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TOMATO SPOTTED WILT VIRUS IN SRI LANKA: EMERGING PROBLEMS OF TOSPOVIRUSES

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ABSTRACT

***Tomato spotted wilt virus* (TSWV), the type species of the genus *Tospovirus* has long been reported infecting economically important horticultural crops such as tomato, groundnut, pepper, potato and soybean, all over the world. In Sri Lanka, characteristic tospovirus symptoms such as bud necrosis, axillary shoot proliferation and ring spots were observed on leaves of groundnut in Angunakolapalassa, and concentric rings on leaves and fruits of tomato, in Ambalantota and Gannoruwa. Sap extracted from both tomato and groundnut plants was serologically positive for TSWV infection in a double antibody sandwich enzyme-linked immunosorbentassay (DAS-ELISA), but not for Groundnut bud necrosis virus (GBNV). Nucleocapsid (N) genes of both isolates were amplified in Reverse Transcription Polymerase Chain Reactions (RT-PCR) using primers specific for TSWV and GBNV. The sequences obtained showed 98% amino acid sequence identity to the N gene of the Brazilian isolate of TSWV-BR-01. This study confirms the presence of TSWV infecting groundnut and tomato in Sri Lanka.**

Key words: DAS-ELISA, TSWV, Serology, RT-PCR

INTRODUCTION

The genus *Tospovirus* represents the only genus infecting plants within the family *Bunyaviridae.* The viruses of the other four genera of this family infect animals. The viruses of the genera *Orthobunyavirus*, *Nairovirus* and *Phleobvirus* are arthropod-borne, while those of the genus *Hantavirus* are spread by excretions of rodents. Some species infect cattle and humans after exposure to the transmitting vectors and cause serious diseases such as Rift valley fever, Hantavirus pulmonary syndrome and Crimean-Congo haemorrhagic fever (Goldbach & Peters 1994). Tospoviruses infect a wide range of economically important vegetables, legumes and ornamental plants, and numerous weed species in subtropical and temperate regions in the world (Pappu *et al.* 2009).

Tospoviruses share similar virion structure with other bunyaviruses. The tripartite ssRNA genome is encapsidated by a lipid envelop forming pleomorphically spherical particles (80-120 nm diameter). The genome consists of three RNA molecules referred to as large (L), medium (M) and small (S) (Nichol *et al.* 2005). The L RNA has a negative-sense nature encoding the RNA-dependent RNA polymerase (RdRp), while the M and S RNAs are ambisense (de Haan *et al.* 1990;de Haan 1991). The M segment encodes a precursor of the two glycoproteins, the G_C and $G_N$proteins on the lipid envelop, in the viral complementary sense and a non-structural protein (NS_M) in a viral-sense RNA which is involved in cell-to-cell movement of the virus within its plant host (Kormelink *et al.* 1992). The S RNA encodes nucleocapsid (N) protein in the viral complementary sense and a non-structural protein (NS_S) in the viral sense which has a function as suppressor of RNA silencing (Hassani-Mehraban *et al.* 2010).

The tospoviruses are transmitted by thrips (*Thripidae*, *Thysanoptera*) in a circulative-propagative manner (Ullman *et al.* 1993;Wijkamp*et al.* 1993).Out of the estimated 5,000 thrips species, only 14 species are known as potential tospovirus vectors. Most of vectors belong to the genera *Frankliniella* and *Thrips* (Riley *et al.* 2011). Species in the genus *Frank-*

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liniella have a neotropical distribution of which majority of species are considered to be endemic to the Americas (Nakahara 1997). The vectors, *F. occidentalis* and *T. tabaci,* have a worldwide distribution (Mound 2002).

Until 2009, nineteen tospovirus species have been described, of which eight are accepted as confirmed species by the ICTV, while the rest are considered as tentative species (Pappu *et al.* 2009). Recently, the tospovirus species list expanded up to 24 species with the identification of Alstroemeria necrotic streak virus (ANSV) from Colombia infecting *Alstroemeria* sp. (Hassani-Mehraban *et al.* 2010), Tomato necrotic rings pot virus (TNRV) infecting tomato in Thailand (Seepiban *et al.* 2011),Pepper necrotic spot virus from Peru (Torres *et al.* 2012), Soybean vein necrosis-associated virus from United States (Zhou *et al.* 2011)and Bean necrotic mosaic virus from Brazil (de Oliveira *et al*. 2011).The major descriptor used to demarcate a new tospovirus species is its N protein amino acid sequence identity with previously reported sequences (Hassani-Mehraban *et al.* 2010).Other descriptors such as vector specificity, serology and host range (Reddy *et al.* 1995) form additional markers. Strains of the same species have a higher amino acid residues identity than 90% in their N proteins, and members of distinct species have a lower identity than 90% (Nichol *et al.* 2005).

