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Impact of the short-term mild and severe ozone treatments on the potato spindle tuber viroid-infected tomato (*Lycopersicon esculentum* Mill.)

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Abstract

This study was the first attempt to find out if the phenotypic plant response to the short-term ozone exposure might be altered by the presence of systemic viroid infection in plants. Model pathosystem used in these experiments was formed from the tomato (*Lycopersicon esculentum* Mill.) cv. 'Micro-Tom' and potato spindle tuber viroid. Viroid-infected plants were exposed to chronic, acute and extreme ozone treatments in order to find out if the elevated concentration of ozone could have any significant impact on the pathogenicity and spread of sub-viral pathogen. The results revealed that phenotypic alterations caused by the presence of viroid infection in plants were highly dependent on the dose of ozone received by plants. Only slight yellowing of the plant top leaves was detected in plants exposed to 80 ppb of ozone for 8 hours. No significant differences between viroid-inoculated and uninoculated plants were observed in the phenotypic appearance as well as in plant growth after application of this treatment suggesting that naturally occurring single short-term peaks of elevated ozone concentration could have no significant impact on viroid and host interaction. There were no significant differences observed in the degree of visible ozone injury between viroid-inoculated and uninoculated plants just after the short-term acute (400 ppb × 6 h) and extreme (300 ppb × 13 h) ozone treatments were applied. However, in the end of the growth period clearly expressed differences in plant growth, degree of defoliation and the regeneration capacity were revealed between viroid-inoculated and uninoculated plants submitted to these treatments, indicating that pathogenicity of potato spindle tuber viroid could be altered by the exposure of inoculated plants to the severe ozone stress.

Key words: pathogenicity, potato spindle tuber viroid, short-term ozone treatment.

Introduction

It has been well documented that high concentration of tropospheric ozone is harmful to plants (Morgan et al., 2003; Hayes et al., 2007; Booker et al., 2009). It affects many aspects of plant life including plant disease development and co-evolutionary relationships between plant and pathogens. Many studies have been done to reveal the impact of ozone on bacterial and fungal diseases. However, much less information about ozone effect on viral diseases is available (Sandermann, 1996; Krupa et al., 2001).

Controversial information about the effect of elevated ozone concentration on virus-caused plant diseases was obtained from the experimental studies. It has been reported that enhanced resistance to ozone injury is induced by bean common mosaic virus (Davis, Smith, 1974), tobacco mosaic virus (Brennan, Leone, 1969; Ormrod, Kemp, 1979), tobacco ring spot virus (Vargo et al., 1978). On the contrary, elevated ozone concentration completely eliminated the effect of barley

yellow dwarf virus on biomass reduction in wild oat (Pollina et al., 2008). According to Reinert and Gooding (1978), ozone reduced the suppression of tobacco etch virus on leaf and stem dry weight. In some studies growth has been significantly reduced in plants exposed to ozone after certain virus incubation period (Reinert, Gooding, 1978; Ormrod, Kemp, 1979). No significant virus and ozone interaction was found for plants inoculated with peanut stunt virus (Heagle et al., 1992) and tobacco streak virus (Reinert, Gooding, 1978). The major reason why such variable results were obtained could be a high number of factors having an impact on the final effect. The variation in ozone-caused injury degree was found to be influenced by growth conditions, virus species, and plant cultivar or even clone, ozone treatment conditions and virus incubation period.

More recent studies have shown many similarities between the plant responses to ozone and pathogens. Oxidative stress induced cell death pattern

in plants resembling the resistance (*R*) gene-mediated host defence response caused by pathogens (Rao, Davis, 2001; Overmyer et al., 2005). Advances in microarray technologies revealed statistically significant changes in a high number of genes that altered transcriptional activity as a result of treatment with elevated ozone concentration of virus-infected plants (Bilgin et al., 2008). Such evidence suggests high importance of ozone, plant and virus interaction on the spread and development of viral disease under the pressure of atmospheric pollution.

