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TOSPOVIRUSES IN THE REPUBLIC OF MACEDONIA DURING 1996-2010, OCCURRENCE AND DISTRIBUTION

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Abstract

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The research was carried out during the period between 1996 and 2010. The presence of two tospoviruses (TSWV and INSV) in the Republic of Macedonia was confirmed by different serological tests and characterization based on the size of N proteins obtained from different viral isolates. The results showed that the dominant tospovirus in Macedonia is TSWV, with the exception of the sample GV-I, in which the presence of INSV was detected for the first time in the Republic of Macedonia.

Key words: tospoviruses, TSWV, INSV

Introduction

The genus *Tospovirus* is a member of *Bunyaviridae,* a family of negative-stranded RNA viruses, which include vector-born viruses (Büchen-Osmond, 2006). Tomato spotted wilt virus (TSWV) as a major member of the *Tospovirus* genus is one of the most economically important plant viruses (Prins and Kormelink, 1998a). Supported in persistent manner of transmission by different types of thrips, TSWV and other tospoviruses have been documented infecting over eight hundred plant species of 82 families (Prins and Kormelink, 1998b). Furthermore, TSWV is known to infect more than 900 species belonging to over 90 monocotyledonous and dicotyledonous plant families and INSV infects at least 300 species in 85 different monocotyledonous and dicotyledonous families. Other tospoviruses, such as Capsicum chlorosis virus (CaCV), have more limited known natural host ranges (Persley et al., 2006; Lebas and Ochoa-Corona, 2007). Important agricultural crops (tomato, pepper, salad, onion, tobacco, potato, peanut etc.) are known as hosts of TSWV and other tospoviruses, as well as many ornamentals and weeds (Prins and Kormelink, 1998b, Pappu, 2009). Until early 1990s, TSWV was the only known plant virus of the Bunyaviridae (Milne and Francki, 1984). Investigations of N-protein and N-genome (Avila et al., 1992; Dewey et al., 1995; Duarte et al., 1995; Iwaki et al., 1984; Yeh et al., 1992; Yeh and Chang, 1995) predicted new taxonomic development within the Tospovirus genus. Thus, according to criteria related to N-gene, Impatiens necrotic spot virus (INSV) was a new tospovirus confirmed and located within Tosposerogroup III (Law and Moyer, 1990, Avila et al., 1992). In the following period a representative number of tospovirus-like isolates reached their taxonomic position based on already established criteria (Goldbah and Kuo, 1996). TSWV, TCSV, GRSV, INSV, WSMV, GBNV, MSWV and GYSV were accepted by the International committee on taxonomy of viruses (ICTV) in 1995 (Murphy et al., 1995). This process is still open. Until today, 19 distinct species have been discovered, but only eight of them are accepted as confirmed species by the ICTV, while the rest are considered as tentative species (Pappu et al., 2009). In Europe, five different tospovirus species (CSNV, INSV, IYSV, PoRSV, TSWV) have been reported (Pappu et al., 2009), but only two of them (TSWV and INSV) are well-established in many European countries (EPPO, 2004; Kazinczi et al., 2007, Pappu et al., 2009). Other three tospoviruses are not so widespread in European countries.

The first reports for the presence of TSWV (tobacco infections) in Macedonia, were in the period between 1960-1970 (Mickovski, 1969, 1970, Mickovski and Todorovski, 1973). Later, TSWV occurrence was reported in tomato and pepper (Rusevski, 1995), and many other crops, weeds and ornamentals (Rusevski, 2002, 2003, 2004, 2005). Since then,

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TSWV and other possible tospoviruses are constant long-term aim for monitoring and investigations.

Materials and Methods

Sampling

Field investigations covered the period from 1996-2010. During this period, presence of TSWV was monitored in 15 different regions in Macedonia (Kocani, Kumanovo, Ohrid, Strumica, Sveti Nikole, Radovish, Skopje, Bogdanci, Gevgelija, Tetovo, Gostivar, Resen, Bitola, Prilep and Kavadarci). In open field production, observations were made twice a year during which samples were collected from plants with tospo-like symptoms, in early spring and in late summer. In protected area production (glasshouse, plastic gardens), observations and sampling were performed throughout the year, depending on plant production.

