way to this isolate, denoted NL-07, but the symptoms on *Impatiens* do not differ from those caused by other TSWV isolates. Except that the S RNA is slightly smaller, the protein and RNA composition is identical to that established for the isolates described. Comparative studies using ELISA, Western blotting and immunogold labelling techniques clearly demonstrate that NL-07 has a distinct nucleocapsid protein from BR-01 (common type TSWV), BR-03 and SA-05 and moreover it is very closely related to TSWV-I. Northern blot analysis, supporting the serological data, revealed a lack of significant nucleotide sequence homology between BR-01, NL-07 and TSWV-I. Cytopathological studies of NL-07 displayed clusters of virus particles in the cisternae of the endoplasmatic reticulum together with paracrystalline arrays of rigid rod-like fibers, formed by the non-structural protein. Our results show that TSWV classification based on ELISA using monoclonal and polyclonal antibodies against the nucleocapsid protein is stable and safe, even when deletions in the L RNA segment occur or when the virus becomes morphologically defective. We propose to name this virus *Impatiens* necrotic spot virus.

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INTRODUCTION

Tomato spotted wilt virus (TSWV) is transmitted by several thrips species in a persistent manner and has one of the broadest host ranges among plant viruses (Peters *et al.*, 1991). It causes severe outbreaks in a large variety of crops grown in tropical and subtropical climatic zones. Interest for this virus has been evoked due to the recent and rapid spread of the thrips *Frankliniella occidentalis* Perg. over the Northern Hemisphere, which is accompanied with severe outbreaks of TSWV infections.

TSWV contains four structural proteins: a nucleocapsid protein N (29 K), two glycoproteins, G1 (78 K) and G2 (58 K), and a large protein L of approximately 200 K (Mohamed *et al.*, 1973, Tas *et al.*, 1977). The viral genome consists of three single stranded RNA molecules, denoted S RNA (2916 nucleotides), M RNA (approximately 5000 nucleotides) and L RNA (8897 nucleotides) (de Haan *et al.*, 1990; 1991; Maiss *et al.*, 1991).

Due to its molecular composition, mode of transmission, particle morphology, genome structure and coding strategy, TSWV has been classified within the Bunyaviridae as a sole member in the newly created genus tospovirus (de Haan *et al.* 1989a, Elliott, 1990; Francki *et al.*, 1991).

Since there were indications of the presence of different strains of TSWV (Best & Gallus, 1953; Best, 1968), serological differentiation of isolates is currently a topic of intensive research. Law & Moyer (1990) described a TSWV-like virus denoted TSWV-I, with a nucleocapsid protein, serologically unrelated to that of the common TSWV type. cDNA clones to TSWV-I S and M RNA did not hybridize with the corresponding RNAs of the common type. Wang & Gonsalves (1990) compared 30 TSWV isolates from several countries using ELISA. Out of these, 11 isolates could not consistently be detected. They were not further classified in terms of serogroups or -types. A TSWV classification using polyclonal and monoclonal antibodies against nucleocapsid protein in ELISA has recently been proposed by de Ávila *et al.* (1990). These authors were able to classify 20 TSWV isolates from several countries into two serogroups and three serotypes using polyclonal and monoclonal antibodies.

Here we describe a new TSWV like isolate, NL-07, found in The Netherlands with

completely different serological properties compared to BR-01, an isolate belonging to serogroup I (de Ávila *et al.*, 1990). Evidence is presented that NL-07 most likely represents TSWV-I reported by Law & Moyer (1990).

METHODS

Virus maintenance and host range

NL-07 was originally recovered from a naturally infected New Guinea impatiens (*Impatiens* sp.) plant in a glasshouse in The Netherlands. Virus was maintained in the originally infected plant and in addition, stored in liquid nitrogen. NL-07 was propagated in *Nicotiana benthamiana* Domin. plants. TSWV-I was kindly provided by Dr J.W. Moyer (North Carolina State University). The other isolates used in this study were BR-01, a serogroup I isolate, BR-03 and SA-05, which are both serogroup II isolates (de Ávila *et al.*, 1990). The characters refer to country codes used for cars and the digits to the isolate from that country in our collection. Test plants were mechanically inoculated using extracts from infected *N. benthamiana* plants in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium sulphite, after dusting the leaves with 500 mesh Carborundum powder.

Nucleocapsid protein and RNA purification

Nucleocapsid preparations of NL-07 and BR-01 have been extracted using the procedure described by de Ávila *et al.* (1990) with some slight modifications. Pellets obtained after centrifugation on 30% sucrose cushions, were resuspended in 0.01 M citrate buffer, pH 6.0, and centrifuged at 149,000 *g* for 18 h in gradients prepared by mixing two solutions of 25 and 50% of Cs_2SO_4. Nucleocapsid bands were collected and dialyzed three times against citrate buffer. The nucleocapsid material was finally collected into pellets by centrifugation, resuspended in 0.01 M citrate buffer, pH 6.0, and used in different studies.

For antiserum production, the nucleocapsid bands collected from the Cs_2SO_4 gradients were centrifuged through a 20% sucrose cushion of 2 ml at 189,000 *g* for 3 h.

The nucleocapsid protein yield was estimated using the BIO-RAD Protein Assay.

RNA was extracted from nucleocapsid preparations after adding SDS to a final concentration of 1% (w/v) followed by phenol and subsequent phenol/chloroform (1:1) (v/v) extractions. The RNA was precipitated with ethanol and analyzed by electrophoresis on 1.2% agarose gels.

Complementary DNA cloning (cDNA) and Northern blot analysis

RNA was transferred from agarose gels to nitrocellulose membranes by capillary blotting and hybridized to six ^{32}P-labelled cDNA probes specific for TSWV strain BR-01 using standard methods (Maniatis *et al.*, 1982). Three cDNA probes directed to the L RNA were used. One, designated 70, was located at the 5' end from nucleotide position 62 to 1243. Probe 806 was located from nucleotide position 3961 to 4455 in the central region and probe 662 was directed to an RNA stretch between the nucleotides 7032 and 8878 at the 3' end (Fig. 6). cDNA probe pTSWV-vcORF is directed to a stretch between the nucleotides 1982 to 2882 of the S RNA, comprising the complete N gene. Probe PTSWV-514 ranges from nucleotides 13 to 1605 covering most of the NSs gene (de Haan *et al.*, 1990). One M RNA-specific cDNA clone of 600 bp (201) was also used in this study.

Two other cDNA clones denoted pNL-07.1L (1.8 Kb) and PNL-07.2S (1 Kb), corresponding to the L and S RNAs, of isolate NL-07 respectively were synthesized, cloned and selected as described previously (de Haan *et al.*, 1989b).

Antisera production

Purified nucleocapsid preparations of NL-07 containing 61, 123 and 300 mg protein were emulsified with incomplete Freund's adjuvant (1:1) (w/v) and injected intradermally into rabbits at two-weekly intervals. Two weeks after the last injection, blood was collected several times over a period of four months. An antiserum against the TSWV-I nucleocapsid fraction was kindly supplied by Dr J.W. Moyer. The production of antisera against the nucleocapsids of BR-01, BR-03 and SA-05 have been described previously (Huguenot *et al.*, 1990; de Ávila *et al.*, 1990). In electron microscopical studies sera were used against purified nucleocapsids of isolates NL-07 and BR-03, against the G1

glycoprotein of BR-01 (Resende, R. de O., unpublished results) and against the non-structural protein NSs of BR-01 (Kormelink *et al.*, 1991).

Serological analysis

The IgG fractions of all antisera were partially purified and conjugated with alkaline phosphatase as described by Avrameas (1969). The polyclonal antisera against nucleocapsid proteins were used in a double antibody sandwich ELISA format (Clark & Adams, 1977; Resende *et al.*, 1991a).

Series of ten-fold diluted extracts of *N. benthamiana* plants infected with NL-07, TSWV-I and BR-01 and purified nucleocapsid protein preparations (10 to 1000 ng) were analyzed in this format.

A panel of six monoclonal antibodies of BR-01, two directed to the nucleocapsid protein and four directed to the glycoproteins were included in this study using a triple antibody ELISA format (Huguenot *et al.*, 1990; de Ávila *et al.*, 1990).

Nucleocapsid protein analysis

Purified nucleocapsid preparations of the TSWV isolates and extracts from healthy and infected *N. benthamiana* plants were analyzed on SDS-PAGE 15% gels (Laemmli, 1970). Proteins were transferred to Immobilon-P transfer membranes (Millipore) (Towbin *et al.*, 1979) and treated with polyclonal antisera against the nucleocapsid proteins of BR-01, NL-07, TSWV-I, BR-03 and SA-05.

Purified nucleocapsid preparations were mixed with dissociation buffer in a ratio of 1:1 (v/v). Plant extracts were ground in a 1:5 (w/v) ratio with PBS-T (phosphate buffered saline supplemented with 0.05% Tween 20) centrifuged for 2 min at 100 *g* and mixed in a 1:1 (v/v) ratio with dissociation buffer.

The IgG fraction of each antiserum and the goat anti-mouse IgG alkaline phosphatase conjugate (1 μg/ml) were used in 1000-fold dilutions in PBS and incubated for 1 h. The bands were visualized using the alkaline phosphatase conjugate-nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Sigma) system according to Leary *et al.* (1983).

Electron microscopy

Leaf-dip preparations from infected plants were negatively stained with a neutral solution of 1% uranyl acetate in water.

In immunolabelling studies leaf tissue was embedded in Spurr's medium after fixation in modified Karnovsky fixative, and post-fixation in osmic acid. The sections made were stained with uranyl acetate and Reynold's lead citrate. The viral antigens were detected in the aldehyde-fixed, LRGold-embedded sections of infected leaf material (van Lent *et al.*, 1990)

RESULTS

Host range and symptoms

Symptoms of NL-07 (Table 1 and Fig. 1A) on naturally infected *Impatiens* sp. plants consisted of necrotic spots, often with concentric necrotic rings on some young but full-grown leaves. However, most of the leaves remained symptomless. Stem necrosis could be observed on some infected plants. Except for *Vigna unguiculata* (L.) Walp. on which NL-07 induced clearly distinct light brown local lesions, the symptoms noted did not differ from those caused by other TSWV isolates.

Various solanaceous species were found to be susceptible to NL-07 and TSWV-I. Mechanical inoculation of young *N. benthamiana, Nicotiana clevelandii* A. Gray and *Capsicum annuum* L. plants resulted in necrotic and chlorotic local lesions on the inoculated leaves followed by vein necrosis, mosaic or mottling and leaf-deformation on full-grown leaves. The virus usually killed infected *N. benthamiana* plants within 12 to 14 days after inoculation in a process in which the leaves apparently wilted (Fig. 1B).

NL-07 and TSWV-I only produced local lesions on the inoculated leaves of all other host plants tested including the solanaceous species *Lycopersicon esculentum* Mill., *Nicotiana tabacum* L. Samsum NN and *Petunia hybrida* Vilm. Plants of *Emilia sonchifolia* (L.) DC. respond with local lesions on the inoculated leaves and a mosaic on systemically infected leaves (Table 1). Compared to TSWV-I, the symptoms induced by NL-07 are identical, but usually slightly more severe (data not shown).

Fig. 1 - (A) Impatiens sp. plant naturally infected with NL-07, a TSWV isolate found in The Netherlands. (B) Nicotiana benthamiana plants mechanically inoculated with NL-07. From left to the right: healthy plant, infected plants 8, 12 and 14 days after inoculation.

Table 1. Symptomalogical responses of several hosts to NL-07 isolate.

Host plants	Reaction on host plants* local	systemic
Amarantaceae		
Gomphrena globosa L.	NS, VN	
Balsaminaceae		
Impatiens sp.	CNR	SN
Chenopodiaceae		
Chenopodium quinoa Wild	CL (pp)	
C. amaranticolor Coste & Reyn	NL (pp)	
Compositae		
Emilia sonchifolia (L.) DC.	ChR	Mo
Zinnia elegans Jacq.(a)	ChR	(VC, YM)
Cucurbitaceae		
Cucurbita sativus L. (b)	CS	
Leguminosae		
Vigna unguiculata (L.) Walp.(c)	NL	
Solanaceae		
Capsicum annuum L. (d)	CS	(Mo)
Datura stramonium L.	CNR	(CS)
Lycopersicon esculentum Mill.(e)	CNR	
Nicotiana benthamiana Domin.	NS	VN, LD, D
N. clevelandii A.Gray	ChR	VC, M, LD
N. glutinosa L.	CNR	VC, M, LD
N. rustica L.	NS	(M)
N. tabacum L. Samsum	CNR	
N. tabacum L. Samsum NN	CNR	
Petunia hybrida Vilm.(f)	NS	

**NL (pp): pin-point necrotic lesions; CL (pp): pin-point chlorotic lesions; ChR: chlorotic rings; CNR: concentric necrotic ring; CS: chlorotic spots; NS: necrotic spots; VC: vein clearing; VN: vein necrosis; M: mosaic; Mo: mottling; YM: yellow mosaic; LD: leaf-deformation; SN: stem necrosis; D: death of the plant; ( ): erratic systemic infection. (a) cultivar Reuzenbloemig; (b) cultivar Lange gele tros; (c) cultivar California Blackeye; (d) cultivar Westlandse zoete; (e) cultivar Moneymaker; (f) cultivar Pink Beauty.*

Protein analysis

SDS-PAGE experiments revealed that the N protein of NL-07 and TSWV-I have molecular masses of approximately 28 K, which is slightly smaller than that of BR-01 (28.8 K)(Fig. 2).

Antisera produced to purified virus preparations of BR-01 (data not shown) and to

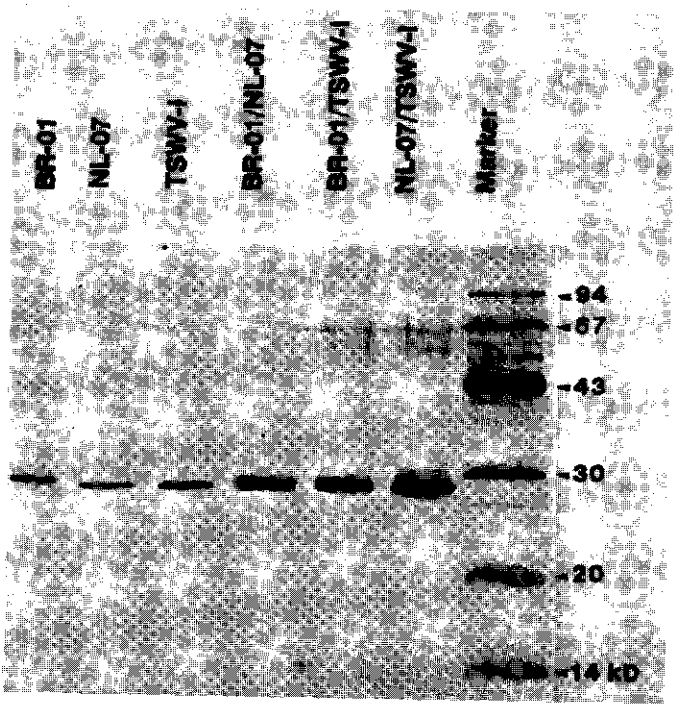

Fig. 2 - SDS-PAGE of the nucleocapsid proteins of three TSWV isolates. From left to the right: BR-01 (lane 1), NL-07 (lane 2), TSWV-I (lane 3), BR-01 and NL-07 (lane 4), BR-01 and TSWV-I (lane 5), NL-07 and TSWV-I (lane 6) and markers (lane 7).

purified nucleocapsids of BR-01, BR-03 and SA-05 did neither react with extracts of plants infected with the isolates NL-07 and TSWV-I (Fig. 3A), nor with nucleocapsid preparations of these viruses (data not shown). Antisera produced to the nucleocapsid proteins of NL-07 and TSWV-I reacted with extracts from plants infected with these viruses in homologous as well as in heterologous ELISA experiments (Fig. 3B). Using infected plant extracts or purified nucleocapsids the isolates BR-01, BR-03 and SA-05 did not react with antisera to the nucleocapsid proteins of NL-07 (Fig. 3B) and TSWV-I (data not shown). Antisera against purified virus preparations of NL-07 and TSWV-I were not tested, as these viruses could not be purified using the method described by Tas *et al.* (1977). The results obtained strongly suggest that the nucleocapsid proteins of NL-07 and TSWV-I are serologically close related, but almost completely distinct from those of BR-01, BR-03 and SA-05. In addition, both isolates did not react with the panel of monoclonal antibodies, directed to the nucleocapsid protein and to the membrane

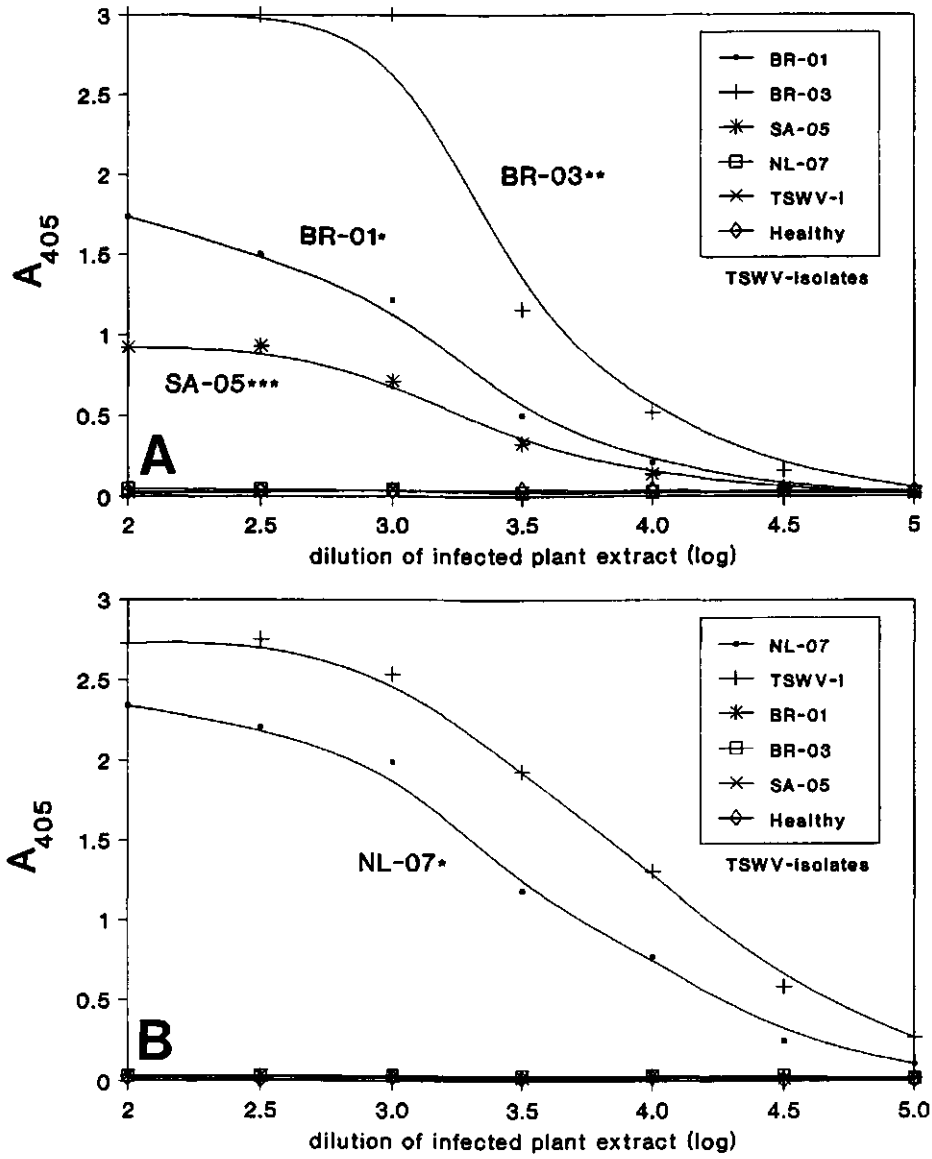

Fig. 3 - ELISA reactions with extracts from plants infected with five TSWV isolates and an extract from a healthy plant. (A) Reaction with polyclonal antisera against nucleocapsid protein of BR-01, BR-03** and SA-05***. (B) Reaction of polyclonal antisera against NL-07* nucleocapsid protein. The A_{405} was measured 45 min after substrate addition.*

glycoproteins of isolate BR-01 (data not shown) (Huguenot *et al.*, 1990; de Ávila *et al.*, 1990). The isolates BR-03 and SA-05 gave positive reactions with these MAbs (de Ávila *et al.*, 1990). Extracts from plants infected with NL-07 and TSWV-I and purified nucleocapsid protein preparations of these isolates reacted on Western blots only with antisera to purified nucleocapsids of NL-07 and TSWV-I (Figs. 4 and 5). However, a very faint reaction was observed between NL-07 nucleocapsid protein and antisera to purified nucleocapsid preparations of SA-05 and BR-03, indicating the existence of some common internal epitopes on the nucleoproteins of these isolates. On the other hand, on Western blots the nucleocapsid proteins of NL-07 and TSWV-I did not cross-react with antisera against nucleocapsid proteins of isolates BR-01, BR-03 and SA-05, supporting the results from the ELISA experiments that these isolates have a nucleocapsid protein which is distinct from that of NL-07 and TSWV-I.