The geographical delineation of tospoviruses is clustered into an Euro-Asian and an American's cluster based on their N protein amino acid sequence phylogeny (Voinnet *et al.* 2000; Cortes *et al*. 2001; Gibbs 1995). To date, 15 species are identified from the Asian continent. The greatest diversity in tospoviruses occurs in South-East Asia infecting a wide range of crop species. *Tomato spotted wilt virus* (TSWV), the type species of the genus *Tospovirus* is geographically the most widely distributed species within this genus. However, in Asia, TSWV has only been reported from some countries such as Japan, Korea, Israel, Middle East, Taiwan (Pappu *et al.* 2009) and Indonesia (Damayanti & Naidu 2009). Groundnut bud necrosis virus (GBNV), although first considered to be TSWV, has been reported to cause severe economic losses in groundnut, tomato, potato and soybean in many Asian countries (Reddy *et al*. 1995). In addition of GBNV, four other tospoviruses are known to occur in India, *i.e.* Capsicum chlorosis virus (CaCV), Groundnut yellow spot virus (GYSV), Irish yellow spot virus (IYSV), and Watermelon bud necrosis virus (WBNV) (Mandal *et al.* 2012). In addition, several other tospoviruses such as Melon yellow spot virus (MYSV), Tomato necrotic ring spot virus, Watermelon silver mottle virus (WSMoV) and Chrysanthemum stem necrosis virus (CSNV) are reported from South -East Asia causing diseases in vegetables and ornamentals (Pappu *et al.* 2009). In Sri Lanka, IYSV infecting leek has recently been reported (Widana Gamage *et al.* 2010) being the first species using PCR and phylogenetic analysis in its identification. This isolate shows a 97% protein identity with the Dutch strain (IYSV-NL) and 92% with the Brazilian strain (IYSV-BR). Earlier in 1988, TSWV has been reported infecting groundnut (*Arachis hypogaea*). Its identification was based on the symptoms produced on *Petunia hybrida*, known to cause local lesions specific for TSWV and all other tospoviruses, thrips transmission studies and serology (Jayasena *et al.* 1988). These properties were the only tools that could be used to identify an infection with TSWV; at that time the only representative of a monotypic virus group. High-quality antisera became available for the detection of tospoviruses, during the early1990s (de Avila *et al*. 1993b; Reddy *et al.* 1995). Later, data from serological comparative studies and subsequently from sequencing of nucleic acids revealed the existence of several distinct tospoviruses (German *et al.* 1992: de Ávila *et al.*1993a). One example of such an understandable misidentification was made in the identification of the pathogen of a disease in groundnut as being caused by TSWV (Ghanekar *et al*. 1979; Amin *et al*.1981; Reddy *et al.* 1991). Reddy *et al*.(1995) identified this pathogen as a new tospovirus named Peanut bud necrosis virus (now known as GBNV) in India and was later also detected in other South -East Asian countries. Hence, the identification of TSWV infecting groundnut in Sri Lanka as

early as 1988 needs to be confirmed with sequence data. However, no information is available in the previous report (Reddy *et al.* 1995) or any other report on the identification of GBNV in Sri Lanka except the occurrence of bud necrosis disease. To date, no gene sequence data is available on tospoviruses in Sri Lanka except the N protein gene sequence of IYSV (Accession No. GU901211) deposited in the NCBI/Genbank (Widana Gamage *et al.* 2010). Further, to our knowledge an island-wide screening has not yet been done on the incidence of tospoviruses and no data on the thrips vector species have been collected so far. But it will be likely that some of the species prevailing in South-East Asia will also available in Sri Lanka. Hence, the primary objective of this study is to identify tospoviruses infecting economically important crop plants in Sri Lanka and to determine its phylogenetic position among the known tospovirus species.

MATERIALS AND METHODS

Samples were collected from plants showing characteristic symptoms of tospovirus infections on plants of different crop species grown in open fields during June-August in 2009 in the Southern, Central, Uva and North-central provinces where most of the vegetable crops are grown. Plants were sampled when they were suspected to be infected with a tospovirus on the basis of symptoms on leaves and fruits. Collected samples were kept on ice in the field in order to prevent rapid desiccation due to the high prevailing temperature (32ºC).