Tomato, pepper, tobacco and lettuce were the main plants on which observations were focused, but we also observed and collected samples from many weeds and ornamentals known to be tospovirus hosts. Monitoring and sampling were not executed in all regions in all years due to various reasons (decreased security, denied access, etc.).

Identification methods

Identification of the tospovirus status in suspected samples were done by bioassay (Krstic and Tosic, 1994), gel diffusion (Krstic and Tosic, 1994) or DAS ELISA (Clark and Adams, 1977), while distinctive identify of different tospovirus species from Macedonian isolates was performed by western blot. The taxonomic positions of tested isolates were determined according to established criteria (Goldbah and Kuo, 1996), by analyzing N-protein (directly related to N-gene).

Virus Purification and Antiserum Production

For virus purification, two TSWV-like isolates (Kc1 and Kc2) were used. Methods described by Roggero et al. (1996), supplemented and upgraded in some details, have been used. Bio-concentration of virus antigens was done in young *Tropaeolum majus* plants, cultivated at 25-30˚C and 15/9h day/night regime. For extraction and purification ice-cold 0.01M phosphate buffer pH=7 containing 0.01M Na_2SO_3 was used. Leaves with systemic symptoms (3:1w/v) were homogenized and filtered through cheesecloth and clarified by 2 centrifugations at 8000 g for 15 min. The pellets were laid over a 60-10% sucrose density gradient and centrifugated for 90 min at 30 000 g. Samples where virus zones appeared inside 30% sucrose density were resuspended in extraction buffer and saturated by centrifugation for 150 minutes at 45 000 g. Virus sediment was collected in ice-cold 0.001M phosphate buffer pH=7 and immediately used for rabbit immunization. Presence of virus antigen during the purification process was controlled by bioassay and western blot.

Serological Analysis

Gel diffusion

These tests were performed using methodology described by Krstic and Tosic (1994). The reactions took place in 1.5-2% agar gel (Difco).

DAS ELISA test

Collected samples were tested for virus infections with DAS ELISA serological method, described by Clark and Adams (1977) and modified as proposed by Bioreba - AG (1999). Used antibodies recognized tosposerogroups I, II and III.

Western – Blot Analysis

This analysis was done as described by O'Donel et al. (1982), Rybicki and Von Wecmar (1982)**,** upgraded by Krstic (1994), and modified by Rusevski (2002). The analysis consists of polyacrilamide gel electrophoresis, antigen adsorption on nitrocellulose paper, and immunology-enzymatic improvement. Samples were prepared in Tris-glicine buffer (pH=8.5; 0.6 g. Tris; 2.28 g Glicine, 0.2 g SDS in 100 ml distillated water) and in sample buffer (pH 8.6; 0.83 g Tris, 10ml Glicerol, 0.8 ml β-merkaptoethanol, 5 ml 20% SDS, 0.3 ml 5% Bromfenol bly in 70% ethanol) and were boiled 3-5 min in water bath. For antigen electrophoresis, discontinued polyacrilamide gels were prepared (5% for sample concentration, and 9% for protein separation). Electrophoresis was performed by EC250-90 mini-vertical gel system at room temperature for 60 minutes (electrical power 20 mA and voltage 100 V – 10 min for sample concentration and 30 mA – 150 V, 50 min for protein separation). Transcription of the antigens from gel to TBS moistened nitrocellulose paper was supported by Tris-glicine-methanol buffer. Immunology-enzymatic processing was performed with primary antiserum obtained from isolates Kc1 and Kc2 and primary IgG related to tosposerogroups I, II and III (Bioreba AG production) and secondary antiserum, Horseradish Peroxidase Conjugatet Goat Anti-rabbit IgG (Sigma). Nitrocellulose paper development was performed by TBS buffer with supplements of methanol – 1ml, to which 4-chlor-1-naphthol – 2 mg (1.11952.0010 – Merk) and 3 µl 30% Perhydrol (H_2O_2) were added. The hydrolysis substrate reaction was interrupted by washing with distilled water.