RNA analysis

The estimated sizes of the genomic RNA segments of NL-07 and TSWV-I are identical to that of BR-01 (Fig. 7). The differences observed on the mobility is due to the source used (purified nucleocapsids or virus preparations) to extract the RNA (data not shown). The studied lines of NL-07 and BR-01 contain one extra RNA segment of approximately 3000 nucleotides in length. Northern blot hybridization experiments revealed that these RNA molecules represent a defective L RNA segment (Fig. 7).

No nucleotide sequence homology could be detected between the M and S RNAs of BR-01 and NL-07 using ^{32}P-labelled cDNA probes of BR-01 and NL-07. A very faint reaction with a small region in the middle of the L RNA segment was observed using the BR-01 probe 806 (Figs. 7 and 8).

Electron microscopy

In electron microscopical studies, typical TSWV-like particles were found in leaf-dip preparations of *N. clevelandii* plants infected with NL-07 (Fig. 9A). In tissue sections, aggregates of virus particles were observed in large amounts in the endoplasmic reticulum cisternae in cells of *Impatiens* plants naturally infected by this isolate (Fig. 9B). Studies on thin sections of *N. clevelandii*, infected with NL-07, showed that virus particles

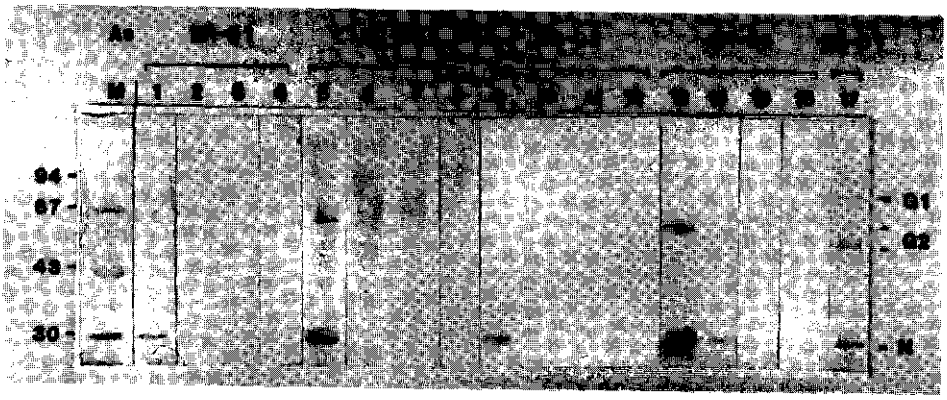

Fig. 4 - Western blot analysis of extracts from healthy and infected TSWV plants. The plants were infected with BR-01 (lanes 1 and 5), NL-07 (lanes 2, 6, 10, 14), TSWV-I (lanes 3, 7, 11, 15), BR-03 (lane 9), SA-05 (lane 13), purified preparation of the BR-01 isolate (lane 17) and healthy plant extract (lanes 4, 8, 12, 16). The antisera (As) used are towards denatured BR-01 nucleocapsid protein (N) (lanes 1 to 4), towards BR-01 native N (lanes 5 to 8), towards BR-03 native N (lanes 9 to 12), towards SA-05 native N (lanes 13 to 16) and towards purified virus (Cv) of the BR-01 isolate (lane 17), marker (M).

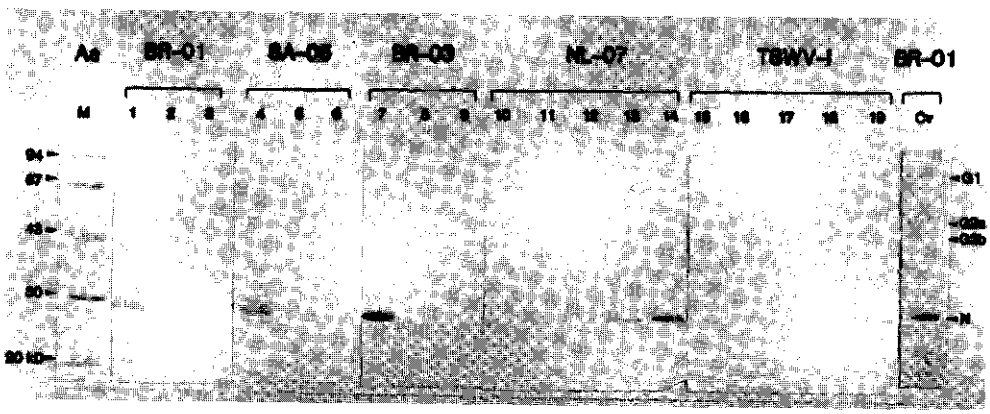

Fig. 5 - Western blot analysis of the nucleocapsid protein of the TSWV isolates BR-01 (lanes 1, 10, 15), NL-07 (lanes 2, 5, 8, 13, 18), TSWV-I (lanes 3, 6, 9, 14, 19), BR-03 (lanes 7, 11, 16) and SA-05 (lanes 4, 12, 17). The antisera used were prepared towards the BR-01, SA-05, BR-03, NL-07 and TSWV-I nucleocapsid fractions. Purified BR-01 preparation (Cv), marker (M).

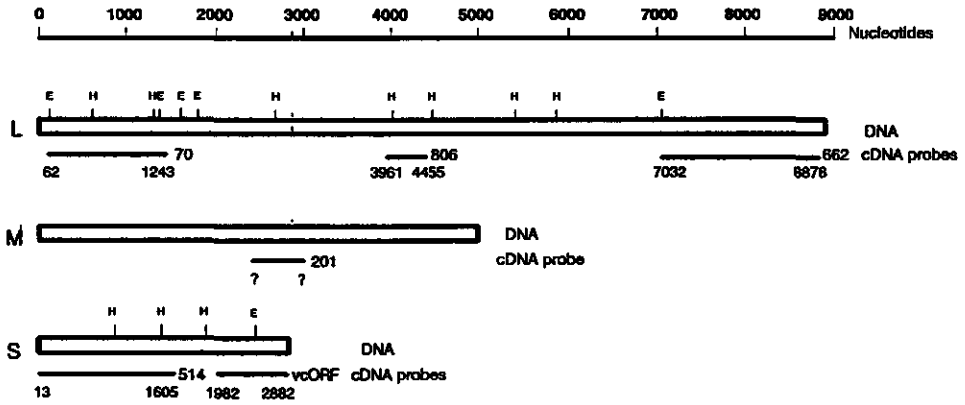

Fig. 6 - Representation of the restriction maps corresponding to TSWV L, M and S RNA of isolate BR-01. The positions of the cDNA probes used are indicated on the restriction map. The sites for the restriction enzymes EcoRI (E) and HindIII (H) are indicated.

and nucleocapsid aggregates, designated earlier as dense masses (Ie, 1982), occurred in mesophyl cells (Fig. 9C). Paracrystalline arrays of rigid rod-like fibers were observed in parenchyma leaf cells of the same host (Fig. 9D).

Immunogold decoration studies

Immunogold decoration experiments supported the results obtained by ELISA, Western and Northern blot studies. Gold label could only be detected in thin sections of plants infected with NL-07 (Fig. 10A) and TSWV-I (data not shown), when antiserum against NL-07 nucleocapsid protein was used. However, antiserum against NSs of BR-01 strongly labelled the fibrous inclusions consisting of paracrystalline arrays in NL-07 infected cells. This indicates that this protein of both isolates may show high amino acid sequence homology (Fig. 10 B, C).

Using antiserum to BR-01 nucleocapsid protein (Fig. 11A), only a few gold particles were observed on the dense aggregates and fibrous inclusions in cells of plants infected with NL-07, and in addition, only a few particles were found to occur on virus particles.

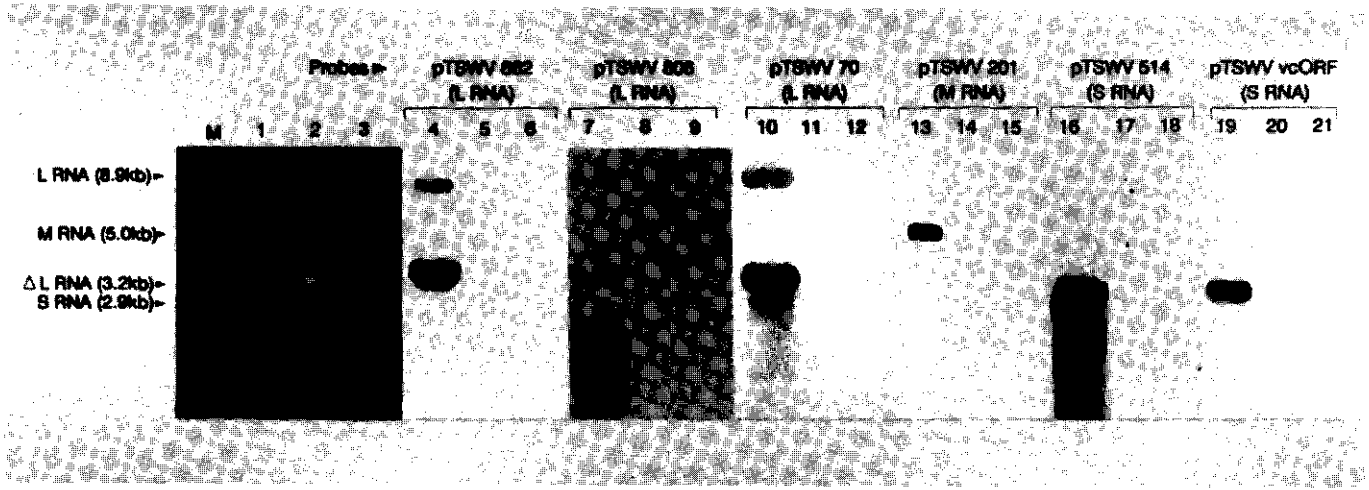

Fig. 7 - Northern blot analysis of the RNA of BR-01 (lanes 1, 4, 7, 10, 13, 16, 19), NL-07 (lanes 2, 5, 8, 11, 14, 17, 20) and TSWV-I (lanes 3, 6, 9, 12, 15, 18, 21). The ^{32}P-labelled cDNA probes used and their positions on the BR-01 RNA segments are indicated in Fig. 6. RNA marker (M).

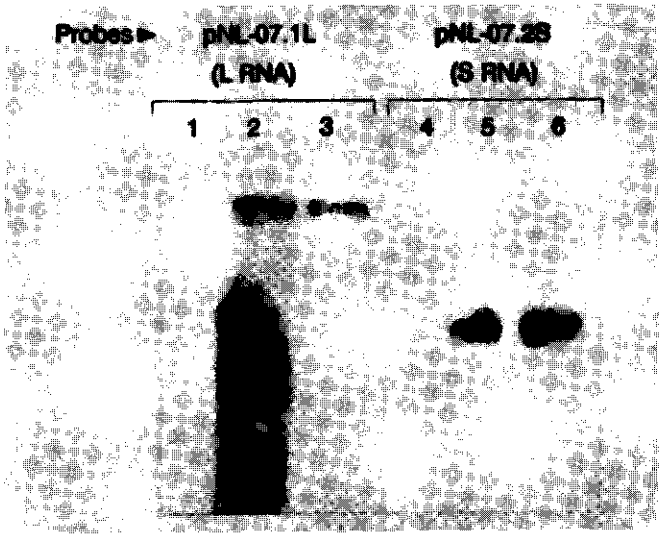

Fig. 8 - Northern blot analysis of RNA of BR-01 (lanes 1 and 4), NL-07 (lanes 2 and 5) and TSWV-I (lanes 3 and 6) using P^{32}-labelled cDNA probes derived from TSWV NL-07.

Antiserum against BR-03 nucleocapsid protein slightly reacted with the nucleocapsid aggregates and not with the fibrous inclusions (Fig. 11B). These results suggest that the nucleocapsid proteins of NL-07 and BR-03 might have a weak amino acid sequence homology. Although the antiserum against virus preparations of BR-01 did not react in ELISA with NL-07 and TSWV-I (data not shown), significant labelling of virus particles occurred when sections were incubated with antiserum against the G1 protein of BR-01 isolated from SDS-polyacrylamide gels (Fig. 11C). Using the same antiserum on Western blots, a faint reaction was observed at the G1 position with NL-07 and TSWV-I (data not shown). These results suggest that some internal epitopes on G1 may be conserved between BR-01, NL-07 and TSWV-I.

DISCUSSION

This study clearly demonstrates that the TSWV-like isolate NL-07 found on *Impatiens* sp. in The Netherlands, is almost identical to TSWV-I, a TSWV-like virus from *Impatiens* in the USA described by Law & Moyer (1990). Both isolates differ in several properties from other TSWV isolates, such as BR-01, (de Haan *et al.*, 1990), BR-03 and SA-05 (de Ávila *et al.*, 1990).

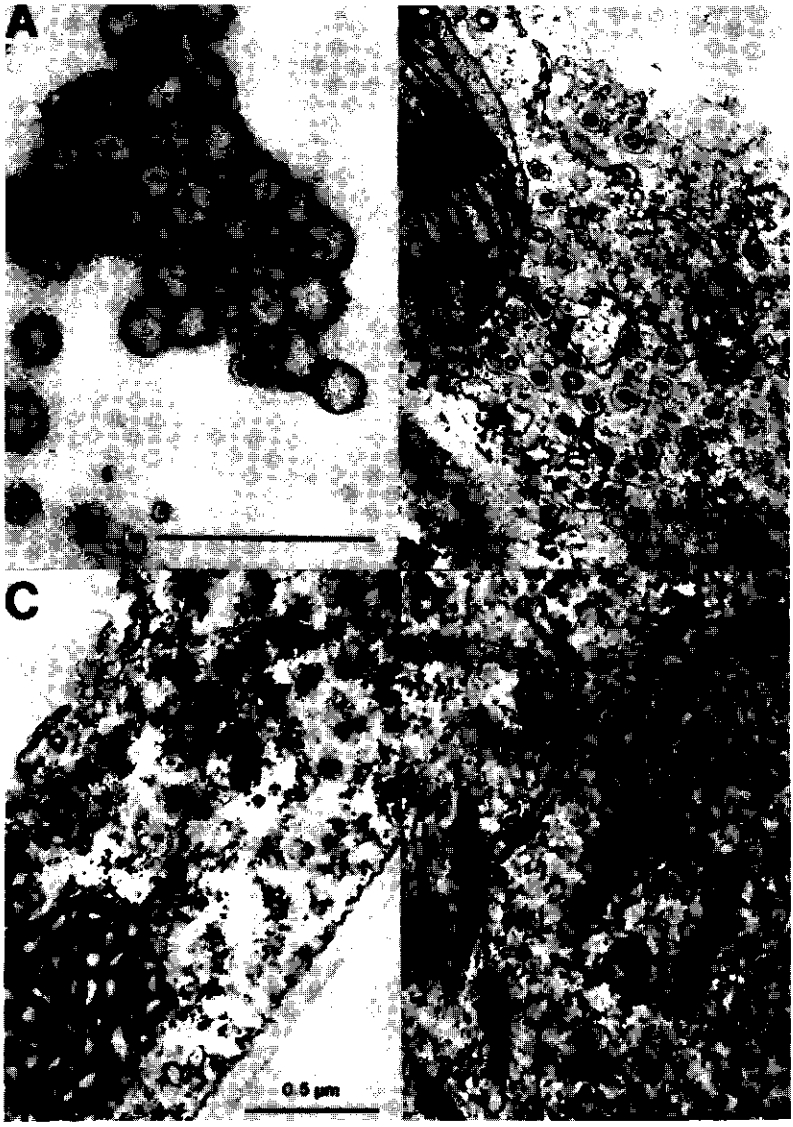

Fig. 9 - Electron microscopical studies of isolate NL-07. (A) Leaf dip preparation, negatively stained with uranyl acetate, from a Nicotiana clevelandii plant infected with NL-07. TSWV-like particles occur in large amounts in the extracts. (B) Virus particles in the endoplasmatic reticulum cisternae of naturally infected Impatiens sp. (C) Particles and nucleocapsid aggregates (D) in a mesophyl parenchyma cell of N. clevelandii, mechanically infected with NL-07. (D) Paracrystalline array of rigid rod-like fibers (F) in a N. clevelandii leaf cell infected with NL-07. M: mitochondrion; P: chloroplast. Bars: 0.5 nm.

Fig. 10 - Immunolabelling of viral products in N. clevelandii cells infected with isolate NL-07. (A) Gold labelling of the nucleocapsid aggregates (D) using NL-07 nucleocapsid protein antiserum. F: fibrous inclusions; V: virus particles; P: chloroplast. (B, C) Gold labelling of the fibrous inclusions (F), forming paracrystalline arrays, using antiserum towards the NSs protein of isolate BR-01. Immunolabelling with gold particles was enhanced by silver staining. V: virus particles; N: nucleus; P: chloroplast. Bars: 0.5 nm.