Viroids are the smallest causative agents inducing virus like disease symptoms in plants. They are classified into two *Pospiviroidae* and *Avsunviroidae* families of sub-viral particles that are formed of small single-stranded RNAs arranged in a closed loop without protein shell and that replicate in host plants where they may or may not be pathogenic. Viroid RNA is not translated and replicates by rolling circle mechanism without coding any proteins. This is in contrast to plant viruses where translation of viral genetic information is crucial for virus replication. Viroid hosts include both herbaceous and woody species – agronomic as well as ornamental. More than 30 species of viroids have been detected in many higher plants since they were discovered in 1971 by Diener. There are still considerable difficulties faced by growers in the control of viroid-caused diseases of economically important crops (Diener, 2007).

No information about the interaction of sub-viral pathogen, plant and elevated ozone concentration is available. Therefore, this study was aimed to find out if the presence of viroid infection can cause any differences in the phenotypic plant response to the short-term treatment with elevated ozone concentration under controlled environmental conditions using a model pathosystem formed from the model plant breed tomato (*Lycopersicon esculentum* Mill.) cv. ‘Micro-Tom’ and potato spindle tuber viroid (PSTVd). Tomato (*Lycopersicon esculentum* Mill.) cv. ‘Micro-Tom’ is widely accepted as a model plant breed in genetic and physiological studies (Matsukura et al., 2008). This model breed is known to be a suitable host in the studies of interaction of plant and the most common bacterial, fungal and viral pathogens (Takahashi et al., 2005; Sturbois et al., 2012). Here we tested the ability of tomato cv. ‘Micro-Tom’ to be exploited as a model host in the studies on viroid, host and ozone interaction. The advantage of this plant breed was susceptibility to potato spindle tuber viroid, short vegetation period, small size of the plants and good growth under controlled environmental conditions using relatively small amount of soil. The disadvantages we faced were low sensitivity of ‘Micro-Tom’ to the short-term ozone treatment in the young age, absence of clear viroid-induced phenotypic symptoms, and slow viroid bioamplification under the optimal growth conditions.

Material and methods

Plant growth and inoculation. Tomato cv. ‘Micro-Tom’ plants were grown in the autoclaved commercially available substrate for tomato cultivation with 10% of perlite added in 2012. Fertilizers N (100–400 mg l⁻¹), P (50–200 mg l⁻¹), and K (100–500 mg l⁻¹) had been already added to the substrate, pH was 5–7. No additional fertilization was applied during the experiments. Plants were kept in the growth chamber

with a photoperiod of 12 h at 20–25°C and watered with tap water 3–4 times per week. Two seeds per pot of 8 × 8 × 12 cm (for chronic and extreme treatment) and 6 × 6 × 8 cm (for acute treatment) were sown and only one seedling was left after germination. Mechanical viroid inoculation was carried out in 2–3 weeks after sowing when the second pair of true leaves appeared and was up to 5 cm long. Two leaves per plant were dusted with carborundum and rubbed after adding 20 µl of tomato sap extract. Infected plant sap was taken from tomato cv. ‘Micro-Tom’ used for viroid bioamplification 70 days post inoculation (dpi). Plants were kept avoiding leaf contact between inoculated and uninoculated individuals.