Results

Epidemiology

Presence or absence of tospoviruses in different plant samples, during 1996-2010 are presented in Table 1 and Figure 1. Data in Table 1 present occurrence, distributions and

natural hosts range of tospoviruses in the Republic of Macedonia during the investigation period. Tospovirus infection in glasshouses near Ohrid, has been present in all years of monitoring and sampling since 1996. Similarly, we detected tospoviruses in glasshouses and the surrounding vegetation near the towns of Bogdanci and Gevgelija, in all years of sampling and monitoring, which started in 2000. On the contrary to these locations which we consider as stable sources of tospoviruses, in most of the other regions, tospoviruses were identified occasionally, i.e. not in all years of monitoring and sampling. Regions around cities of Bitola, Resen, Tetovo and Gostivar were tospovirus-free during the whole investigated period. Overall, we found significant incidence of tospoviruses in most regions which were subject to monitoring.

Regarding hosts, we identified tospoviruses in pepper (different varieties), tomato (different varieties), tobacco, lettuce, as well as in different weeds and in many ornamental plants (Table 1).

Identification of tospovirus species

Western blot tests (Figures 2a, 2b and 3) confirmed presence of TSWV in plant samples. Serological processing of typical TSWV-like isolates, particularly a sample from the Kocani region, showed presence of 3 different structural proteins: G1 (≈75-78 kDa), G2 (≈56-58 kDa) and the most common N-protein (≈30 kDa) (Figure 2a).

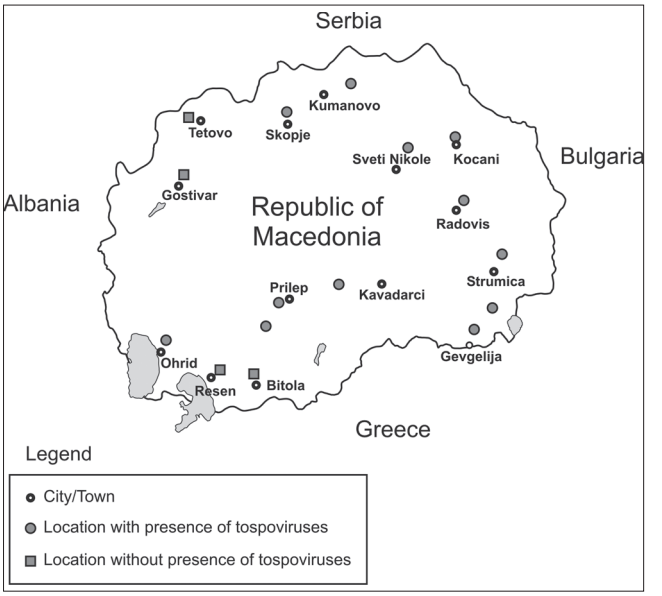

Fig. 1. Locations of monitoring for Tospoviruses (1996-2010)

Table 1
Occurrence, distribution and natural plant hosts of tospoviruses in Macedonia (1996-2010)

Region	Year of investigation and used method for detection														
	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
	B	B+GD	B+GD	B+DE	B+DE	DE	DE	DE	DE	DE	DE	DE	DE	DE	DE
Kocani	P, To	P, To	P, To, W	-	-	-	-	-	P	*	*	*	P	P	*
Kumanovo	T, To	T, To, W	T, To, W	T, To, W, P	T, To, W	*	*	T, To, W	To	*	*	*	*	To	*
Ohrid	O, W	O, W	O, W	O, W	O, W	O, W	O, W	O, W	-	O, W	O, W	O, W	*	*	*
Strumica	To, P, T	To, P, T	-	-	-	-	-	-	-	-	To, P, T	To, P	To	To	To
Sv. Nikole	T	T	-	-	-	-	-	-	-	-	-	*	*	*	*
Radovish	T	T	-	-	-	-	-	-	-	-	-	-	*	T	*
Skopje	-	-	P, To, W	-	P, To, W	P, To, W	-	-	P, To, W	P, To, W	-	-	-	P, To, W	-
Bogdanci	-	-	-	To, S, T, W	To, S, T, W	To, S, T, W, O	To, S, T, W,O	To, S, T, W, O	To	To, P	To, P, W	To, P, W	To, P, W	To, P, W	To, P, W
Gevgelija	-	-	-	O, W	O, W	O, W	O, W, To	O, W	W	*	O, W	W	W	W	W
Kavadarci	-	-	-	-	-	*	*	*	*	*	-	-	*	P	-
Prilep	-	-	-	-	-	-	-	-	-	P	-	-	-	P	-
Bitola	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*
Resen	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*
Tetovo	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*
Gostivar	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*