Young leaves with clear symptoms were cut into 3-4 mm wide strips and placed in Petri plates on top of a layer of anhydrous Calcium chloride ($CaCl_2$). Leaf material and $CaCl_2$ were separated by a filter paper. The Petri plates were tightly closed with parafilm and placed in a refrigerator (4ºC). After 2 days, the dried crispy leaf material was transferred into a tube. Tubes were filled from bottom to top with subsequent layers consisting of anhydrous $CaCl_2$, a small plug of cotton wool, dried leaf material, a small plug of cotton wool, and finally a small amount of anhydrous $CaCl_2$. Tubes were sealed with parafilm wrapped around the lid and stored at -80ºC. Samples containing dried leaf materials sealed in plastic bags were brought to the laboratory of Virology, Wageningen, for further studies.

Crude virus extracts were prepared by grinding dried leaf material in 1 x PBS buffer (0.14M NaCl, 0.002M KH_2PO_4, 0.008M $Na_2HPO_4.2H_2O$, 0.002M KCl, 0.003 NaN_3 at pH 7.4) and mechanically inoculated onto carborandum powder dusted leaves of *Nicotiana benthamiana* in order to recover and propagate viruses. All samples that were able to infect *N. benthamiana* were subsequently inoculated onto *Petunia hybrida,* which produces dark brown local lesion within two to three days after inoculation (Brunt 1959). Samples which were able to infect both *N. benthamiana* and *P. hybrida* were used in further analysis. The selected isolates and other tospoviruses used in this study were maintained in *N. benthamiana*. However, mechanical serial passages over maintenance hosts were limited to maximum four passages in order to prevent the generation of defective intermediates (Inoue-Nagata *et al.* 1997). Inoculated plants were placed for symptom development in the greenhouse at 25ºC under normal day-light conditions and in winter additional light was given.

Virus extracts of systemically infected *N. benthamiana* plants were serologically compared with other tospovirus species by DAS-ELISA (Clark & Adams 1977) after slight modifications, using polyclonal antisera directed against N protein of each virus tested. Microtitre plates were coated with immuno-gamma globuline (IgG) (1 µg/ml) of TSWV, Tomato chlorotic spot virus (TCSV), GBNV, Groundnut ring spot virus (GRSV), MYSV, WSMoV, Tomato yellow ring virus (TYRV) and IYSV. Un-occupied sites in IgG coated wells were blocked with 2% Elk (dried

skimmed milk) dissolved in 1 x PBS in order to prevent any a-specific protein attachment. Virus sap extracts were prepared by macerating 0.03g of systemically infected leaves in 1 ml of 1x PBS-Tween-20. Subsequently, IgG conjugated with alkaline phosphatase was added as a secondary antibody (1 µg/ml) to the plate. Finally, when the substrate p-nitrophenyl phosphate (1 µg/ml) in substrate buffer (96 ml diethanolamine, 600 ml Mili Q, 0.003 M NaN_3 at pH 9.8) was added. Plates were incubated at 37ºC or at room temperature. The development of yellow colour due to the release of the phosphate group from the substrate indicates the presence of the virus to be detected. Absorbances were measured at A_{405nm} using a FLUOstar OPTIMA (BMG LABTECH GmbH, Offenburg, Germany). ELISA readings were made 30, 60, 90 and/or 120 min of incubation. A sample was considered positive when the A_{405nm} value was higher than the average of the healthy value plus three times the standard deviation.

The results obtained in DAS-ELISA were confirmed by RT-PCR. Total RNA was extracted from systemically infected *N. benthamiana* leaves using TRIzol® method (Invitrogen) according to the manufacturer's protocol. The nucleocapsid genes were reversely transcribed by AMV (HC) reverse transcriptase (Promega Corporation, USA) using primers specific for TSWV (Table 1) and GBNV. The primer combinations used in first strand cDNA synthesis were also used in PCR.