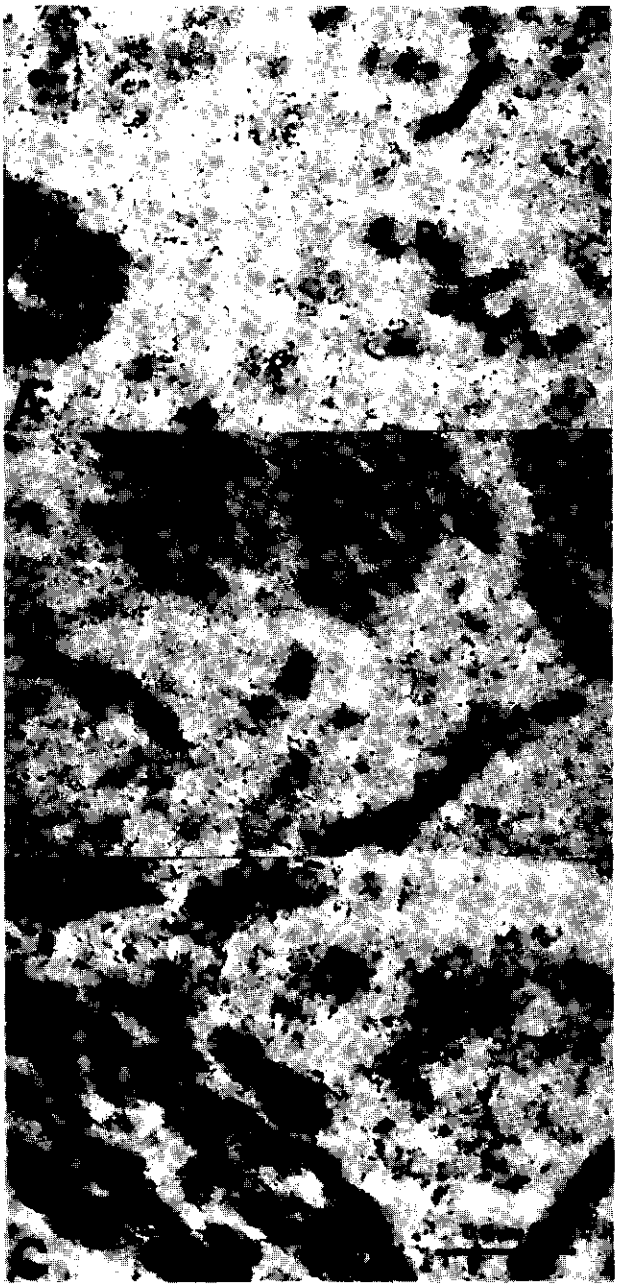

Fig. 11 - Immunolabelling of viral products in cells infected with isolate NL-07. (A) Section incubated with antiserum against nucleocapsid protein of BR-01. Practically no label occurs on dense aggregates (D) or fibrous inclusions (F). Virus particles (V) are slightly labelled. (B) Immuno-labelling with anti-nucleocapsid serum of BR-03. A weak labelling of the nucleocapsid aggregates occurs (D), but not of the fibrous inclusions (F). (C) Significant labelling in virus particles (V) only in sections incubated with antiserum against the G1 glycoprotein of BR-01. Bar: 0.5 nm.

Both NL-07 and TSWV-I induce similar symptoms on the host plants tested, but notable differences were found between these isolates and BR-01 in terms of symptom expression on a number of plants, widely accepted as test plants for TSWV (Francki & Hatta, 1981). Some of the solanaceous hosts do not react with systemic infection (Table 1) which means that more plant species are hypersensitive for these novel isolates than for the established strains. The cytopathology of NL-07 resembles that of TSWV (Fig. 9A, D)(Francki & Grivell, 1970). Nevertheless, some cytopathological differences were observed between NL-07 and TSWV-I. Virus particles are almost completely absent in TSWV-I infected cells (Law & Moyer, 1990; Urban *et al.*, 1991). This observation shows that TSWV-I is a morphologically defective virus, a phenomenon previously reported by Ie (1982); de Ávila *et al.* (1990) and Resende *et al.* (1991b).

ELISA studies demonstrated that the NL-07 and TSWV-I nucleocapsid proteins are serologically closely related but distinct from those of BR-01, BR-03 and SA-05 (Fig. 3A, B). De Ávila *et al.* (1990) proposed to classify TSWV in serogroups according to their reaction with polyclonal nucleocapsid protein antisera. The novel isolates studied now have to be considered as representatives of a new serogroup, designated III, since they do not react with nucleocapsid antiserum prepared to BR-01 (a serogroup I virus), and to BR-03 and SA-05 (serogroup II viruses). In addition both viruses do not react with two monoclonal antibodies directed to the nucleocapsid protein of isolate BR-01. The serological differences between the nucleocapsid proteins of the serogroup I, II and III isolates is confirmed by the results obtained in Western blotting and immunogold labelling studies.

Our results on the nucleocapsid proteins confirm those reported by Law & Moyer, (1990), who demonstrated in Western blotting studies that the N protein of TSWV-I differed serologically completely from that of another isolate they studied.

Kameya-Iwaki *et al.* (1988) reported a TSWV isolate in watermelon and Hayati *et al.* (1990) in *Verbesina alternifolia* with nucleocapsid proteins which were serologically different from the other isolates studied by these authors. Whether these viruses can be classified in any of the groups established by de Ávila *et al.* (1990) needs further studies.

The faint reaction of BR-03 and SA-05 nucleocapsid protein antisera with the nucleocapsid protein of the serogroup III viruses in Western blots (Figs. 4, 5) suggests

that the nucleocapsid proteins of these viruses have some internal epitopes in common.

Immunogold decoration experiments indicate that epitopes on the G1 glycoprotein are shared between NL-07, TSWV-I and BR-01. This is in agreement with the results obtained by Law & Moyer (1990).

The N protein of the TSWV serogroup I isolates has a Mr of 28.8 K (de Haan *et al.*, 1990; Maiss *et al.*, 1991). The N protein of the serogroup III isolates is approximately 1 K smaller as shown in SDS-PAGE co-migration studies (Fig. 2). The differences found in the serological studies between the nucleocapsid protein of the isolates of serogroup I and III is supported by the results of the Northern blot hybridization experiments. Using ^{32}P labelled cDNA probes of BR-01 and NL-07 differences could not be detected between both isolates, except for a small region in the middle of the L RNA. Sequence analysis studies have revealed that this region contains the putative polymerase amino acid motifs (de Haan *et al.*, 1991). The studies presented here furthermore demonstrated that the isolates NL-07 and TSWV-I are almost identical, except that NL-07 contains a defective L RNA molecule of approximately 3000 nucleotides long. Resende *et al.* (1991) demonstrated the presence of defective L RNAs in some lines of the BR-01 isolate. Such defective L RNA molecules were also detected in the lines of the isolates BR-01 and NL-07 used in this study. The occurrence of defective L RNA segments in NL-07 indicates that the generation of defective L RNA molecules is not restricted to the L RNA of serogroup I isolates.

Law & Moyer (1990) did not detect any homology between S RNA of TSWV-I and their common type TSWV comparing their TSWV isolates using a S RNA-specific clone. These results were confirmed in this study. We detected no homology between the N gene of serogroup I and III viruses using BR-01 and NL-07 probes (Fig. 7, 8). In addition, no hybridization could be detected between NL-07, TSWV-I and BR-01 using a NSs probe (Fig. 7). However, sequence analysis has shown that both serogroups share considerable amino acid sequence homology of the NSs gene (data not shown). Also, immunogold labelling experiments revealed that the fibrous structures, induced by NL-07 (Fig. 10B, C) are recognized by BR-01 NSs antiserum, indicating that this protein of both isolates possesses significant homology in the amino acid sequences. These results have been confirmed by Kormelink *et al.* (1991) in a study in which the NSs protein of the

TSWV isolates NL-07 (denoted then H7), E-01 (S1) and BR-01 (CNPH1) were characterized.

Law & Moyer (1990) demonstrated that a M RNA-specific cDNA clone of TSWV-I did not cross-hybridize with the common type. Similar results were obtained by us using a M probe in studies with NL-07 and TSWV-I and suggest that no extensive homology exists between the isolates of serogroup I and III.

Classification of TSWV isolates using antigenic determinants on the internal nucleocapsid proteins seems to be stable and reliable (de Ávila *et al.*, 1990) and is not affected when isolates become morphologically defective or when defective L RNAs appear.

Both viruses are so distinct from the serogroups I and II isolates that they can considered to be new virus species, for which the name *Impatiens* necrotic spot virus may be used. A similar proposal has been made by Law *et al.* (1991). More molecular data are required to answer the question whether they have to be placed in a new genus or deserve a real place in the genus tospovirus.

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CHAPTER 5 DISTINCT LEVELS OF RELATIONSHIPS BETWEEN TOSPOVIRUS ISOLATES

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SUMMARY

The taxonomic relations of a number of tospovirus isolates, collected in different geographical areas and from different host plants, were studied. To delineate these isolates, properties such as susceptibility of a limited range of host plants, symptomatology, cytopathology, nucleocapsid composition, serology of their nucleocapsid proteins and nucleotide sequence homology were compared. The results show that isolates which have previously been discriminated as members of three different serogroups, should in fact be regarded as representatives of at least three distinct virus species in the tospovirus genus.

This chapter is *in press* in a slightly modified version as: de Ávila *et al.* (1992). Distinct levels of relationships between tospovirus isolates. *Archives of Virology.*

INTRODUCTION

Tomato spotted wilt virus (TSWV) causes worldwide, but mainly in tropic and subtropic regions, serious diseases in many agricultural, horticultural and ornamental crops. It is the only virus which is biologically transmitted by thrips species (Peters *et al.*, 1991). The enveloped virus particles have a roughly, isometrical morphology with a diameter of 70-110 nm. The virion contains four proteins: a nucleocapsid (N) protein associated with three genomic RNA segments, two glycoproteins (G1, G2) associated with the viral envelope and a large (331.5 K) protein which has been proposed to be the viral polymerase (de Haan *et al.*, 1990; de Haan, 1991). Its genome consists of three single stranded RNA molecules denoted small (S) RNA (2916 nucleotides), medium (M) RNA (approximately 5000 nucleotides) and large (L) RNA (8897 nucleotides). The S RNA encodes the N and a non-structural protein (NSs) in an ambisense gene arrangement, while the L RNA encoding the viral polymerase has a negative polarity (de Haan *et al.*, 1990; de Haan *et al.*, 1991; Kormelink *et al.*, 1990). The M RNA codes for the two glycoproteins and presumably another non-structural protein (Kormelink *et al.*, 1992).

Based on its genome organization, TSWV has recently been classified as the sole member of the newly created genus, *Tospovirus*, within the Bunyaviridae (Francki *et al.*, 1991). Thus far, the various isolates found in infected plants and crops could not reliably be differentiated in strains or species using biological and morphological properties or classical serology. Attempts by Norris (1946) and by Best and Gallus (1955) to classify TSWV isolates by stable symptom characteristics have not been followed up, partly due to the lack of any other property to support this classification. Applying polyvalent antisera and monoclonal antibodies to the N protein of the TSWV isolate BR-01, de Ávila *et al.* (1990, 1992) distinguished three serogroups, denoted I, II and III. Members of serogroup III deviate substantially from the serogroup I and II isolates in both biological and serological properties as well as in nucleotide sequence homology of the N gene. Hence, the serogroup III viruses are considered to form a separate species for which the name *Impatiens* necrotic spot virus (INSV) has been proposed (Law *et al.*, 1991a,b and c; de Ávila *et al.*, 1992; de Haan *et al.*, 1992).

In the present study, we show that serological and molecular properties can be used to define criteria by which species can be discriminated within the genus *Tospovirus*. With these criteria the taxonomical status of the seven selected isolates, previously placed into three serogroups, will be revised.

METHODS

Virus isolates, maintenance and nomenclature

The tospovirus isolates studied are listed in Table 1 indicating to which serogroup they belong (de Ávila *et al.*, 1990, 1992) with their original designations and the new ones using the country code for automobiles while the number refers to the isolate from that country in our collection. However, SA has been used for South Africa instead of ZA. Although NL-07 has already been recognized as a distinct virus, for which the name *Impatiens* necrotic spot virus has been proposed (Law *et al.*, 1991b, c; de Ávila *et al.*, 1992; de Haan *et al.*, 1992), the notation NL-07 will be used throughout this study.

The isolates in desiccated infected leaf material were stored in liquid nitrogen and, when required, recovered by mechanical inoculation on *Nicotiana benthamiana* Domin. or *Nicotiana rustica* L. using 0.01 M phosphate buffer, pH 7.0, containing 0.01 M Na_2SO_3.

Studies on symptom expression

Symptom expression of the isolates was studied on a few plant species belonging to seven botanical families (Table 2). The plants were mechanically inoculated using inocula from systemically infected *N. rustica* plants infected two weeks before.

Antisera preparation, serological studies and immunoblotting

Nucleocapsid fractions of BR-01, E-01, BR-03, BR-06, SA-05, BR-08 and NL-07 were extracted as described by de Ávila *et al.* (1991). Approximately 500 μg of purified protein emulsified with incomplete Freund's adjuvant (1:1) was used to immunize rabbits with three intradermal injections at two-weekly intervals. Blood was collected, starting two weeks after the last injection, over a period of four months.

Table 1. Serogrouping, notation and origin of the tospovirus isolates compared in this study.

Serogroup	Notation	Origin	
		Country	Host
I	BR-01	Brazil	tomato
	E-01	Spain	pepper
II serotype I	BR-03	Brazil	tomato
	BR-06	Brazil	tomato
serotype II	SA-05	South Africa	groundnut
	BR-08	Brazil	tomato
III	NL-07	Netherlands	*Impatiens*

The isolates were analyzed in a double antibody sandwich ELISA format (DAS-ELISA) (Clark & Adams, 1977). Serially diluted extracts (log 1.5 up to log 4.5) from infected plants and purified nucleocapsid preparations, containing 5 to 100 ng protein/ml, were used as antigen samples. An identical series of diluted extracts from healthy plants was used as a negative blank in these assays. The isolates were also tested in a triple antibody sandwich format (TAS-ELISA) using two monoclonal antibodies (MAbs) directed to the N protein of BR-01 (Huguenot *et al.*, 1990). In these tests, 30-fold diluted extracts from infected and healthy plants and 100 ng/ml of purified nucleocapsid protein were used as antigen preparations.

In addition, the isolates compared were analyzed by immunoblotting as described by de Ávila *et al.* (1992) using antisera against native N protein and antisera against denatured G1 and N protein of the BR-01 isolate. Purified nucleocapsid preparations and extracts from infected plants were used as antigen sources. Virus preparations were obtained using the protocol of Tas *et al.* (1977).

Polyacrylamide gel electrophoresis

The molecular masses of the nucleocapsid proteins of all isolates studied were estimated after analysis by electrophoresis on a sodium dodecyl sulphate (SDS)-15% polyacrylamide gel.

Nucleocapsid profiles

Nucleocapsid pellets of the isolates BR-01, E-01, BR-03, SA-05 and BR-08 were resuspended in 0.01 M sodium citrate buffer, pH 6.0, and centrifuged at 150,000 *g* for 2.5 h in 20-40% sucrose gradients prepared with the same buffer (Peters *et al.*, 1991). The position of the nucleocapsid bands were recorded using an ISCO, model 185.

Electron microscopy

Electron microscopical studies were made on thin-sectioned *N. rustica* plant material mechanically inoculated with BR-01, E-01, BR-03, BR-06, SA-05, and BR-08 as described by Kitajima *et al.* (1992).

RNA extraction and Northern blot analysis

RNA was extracted from nucleocapsid preparations of each isolate after adding SDS to a final concentration of 1% (w/v) followed by one phenol and two subsequent phenol/chloroform (1:1) extractions and ethanol precipitation. The RNA extracts were analyzed by electrophoresis on 1% agarose gels, transferred to nitrocellulose membranes by capillary blotting (Laemmli, 1970) and subsequently hybridized to ^{32}P-labelled tospovirus-specific cDNA fragments. To consider stringency conditions in heterologous comparisons, the hybridizations were performed at 55, 60 and 65°C and the membranes were washed in 2xSSC for 30 min, followed by another washing step for 20 min in 0.1xSSC containing 0.1% SDS. Two probes, complementary to certain regions in the S RNA of isolate BR-01, were used: one probe (S1) corresponded to a stretch within the NSs protein gene between nucleotides 12 to 184, the other probe (S2) to a stretch within the N protein gene between nucleotides 2279 - 2885 of the S RNA (Kormelink *et al.*, 1992). Three, approximately 0.8 kb long N protein specific probes prepared by PCR from the 3' ends of the S RNAs of BR-03, SA-05 and NL-07 were also included in this study. These probes were denoted BR-03N, SA-05N and NL-07N, respectively. In addition, a probe, PNL-07-2, derived from the 5' end of the NL-07 S RNA segment was used. The homology of the L RNA of the isolates was studied using a BR-01 L derived cDNA clone (PTSWV-331) ranging from nucleotide position 7212 to 8284 (de Haan *et al.*, 1991).

Table 2. Symptomatological responses of several hosts to seven tospovirus isolates.

