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Mixed viral infections in tomato as a precondition for economic loss

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Abstract. Tomato is one of the economically important crops in Bulgaria. Low tomato yields in the country are mostly due to lack of knowledge on sustainable agronomic practices, lack of improved varieties, which are well suited for high yield and resistance to diseases, and also due to damage caused by pests and diseases. Many viruses infect the tomato crop. The object of this research was to identify viruses infecting tomato crops in Bulgaria. The presence or absence of a viral infection in tomato plants was carried out with DAS-ELISA serologic assays with specific polyclonal immunoglobulin G for the relevant plant virus. Most of the tomato plants were infected with one or two viruses and in rare cases with more than two viruses. We found that mixed viral infections caused more severe necrotic damages in the tomato fruits and increased the economic loss compared with mono infections.

Keywords: tomato, mixed plant viral infections

Introduction

Tomato Spotted Wilt Virus (TSWV), Tomato Mosaic Virus (ToMV), Cucumber Mosaic Virus (CMV), Potato Virus Y (PVY) and Pepper Mild Mottle Virus (PMMV) infect different species of the family Solanaceae including tomatoes. These viruses can infect either singly or in combination and infection results in various symptoms, which range from a mild to severe mottle, leaf distortion, necroses of leaves and fruit to extreme plant stunting.

PVY, a member of the genus Potyvirus that infects tomato occurs in several pathotypes (Jones et al., 1991), which are transmitted by aphids in a non-persistent manner. Typical symptoms of PVY in tomato include mosaic, vein chlorosis, mild mottling, dark brown necrosis on leaflets, severe necrosis, leaf curling, and drooping (Jones et al., 1991).

The genus Tospovirus contains viruses, which are very unstable, especially at pH values below 5.5. Viruses of this genus have a characteristic membranous lipoprotein envelope and form cytoplasmic inclusions in plant cells (Green and Kim 1991). TSWV is the only known member of this genus that infects tomato. Mild mutant strains of TSWV exist and have been inoculated in tomato for cross-protection against more severe strains (Gonslaves and Providenti, 1989). The virus is known to cause chlorosis and yellow rings on tomato leaves and fruits. It is transmitted persistently by thrips. Seed transmission also occurs (Jones et al., 1991; Ullman et al., 1996).

PMMV was first described in Italy in 1984. Since then, it has spread and become a significant pathogen of tomato and pepper crops worldwide (Wetter, 1984). It is a member of the genus Tobamovirus (Fauquet, 2005). PMMV infects cultivated pepper plants through seed and soil transmission in the fields, causing severe mosaic symptoms. PMMV is not transmitted by insects. It can be seed borne, consequently, the seedlings can be infected by mechanical contamination from their seed coats during transplanting or other cultural procedures. This is a primary source of infection. Foliar symptoms of PMMV consist of mottling and yellow/green mosaic, while fruit may be small, malformed and mottled, with sunken or raised necrotic spots. Yield loss is considerable when young plants become infected. When the virus contaminates once in a green pepper field by carrying over seed, it is extremely difficult to get rid of it. The virus in infected plants remains as long as green pepper is cultivated continuously in that field, because the plants later become sources to infect newly transplanted young seedlings. The virus is quite stable and highly infectious and is easily spread from plant to plant during normal crop maintenance. Also, the virus can persist in the previous crop in infected pepper debris such as leaves, stems or roots in soil for several months (Agrios, 2005).
Therefore, is necessary to detect viruses on tomato crops in order to take relevant measures to limit their spread and reduce losses from poor quality production caused by viral infections. The object of this research was to identify viruses infecting tomato crops in Bulgaria and to determine damages on plants caused by the virus infections.

Material and methods

The tested tomato plants were from cv. Ideal. DAS-ELISA. The analysis was conducted by the method of Clark and Adams (1977). We used a commercial kit of LOEWE Biochemica GmbH, Sauerlach, Germany. ELISA plates were loaded with antiserum (IgG) for the relevant virus, with dilutions (according to the instructions of the manufacturer) in 0.05 M carbonate buffer. The samples were incubated for 4 hours at 37°C, and the unbound components were washed out with PBS-T buffer for 5 min. All samples were grounded in extraction buffer containing 1% PVP (polyvinyl pyrrolidone) at a ratio of 1:10. The plates were incubated at 4°C for 16 hours. Following the third wash step alkaline phosphatase conjugate for the relevant virus was added and the plates were incubated for 4 hours at 37°C. The used substrate was p-nitrophenyl phosphate (p-nitrophenyl phosphate, Sigma) in diethanolamine buffer (pH 9.8) at a ratio of 1mg/ml. The reaction proceeded in the light at room temperature and was stopped with 3N NaOH. The adsorption of the color reaction was measured at multifunctional detector (DTX 880) at a wavelength of 405 nm. The positive samples had optical density (OD) over the threshold (Cut-off) which was two times the value of the negative control.