Viroid detection. Systemic spread of viroid infection in inoculated plants was detected by two-step reverse transcription and polymerase chain reaction (RT-PCR) technique. Plant total ribonucleic acid (RNA) extraction was made from 0.1 g tissue taken from the top leaves of the plant. Samples for RNA extraction were taken 50–70 dpi. Plant total RNA was extracted using silica-based RNA extraction method (Menzel et al., 2002). Reverse transcription (RT) step to produce complementary deoxyribonucleic acid (cDNA) from template RNA was done as follows: a mixture of plant total RNA, reverse primer (20 pmol) and nuclease free water (Thermo Fisher Scientific, Lithuania) was heated at 70°C for 10 min and chilled on ice. Then a mixture of 5 × reaction buffer, 10 mM mix of nucleotides (dNTP), 20 U ribonuclease inhibitor and 200 U of Premium Revert Aid™ Reverse Transcriptase (Thermo Fisher Scientific) was added and RT step was continued at 50°C for 60 min. After the RT step, PCR reactions were set up containing 2 µL of cDNA, antisense and sense primer (each of 20 pmol), MgCl₂ (0.7 mM), dNTP mix (0.2 mM), 10 × PCR reaction buffer and 1 U of Hot start *Taq* polymerase (“Thermo Fisher Scientific”). Amplification (PCR) step was done as follows: 35 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C were followed by cDNA denaturation for 3 min at 94°C and finished with a final extension for 5 min at 72°C. Primers by Weidemann and Buchta (1998) were used for whole length PSTVd amplification. As negative control, reactions containing no template RNA and RNA extracted from healthy plants were used in RT-PCR reactions. To test quality of RT-PCR with the infected samples whether the reactions were not inhibited by remains of silica, polysaccharides, etc., a series of RT-PCR reactions with the samples from the same plants was performed with universal plant 5S rRNA specific primers (Kolchinsky et al., 1991). Products of RT-PCR reactions were analyzed on 5% polyacrylamide gel after staining with ethidium bromide.

Ozone treatment. Chronic treatment was applied by exposing plants to 80 ppb ozone concentration for 8 hours during the light period. In this experiment 15 viroid-uninoculated and 15 inoculated plants were ozone-exposed on day 7 post inoculation while 10 inoculated plants and 10 uninoculated plants were kept in ambient ozone concentration under the same environmental conditions. Acute treatment was applied by exposing plants to 400 ppb ozone concentration for 6 hours during the light period. In this experiment 20 uninoculated and 20 inoculated plants were ozone-exposed on day 7 post inoculation while 20 inoculated plants and 20 uninoculated plants were kept in the ambient ozone concentration under the same environmental conditions.

Extreme treatment was applied by exposing plants to 300 ppb ozone concentration for 13 hours (6, 4 and 3 h, respectively on the first, second and third day) during the light period. In this experiment 5 uninoculated and 5 inoculated plants were submitted to ozone treatment 60 days post inoculation while 5 inoculated plants and 5 uninoculated plants were kept in the ambient ozone concentration under the same environmental conditions. Ozone fumigation was carried out in the closed top chamber under conditions that were very close to the ones in the growth chamber. Control plants were kept in the separate chamber at ambient ozone concentration during the time of ozone fumigation. Ozone concentration was measured continuously with the UV absorption ozone analyzer O341M (Environnement S. A., France) by averaging data of 1 minute. Limits of the measurements with the ozone analyzer were 0–1000 ppb and sensitivity – 1 ppb. Ozone in the chamber was generated by ozone generator OZX-B300T (Enaly Ozone Ltd., Canada).

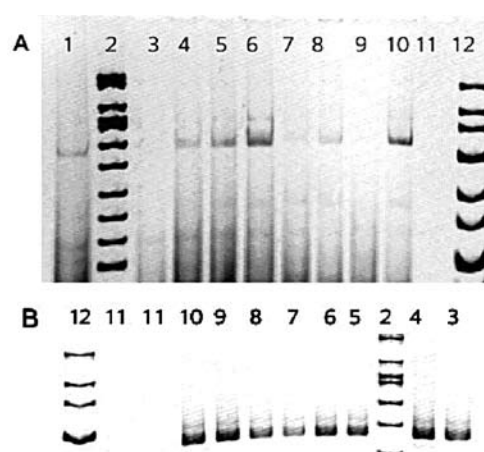
Statistical analysis. Height of viroid inoculated and uninoculated plants was measured at the end of the growth period. The mean, variance and t-test were calculated using *Excel* software of *Microsoft Office 2003*. The significance of the difference between means within each treatment was examined applying Student's *t*-test considering unequal variance of two-samples and two-tailed distribution.