Host plants	Reaction on host					
	TSWV					
	BR-01		E-01		BR-03	
	L**	S***	L	S	L	S
AMARANTACEAE						
Gomphrena globosa L.	NL	-	NL	-	NL	-
BALSAMINACEAE						
Impatiens sp.	NR	VC	NR	VC,Y	NR	VC,Y
CHENOPODIACEAE						
Chenopodium quinoa Wild	N(pp)	-	N(pp)	-	N(pp)	-
Ch. amaranticolor Coste & Reyn	N(pp)	-	N(pp)	-	N(pp)	-
COMPOSITEAE						
Emilia sonchifolia (L.) DC.	NL	Mo	-	Mo	-	Mo
Zinnia elegans Jacq. (a)	-	-	CS	Mo	-	-
CUCURBITACEAE						
Cucurbita sativus L. (b)	CL	-	CL	-	CL	-
LEGUMINOSEAE						
Vigna unquiculata (L.) Walp (c)	CL	Mo,LD	CL	Mo,LD	CL	Mo,LD
Phaseolus vulgaris	CL	VC	CL	VC	CL	VC
Pisum sativum	NR	Mo,B,W	NR	Mo,B,W	NR	Mo,B,W
Arachis hypogaea L.	CR	Mo	CR	Mo	CR	Mo
SOLANACEAE						
Capsicum annuum L. (d)	NR	Mo	NR	Mo	NR	Mo
Datura stramonium L.	-	C	-	Mo	N(pp)	Mo
Lycopersicon esculentum Mill. (e)	-	Mo,B	CS	Mo,B	NR	Mo,B
Nicotiana benthamiana Domin.	CS	VC,M,LD	-	M	CS	M,LD
N. clevelandii A. Gray	NR	M,LD	NR	VN,M,LD	NR	VN,M,LD
N. glutinosa L.	NL	VN,LD	NL	VN,M,LD	NL	VN,M,LD
N. rustica L.	CS	VC,M	CS	M	CS	VC,M
N. tabacum L. Samsum	NL	VN,CS,M	NL	CS	NR	VC,M,LD
N. tabacum L. Samsum NN	NR	VC	NR	VC	NR	VC,M
Petunia hybrida Vilm. (f)	NL	-	NL	-	NL	-
Physalis floridana Rydberg	NL	M,TN	NL	M	NL	Mo,VN
Impatiens sp.	-	CR	-	CR,M	-	CR,Mo

L**: local; S***: systemic.
*B: bronzing; CL: chlorotic lesions; CS: chlorotic spots; CL(np) chlorotic lesions with Mo: mottling; NL(b): necrotic local lesions (light-brown); W: wilting; N(pp): pin-point VC: vein clearing; VN: vein necrosis; TN: top necrosis; Y: yellowing. (a) cv Renzenbloemig (e) cv Moneymaker (f) cv Pink Beauty.

plants*							
isolates							
BR-06		SA-05		BR-08		NL-07	
L	S	L	S	L	S	L	S
NL	-	NL	-	NL	-	NL	-
NR	VC,Y	NR	VC,Y	NR	Y	NR	Y
N(pp)	-	N(pp)	-	N(pp)	-	N(pp)	-
N(pp)	-	N(pp)	-	N(pp)	-	N(pp)	-
CR	Mo	-	Mo	-	Mo	CR	Mo
CR	Mo	-	CS	CS	Mo	-	-
CL	-	CL	-	CL	-	CL(np)	-
CL	Mo,LD	CL	Mo,LD	CL	Mo,LD	NL(b)	-
CL	VC	CL	VC	CL	VC	NL(b)	-
NR	Mo,B,W	NR	Mo,B,W	NR	Mo,B,W	-	NL
CR	Mo	CR	Mo	CR	Mo	-	-
NR	Mo	NR	Mo	NR	Mo	NR	Mo
N(pp)	Mo	N(pp)	M	-	M	CS	-
NR	Mo,B	CS	Mo	CS	Mo	NS,VN	-
CS	VC,M,LD	CS	M,LD	CS	VC,M,LD	NR	M,LD,D
NR	M,LD	NR	VC,M,LD	NR	M,LD	NR	VC,M,LD
NR	VN,M,LD	NL	M,LD(pp)	NR	VN,M,LD	NL	-
NL	VC,M	NR	M	CS	M	NL	-
NR	VC,M	NR	VC,Mo	NR	VN,M,LD	NR	-
NR	VC,Mo	NR	VC,Mo	NR	VN,M,D	NR	-
NL	-	NL	-	NL	-	NL	-
NL	M	NL	M	NL	M	NR,VN	M
-	NR	-	CR	NR	Mo	-	NR,VN

necrotic centers **D**: death of the plant; **LD**: leaf-deformation; **M**: mosaic; necrosis; **NR**: necrotic rings; **NS**: necrotic spots; **SN**: stem necrosis; (b) cv Lange gele tros (c) cv California Blackeye (d) cv Westlandse Zoet

RESULTS

Host range and symptomatology

All plant species tested were susceptible to the seven isolates studied (Table 2). Except NL-07, the isolates could not be differentiated by the symptoms induced although they differed in severity and length of the incubation period. They induced necrotic or chlorotic spots and rings, followed by vein necrosis on the inoculated leaves of most solanaceous hosts tested; a mosaic, mottling and leaf deformation appeared on the top leaves followed by necrosis as the leaves of these hosts aged. Severe vein necrosis occurred mainly on some hosts infected by BR-01, E-01, BR-03, BR-06 and BR-08 isolates which originated from tomato or pepper. Necrosis rarely occurred when the solanaceous hosts were infected with SA-05, an isolate from groundnut. Remarkable differences in symptomatology, however, were found between these isolates and NL-07. The latter isolate induced local lesions on the inoculated leaves of *Datura stramonium*, *L. esculentum*, *Nicotiana tabacum* L. "Samsun" and "Samsun NN", and failed to infect these species systemically. NL-07 developed numerous local lesions on inoculated leaves of *N. benthamiana* and severe vein necrosis on young leaves. Plants died within two weeks post-infection by a process that could be described as wilting induced by an apparently systemic spread of the virus. Systemic infections on *Capsicum annuum* L. and *N. rustica* are occasionally caused by NL-07. Of the solanaceous hosts tested, *Petunia hybrida* Vilm. reacted only with local lesions on leaves inoculated with all seven isolates. NL-07 induced only light brown local lesions on the inoculated leaves of *Vigna unguiculata* (L.) Walp. whereas the other isolates induced chlorotic spots on inoculated leaves followed by vein clearing, mottling and severe leaf deformation on full-grown leaves.

All isolates tested induced dark brown necrotic spots, often with concentric necrotic rings, on the leaves of *Impatiens* plants while a number of leaves usually remained symptomless. In addition, colour-breaking was noticed in the flowers. The symptoms induced by NL-07 were consistently found to be more severe.

Cytopathology

Basically, all isolates studied exhibited similar cytopathological effects. In ultrathin sections (Fig. 1), roughly spherical enveloped virus particles were observed within membrane bound cavities of the endoplasmic reticulum system. The mature particles of most isolates occurred as clusters in a single cavity, with the exception of those of BR-08 which were often found singly in a vesicle. Aggregates of dense material representing free nucleocapsids (NA) and inclusions of fibrous material (F), which contain the non-structural protein (NSs) encoded by the S RNA, were found in varying amounts in cells infected with all isolates studied. Nucleocapsid aggregates were rarely found in field isolates but increased in amount after some mechanical transfers of the isolates. The occurrence of fibrous inclusions seemed to be time and host dependent as they could not be detected in samples collected at different times from some species, and were consistently absent in plants of other host species.

Serological relationships

Serological relations between the isolates were studied in DAS-ELISA using serial dilutions of purified N protein and of extracts from infected plants. Strongly positive responses were obtained with the N protein of all isolates in homologous reactions (Fig 2 A - F). No reaction was observed between the N protein of NL-07 and that of the other isolates (Fig. 2F). The 100-fold lower response between the serogroup I and II isolates and the absence of any response with NL-07 from serogroup III, confirms the serological distinction previously made among the different serogroups. The 10-fold lower responses between BR-03 or BR-06 on one hand and SA-05 and BR-08 on the other (Fig. 2 B - E) confirm that the serogroup II isolates can be differentiated further as has previously been shown (de Ávila *et al.*, 1990, 1992).

Since similar results were found when serially diluted extracts from plants infected with these isolates were tested in DAS-ELISA, the observed differences are not due to different virus concentrations in the plants (Fig. 3).

The serological interrelationships were further tested using two MAbs, N1 and N2, directed to the N protein of BR-01. High responses were obtained in reactions with the N protein of BR-01, while no reaction was obtained with BR-03, BR-06 and NL-07.

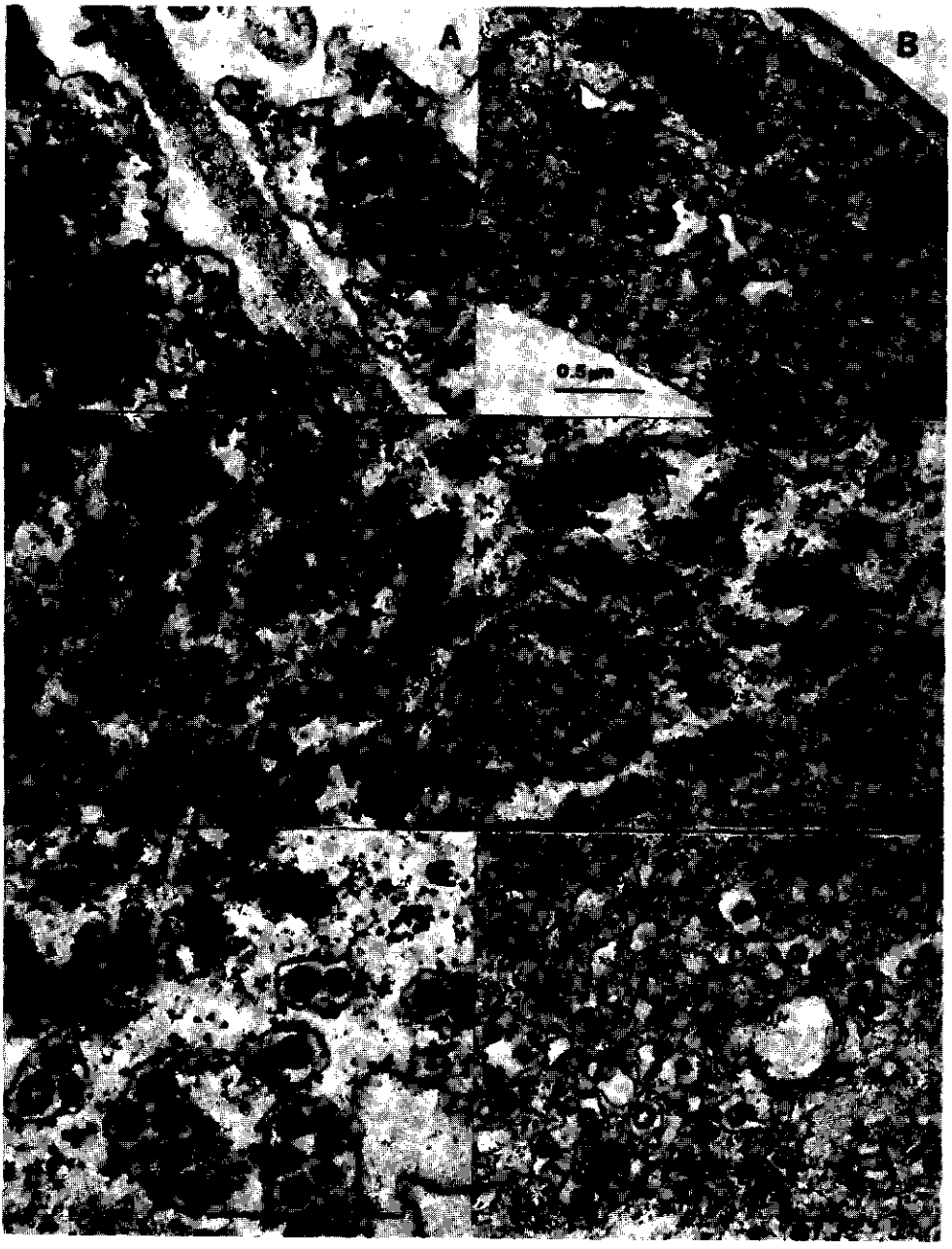

Fig. 1 - Electron micrographs of six tospovirus isolates in the cytoplasm of leaf mesophyll cells of infected Nicotiana rustica. Enveloped virus particles (V) could be observed in various numbers, sometimes interspersed with nucleocapsid aggregates (NA) or inclusions of fibrous material (F), BR-01 (A), E-01 (B), BR-03 (C), BR-06 (D), SA-05 (E), BR-08 (F). M: mitochondrion, G: Golgi complex. Bars: 0.5 nm.

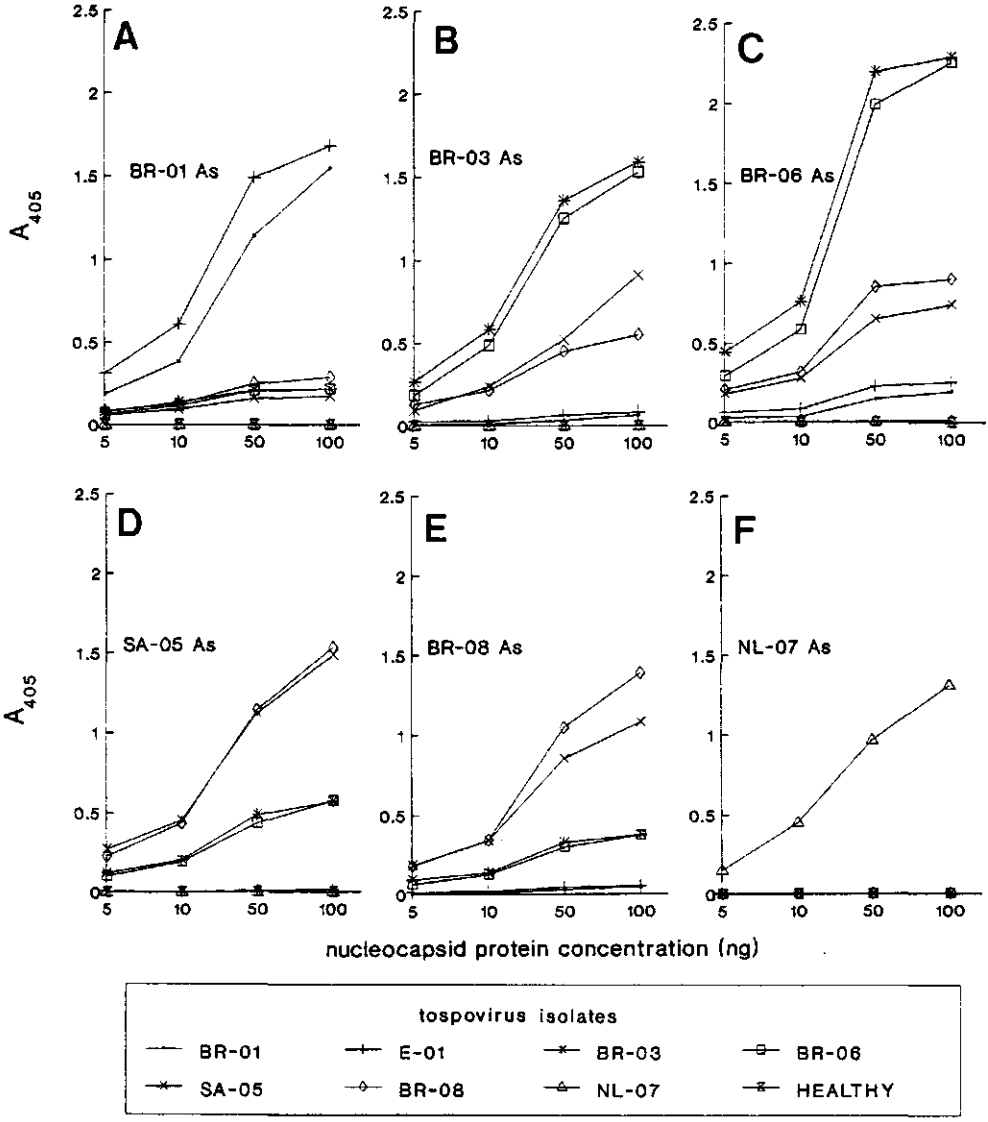

Fig. 2 - Comparison of seven tospovirus isolates by ELISA using a dilution series of N protein as antigen and polyclonal antisera (As) produced against the N protein of each isolate. A: BR-01 As, B: BR-03 As, C: BR-06 As, D: SA-05 As, E: BR-08 As and F: NL-07 As. The absorbance values (405 nm) were measured 1 h after substrate addition.

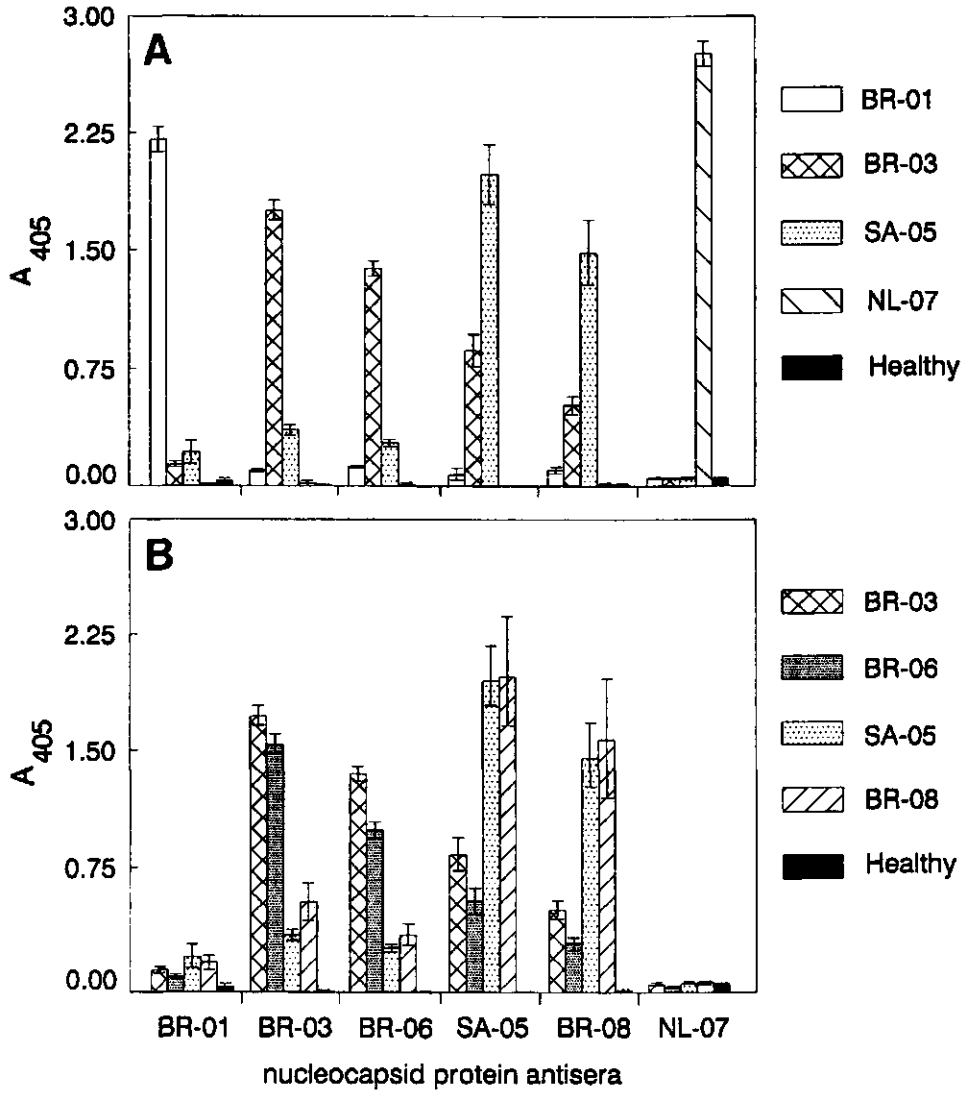

Fig. 3 - Serological differentiation of six tospoviruses. (A) Reaction of these viruses using polyclonal antisera against their respective N proteins and extracts from infected plants as antigen source. (B) Reaction of serogroup II viruses using the same sera and infected plants as antigen source. The identity of virus isolates tested are given at the right. The absorbance values (405 nm) were measured 1 h after substrate addition. The isolates SA-05 and BR-08 reacted only with MAb N1, but not with N2 (Fig. 4).

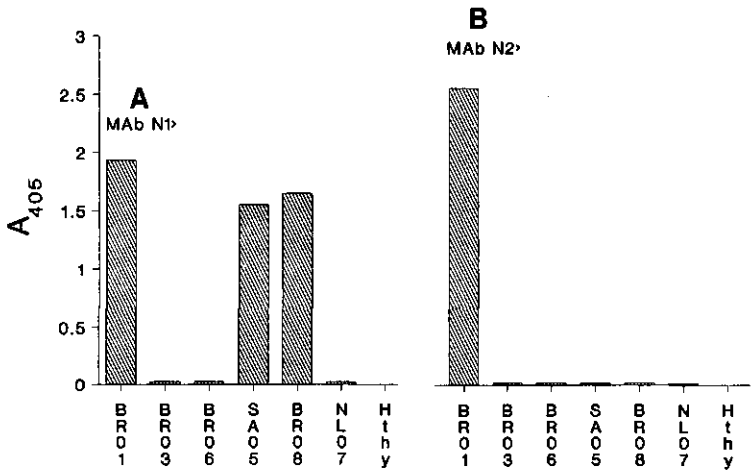

Fig. 4 - ELISA reactions of six tospoviruses using a panel of two monoclonal antibodies (N1 and N2) raised to the N protein of isolate BR-01. Purified N protein samples (100 ng) of each isolate were used as antigen source. The identities of tospovirus isolates tested are indicated on the X-axis; the absorbance values (405 nm) were measured 1 h after substrate addition.