**RNA extraction from potatoes infected with PVY.** Extraction of total RNA was performed with RN Easy Plant Mini Kit (Qiagen, Germany). Extraction was carried out according to the instructions of the manufacturer.

**Touch-Down RT-PCR.** We used primers PVY Primer 1, 7 and 8 for P1 gene region of the virus (Petrov, 2012), with program modification touch-down. Copy DNA synthesis: denaturation of total RNA (0.05-0.5 μg) at 95°C for 5 min with 10 μl PVY Primer1 primer in a final volume of 10 μl; cooling on ice to avoid renaturation; preparation 15 μl of master mix: 5 μl of 5MMLV-buffer, 2 μl of dNTPs (2mM), 0.5 μl of M-MuLV Reverse transcriptase (200 U/μl), 7.5 μl H2O. Incubation step at 42°C for 60 min. Master mix for the PCR is: 1 μl cDNA, 2.75 μl 10 PCR buffer, 2.2 μl MgCl2 (25 mM), 2.2 μl dNTPs (2 mM), 1 μl PVYPrimer 1 (10 μM), 1 μl PVYPrimer 7 (10 μM), 1 μlPVYPrimer 8 (10 μl), 1 μl Taq DNA-Polymerase (5 U/μl), 12.85 μl H2O. PCR was done in thermo cycler Auto-Q Server (LKB, UK) with following programme: initial denaturation step 3 min 95°C; five cycles 30 sec 92°C, 30 sec 62°C, 90 sec 72°C; five cycles 30 sec 95°C; modification touch-down. Copy DNA synthesis: denaturation of total from TSWV and PMMV. The damages of the tomato plants were

**Results**

From all tested samples from tomato plants cv. Ideal we received different symptoms. Most of them were mosaics, chlorosis and necrosis of the plant leaves, necrosis on the stems and different necrotic patterns on the fruits. For some of the tomato plants cv. Ideal there were mixed infections from PVY, ToMV and CMV. These three viruses in mix infection caused uneven ripening of tomato fruits and green islands on the surface of the fruits (Figure 1). When peeling the skin of the fruit we noticed clear necrosis under the skin. Later on when the fruits ripen completely the necrotic spots grew in surface, became darker and deeper in the fruits, making the fruits unusable for fresh consumption or the canning industry (Figure 2). Thus damages of the tomato fruits from these plants strongly increased. PVY and CMV mono-infections of tomato plants caused only mosaic and chlorotic symptoms of the leaves, but fruits remained symptomless. In contrast, ToMV mono-infection can cause necrotic symptoms of the fruits. Not of less importance is early fall of the fruits of the infected plants with these three viruses. Thus, both the quantity and quality of the obtained production considerably decreased.

For other tomato plants cv. Ideal there were mixed infections from TSWV and PMMV. The damages of the tomato plants were

**Figure 1. Symptoms of tomato fruits infected with mix infection of PVY, CMV and ToMV**

**Figure 2. Necrosis of tomato fruits infected with mix infection of PVY, CMV and ToMV**
milder, but in the fruits these viruses caused severe necrotic patterns which significantly reduced the quality of the tomato fruits and increased losses for the farmers (Figure 3). Necrosis grew and darkened, going into depth of these contaminated fruits.

We found that in mix infections DAS-ELISA values of the tested plant viruses were reduced compared with mono infections. Some tomato samples were infected with PVY, ToMV and CMV (Figure 2), where PVY had the highest virus titer 1.553 and repressed the other two viruses (Figure 4), but they were also present in the same plant in spite of their low virus titer compared with PVY. Thus DAS-ELISA values for ToMV was reduced to 1.234 and for CMV to 0.895. In spite of this reduction of DAS-ELISA values of the different viruses they remained over the cut off value (0.450) proving the presence of mixed viral infection. DAS-ELISA value of the used negative control for the sample buffer was 0.092 showing no virus contamination between samples. PVY in this case dominated as an aphid transmitted virus and suppressed the movement in the same plant of another aphid transmitted virus (CMV) and mechanically transmitted ToMV.

In the plants infected with PMMV and TSWV we received different results from the previous group of samples infected with the three viruses. DAS-ELISA value of PMMV was higher in range –

Figure 3. Necrotic patterns on tomato fruits from cv. Ideal infected with mix infection of TSWV and PMMV

Figure 4. DAS-ELISA results of tomato samples infected with mix infection of PVY, ToMV and CMV

Legend: 1 – tomato leaf sample tested for PVY; 2 – negative control for PVY from the kit; 3 – positive control for PVY from the kit; 4 – negative control for PVY for Sample buffer used; 5 – tomato leaf sample tested for ToMV; 6 – negative control for ToMV from the kit; 7 – positive control for ToMV from the kit; 8 – negative control for ToMV for Sample buffer used; 9 – tomato leaf sample tested for CMV; 10 – negative control for CMV from the kit; 11 – positive control for CMV from the kit; 12 – negative control for CMV for Sample buffer used