Results and discussion

Spread of the viroid infection in plants. No significant differences in phenotypes between viroid inoculated and uninoculated plants of tomato cv. 'Micro-Tom' under the optimal growth conditions had been observed. Systemic spread of viroid infection and bioamplification of viroid RNA in the tomato cv. 'Micro-Tom' grown in the temperature of 20–25°C was very slow. The presence of viroid infection by RT-PCR was hardly detectable on 21 dpi. Better results were obtained using RNA extracted on day 30 dpi. However, the viroid titers obtained were highly variable among the plants, in contrast to the titers of plant ribosomal RNA used as an internal control (Pic. 1). Variation in viroid titers could be caused by different viroid concentration in the inoculum.

Effect of chronic ozone treatment. Only slight yellowing of the plant top leaves was observed in the plants exposed to 80 ppb of ozone for 8 hours. There were no significant differences observed in plant response to this ozone treatment in the phenotypic appearance as well as in plant growth (Table 1). These results indicated that naturally occurring single short-term peaks of elevated ozone concentration would have no any significant impact on viroid and host interaction at least in the model pathosystem exploited for this study.

Effect of acute ozone treatment. Typically ozone-caused necrotic spots appear on leaves of ozone-sensitive plants shortly after the exposure under acute ozone treatment (Sanderman, 1996; Krupa et al., 2001). In our experiment, acute ozone treatment carried out on 2–3 week old plants, never caused the appearance of necrotic spots on the treated leaves. However, the differences in leaf colour between viroid-infected and uninfected plants started to show two weeks after ozone exposure. Initially, an outbreak of red colour appeared on



Notes. A – whole length of potato spindle tuber viroid (PSTVd) amplicons obtained using PSTVd-specific primers; B – amplicons obtained using primers for internal ribonucleic acid (RNA) control. Lane 1 – positive PSTVd control, lane 2 – deoxyribonucleic acid (DNA) marker pUC mix 8 (Thermo Fisher Scientific, SM0301), lane 12 – low range DNA marker (Thermo Fisher Scientific, SM0383), lanes 3 and 9 – samples from uninoculated plants (negative controls), lanes 4–8 and 10 – PSTVd-infected plants, lane 11a and 11b – water negative controls of PCR and RT with PCR reactions, respectively.

Picture 1. Amplicons obtained after reverse transcription (RT) and polymerase chain reaction (PCR) using samples picked up from the plants 30 days post inoculation (dpi)

Table 1. Effect of ozone treatment on the height of the viroid inoculated and uninoculated plants at the end of growth season

Ozone treatment	Average height (cm) of		Significance level
	viroid-infected plants	viroid-uninfected plants	
Chronic	16.9	17.3	n.s.
Acute	9.1	12.9	***
Extreme	12.7	16.8	*

Notes. Differences between viroid-inoculated and uninoculated plants revealed by Student's *t*-test are: n.s. – non significant, * – significant at $p < 0.05$ level and *** – significant at $p < 0.001$ level. Chronic, acute and extreme ozone treatments were fulfilled by keeping plants in the chamber with ozone concentration 80, 400 and 300 ppb for 8, 6 and 13 hours, respectively.

the leaves of viroid-infected plants. At the end of growth season, ozone-exposed viroid-uninoculated plants had a higher degree of defoliation than viroid-inoculated ozone-exposed or uninoculated ozone-untreated plants (Pic. 2).

It was observed a long time ago that presence of virus infection can reduce air pollution-caused injury in commercially-grown plants that are sensitive to ozone (Davis, Smith, 1974; Bisessar, Temple, 1977). However, only several plant species sensitive to ozone and susceptible to certain virus species were used as experimental hosts in the studies of ozone stress effect on virus-infected plants. Tobacco (*Nicotiana tabacum* L.), woodland tobacco (*Nicotiana sylvestris* Sp. and S. Comes), soybean



Picture 2. Differences in the plant height and the degree of defoliation observed between viroid-infected (on the left) and uninfected (on the right) tomatoes (*Lycopersicon esculentum* Mill.) cv. 'Micro-Tom' in six weeks after acute ozone treatment (400 ppb \times 6 h) carried on young plants