Legend: P- Pepper; To- Tomato; T - Tobacco; S- Lettuce; W - Weeds; O- Ornamental; * - No data; B- Bioassay; DE- DAS ELISA; B+GD - Bioassay + Gel diffusion; B+DE - Bioassay + DAS ELISA; –- Absence of tospovirus

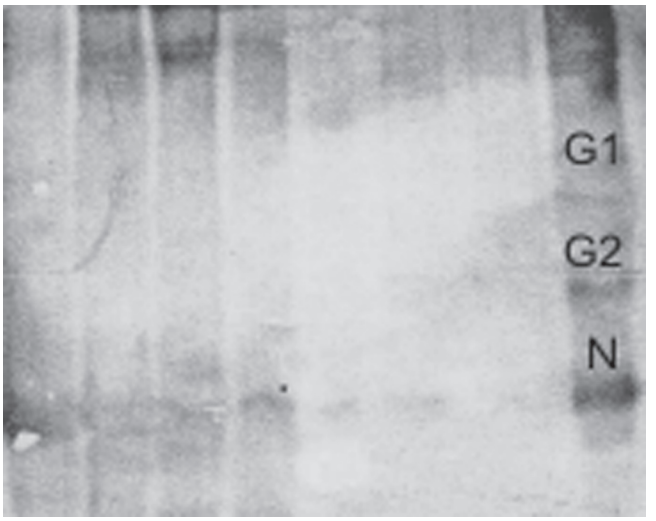

Fig. 2a. Western blot analysis of Macedonian tospovirus isolates (performed with comercial antisera – Bioreba AG)
Legend: G1- Viral glycoprotein; G2- Viral glycoprotein; N- Viral coat protein

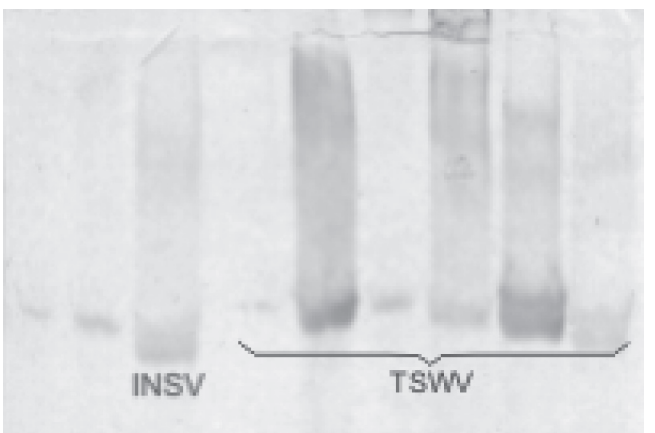

Fig. 2b. Western blot analysis of Macedonian tospovirus isolates (performed with comercial antisera – Bioreba AG)

The size of N-nucleoprotein determined in sample GV-I (≈27 kDa, Figure 2b), obtained from *Impatiens walleriana*, showed significant difference compared to all other isolates. In this case, processing of samples was performed with standard antiserum that recognizes viruses from serogroups I, II and III. These isolates represent infections with INSV, which is the first report of presence of this virus in the Republic of Macedonia.

Overall, based on the size of the structural (N) protein in all isolates during the investigated period, the dominant tospovirus in the Republic of Macedonia was TSWV, with the exception of the sample GV-I, in which only the INSV was detected.