Figure 5. DAS-ELISA results for tomato samples infected with mix infection of TSWV and PMMV

Legend: 1 – tomato leaf sample tested for TSWV; 2 – negative control for TSWV from the kit; 3 – positive control for TSWV from the kit; 4 – negative control for TSWV for Sample buffer used; 5 – tomato leaf sample tested for PMMV; 6 – negative control for PMMV from the kit; 7 – positive control for PMMV from the kit; 8 – negative control for PMMV for Sample buffer used

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3.000 (Figure 5). This was the maximum value for this method. PMMV DAS-ELISA value was three times higher than TSWV and great economic relevance of such mixed infections, there are no suppressed the thrips transmissible TSWV. only available data in tomato focused on with
For confirmation of the results from DAS-ELISA for PVY and for determination of the PVY virus strain we performed Touch-down RT-PCR with specific primers for P1 gene region of the PVY genome. The chosen fragment from P1 enclosed by PVY Primer 1 and PVY Primer 7 gave the product 640 bp for PVYN strain and the gene fragment enclosed by PVY Primer 1 and PVY Primer 8 – 445 bp product for PVYN/NTN strain or 280 bp for PVYNTN strain. We used three samples – from PVY infected tomato leaf, fruit and seed. From these samples we extracted RNA with RNeasy plant mini kit. From this RNA we received cDNA with the PVY Primer. This cDNA we used for Touch-down RT-PCR for determination of the different PVY strains. From all tested samples we received a single positive band of 443 bp product, compared with DNA ladder, which corresponded to PVYN/NTN necrotic group of virus strains (Figure 6). All the controls used in the PCR were clear (without any product) showing that there were no contaminations between the different samples. The result confirmed that tomato cultivar Ideal was infected with PVYN/NTN group strain. The virus strain PVYN/NTN was identified in the leaves, fruits and seeds of the infected tomato plant. This shows a smooth spread of the virus in all parts of the host plant, and especially in the seed which is a prerequisite for its mass distribution in the next year if it is used for planting the seeds of this infested plant.

Discussion

In most cases in the literature scientists report mono infection of the plants. There were some reports of mixed infections in tomato. Mixed infections of PVY and CMV, often carrying its satellite RNA (CMV-satRNA), were detected in commercial fields of tomato crops during the CMV outbreaks that occurred in Italy in the mid-1980s (Gallitelli et al., 1988; Gallitelli, 2000). In spite of the high frequency and great economic relevance of such mixed infections, there are no reports on the interactions between CMV and PVY in this host. The only available data in tomato focused on plants mix-infected with CMV and the Begomovirus sp. Abutilon mosaic virus (Wege and Siegmund, 2007). The amount of damage that plant viruses cause to tomatoes varies, depending on the particular virus or combination of viruses present, the virulence of the virus strains, the susceptibility of the variety, the timing of infection, the abundance of insect vectors, and environmental conditions. Disease incidence can range from a few scattered plants in a field to total crop failure. Mixed infections may cause symptoms that are more severe than either virus might cause alone. Virus diseases are difficult to control because of complex interrelationships among virus, host, vector, virus source, RNA we received cDNA with the PVY Primer. This cDNA we used for Touch-down RT-PCR for determination of the different PVY strains. From all tested samples we received a single positive band of 443 bp product, compared with DNA ladder, which corresponded to PVYN/NTN necrotic group of virus strains (Figure 6). All the controls used in the PCR were clear (without any product) showing that there were no contaminations between the different samples. The result confirmed that tomato cultivar Ideal was infected with PVYN/NTN group strain. The virus strain PVYN/NTN was identified in the leaves, fruits and seeds of the infected tomato plant. This shows a smooth spread of the virus in all parts of the host plant, and especially in the seed which is a prerequisite for its mass distribution in the next year if it is used for planting the seeds of this infested plant.

Figure 6. Touch down RT-PCR for P1 gene region of PVY, 443 bp (N/NTN strain)
Legend: 1 – GeneRuler 100bp Plus DNA Ladder; 2 – 443 bp product from P1 gene region of PVY received from infected tomato leaf sample; 3 – 443 bp product from P1 gene region of PVY received from infected tomato fruit sample; 4 – 443 bp product from P1 gene region of PVY received from infected tomato seed sample; 5 – negative control from PCR master mix; 6 – negative control from healthy tomato leaf; 7 – negative control from healthy tomato seed

Conclusion

From all infected tomato plants from the cv. Ideal we received different results. There were some plants infected with one virus but most of them were infected with more than one virus. Mono infections did not affect so seriously quality and quantity of the fruit production, but the infected plants were virus reservoirs. Mixed viral infections in tomato led to major damage on fruit and loss of production. Testing the seeds and seedlings could prevent
distribution of these infections.

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