(*Glycine max* L.), white clover (*Trifolium repens* L.), pinto bean (*Phaseolus vulgaris* L.), wild oats (*Avena fatua* L.) and tomato (*Lycopersicon esculentum* Mill.) were among them. Tomato is an economically important crop. It is sensitive to ozone and many pathogens but sensitivity to ozone and viral infection was found to differ remarkably among the cultivars (Ormrod, Kemp 1979; Arie et al., 2007; Calvo et al., 2007). No study on the response of tomato cv. 'Micro-Tom' to ozone treatment was found to be done; however, the effect of oxidative stress and activity of antioxidant enzymes were examined by several authors (Li et al., 2004; Gratao et al., 2008). The results of our experiment revealed no visible injury caused by ozone treatment on young tomato cv. 'Micro-Tom' plants. However, statistically significant (at 99.9% level) differences in plant height were found at the end of growth period after exposure of plants to the acute ozone treatment (Table 1) which well corresponds to the other reports that ozone could inhibit growth and development of plants.

Extreme ozone treatment. Extreme ozone treatment was carried out three days in a row exposing mature plants to 300 ppb ozone concentration during light period for 6 hours on day 1, for 4 hours on day 2 and for 3 hours on day 3. Rapid appearance of necrotic spots on plant leaves was observed within the hours after treatment on day 3. All leaves that had been exposed to ozone were dropped in few days. One infected plant died after this treatment while the others survived and regenerated new shoots, leaves, flowers and fruits. Clear differences between viroid-inoculated and uninoculated plants were noticed in regeneration capacity. Uninoculated plants fully recovered after the stress: they produced new shoots, leaves, flowers, fruits and seeds. Necrotic spots were visible even on the newly emerged leaves of viroid infected plants. Most interestingly, the regenerated shoots of ozone-treated viroid-infected plants were much shorter had smaller flowers, fruits and produced no seeds as compared to the old ozone-exposed shoots of the same plants (Pic. 3). However, viroid infection in regenerated leaves of the exposed plants was still present only in very low titers and was hardly detectable by RT-PCR.



Picture 3. Regeneration of tomato (*Lycopersicon esculentum* Mill.) cv. 'Micro-Tom' plants exposed to extreme ozone treatment (400 ppb \times 13 h): potato spindle tuber viroid-infected plant (on the left) and uninfected plant (on the right)

Tomato cv. 'Micro-Tom' harbours some mutated genes yielding the size reduction and dwarf appearance (Marti et al., 2006). The latest research on viroids revealed that the marked upregulation of genes involved in the response to viroid infection of the dwarf cv. 'Micro-Tom' was noticed only when exogenous brassinosteroid was applied to infected plants, thereby providing the first evidence for the involvement of brassinosteroid-mediated signalling in viroid disease induction (Owens et al., 2012). This is why different response in host and pathogen interaction might be observed in the case of different experimental pathosystem. Our results in Table 2 show that appearance of phenotypic alterations caused by the presence of viroid infection in plants was highly dependent on the dose of ozone received by plants during the short-term ozone treatment.

A huge number of soybean transcripts showing significant changes in general expression as a response to combined effect of elevated ozone and soybean mosaic virus infection is known (Bilgin et al., 2008). This combination caused statistically significant changes

in differential expression to virus and ozone of 369 and 231 of soybean transcripts respectively at 8 and 72 hours post infection. Viroid is an obligate parasite of the host transcriptional machinery. Changes in the transcription level of the big amount of host genes could be the reason why changes occurring in viroid pathogenicity included different phenotypic traits depending on the level of stress caused by oxidative stress.