Table 1.Primers used to amplify N gene TSWV. Non-viral sequences are indicated in bold fase

Primer	sequence 5' to 3'
T S W V start p19	**CCCGGATCC**ATGTCTAAGGTTAAGCTCACTAAGG
T S W V stop p15	**CCCGGATCC**TCAAGCAAGTTCTGCGAGTTTTG

The resulting PCR amplicons with an expected size (~ 800 bp) were analysed on 1% agarose gels and extracted using home-made columns (Borodina *et al.*2003) prepared by silica membranes cut out from GF/F borosilicate glass fibre paper (Whatmann™, England) and cloned into pGEM-T easy (Promega Corporation, USA) vector system. Ligations were transformed into *E. coli* (DH5α) competent cells by electrophoration. Recombinant plasmids were purified using home-made columns (Borodina *et al.*2003) with slight modifications and digested with *Not1* restriction enzyme. Presence of cloned fragments was checked on 1% agarose gels. Plasmids containing expected size inserts were sequenced. Obtained sequences were compared (BLAST) with sequences available in NCBI/GenBank.

RESULTS AND DISCUSSION

Samples were collected from different vegetable crops showing symptoms resembling of those caused by tospoviruses as could be judged from pictures on the internet. However, it was difficult to distinguish between tospovirus infections and infections by any other virus by visual inspection only.

Tomato (var. Thilina) showing characteristic TSWV symptoms of necrotic spots surrounded by concentric rings or ring spots on leaves and fruits (Fig 1 A, B) were observed in Ambalantota in Southern, and Gannoruwa in Central Provinces. Severe stunting of plants associated with bud necrosis, axillary shoot proliferation and brownish rust on leaves (Fig 1 C, D) were observed in groundnut (var. Walawa) in Angunakolapalassa in Southern Province. The symptoms of groundnut resemble those as described by Reddy *et al.* (1995). However, the symptoms caused by TSWV and GBNV on groundnut cannot be distinguished from each other or with difficulties. In addition, severe chlorosis and irregularly shaped chlorotic eye-like spots surrounded by distinct margins were also observed on leaves.

Most of the cucurbits including watermelon were found to be heavily infected with one or more viruses in several fields but, did not resemble to any characteristic tospovirus-like symptoms. No any cucurbit sample gave a positive response on *N. benthamiana* indicating that none of the samples were infected with Cucumber mosaic virus. As an advice for further studies, cucumber seedlings have to be used to isolate possibly infecting viruses. Severe damages caused by thrips feeding were observed on onion and leek leaves in several fields in major growing areas. These observations lead us to look for possible IYSV infection in onion and leek. Typically, IYSV infection results in local chlorotic spots and diamond shaped lesions on leaves. Of the onion samples showing local chlorotic spots, we were not able to detect IYSV infection. However, IYSV infection was detected in leek showing diamond shaped lesions on leaves. Often, in both onion and leek, thrips damages on leaves were observed larger than the damage that could be caused by IYSV.

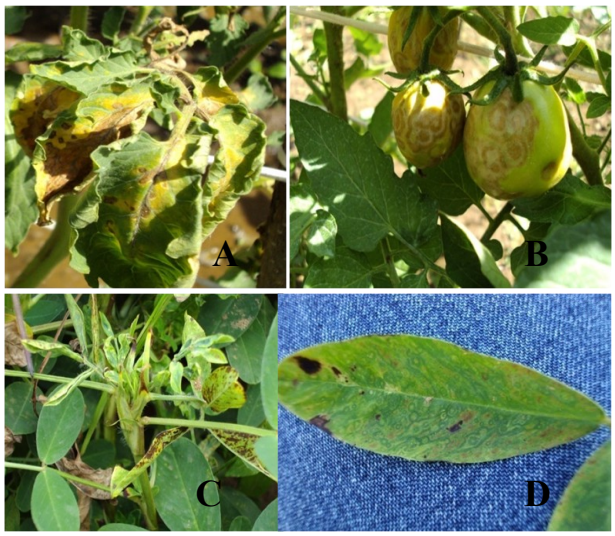

Fig1.Tomato spotted wilt virus on tomato (A and B) and groundnut (C and D). A-necrotic lesions on leaves, B- brownish concentric rings on fruits of tomato, C- bud necrosis, axillary shoot proliferation and brownish rust on the leaves, D-chlorotic eye-like spots on a leaf of groundnut.

Viruses were recovered on mechanically inoculated *N. benthamiana* plants. Inoculated plants showed chlorotic spots on locally infected leaves after three to four days of inoculation while systemic infections were observed after one week. Three of the isolates that were able to systemically infect *N. benthamiana* plants were also able to produce necrotic local lesions on *P.hybrida*. Samples which produce necrotic lesions on inoculated leaves of *P.hybrida* can be considered infected with a tospovirus, as the local lesions appearing within three days given as indication of a tospovirus infection. Accordingly, two tomato samples from Ambalantota and Gannoruwa and a groundnut sample from Angunakolapalassa were found to be infected with tospoviruses and selected for further characterization.