The isolates SA-05 and BR-08 only reacted with MAb N1, but not with N2 (Fig. 4). These results underline the previous conclusion that isolates BR-03 and BR-06 differ distinctly from SA-05 and BR-08.

Western blot analysis confirmed and substantiated the results obtained in ELISA (Fig. 5). The BR-01 and E-01 N proteins strongly reacted with the antiserum against the N protein of BR-01, while only a faint reaction was observed with BR-03, BR-06, SA-05 and BR-08, and no reaction at all with NL-07. The serological distinction between BR-03 and BR-06 isolates and the SA-05 and BR-08 isolates, all belonging to serogroup II, could again be demonstrated in these studies (Fig. 5). As found with ELISA, the antiserum to NL-07 N-protein showed only strong responses in the homologous reaction (Fig. 5, lane 7) and did not react at all with the N proteins of the other isolates, except for SA-05 and BR-08, which gave a faint response (Fig. 5). Since both ELISA and Western blot studies gave identical results it is therefore concluded that either technique is suitable to differentiate the isolates using antisera to their N proteins. On the contrary, when using an antiserum against denatured BR-01 G1 glycoprotein, the isolates studied could not unequivocally be distinguished by Western blot (Fig. 6), suggesting that this antigen is more conserved than the N protein.

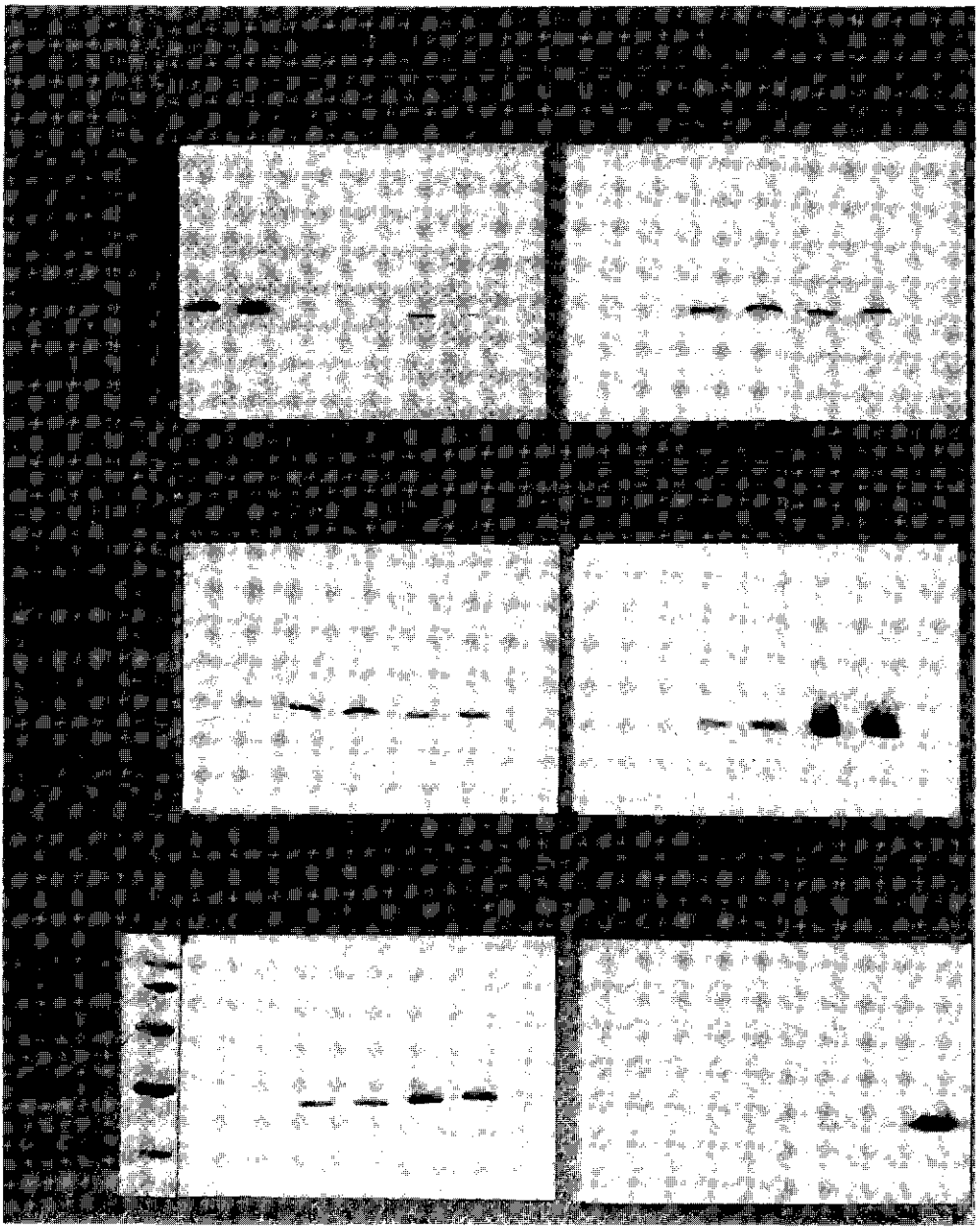

Fig. 5 - Western blot analysis of six tospovirus isolates using antisera raised to their respective N proteins. From left to right: marker proteins (M), isolate BR-01 (lane 1), E-01 (lane 2), BR-03 (lane 3), BR-06, (lane 4), BR-08 (lane 5), SA-05 (lane 6) and NL-07 (lane 7).

The apparent size of the G protein seems to vary from 78 to 82 K (Fig. 6). This variation may, however, be caused by differences in glycosylation of this envelope protein.

Analysis of protein composition

On SDS-polyacrylamide gels, the N proteins of BR-01, E-01, SA-05 and BR-08 migrated slower than those of BR-03, BR-06 (Fig. 7) and NL-07 (de Ávila *et al.*, 1992). The migration rates correspond with a molecular mass of 29 K and 28 K, respectively. The value of 29 K found, for the first isolates, matches rather well with the molecular weights of the BR-01 N protein (28.8 K) as directly deduced from sequence data (de Haan *et al.*, 1990). However, the differences in molecular mass of the N protein does not correspond with the serological partition of the isolates.

Nucleocapsid profiles

The sedimentation profiles of the nucleocapsids of each isolate extracted from *N. rustica* displayed its own characteristic profile in sucrose gradients (data not shown). Three nucleocapsid fractions were recognized after centrifugation of the nucleocapsid material of BR-01, E-01, BR-03, SA-05 and BR-08. The top fraction contained S RNA, the middle fraction M RNA and traces of S RNA, whereas the bottom fraction mainly contained L RNA and readily detectable amounts of S and M RNA (Peters *et al.*, 1991). When the isolates were transferred several times by mechanical inoculation, one or more additional bands could be discerned in the gradient. These bands consisted of sub-genomically sized, defective L RNA segments (Resende *et al.*, 1991). Using the extraction method described, it was not possible to extract the nucleocapsids of isolates BR-06 and NL-07, and their profiles could therefore not be studied.

Northern blot analysis

All isolates contained the three genomic RNA segments characteristic of all Bunyaviridae (Fig. 8). As they comigrated with the L, M, and S RNA of BR-01, their sizes are about 8.9 kb, 5.0 kb and 2.9 kb (de Haan, 1991). However, additional RNA specimens, approximately 3.0 to 2.0 kb long, were found in RNA extracts of BR-01,

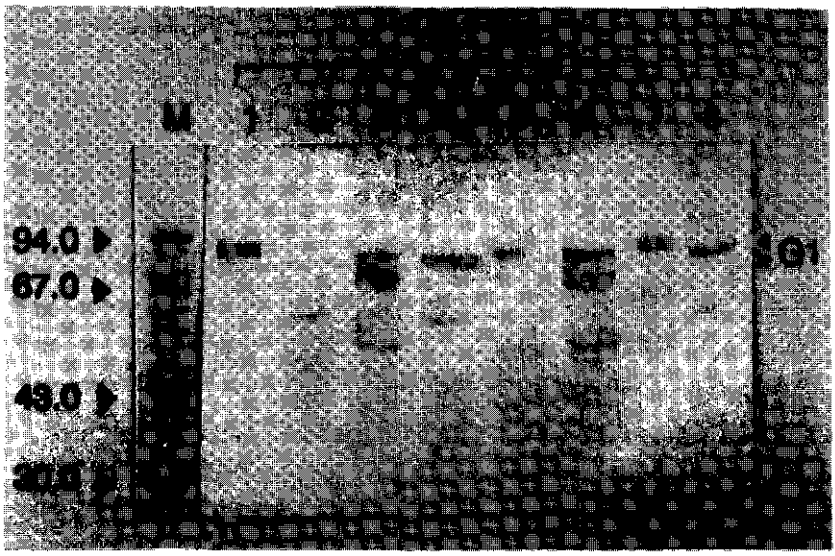

Fig. 6 - Western blot analysis of six tospovirus isolates using an antiserum against denatured G1 glycoprotein of isolate BR-01. Extracts of infected plants served as antigen sources. Marker proteins (lane M), isolate BR-01 (lane 1), healthy plant extract (lane 2), BR-03 (lane 3), BR-06 (lane 4), E-01 (lane 5), SA-05 (lane 6), purified virus preparation of BR-01 isolate (lane 7) and BR-08 (lane 8).

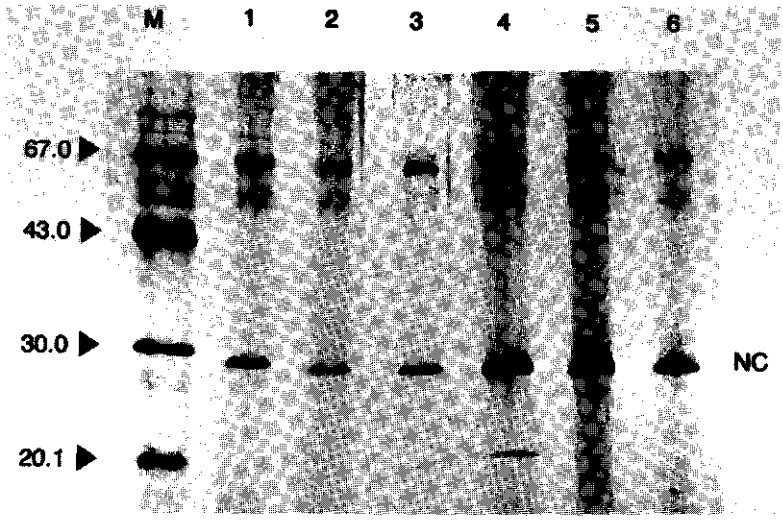

Fig. 7 - Electrophoretic mobilities in a SDS-polyacrylamide gel of the N proteins of three different tospovirus isolates. Marker proteins (M), isolate BR-01 (lane 1), BR-03 (lane 2), BR-06 (lane 3), BR-01 and BR-03 (lane 4), BR-01 and BR-06 (lane 5), BR-03 and BR-06 (lane 6).

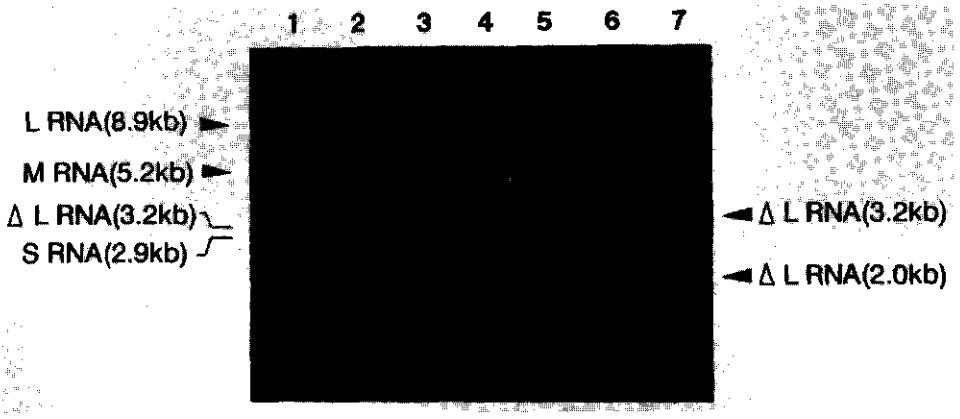

Fig. 8 - Electrophoretic analysis of genomic RNA of seven tospovirus isolates: isolate BR-01 (lane 1), E-01 (lane 2), BR-03 (lane 3), BR-06 (lane 4), SA-05 (lane 5), BR-08 (lane 6) and NL-07 (lane 7). Defective interfering L RNA segments are indicated by open upright triangles.

NL-07 and SA-05, respectively. Northern hybridization studies revealed that these RNA molecules represented deletion-containing, defective derivatives of the L RNA segment (Resende *et al.*, 1991). The differences in migration rates of S RNAs found between the isolates (Fig. 8) were possibly due to the source (purified virus particle preparations or nucleocapsid extracts) from which the viral RNA was extracted and hence reflect the difficulty of completely removing the N protein during the RNA extraction.

The hybridization studies revealed significant divergence in sequence homologies between the N genes of the isolates studied (Fig. 9). These studies show that the probe to the N gene of BR-01 (probe S2) weakly detected the N gene of the serogroup II viruses, while probes derived from the N genes of serogroup II viruses (probes BR-03N and SA-05N) did not, or only very weakly, recognize the BR-01 N gene (Fig. 9). No homology was detected between the N gene of NL-07 and those of the serogroup I and II viruses.

Almost similar results were obtained testing the NSs region of these viruses with probes, S2 and PNL-07-02, corresponding with the NSs genes of BR-01 and NL-07, respectively (Fig. 9). These results demonstrate that the NSs genes of these isolates have

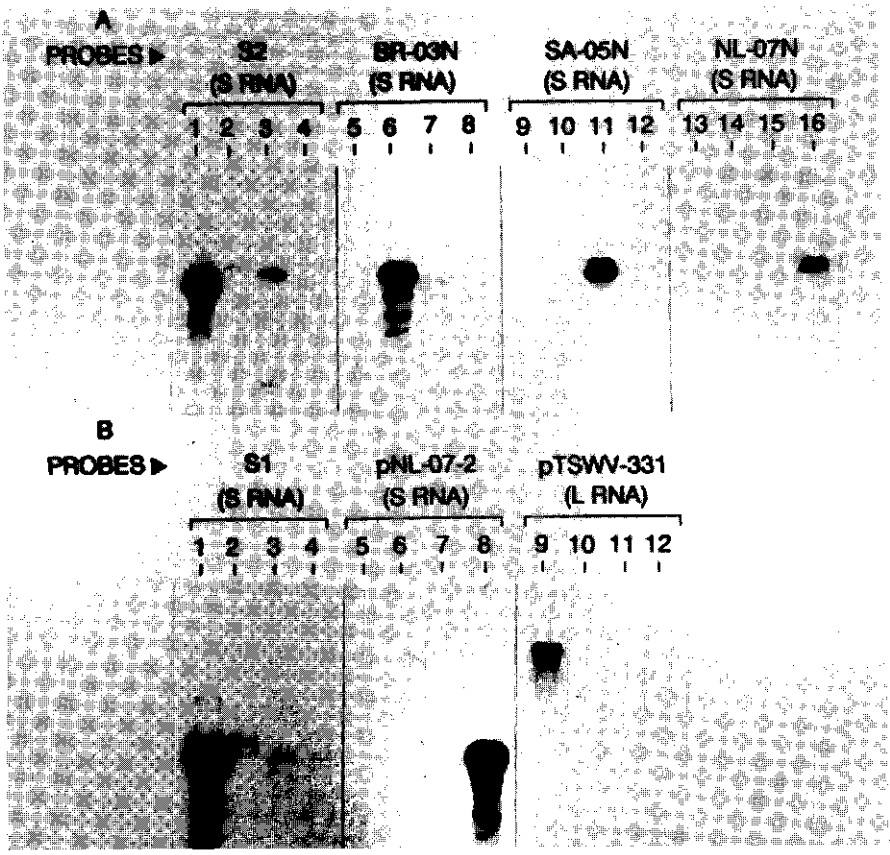

Fig. 9 - Northern blot analysis of four tospovirus isolates using S and L RNA specific cDNA probes. After electrophoresis under denaturating conditions, RNA was transferred to Hybond-C membrane and hybridized to ^{32}P-labelled DNA fragments. (A) Hybridization using probes S2, BR-03N, SA-05N and NL-07N, which are specific for the N genes of the isolates BR-01, BR-03, SA-05 and NL-07, respectively. (B) Hybridization using probes S1 and PNL-07-2, both derived from the non-structural gene (NSs) of isolates BR-01 and NL-07, respectively. The DNA probe PTSWV-331 is L RNA specific and derived from the BR-01 isolate. BR-01 (lanes 1, 5, 9, 13), BR-03 (lanes 2, 6, 10, 14), SA-05 (lanes 3, 7, 11, 15) and NL-07 (lanes 4, 8, 12, 16).

diverged at a slightly lower rate than the nucleocapsid gene. This conclusion is supported by immuno-decoration studies (Kitajima *et al.*, 1992; Kormelink *et al.*, 1991).

Also a large divergence may be expected for the L gene as the BR-01 L RNA-specific probe, PTSWV-331, did not hybridize with the L RNA segments of the other isolates.

DISCUSSION

In this study seven different tospovirus isolates, previously placed into three serogroups on the basis of ELISA studies (de Ávila *et al.*, 1990, 1992; Kitajima *et al.*, 1992), have been further compared at the level of host range, symptom induction, cytopathology, serology, nucleocapsid composition, and Western and Northern blot analyses. All plant species tested were susceptible for the isolates compared. However, isolate NL-07, a serogroup III member, produced only local lesions on the inoculated leaves of most solanaceous species tested, while the other isolates systemically infected these species (Table 2). Differences in host response can be used as a criterion, though certainly not as an ultimate one, to recognize isolates belonging to serogroup III. Differences in the local lesions produced by NL-07 and the other isolates on *V. unguiculata* can likewise be used as a criterion to distinguish serogroup III isolates from those in serogroups I and II. Since symptoms of tospoviruses can attenuate during mechanical transfers (Resende *et al.*, 1991), or may change due to point mutations as has been shown for tobacco mosaic virus (Knoor & Dawson, 1988), the use of symptoms is not without risk in the classification of viruses. The nucleocapsid composition of the isolates, as revealed by the profiles obtained after centrifugation of purified preparations on sucrose gradients, shows that each isolate sediments in a characteristic pattern (Peters *et al.*, 1991). This patterns can not be used as a taxonomical criterion as the isolates within a serogroup show a large variety of patterns.

The ELISA and Western blot analysis using polyclonal antisera to the N protein of the different isolates and monoclonal antibodies raised against the N protein of BR-01 confirm the earlier results of de Ávila *et al.* (1990, 1992) which demonstrated that the tospoviruses can be divided into three serogroups, one of them contained two serotypes (Figs. 2, 4). The serogroups were defined by antigenic differences between the nucleocapsid proteins of different viruses using polyclonal antisera, and serotypes by antigenic differences between the N proteins of viruses within a serogroup using MAbs.