Table 2. Changes in phenotypic appearance of tomato cv. 'Micro-Tom' caused by potato spindle tuber viroid (PSTVd) infection depending on the dose of ozone received by plants during the short-term ozone treatment under controlled environment

Ozone treatment	Concentration × exposure time ppb × hours	Phenotypic effect on submitted plants	
		viroid-inoculated	viroid-uninoculated
Chronic	80 × 8	non-significant effect	non-significant effect
Acute	400 × 6	discolouration, growth reduction,	leaf defoliation
Extreme	300 × 13	poor regeneration, visual injury on new leaves, no seed production	full recovery, regeneration of healthy leaves

The results of our study indicated that the experimental pathosystem of tomato cv. 'Micro-Tom' and potato spindle tuber viroid could be useful in the studies of pollution pathology and could provide new evidence on changes in viroid pathogenicity under the very high environmental pressure of elevated ozone concentration.

Conclusions

1. The results of our investigation indicated that the experimental pathosystem of tomato (*Lycopersicon esculentum* Mill.) cv. 'Micro-Tom' and potato spindle tuber viroid (PSTVd) could be useful in the studies of pollution pathology and could provide new evidence on the presence of interaction among sub-viral pathogen, plant and elevated ozone concentration considering the phenotypic response during the whole growth period.

2. There were no significant differences observed in the degree of visible ozone injury between viroid-inoculated and uninoculated plants shortly after the short-term chronic (80 ppb × 8 h), acute (400 ppb × 6 h) or extreme (300 ppb × 13 h) ozone exposure.

3. Significant differences in plant growth, degree of defoliation and regeneration capacity were found between viroid-inoculated and uninoculated plants after acute and extreme ozone treatments had been applied, indicating that pathogenicity of PSTVd could be altered under very high environmental pressure caused by elevated ozone concentration.

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Skirtingų ozono dozių poveikis bulvių verpstiškumo viroidu užkrėstiems valgomojo pomidoro (*Lycopersicon esculentum* Mill.) augalams

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Santrauka

Tyrimo metu siekta išsiaiškinti, ar užkrėtimas viroidu gali pakeisti augalo fenotipinį atsaką į trumpalaikį poveikį didelės koncentracijos ozonu. Siekiant nustatyti, ar didelė ozono koncentracija gali turėti esminės įtakos šio ligų sukėlėjo patogeniškumui ir plitimui, buvo atlikti trys viroidu užkrėstų augalų trumpalaikio poveikio skirtingomis ozono koncentracijomis (80 ppb × 8 val., 400 ppb × 6 val. ir 300 ppb × 13 val.) eksperimentai. Tam buvo pritaikyta modelinė patosistema, kurioje veislės 'Micro-Tom' pomidorai buvo panaudoti kaip subvirusinio patogeno, bulvių gumbų verpstiškumo viroido, šeimininkas.

Tyrimų rezultatai parodė, kad viroidu užkrėstų augalų fenotipiniai pokyčiai buvo labai priklausomi nuo ozono dozės, kuria buvo paveikti augalai. Užkrėstus augalus 8 valandas paveikus 80 ppb ozono koncentracija, buvo pastebėtas tik nežymus viršūnės pageltimas. Nei esminių augalų fenotipo, nei augimo pokyčių, nulemtų tokio ozono poveikio nebuvo nustatyta. Tai rodo, jog trumpalaikiai gamtoje aptinkami ozono koncentracijos pakilimai neturi didesnės įtakos augalo šeimininko ir viroido sąveikai. Trumpalaikis augalų poveikis didelėmis koncentracijomis (400 ppb × 6 val. ir 300 ppb × 13 val.) ozono tuojau pat po poveikio nesukėlė esminių skirtumų tarp viroidu užkrėstų ir neužkrėstų augalų matomų pažeidimų. Tačiau augimo laikotarpio pabaigoje tarp augalų, kuriems buvo taikytas toks poveikis, išryškėjo žymūs augimo, defoliacijos ir atžėlimo skirtumai, rodantys, jog stiprus ozono streso poveikis gali turėti didelės įtakos bulvių verpstiškumo viroido patogeniškumui.

Reikšminiai žodžiai: bulvių gumbų verpstiškumo viroidas, patogeniškumas, trumpalaikis ozono poveikis.