All the three isolates were serologically positive for TSWV infections in DAS-ELISA, whereas no positive reactions were obtained with GBNV antiserum. Serological cross-reactions with GRSV and TCSV polyclonal antisera were also positive with lower signals as shown by de Ávila *et al.* (1993b), indicating that isolates were closely related to TSWV and not to GRSV and TCSV.

RT-PCR reactions with TSWV specific primers (Table 1) for its entire N gene amplified around 800 bp fragments corresponding to the size of the TSWV N gene (Figure 2). These RT- PCR results confirmed that the tomato and groundnut plants in the field were certainly infected with TSWV. No amplification was given with GBNV specific primers.

The sequences of tomato and groundnut isolates compared with other available TSWV N gene sequences in the Genbank showed a 98% protein identity to the N gene of Brazilian isolate (BR-01). The non-identical amino acid residues of the tomato TSWV isolate which differed from the BR-01 isolate, also differed from the groundnut isolate. The complete sequences of TWSV N gene of tomato

and a partial sequence TSWV N gene of groundnut have been deposited at NCBI/ GenBank under accession numbers HM231305, HM231307 and HM231306respectively. To our knowledge, previously reported serological detection of TSWV infecting groundnut in Sri Lanka (Jayasena *et al.*1988) might have been a mis-identification due to the use of inadequate tools to discriminate the different tospoviruses species. Therefore, we cannot exclude that the latter authors would have been working with GBNV as the virus they analysed was isolated from groundnut, probably the most prevailing tospovirus on groundnut.

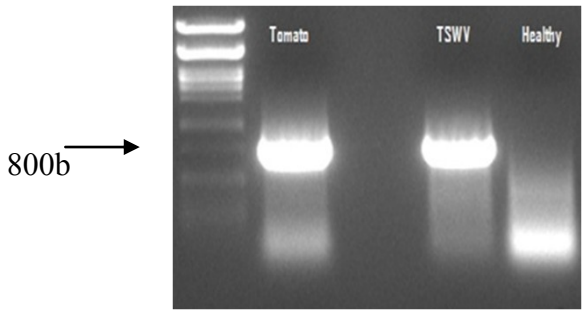

Fig2. RT-PCR showing bands amplified (~800 bp) with TSWV specific primers. L_1: Lambda DNA ladder (*Pst1*), L_2: Tomato isolate, L_3: TSWV BR-01 strain (positive control) and L_4: healthy *N. benthamiana* (negative control)

Although TSWV was found infecting only tomato and groundnut so far in Sri Lanka, its natural host range may expand in the future with extended survey thought out the island since TSWV is known to infect more than 900 plant species worldwide (Pappu *et al*. 2009). It is important to point out that our samples were collected from a limited number of crop species from some fields in our crop growing regions. No samples were collected from ornamental or weed plant species.

Further, no data have been collected on the thrips vector population transmitting either TSWV or IYSV in Sri Lanka. Although *F. occidentalis* is the principal vector of TSWV, this virus can also be transmitted by several other thrips species including *T. tabaci* which is the only vector of IYSV but may transmit TSWV as well and probably the other viruses prevailing in South-East Asia (Riley *et al.* 2011). Due to the inadequate sample collection representing whole crop range and growing regions in Sri Lanka, and the absence of data on vector prevalence, abundance or damage caused by vectors, we cannot conclude that only two tospoviruses; TSWV and IYSV are pathogens of Sri Lanka. It is highly possible that other tospoviruses prevailing in South -East Asia might also available in Sri Lanka infecting various crops, ornamental and weed species.

Tospoviruses are becoming an emerging problem in the agriculture sector of many countries like Bangladesh, India, Nepal, Pakistan and Sri Lanka in the Indian sub-continent (Mandal *et al.* 2012). Systematic and regular surveys for disease identification, indexing and controlling of transmitting vectors although helps to some extent in controlling the virus to minimize the present and future economic impact, survival of the virus between crops has also to be understood. In addition, regular monitoring for the species crossing borders through international trade is considered prime importance.

CONCLUSION

The present study confirms presence of three isolates of TSWV infecting tomato and groundnut in Sri Lanka. Further, the identified TSWV isolates were found closely related (98%) to the Brazilian isolate (BR-01) according to the N gene phylogeny. This is the first report providing N gene sequence data of TSWV isolates infecting crops in Sri Lanka.

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