The absence of any serological cross-reactivity between the N protein of NL-07 and those of the other isolates suggests a large divergence between these N proteins. This conclusion is also supported by the lack of any detectable cross-hybridization between

BR-01 (serogroup I) and NL-07 (serogroup III) N gene probes. Sequence data revealed that the N protein of BR-01 (de Haan *et al.*, 1990) and those of NL-07 (de Haan *et al.*, 1992) and TSWV-I (Law *et al.*, 1991a, c) which is almost identical to NL-07 (de Ávila *et al.*, 1992; de Haan *et al.*, 1992) share an overall identity of 67% in their amino acid sequences. These serological and molecular studies provide sufficient data to consider the serogroup I and III viruses as different species within the genus *Tospovirus*, denoted as tomato spotted wilt virus and *Impatiens* necrotic spot virus, respectively (Law *et al.*, 1991a, b and c; de Ávila *et al.*, 1992; de Haan *et al.*, 1992). In addition, the serogroup I and II viruses are less distinct. Using ELISA, the N protein of these isolates are consistently at least 100 fold less reactive in heterologous reactions (Fig. 2). Moreover, one of the two MAbs produced to the N protein of BR-01 does not react with two isolates of serogroup II, while both do not react with the other two studied isolates of this serogroup II (Fig. 4). These observations, confirmed by Western blot analysis (Fig. 5), prompted de Ávila *et al.* (1990) to distinguish two serotypes in serogroup II (Table 1). Although it is clear from the present study that the serogroup II isolates do not belong to the species TSWV and INSV, it remains to be established whether, serogroup II represents one or two additional tospovirus species. This means that sequence data are required to distinctively classify the taxonomic position of these isolates.

The serological data using antisera against denatured G1 glycoprotein (Fig. 6) and the results obtained by immunogold decoration of the virus particles (de Ávila *et al.*, 1992; Kitajima *et al.*, 1992), show that this protein is more conserved than the nucleocapsid proteins within the genus *Tospovirus*, an observation also found for the genus *Phlebovirus* (Shope, 1985; Pifat *et al.*, 1988). A similar conclusion has also been made by Law *et al.* (1991a).

The present study clearly shows that most phenotypic tospovirus characteristics such as cytopathology, nucleocapsid composition and sizes of RNA segments are genus-specific and cannot, thus, be used to define species. Other characteristics such as nucleotide sequences of certain genes, amino acid sequences or derivatives thereof such as serology are required to distinguish possible species. Distinction of tospoviruses on the basis of N protein sequence divergence may provide a reliable approach. The N protein, part of the replication complex, has a central function during the infection in

regulating the switch from transcription to replication, and encapsidating genomic and anti-genomic RNA (Beaton & Krug, 1984, 1986). Thus we anticipate that this protein, serving in different processes and showing significant diversity, will be useful in establishing strains and species in the tospoviruses on the base of its serology and primary sequences.

After some mechanical transfers aggregates consisting of nucleocapsid protein are discerned in the infected cells. Since they lack a membrane envelope it is difficult to reconcile that they are transmitted by thrips. In addition, transmission experiments with lines consisting of morphological defective isolates were unsuccessful (data not published). These defects either caused by internal deletions in the L RNA or by morphological defects do not affect the serology using N-protein antisera or species relationships.

Although not fully characterized, other tospovirus isolates currently reported as TSWV may turn out to be new species. For instance, two viruses isolated from groundnut, denoted as groundnut bud necrosis virus (Chanekar *et al.*, 1979; Reddy *et al.*, 1992) and peanut yellow spot virus (Reddy *et al.*, 1991) are likely candidates for new species. Another one may be watermelon silver mottle virus, since this virus appears serologically different from TSWV (Kameya-Iwaki *et al.*, 1988). Likewise, a tospovirus isolated from *Verbesina alternifolia* (Hayati *et al.*, 1990) seems to lack any serological relationship with TSWV and INSV. Thorough studies, however, in which these isolates are compared with well characterized TSWV and INSV isolates have thus far not been reported.

In conclusion, our results show that various tospovirus isolates can be differentiated by serological and molecular hybridization studies. The clear and consistent differences observed among the isolates studied indicate that different species exist within the tospoviruses. For a conclusive classification, however, sequence data of appropriate genes or genome segments are required.

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CHAPTER 6 CLASSIFICATION OF TOSPOVIRUSES BASED ON PHYLOGENY OF NUCLEOPROTEIN GENE SEQUENCES

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SUMMARY

The nucleotide sequences of the nucleoprotein (N) genes of seven tospovirus isolates representing three serogroups were determined and used to establish phylogenetic parameters to delineate species within the *Tospovirus* genus of the Bunyaviridae. A high sequence divergence (55.9% homology on nucleotide level) was observed between isolates of serogroup I (tomato spotted wilt virus, TSWV) and isolates of serogroup III (*Impatiens* necrotic spot virus, INSV). The serogroup II isolates take an intermediate position. Their N genes have a 75% homology with the N genes of serogroup I isolates and 57% with those of serogroup III isolates. Whereas the isolates within serogroup I or serogroup III showed almost identical sequences, the two isolates BR-03 and SA-05 of serogroup II significantly diverged from each other (82.1% sequence homology). The results obtained support the conclusion that, in addition to the species TSWV and INSV, both the serogroup II isolates BR-03 and SA-05 have to be considered as distinct species within the genus *Tospovirus* for which the names tomato chlorotic spot virus and groundnut ringspot virus are proposed respectively.

This chapter is *in press* in a slightly modified version as: de Ávila *et al.* (1993). Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. *Journal of General Virology.*

INTRODUCTION

Virus isolates described as tomato spotted wilt virus (TSWV) cause worldwide serious diseases in many crops and infect a considerable number of different plant species (Peters *et al.*, 1991). Whereas in the past TSWV infections were mainly found in (sub)tropical regions, devastating outbreaks also occurred in non-solanaceous crops in the Northern Hemisphere in the last decade. This expansion is caused by the spread of the thrips *Frankliniella occidentalis* (Perg.), an efficient vector of TSWV, over the USA and Canada, and its subsequent invasion in Europe (Cho, 1986; Marchoux, 1990; Vaira *et al.*, 1992).

Virus particles of TSWV isolates are spherical (70 to 110 nm in diameter) with a lipid membrane covered with surface projections formed by glycoproteins. The viral genome consists of three linear single stranded RNA segments, denoted L, M and S, complexed with nucleocapsid protein (N) and presumably with a viral transcriptase. The complete nucleotide sequences have become available for the genome of the Brazilian isolate (BR-01). The L RNA (8.9 kb) is of negative polarity and encodes a putative RNA polymerase of 331.5 K (de Haan *et al.*, 1991). The two other genomic RNAs use ambisense coding strategies. The M RNA (4.8 kb) codes for a precursor to the two envelope proteins G1 (78 K) and G2 (58 K) and a non-structural protein denoted NSm (Kormelink *et al.*, 1992a, b). The S RNA (2.9 kb) encodes the N protein (28.8 K) and another non-structural protein (NSs, 52.4 K) (de Haan *et al.*, 1990; Kormelink *et al.*, 1991).

Based on the detailed knowledge of the BR-01 isolate, TSWV has been classified as a sole member of the newly created genus *Tospovirus* within the family of Bunyaviridae (Francki *et al.*, 1991). In view of the worldwide spread of tospovirus isolates, able to infect a high number of plant species (more than 500 are reported), one may question whether these virus isolates do not show enough variation to consider them as belonging to more than one single virus species. De Ávila *et al.* (1990, 1992a, b) indeed showed that a selection of 20 isolates originating from different geographical areas and crops can be divided into three distinct serogroups using polyclonal antibodies directed to their N proteins. Most isolates studied belong to serogroup I, including type isolate BR-01. The serogroup I isolates reacted only weakly with antibodies to serogroup II viruses, and not

at all with antibodies raised against serogroup III viruses. This serogroup consists of almost completely identical isolates from *Impatiens* plants in the USA (TSWV-I, Law & Moyer, 1990, Law *et al.*, 1991) and in The Netherlands (NL-07, de Ávila *et al.*, 1992a, de Haan *et al.*, 1992) differing entirely from the serogroup I and II viruses in a serological way (Law & Moyer, 1990; Law *et al.*, 1991; de Ávila *et al.*, 1992a, b). Therefore, serogroup III isolates are being considered as belonging to a different species, which was denoted *Impatiens* necrotic spot virus (INSV), while serogroup I may represent a species for which the name tomato spotted wilt virus should be reserved. Furthermore, serogroup II splits into two distinct serotypes (de Ávila *et al.*, 1990), both of which possibly represent an additional species. Other distinct TSWV-like isolates have been found in groundnut (Chanekar *et al.*, 1979; Reddy *et al.*, 1991, 1992) and watermelon (Kameya-Iwaki *et al.*, 1988) but their relationship with the serogroup I, II and III have not fully been characterized yet.

To establish criteria to define the taxonomic status of the various tospoviruses into species, the nucleotide sequences of their N genes and their amino acid composition of seven isolates, preliminary classified into three serogroups, were determined and compared.

METHODS

Virus isolates

Isolate BR-03 was collected from tomato in Brazil, and SA-05, kindly supplied by Dr G. Adam (Braunschweig, FRG), isolated by Dr. Pietersen from groundnut in South Africa. Both isolates, classified as serogroup II members (de Ávila *et al.*, 1991, 1992b) were multiplied in *Nicotiana rustica* L. var. America. RNA was extracted from purified nucleocapsids as described previously (de Haan *et al.*, 1989; de Ávila *et al.*, 1990).

Complementary DNA clones and nucleotide sequence analysis

Complementary DNA (cDNA) was synthesized according to Gubler & Hoffman (1983). The RNA was primed by a synthetic oligonucleotide (5'-CCCGGATCCTG-CAGAGCAATTGTGTCA-3') containing a BamHI site, complementary to the first 15

nucleotides at the 3' end of S RNA which is conserved between isolate BR-01 and isolate NL-07 (de Haan *et al.*, 1992). Double-stranded cDNA was made blunt-end with T4 DNA polymerase and subsequently digested with BamHI, resulting in BamHI/blunt cDNA fragments. These fragments were cloned in pUC19 plasmid vectors, digested with BamHI and SmaI (Yanisch-Perron *et al.*, 1985). The specificity of clones was confirmed by Northern blot hybridization (data not shown). Clones covering the N gene of BR-03 and SA-05 were selected and their nucleotide sequences determined with alkaline-denatured plasmid DNA as templates, using either the standard M13 forward and reverse sequencing primers (Zhang *et al.*, 1988) or synthetic oligonucleotides complementary to previously determined sequences. Sequence alignments were performed using the GCG Wisconsin software package (Devereux *et al.*, 1984).

RESULTS

Molecular cloning and sequence analysis of the N genes of the isolates BR-03 and SA-05

Sequences of the N genes of various serogroup I and III isolates have previously been been reported (de Haan *et al.*, 1989; Maiss *et al.*, 1991; Law & Moyer, 1991). To obtain sequence information of two distinct serogroups II isolate (BR-03 from Brazil and SA-05 from South Africa) their N genes were cloned. Using a specific primer complementary to the 3'-termini of the genomic S RNA of BR-01 or NL-07, several cDNA clones of BR-03 and SA-05 isolates, approximately 1 kb long, containing the complete coding regions of the respective N proteins were obtained. Sequence determination of these clones revealed that for both isolates the N gene ranged from nucleotide 153 to 942 (numbered from the 5' end of the vc strand (Fig. 1), corresponding with a N protein of 258 amino acid residues and a molecular weight of 28,677 K (BR-03) or 28,836 K (SA-05). Both these figures and their homology with the predicted products of the N protein gene of isolate BR-01 (see below) confirm that the cloned sequences represented the N genes of the respective isolates. An alignment of the N gene sequences of serogroup II isolates, BR-03 and SA-05, with those of serogroup isolate BR-01 and serogroup III isolate NL-07 is presented in Fig. 1.

Divergence among S RNA sequences of different tospovirus isolates

The determined nucleotide sequences of the S RNA of BR-03 and SA-05 were compared with those of BR-01 (TSWV) and NL-07 (INSV). The alignment shown in Fig. 1 reveals that only the first fifteen nucleotides at the 3'-termini are fully conserved. The remaining part of the 3' noncoding sequence shows a remarkable lower homology. In this region (till the start of the N gene) the serogroup II isolates display a homology of 65.8 (BR-03) to 70.9% (SA-05) with serogroup I and only 46 to 47% with serogroup III. The homology in the 3' non-translated region of the S RNA of serogroup I and III isolates was also estimated at 50.7% (de Haan *et al.*, 1992). On the other hand, the sequence homology of this region is highly conserved (99%) among isolates within serogroup I or III, whereas it is less conserved between the two serogroup II isolates (88.1%, Fig. 1 and Table 1).

The same conclusions, as derived from the noncoding region, can be drawn from the translated region within the various determined S RNA sequences, i.e. the N gene, showing similar levels of divergence and conservation between and within serogroups, respectively (Fig. 1 and Table 1).

Divergence among N protein sequences of different tospovirus isolates

Since the serogrouping of tospoviruses (de Ávila *et al.*, 1990, 1992a, b) has been based on analyses using poly- and monoclonal antibodies directed to the N protein, it is worthwhile to determine whether this grouping fits with the rates of divergence in the N protein sequences. Moreover, this sequence divergence can possibly be used as a molecular parameter, and thus a true phylogenetic criterion for the classification of tospoviruses into species and strains. To this end an alignment was made of the newly determined serogroup II N protein sequences with those of previously published sequence data from serogroup I isolates BR-01, Haw (from Hawaii) and L3 from Bulgaria (de Haan *et al.*, 1990; Maiss *et al.*, 1991; German, personal communication) and NL-07 (Law *et al.*, 1991; de Haan *et al.*, 1992). The alignment is shown in Fig. 2 and the calculated percentages of sequence homology in Table 1.

The N protein sequences of the two serogroup II isolates was for 76.4 (BR-03) to 78.2 % (SA-05) homologous to all serogroup I isolates; the latter showing 99% or more

Table 1. Nucleotide and amino acid sequence homologies (%) of the 3' end of the S RNA molecules of four tospovirus isolates encoding the N protein.

	Tospovirus isolates			
	BR-01	BR-03	SA-05	NL-07
BR-01		*65.8	70.9	50.7
		#74.2	75.9	55.9
		$76.4	78.2	55.4
BR-03			88.1	46.0
			82.1	57.6
			81.0	56.0
SA-05				47.0
				57.3
				54.0

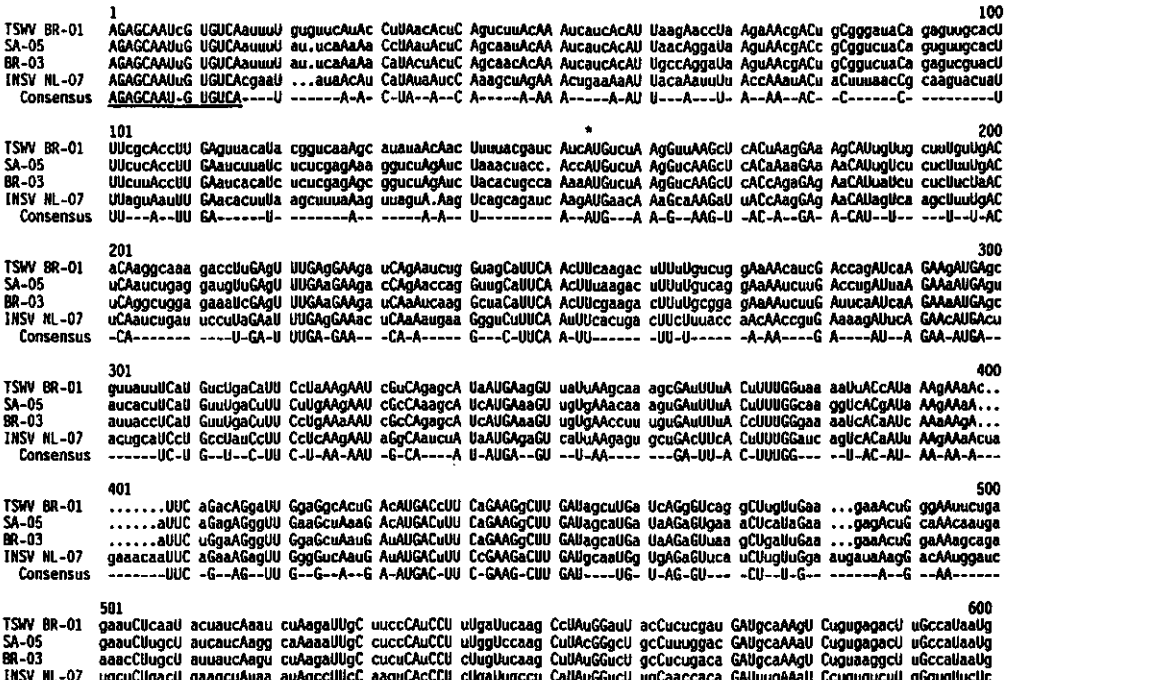
(*) % of nucleotide sequence homology at 3' non-coding region of the S RNA. (#) % of nucleotide sequence homology of the N gene. ($) % of amino acid sequence homology of the N protein.

homology to each other. The homologies in the protein sequences is considerably lower (56-54%) between serogroup II and serogroup III isolates, and is of the same level as the homologies between serogroup I and III N protein sequences (Table 1). As found for the N gene, also the N protein sequences of both serogroups II isolates significantly diverged, showing only 81% homology. Despite this clear distinction, both serogroup II isolates are serologically closer related to each other than to the isolates of the other serogroups (De Avila *et al.*, 1991; 1992b).