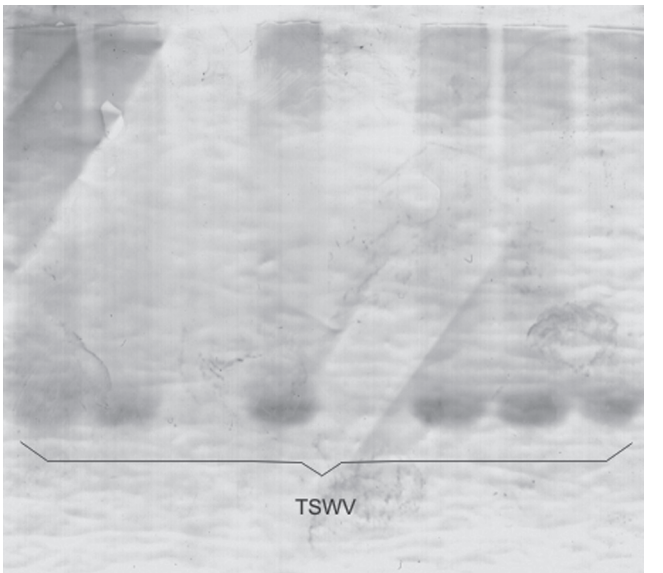

Fig. 3. Western blot analysis of Macedonian tospovirus isolates (performed with antisera obtained from isolates Kc1 and Kc2)

Discussion

Considering the polyfagous nature of tospoviruses, especially of TSWV, which can cause damage to a wide range of agricultural crops worldwide (Iwaki et al., 1984; Cho et al., 1986; Cho et al., 1987; Bertaccini and Bellardi, 1990; Camann et al., 1995; Padgett et al., 1995; Chatzivassiliou et al., 1996; Chen and Chiu, 1996; Culbreath et al., 1996; Daughtrey, 1996; Janser et al., 1996; Resende et al., 1996; Singh and Krishnareddy, 1996; Verhoeven et al., 1996; Peters et al., 1996; Daughtrey et al., 1997, Büchen-Osmond, 2006; Persley et al., 2006; Lebas and Ochoa-Corona, 2007; Pappu, 2009), the obtained results regarding the dispersion and epidemiology of tospoviruses in the Republic of Macedonia, were expected.

Results regarding the presence and prevalence of different tospovirus species are consistent with the results obtained in many other studies, in which authors have revealed dominant presence of TSWV (Avila et al., 1992; Yeh et al., 1992; Dewey et al., 1995; Heinze et al., 1995; Yeh and Chang, 1995; Adkins et al., 1996; Roggero et al., 1996; Kikkert et al., 1997; Prins and Kormelink, 1998; Duijsings et al., 1999; Kikkert et al., 1999; Jan et al., 2000). Moreover, it should be highlighted that two tospovirus species detected in our study (TSWV and INSV) are widespread and well-established in many European countries (Mumford et al., 1996; EPPO, 2004; Kazinczi et al., 2007; Pappu et al., 2009). Other three tospoviruses (CSNV, IYSV, PoRSV) that are not so wide-

spread in European countries (Pappu et al., 2009), were not detected in our study.

Conclusions

The presence of tospoviruses during the period of research (1996-2010), has been verified in 15 different regions in the Republic of Macedonia (Kocani, Kumanovo, Ohrid, Strumica, Sveti Nikole, Radovish, Skopje, Bogdanci, Gevgelija, Tetovo, Gostivar, Resen, Bitola, Prilep and Kavadarci).

Tospovirus infections were observed in all years of monitoring in the regions of Ohrid, Bogdanci and Gevgelija. In the regions of Kocani, Kumanovo, Strumica, Sveti Nikole, Radovish, Skopje, Prilep and Kavadarci, the tospovirus infections were detected occasionally. Regions around the cities of Bitola, Resen, Tetovo and Gostivar were tospovirus-free during the whole period of research (1996-2010).

Prevalent tospovirus in all tested samples was TSWV, with the exception of sample GV-I, in which only INSV was detected. Detection of tospovirus INSV in sample GV-I represents the first report of presence of this virus in the Republic of Macedonia.

Further investigations, in which bigger number of plant hosts and samples will be included, is needed to see whether the tospovirus INSV is more widely spread in the territory of the Republic of Macedonia.

At the end, we believe that our results will help to solve the puzzle of tospoviruses occurrence, distribution and diversity in the Balkan region and Europe.

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