Finally, examination of the alignment of the N protein sequences (Fig. 2) of the three serogroups reveals that four main domains in the N proteins are highly conserved particularly between residues 51 and 204. Analysis of the N proteins showed that two hydrophilic domains are located between the residues 30 to 40 and 160 to 190, respec-

*Fig. 1 - Alignment of the 3' end of the S RNA molecules carrying the N gene of four tospovirus isolates representing three serogroups. Nucleotides are numbered from the 5' end of the vc strand. The nucleotide sequences of the isolates TSWV-L3 and TSWV-Haw (serogroup I) and TSWV-I (serogroup III) are not shown since they are almost identical to that of BR-01 (serogroup I) and NL-07 (serogroup III), respectively. The asterisks * indicate translational start and termination codons. Dots representing gaps are introduced to reach an optimal alignment. The 3' terminal consensus sequence is underlined.*

ALIGNMENT OF THE 3' ENDS OF THE S RNA MOLECULES OF DISTINCT TOSPOVIRUS SPECIES



ALIGNMENT OF THE N PROTEINS OF DISTINCT TOSPOVIRUS SPECIES

```
                1                                                                                                  100
TSWV BR-01 MsKvKlTkEs IvaLLTQgkd lEFEEdQnlv aFNFktFcle NldqIkkNsv iSCLtFLKNR QSIMkVikqs DFTFGkiTIK Kt...SdRig gTDNTFRRLD
TSWV L3    MsKvKlTkEs IvaLLTQgkd lEFEEdQnlv aFNFktFcle NldqIkkNsv iSCLtFLKNR QSIMkVikqs DFTFGkiTIK Kt...SdRig aTDNTFRRLD
TSWV Haw   MsKvKlTkEs IvaLLTQgkd lEFEEdQnlv aFNFktFcle NldqIkkNsi iSCLtFLKNR QSIMkVikqs DFTFGkiTIK Kt...SdRig aTDNTFRRLD
SA-05      MsKvKlTkEn IvsLLTQsed vEFEEdQnqv aFNFktFcqe NldlIkkNsi tSCLtFLKNR QSIMkVvkqs DFTFGkvTIK Kn...SeRve akDNTFRRLD
BR-03      MsKvKlTrEn IisLLTQage tEFEEdQika tFNFedFcge NldsIkkNsi tSCLtFLKNR QSIMkVvnlc DFTFGkiTIK Kn...SgRvg anDNTFRRLD
INSV NL-07 MnKaKiTkEn IvkLLTQsds lEFEEtQneg sFNFtdFftn NrekIqrMtt aSCLsFLKNR QSIMrViksa DFTFGsvTIK KtrnnSeRvg vrDMTFRRLD
INSV I     MnKaKiTkEn IvkLLTQsds lEFEEtQneg sFNFtdFftn NrekIqrMtt aSCLsFLKNR QSIMrViksa DFTFGsvTIK KtrnnSeRvg vrDMTFRRLD
Consensus  M-K-K-T-E- I--LLTQ--- -EFEE-Q--- -FNF--F--- N---I--M-- -SCL-FLKNR QSIM-V---- DFTFG--TIK K----S-R-- --DNTFRRLD

                101                                                                                                200
TSWV BR-01 slIRVrLve. etgnsenLnt ikskIaSHPL iqaYGLpldD aKSvrLaimL GGSlPLIASV dsfEmisvVL AIYQDakykd LGIdpkKyDT kEAlGKVCTV
TSWV L3    slIRVrLve. etgnsenLnt ikskIaSHPL iqaYGLpldD aKSvrLaimL GGSlPLIASV dsfEmisvVL AIYQDakykd LGIdpkKyDT kEAlGKVCTV
TSWV Haw   slIRVrLve. etgnsenLnt ikskIaSHPL iqaYGLpldD aKSvrLaimL GGSlPLIASV dsfEmisvVL AIYQDakykd LGIdpkKyDT rEAlGKVCTV
SA-05      smIRVkLie. etannenLai ikakIaSHPL vqaYGLpldD aKSvrLaimL GGSlPLIASV dslEmisvVL AIYQDsqvqe LGIeptKynT kEAlGKVCTV
BR-03      smIRVkLie. etgkaenLai ikskIaSHPL vqaYGLpltD aKSvrLaimL GGSiPLIASV dsfEmisiIL AIYQDakykd LGIepsKynT kEAlGKVCTV
INSV NL-07 amvRVhLvgm ikdngsaLte ainslpSHPL iasYGLattD lKScvLgvlL GGSlPLIASV lnfEiaalpL AIYQDakhve LGIdmsKfsT kEAvGKVCTV
INSV I     amvRVhLvgm ikdngsaLte ainslpSHPL iasYGLattD lKScvLgvlL GGSlPLIASV lnfEiaalvL AIYQDakhve LGIdmsKfsT kEAvGKVCTV
Consensus  ---RV-L--- -------L-- ----I-SHPL ---YGL---D -KS--L---L GGS-PLIASV ---E-----L AIYQD----- LGI---K--T -EA-GKVCTV

                201                                                                                                262
TSWV BR-01 LKSKafeMne dqvkKgKeYA aILsssnPnA KGsvAMehYs EtLnkfYeMF gvkkqaklae la
TSWV L3    LKSKafeMne dqvKKgKeYA aILsssnPnA KGsiAMehYs EtLnkfYeMF gvkkqaklte la
TSWV Haw   LKSKafeMne dqvkKgKeYA aILsssnPnA KGsiAMehYs EtLnkfYeMF gvkkqaklte la
SA-05      LKSKgftMdd aqdnKgKeYA kILsssnPnA KGsiAMdyYs DnLekfYeMF gvkkeaklag va
BR-03      LKSKgftMde eqvqKgKeYA tILsscnPnA KGsiAMehYs EhLdkfYaMF gvrkeaklsg va
INSV NL-07 LKSKgysMns veigKaKqYA dILkacsPkA KGlaAMdhYk EgLtsiYsMF natidfgknd si
INSV I     LKSKgysMns veigKaKqYA dILkacsPkA KGlaAMdhYk EgLtsiYsMF natidfgknd si
Consensus  LKSK---M-- ----K-K-YA -IL----P-A KG--AM--Y- E-L---Y-MF ----------
```

Fig. 2 - Alignment of the N protein sequences of seven tospovirus isolates representing three serogroups. Dots are introduced to reach optimal alignment.

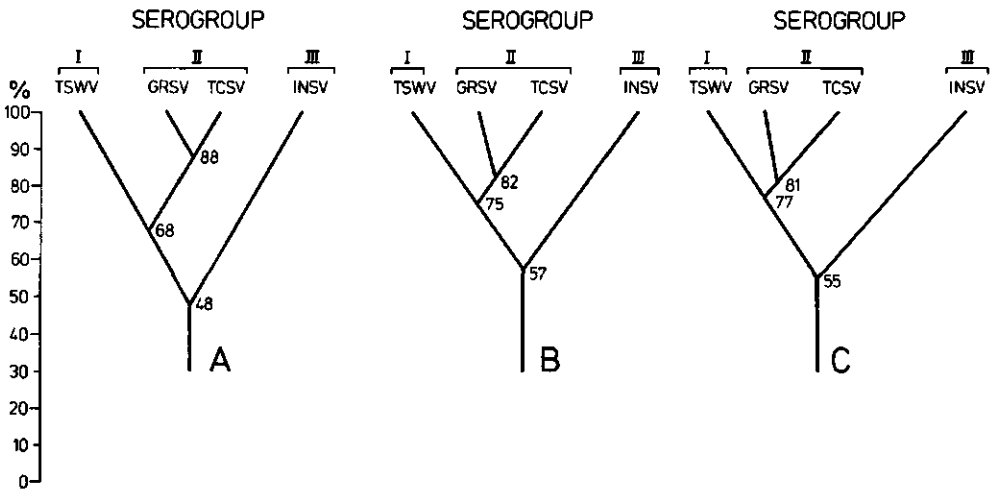


Fig. 3 - Putative phylogenetic tree of tospoviruses based on the homology (%) of the nucleotide sequence of the non-coding (A) and coding region (B) of the N gene encoded by the S RNA segment, and amino acid (C) homology (%) of the nucleocapsid protein.

tively. A highly conserved hydrophobic domain was identified in the middle (125 to 160 residues). It is noteworthy that the position of the methionine residues in the N protein of all isolates studied are conserved and that mainly alpha helix forming amino acids are present.

DISCUSSION

Traditionally, the classification of Bunyaviridae has mainly been based on serology, which resulted in a categorization of these viruses into serogroups and serotypes (Elliot, 1990). Following this classification, and using the antigenic properties of the nucleocapsid protein three serogroups (I to III) have thus far been recognized within the *Tospovirus*, a genus of the Bunyaviridae (Francki *et al.*, 1991). Since the International Committee on Taxonomy of Viruses has recently introduced the species concept in virus taxonomy, it is important to define criteria to delineate species and to avoid the use of less discriminative concepts such as serogroup and -types. In this paper the nucleotide sequences of the N protein gene of two serogroup II isolates, BR-03 and SA-05, have been determined and compared with those of serogroup I and III isolates as to establish discriminative and phylogenetic criteria to define tospovirus species. The crucial role played by this protein in processes as regulating the switch from transcription to replication, functioning in the replication complex and encapsidating genomic and anti-genomic RNA (Beaton & Krug, 1984; 1986) support its selection to define taxonomic criteria for tospoviruses.

The phenotypic and molecular characteristics of the serogroup I and III isolates are basically so much different (Table 1, Law & Moyer, 1990; Law *et al.*, 1991; de Haan *et al.*, 1992; de Ávila *et al.*, 1992a) and their N protein sequences diverged to such an extent (55.4% homology) that these two groups can certainly be defined as two different species. Serogroup I contains the original tomato spotted wilt virus isolates including the type isolate BR-01 (de Ávila *et al.*, 1990) and therefore we propose to employ the name TSWV to replace the term serogroup I (Table 2). The isolates of serogroup III represent a second species, recently proposed as *Impatiens* necrotic spot virus (INSV) (Law & Moyer, 1990; Law *et al.*, 1991, 1992; de Ávila *et al.*, 1992a, b; de Haan *et al.*, 1992). With

respect to serogroup II isolates it was evident from serological studies that they differ notifiable from TSWV and INSV (de Ávila *et al.*, 1992b). Their taxonomic status could not unequivocally be clarified due to overlapping phenotypic characteristics, like host range, symptom expression and cytopathology. Serology using monoclonal antibodies against the N protein showed that these isolates could be divided into two serotypes. Analysis of the nucleotide sequences of the N gene, the amino acid sequence of the N protein, and the nucleotide sequences of the untranslated 3' end regions of the S segment showed that these sequences are 82.1, 81 and 88.1% homologous between BR-03 (serotype I) and SA-05 (serotype II) (Table 1). Although the rate of divergence between these two isolates is less than between TSWV and INSV isolates, we propose to consider them as two distinct species. This proposal is supported by the observation that the N genes within the TSWV and INSV species, with isolates originating either from Brazil, Hawaii and Bulgaria or from the USA and The Netherlands, have an almost 100% nucleotide homology. The two novel species may be named tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV) for the serotype I (BR-03) and II (SA-05) viruses, respectively (Table 2).

Other, not fully characterized, tospoviruses are currently reported as possible new species (Chanekar *et al.*, 1979; Kameya-Iwaki *et al.*, 1988; Reddy *et al.*, 1991, 1992) but clarification of their taxonomic position awaits further studies.

It is noteworthy that the similarity in N proteins among the three serogroups (now species) of the *Tospovirus* genus is higher than among serogroups of the *Bunyavirus* and *Phlebovirus* genera. The N proteins of six viruses representing three serogroups in the genus *Bunyavirus* show an overall sequence similarity of 40%, whereas a homology of 80% or more occurred within a serogroup (Elliot, 1990). The *Phlebovirus* genus N proteins show a divergence which rates from 54 to 30% among serogroups, and a higher relatedness within a single serogroup (Simons *et al.*, 1990; Giordi *et al.*, 1991). However, the amino acid homology of the *Hantavirus* genus N proteins varies from 61 to 83% (Arikawa *et al.*, 1990; Stohwasser *et al.*, 1990). These values resemble those now found for the *Tospovirus* genus. The similar homologies found in both genera might be explained by stronger constraints on the evolution of the N protein genes.

The limited number of vectors used by tospoviruses (Sakimura, 1962) and the non-

Table 2. Proposed species within the genus Tospovirus (Bunyaviridae).

Isolate	Origin		Sero -		Species
	Country	Host	group	type	
BR-01	Brazil	Tomato	I		tomato spotted wilt[1] (TSWV)
BR-03	Brazil	Tomato	II	I	tomato chlorotic spot[2] (TCSV)
SA-05	South Africa	Groundnut	II	II	groundnut ringspot[3] (GRSV)
TSWV-I	USA	Impatiens	III		*Impatiens* necrotic spot[4] (INSV)
NL-07	Netherlands	Impatiens			

(1) de Haan et al. (1990); de Ávila et al. (1990) (2) de Ávila et al. (1990, 1992b) (3) de Ávila et al. (1990, 1992b) (4) Law et al. (1991, 1992); de Ávila et al. (1992a, b); de Haan et al. (1992).

biological transmission of the hantaviruses between rodents and humans (Gonzales-Scarano & Nathanson, 1990) may be one of these constraints. The viruses of the other two genera, *Bunyavirus* and *Phlebovirus*, displaying higher divergence in the N protein, are transmitted by vector species belonging to different families or orders such as mosquitoes, *Culicoides*, phlebotomines and ticks (Gonzales-Scarano & Nathanson, 1990; Peters, 1991).

The Bunyaviridae are classified into five genera based on their mode of transmission, coding strategy and composition of the 3' and 5' end terminal sequences (Francki *et al.*, 1991). The viruses, within the four genera consisting of viruses infecting animals, are basically classified into antigenic groups using hemagglutinin and neutralizing antigenic determinants present on virus glycoproteins and complement fixation associated with nucleocapsid protein (Gonzales-Scarano & Nathanson, 1990). These serological data, and also molecular data found for some, support the existence of several virus species within each genus of this family (Gonzales-Scarano & Nathanson, 1990; Elliot, 1990). However, parameters or criteria by which species could taxonomically be distinguished within the four genera, as now proposed for tospoviruses, still await to be formulated.

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CHAPTER 7 CONCLUDING REMARKS

Tomato spotted wilt virus (TSWV) has been considered for years to form a monotypic plant virus group, not related to any other taxonomic group of plant viruses. Based on the morphological and molecular properties as determined in our laboratory during the past four years, the virus has been identified as a member of the Bunyaviridae, a large family of viruses thus far only regarded to infect animals. Since TSWV is unique in its property to infect plants, it has been placed into a newly created genus, called *Tospovirus* (Francki *et al.*, 1991). As TSWV occurs worldwide, using several thrips species as vector (Sakimura, 1962; Best, 1968) and - moreover - infecting an impressive host range, it may be wondered whether all TSWV isolates belong to a single virus species. This thesis reports studies aimed to analyze and to establish a number of possible, but taxonomically stable descriptors to classify the various isolates from different geographical regions and crops by their phenotypic and molecular characteristics. Such a classification is required to analyze the ecology and epidemiology of tospoviruses and to sustain resistance breeding in susceptible crops.

The first attempts to classify TSWV isolates were based on host responses. Some isolates were differentiated into strains evoking different symptoms on a series of indicator plants. This classification has not been followed up in the past due to the lack of other descriptors. However, this situation has drastically been changed with the development of reliably serological methods, the production of specific and high tittered antisera, and the elucidation of the nucleotide sequences of the genome.

Like for the other genera within the Bunyaviridae, serological parameters can be applied to delineate antigenic groups within the *Tospovirus* genus. The results reported in this thesis show that the nucleocapsid (N) protein represents a useful parameter to discriminate between tospoviruses in serological studies, using either polyclonal or monoclonal antibodies directed to this protein. Thus three distinct serogroups (I, II and III) could be distinguished (Chapters 2, 4 and 5), while, based on different reactivities with monoclonal antibodies, two serotypes (I and II) could be recognized within serogroup II. Most phenotypic tospovirus characteristics such as host range, symptom expression, particle morphology, cytopathogenicity, morphology of inclusion bodies,

nucleocapsid composition and sizes of RNA segments turned out to be genus specific and could, therefore, not be used to differentiate species unambiguously (Chapters 3, 4 and 5). The serogroup I and II isolates evoke systemic infections on most solanaceous species tested, whereas serogroup III viruses induce necrotic symptoms in these hosts, which remain either restricted to the inoculated leaves or expand into the neighboring parts followed by death of the plant. This difference in symptom response is more of epidemiological than of taxonomical interest.

However, the absence of any serological cross-reactivity between the viruses of serogroups I and III justifies to consider them as different species. This conclusion is backed by the sequence divergence of their N protein sequence (55.4% amino acid sequence homology). The serogroup III viruses are therefore proposed to represent a novel species for which the name *Impatiens* necrotic spot virus (INSV) has been coined, while for serogroup I, comprising the most detailed studied Brazilian isolate BR-01, the name tomato spotted wilt virus has been reserved (Chapter 4).

The serogroup II isolates do not only significantly differ from serogroups I and III but also exhibit intra-group variation within the serogroup. They could be split into two serotypes using two monoclonal antibodies directed to the N protein of the isolate BR-01 (Chapters 2 and 5). Analysis of the S RNAs of BR-03 (serotype I) and SA-05 (serotype II) showed that the N genes, and non-translated 3' regions are only homologous for 82.1 and 88% (Chapter 6). A homology of 81% was found for the N proteins of these two isolates. These values are significantly lower than the identity of 99% or more of the N genes of isolates which belong to one of the other two serogroups. This strong conservation of sequences among isolates belonging to serogroup I or serogroup II justifies to consider the serotype I and II isolates of serogroup II also as two different species for which the names tomato chlorotic spot virus (TCSV) for the isolate BR-03 (serotype I) and groundnut ringspot virus (GRSP) for the isolate SA-05 (serotype II) are coined (Chapter 5). The complete absence of any protection against these viruses in transgenic tobacco lines which are highly resistant to TSWV supports this conclusion (de Haan *et al.*, 1992).

The proposed taxonomy of the tospoviruses is thus mainly based on the divergence of the N protein sequence as established by serology and/or sequence determination of the

N gene. This seems to be the most reliable and feasible approach to classify tospoviruses as this protein indeed displays a clear divergence. An additional advantage of selecting the N protein for taxonomic purpose, is that identification is still possible even when eventually an isolate becomes morphologically defective during its maintenance under laboratory conditions, i.e. does not produce glycoproteins (Resende *et al.*, 1991), or generate defective interfering L RNA (Resende *et al.*, 1992).

The rate of N protein sequence divergence among the different tospovirus serogroups is significantly lower than within the *Phlebovirus* and *Bunyavirus* genera (Simons *et al.*, 1990; Giordi *et al.*, 1991; Elliot, 1990) but is comparable to the N sequence divergence found within the genus *Hantavirus* (Arikawa *et al.*, 1990; Stohwasser *et al.*, 1990). One plausible explanation for these discrepancies is that viruses of the *Phlebovirus* and *Bunyavirus* genera are transmitted by vector species belonging to different dipteran families such as the Psychodidae (sandflies), Culicidae (mosquitoes) and Ceratopogonidae (biting midges) and an acarian family Ixodidae (ticks) which may have led to distinct adaptations and therefore further divergence. Along this line the evolution of the tospoviruses would be more restricted as they can only pass through a limited number of thrips species during their transmission. The niche of hantaviruses is also narrow as their occurrence is restricted to rodents while they are transmitted in a spin-off process by contact to human beings.

However, it remains difficult to explain why tospoviruses developed the existing variations in their N protein sequence during their evolution. This variation may reflect the biovariation needed to cover their very broad host range, comprising a large number of both mono- and dicotyledons. Thus far more than 550 plant species, belonging to at least 70 families, have been reported to be susceptible to tospoviruses (Peters, unpublished data). Many belong to the Compositae and Solanaceae. However, as INSV causes usually necrotic responses on the solanaceous species, the number of solanaceous species involved in the spread of this virus will be limited. The niche of this species seems to consist of other plant species, e.g those used as ornamentals (Vaira *et al.*, 1992). The host range of the other two proposed species, TCSV and GRSV, greatly overlaps with the host range of TSWV (Chapter 5).

Analysis of the global distribution of the tospoviruses shows that the isolates belonging

to serogroup I (i.e. the species TSWV), wherever collected, are serologically almost identical. The INSV isolates Type-I and NL-07 (Chapter 4), originating from in the USA and The Netherlands, respectively, and a number of less well characterized INSV isolates found in France and Italy are also serologically indistinguishable. Finding of virtually identical tospoviruses worldwide may mean that no "Old World" or "New World" isolates can be distinguished, as found for the Arenaviridae. Thus far, the spread of TCSV and GRSV seems to be restricted to the (sub)tropics as they have only been found in Brazil and South Africa. Finally, groundnut seems to be an allopathic host for tospoviruses. In the USA, TSWV seems to be the most abundant virus in this crop (Mitchell & Smith, 1991), while groundnut ring spot virus is found in South Africa. In India, groundnut hosts bud necrosis virus (GBNV), which presumably represents an additional other tospovirus species (Reddy *et al.*, 1992). The prevalence of different tospoviruses in groundnut may be explained by the different thrips species prevailing in groundnut in these countries. GBNV is transmitted by *Thrips palmi* Karny (Palmer *et al.*, 1990), whereas *Frankliniella fusca* Hinds seems to be the main vector of TSWV in groundnuts in the USA (Mitchell & Smith, 1991). No information is available on the thrips species transmitting TCSV and GMSV.

Although not fully characterized, other tospovirus isolates currently reported as TSWV may turn out to be further new species in the genus *Tospovirus*. A serological comparison showed that GBNV is serologically unrelated with TSWV and INSV, confirming the idea that GBNV is indeed another tospovirus species (Reddy *et al.*, 1992). Other novel tospoviruses may include watermelon silver mottle virus (WSMV) (Kameya-Iwaki *et al.*, 1988; Yeh *et al.*, 1992) and a tospovirus isolate from *Verbesiana alternifolia* (Hayati *et al.*, 1990). Finally, peanut yellow spot virus (PYSV) may represent another species (Reddy *et al.*, 1991). Detailed studies, in which all these isolates are compared with the more thoroughly studied species have thus far not been reported.

The present study demonstrates that TSWV is not the sole member of the Tospovirus genus, but that several species make up this genus (Chapter 6). The proposed classification for tospoviruses based on phenotypic characteristics and molecular parameters (primarily sequence data of the nucleocapsid gene) can be used to describe the taxonomic and phylogenetic relations between the tospoviruses.

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SAMENVATTING

Het bronsvlekkenvirus van de tomaat, dat in het Engels "tomato spotted wilt virus" (afgekort: TSWV) wordt genoemd, is een wereldwijd voorkomend plantepathogeen. Het veroorzaakt - met name in de tropen - veel schade aan een groot aantal gewassen. Het virus wordt uitsluitend door tripsen verspreid. Recentelijk is het virus veel aandacht gaan trekken nadat de Californische trips, *Frankliniella occidentalis*, één van de belangrijkste vectoren van dit virus, zich is gaan verspreiden over het noordelijk halfrond, en wellicht zelfs over de gehele wereld, vanuit de westelijke staten van de Verenigde Staten van Amerika.

Het ontbreken van goede antisera en moleculaire informatie heeft lange tijd een goede classificatie van dit virus in de weg gestaan. Dit leidde er toe dat het virus aanvankelijk in een monotypische plantevirusgroep werd ondergebracht. Op grond van morfologische eigenschappen, wijze van overdracht en genoomstructuur is het virus thans ondergebracht bij de Bunyaviridae, een grote familie van diervirussen die biologisch veelal door tal van insecten en teken overgebracht worden. Vanwege het afwijkend gastheerbereik (planten in plaats van dieren) is TSWV binnen deze familie in een apart genus (Tospovirus) ondergebracht.

De virusdeeltjes hebben een diameter van 70 tot 110 nm. Zij bestaan uit drie nucleocapsiden omgeven door een lipidemembraan. Deze membraan bevat twee viraal gecodeerde glycoproteinen, G1 en G2, genoemd. Het genoom bestaat uit drie enkelstrengs lineaire RNA segmenten, die S (small), M (medium) en L (large) RNA worden genoemd en met het nucleocapside eiwit de drie nucleocapsiden vormen.

Op grond van het grote waardplantenbereik van TSWV en het vermogen te worden overgebracht door verschillende tripssoorten mag verwacht worden dat er een grote variatie tussen verschillende isolaten van dit virus kan bestaan. Tot voorkort was het niet mogelijk om de diverse isolaten op welke manier dan ook te onderscheiden. Dit proefschrift beschrijft een studie waarin het onderscheiden en classificeren van TSWV isolaten centraal stond. Kennis van de variabele eigenschappen waarin TSWV isolaten kunnen verschillen is niet alleen louter van taxonomisch belang maar ook essentieel om tot een gerichte en verantwoorde bestrijding van het virus te komen, zijn epidemiologie

te leren kennen en over de juiste toetsen te kunnen beschikken in de veredeling van resistente gewassen.

Allereerst is er een analyse gemaakt van een twintigtal verschillende TSWV isolaten, gebruikmakend van een polyklonaal antiserum dat bereid was tegen gezuiverde nucleocapside-eiwit (N) preparaten van een TSWV-isolaat uit Brazilië (BR-01), en twee monoklonale antisera tegen ditzelfde eiwit. Uit deze studie bleek (Hoofdstuk 2) dat de getoetste isolaten in twee serogroepen uiteen vielen. Zestien van de twintig isolaten vertoonden eenzelfde reactie met de gebruikte antilichamen (serogroep I). De overige vier isolaten reageerden slechts zwak met het BR-01 antiserum. Twee van deze isolaten reageerden met geen van beide monoklonalen, de twee andere slechts met een van beiden. Deze vier isolaten werden in een tweede serogroep (serogroep II) met twee verschillende serotypen, I en II, ondergebracht. De zestien serogroep I isolaten die met BR-01 anti-N-serum reageerden, worden - omdat de meeste in het veld op tomaten en peper zijn gevonden - verder als isolaten van TSWV aangeduid.

In het derde hoofdstuk worden de resultaten van een cytopathologische studie aan een dertigtal TSWV isolaten beschreven. Dunne weefselcoupes van geïnfecteerd plantemateriaal werden bestudeerd door middel van elektronenmicroscopie en immunogoud analyse. Tussen de verschillende isolaten werden in cytopathologisch opzicht geen principiële verschillen gevonden. Naast de virusdeeltjes die meestal in clusters in de cisternae van het endoplasmatisch reticulum van geïnfecteerde cellen voorkomen, worden in het cytoplasma ook elektronenmicroscopisch dichte aggregaten en fibrillaire structuren aangetroffen. De aggregaten bleken ondermeer uit nucleocapside-eiwit te bestaan, terwijl de fibrillaire structuren, die in verschillende vormen voorkomen, specifiek reageerden met antisera tegen het non-structurele eiwit (NSs) dat door het S RNA gecodeerd wordt.

In het vierde hoofdstuk zijn de biologische eigenschappen beschreven van een sterk afwijkend Nederlands isolaat (NL-07), dat echter grote overeenkomst vertoont met een isolaat dat in de Verenigde Staten van Amerika door Law en Moyer (J. Gen. Virol. 71: 933-938, 1990) is beschreven. Dit Nederlandse isolaat reageerde totaal niet met het voor serogroep I of serogroep II specifieke antiserum. Op grond van deze serologische resultaten wordt dit isolaat als een nieuw tospovirus beschouwd, waarvoor de naam "*Impatiens* necrotic spot virus" (INSV) is gekozen. Dit virus verschilt echter niet alleen

serologisch van serogroep I en II, maar ook in haar reactie op waardplanten. INSV geeft vaak systemische infecties op bepaalde plantesoorten die als sierplanten, bijv. *Impatiens*, gebruikt worden. Echter de meeste nachtschadeachtigen die vaak systemisch met TSWV reageren geven na infectie met INSV locale necrotische vlekjes op het geïnoculeerde blad. In een gering aantal soorten breiden deze infecties zich uit tot plantedelen die aan het geïnoculeerde blad grenzen, waarna de plant meestal afsterft.

De biologische en de moleculaire eigenschappen van het Braziliaanse isolaat BR-03, en het Zuid-Afrikaanse isolaat SA-05, die als vertegenwoordigers van de beide serotypen in serogroup II worden beschouwd, worden in het vijfde hoofstuk beschreven. De reacties van deze isolaten verschillen op diverse waardplanten in principe niet van die, welke door de serogroep I virussen veroorzaakt worden. Ook met betrekking tot een aantal andere eigenschappen, zoals de verhouding waarin de nucleocapsiden onderling voorkomen, werden geen verschillen gevonden die voor classificatie bruikbaar waren. De conclusies zoals die op grond van serologische analyses in het tweede hoofdstuk zijn getrokken, werden bevestigd in experimenten, waarin antisera tegen het nucleocapside-eiwit van elk van deze isolaten gebruikt werden. In deze studie waarin genoemde isolaten, maar ook andere serotype I- en II isolaten werden getoetst, konden deze serotypen duidelijk van elkaar onderscheiden worden. Bovendien toonden Northern blot hybridisatie experimenten aan dat het RNA van deze isolaten in geringe mate met nucleïnezuur probes van BR-01 reageerden. Ook onderling bleek het RNA van de serotype I isolaten duidelijk van de serotype II isolaten te verschillen. Op grond van deze bevindingen werd gesuggereerd dat de virussen die tot een van deze serotypen behoren als vertegenwoordigers van een aparte virussoort moet worden aangemerkt.

In het laatste hoofdstuk (Chapter 6) wordt aangetoond dat de twee verschillende serotypen uit serogroep II op basis van de verschillen tussen de nucleocapside eiwitten inderdaad als twee verschillende virussoorten beschouwd moeten worden. Vergelijking van de nucleotidenvolgorden toonde aan dat er tussen de nucleocapside-eiwit (N) genen van de isolaten uit serogroup I (TSWV) en III (INSV) een homologie van slechts 55.9% bestaat. Voor de serogroep II isolaten vertoont dit gen meer homologie met de N genen van de serogroep I isolaten (75% homologie) dan met die van de serogroep III isolaten (56% homologie). Terwijl de isolaten uit serogroep I of uit III onderling nagenoeg

dezelfde N gen sequentie blijken te hebben, wijken die van de serogroep II isolaten sterk van elkaar af (82% homologie). Deze resultaten ondersteunen de conclusie dat de serotype I en II isolaten als twee verschillende virussoorten opgevat moeten worden. Voor deze twee nieuwe virussoorten worden de namen "tomato chlorotic spot virus" (TCSV, serotype I) en "groundnut ringspot virus" (GRSV, serotype II) voorgesteld.

Als resultaat van deze studie kunnen we naast TSWV thans drie additionele soorten in het genus *Tospovirus* onderscheiden. Hoogstwaarschijnlijk zullen andere afwijkende isolaten die tot nu toe door andere auteurs minder diepgaand zijn bestudeerd, zoals het groundnut bud necrosis virus en het watermelon silver mottle virus te zijnertijd ook als aparte soorten aan dit genus toegevoegd kunnen worden.

RESUMO

"Tomato spotted wilt virus" (TSWV), denominado no Brasil como virus de vira-cabeça do tomateiro (VCT), apresenta uma distribuição mundial e tem sido responsável por enormes prejuízos nos trópicos em várias culturas importantes. Atualmente este é o vírus de maior importância econômica em hortaliças no Brasil. Nos últimos anos VCT tem recebido grande atenção a nível de pesquisa em todo o mundo, principalmente após a introdução no Hemisfério Norte da espécie de tripes *Frankliniella occidentalis* Perg., que rapidamente disseminou o VCT nessa região.

Devido, à falta de anti-soro com boa sensibilidade e poucas informações a nível molecular, até recentemente, a posição taxonômica do VCT se manteve confusa e inconsistente. Este vírus foi primeiramente classificado no grupo de vira-cabeça do tomateiro tendo o mesmo como único representante. Atualmente, tomando como base as suas características morfológicas, modo de transmissão e organização do genoma, o VCT está classificado como parte de uma grande família de vírus animais denominada Bunyaviridae. Esta família é representada por cinco gêneros cuja grande maioria é transmitida biologicamente por várias espécies de insetos, acarídeos, roedores, etc. No caso do VCT, cujo círculo de hospedeiras se restringe somente à plantas, este virus está classificado em um gênero específico denominado *Tospovirus*.

O VCT apresenta partículas quase isométricas com diâmetro variando entre 70-110nm circundadas por uma membrana de lipídeos. Externamente o virus contém duas glicoproteínas denominadas, respectivamente, G1 e G2. O genoma do virus consiste de três fitas simples de RNA denominadas L, M e S, que associadas com uma outra proteína (28 K) codificada pelo S RNA, formam os nucleocapsídeos.

Este virus apresenta um dos mais amplos círculo de hospedeiras entre os virus de plantas, sendo transmitido por, pelo menos, oito espécies de tripes. Portanto, sendo um virus com tamanha diversidade biológica e encontrado nos mais diversos nichos ecológicos, é de se esperar uma larga diversidade entre os isolados de VCT.

Esta tese tem como objetivo principal explorar a variabilidade existente entre isolados do VCT definindo parâmetros biológicos, serológicos e moleculares que possam caracterizar esta ampla diversidade. Tal conhecimento é importante, não somente do

ponto de vista taxonômico, como também estabele as bases para o entendimento da epidemiologia do virus, identificação e desenvolvimento de variedades resistentes.

As primeiras análises serológicas foram feitas com diversos isolados de vira-cabeça provenientes de várias regiões do mundo, utilizando-se anticorpos policlonais e monoclonais contra a proteína de nucleocapsídeo do isolado BR-01 (capítulo 2). Baseando-se na divergência desta proteína, dois serogrupos foram identificados. Dos 20 isolados estudados, 16 foram agrupados em um único serogrupo denominado serogrupo I. Os quatro isolados restantes tiveram fraca reação cruzada com o anti-soro policlonal proveniente do isolado BR-01. Dentre eles, dois não reagiram com ambos anticorpos monoclonais e os demais com somente um deles. Estes quatro isolados foram então agrupados em um outro serogrupo (serogrupo II), subdividido em dois serotipos (I e II), utilizando-se o painel com dois monoclonais. Os isolados do serogrupo I representam o VCT e sua maioria foi encontrada em plantas solanáceas como tomate e pimentão.

O capítulo 3 descreve um estudo citopatológico de 30 isolados de VCT. Preparações ultra-finas de tecido de plantas infectados foram estudados a nível de microscopia eletrônica e imunomarcação com ouro. Basicamente os diversos isolados não puderam ser diferenciados, pois apresentaram efeitos citopatológicos muito semelhantes. Partículas virais, normalmente agregadas em vesículas, foram sistematicamente encontradas na cisterna do retículo endoplasmático de células infectadas. Além disto, dois tipos de inclusões puderam ser observadas no citoplasma. A primeira consiste de material densamente agregado, que representa proteína livre de nucleocapsídeo. Outra inclusão viral, formada por estruturas fibrilares, presentes em diferentes formas, representam a proteína não estrutural (NSs) codificada pelo S RNA, uma vez que reagem especificamente com anti-soro para esta proteína.

O capítulo 4 descreve a caracterização de um isolado holandês de vira-cabeça (NL-07), originado de *Impatiens* sp. Os resultados mostraram quer este virus é idêntico a outro descrito na mesma hospedeira nos Estados Unidos (Law & Moyer, 1990, J. of Gen. Virol., 71: 933-938). O isolado holandês (NL-07), a nível serológico, não apresentou nenhuma reação cruzada com os anti-soros dos isolados pertecentes aos serogrupos I e II. Com base nos resultados serológicos e moleculares (capítulo 6), este isolado foi proposto como uma nova espécie de virus no gênero *Tospovirus* denominada como

"*Impatiens* necrotic spot virus" (INSV). Tal virus tem sido encontrado principalmente em um grande número de espécies ornamentais e dificilmente infecta sistemicamente solanáceas, mantendo-se restrito às folhas inoculadas. Tal limitação a nível de círculo de hospedeiras confirma-se pelo fato do INSV ser naturalmente detectado somente em plantas ornamentais, apesar da mesma espécie de tripes *Frankliniella occidentalis* transmitir com eficiência VCT e INSV.

A caracterização biológica e molecular de dois tospovirus, um brasileiro e outro sul-africano (BR-03 e SA-05), respectivamente classificados nos serotipos I e II (serogrupo II), é discutida no capítulo 5. Os resultados mostraram que estes isolados são biologicamente muito semelhante ao VCT. A comparação do padrão de RNA e proteínas dos vírus mostraram ser parâmetros não adequados para a discriminação entre isolados. Entretanto, as análises serológicas anteriormente descritas no capítulo 2 puderam ser reconfirmadas utilizando-se anti-soros contra a proteína de nucleocapsídeo específico para cada isolado dentro de cada serogrupo. Os isolados nos três serogrupos apresentam uma divergência significativa na proteína de nucleocapsídeo, e portanto, mostrou-se como melhor parâmetro para definir espécies de vírus dentro do gênero *Tospovirus*. O estudo serológico dos isolados BR-03 (serotipo I) e SA-05 (serotipo II), mostrou que os mesmos não somente divergem entre si como também com relação aos serogrupos I (VCT) e III (INSV). A análise molecular destes isolados, através da técnica "northern blot" utilizando-se sondas de cDNA específicas para o gene de nucleocapsídeo de cada isolado dos diferentes serogrupos e tipos, confirmou as divergências serológicas encontradas na proteína de nucleocapsídeo.

Finalmente, o capítulo 6 define os dois isolados representantes dos serotipos I (BR-03) e II (SA-05) como duas novas espécies de vírus no gênero *Tospovirus*. Estudo comparativo das sequências dos genes que codificam para a proteína de nucleocapsídeo nos diversos isolados pertencentes aos serogrupos I (VCT) e III (INSV), mostrou que ambos serogrupos apresentam uma baixa homologia a nível de aminoácidos (55.9%). Portanto tais isolados são considerados duas espécies diferentes de vírus denominadas de VCT e INSV. Praticamente 100% de homologia foi observada entre os isolados pertencentes a estas duas espécies de vírus. No entanto, os isolados dentro do serogrupo II mostraram resultados diferentes. Os isolados BR-03 e SA-05 apresentaram homologia

de aminoácidos de apenas 82% entre si. Este serogrupo mostrou ser geneticamente mais relacionado com o serogrupo I (75% de homologia de aminoácidos) do que em relação ao serogrupo III (56% de homologia). Estes resultados deram suporte à conclusão de que os isolados presentes nos serotipos I e II (serogrupo II) são de fato duas novas espécies de virus sendo propostos os nomes "tomato chlorotic spot virus" para o isolado BR-03 e "groundnut ring spot virus" para SA-05.

Além das quatro espécies de virus descritas neste trabalho, outros isolados de tospovirus, ainda não completamente caracterizados como "groundut bud necrosis virus" e "watermelon silver mottle virus", provavelmente representarão novas espécies dentro do gênero *Tospovirus*.

CURRICULUM VITAE

Antonio Carlos de Ávila was born 11 November, 1952 in Araxá, Minas Gerais, Brazil. He obtained his bachelor degree in Agronomy at University of Brasília, Brasília, 1976 and his master degree in Phytopathology was succefully completed in 1979.

Since january 1979, he has been working as a plant virologist at the National Center for Vegetable Research CNP-Hortaliças/EMBRAPA, Brasília, Brazil. His research programme focuses on viruses in (seed) potato, papaya ringspot virus and tomato spotted wilt virus. The studies made involve characterization and detection of viruses and also support breeding programmes.

From January, 1989, until December 1992, he studied at the Department of Virology, Wageningen Agricultural University. The results of this study are described in this thesis.

The A.C. de Ávila will pursue his career as plant virologist at EMBRAPA.

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