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Experiments on *Plum pox virus* inactivation from micropropagated plum plants through non-thermal plasma treatment

Termal olmayan plazma tedavisi ile mikro çoğaltılmış erik bitkilerinde *Plum pox virus* inaktivasyonu çalışmaları

Snezhana MILUSHEVA[a*], Lilyana NACHEVA[a], Evgenia BENOVA[b], Plamena MARINOVA[c], Nataliya DIMITROVA[a], Anka GEORGIEVA-HRISTEVA[a]

[a]*Fruit Growing Institute, 12 Ostromila str., 4004 Plovdiv, Bulgaria*

[b]*Sofia University, DLTIS, 27 Kosta Loulchev str., 1111 Sofia, Bulgaria*

[c]*University of Forestry, Faculty of Forest Industry, 10 Kliment Ohridski Blvd., 1797 Sofia, Bulgaria*

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* Corresponding author: Snezhana Milusheva
snezhanamilusheva@gmail.com

A B S T R A C T

Recently, cold atmospheric plasma (CAP) is under investigation for possibility to be applied for inactivation of pathogens in medicine, food technologies, water cleaning technologies and agriculture. The aim of the current study is to investigate the effect of CAP on microplants, propagated *in vitro* from plum tree (*Prunus domestica* L., cv. 'Kyustendilska sinya') naturally co-infected by M and D strains of *Plum pox virus* (PPV) and in that respect the possibility for CAP application for virus inactivation. In the present work, we have used two types of plasma sources for biological systems treatments: a surface-wave-sustained Argon plasma torch and an underwater diaphragm discharge. These enabled several variants of plasma treatment to be performed. Based on the data of IC-RT-PCR tests of the microplants on the third subculture after treatment, it was found the most effective variant was the reiterated plasma torch tip treatment to nodal segments without leaves in gas medium. The strain specific RT-PCR analysis results of PPV positive CAP-treated microplants showed that only PPV-M strain was identified after treatment, although the starting material was co-infected by both strains. The results obtained from IC-RT-PCR and strain specific RT-PCR of the acclimatized *ex vitro* plants have been in agreement with the data from molecular analyses of the microplants tested. These are the first experiments on CAP ability for inactivation of PPV from tissue of living woody plants even if in *in vitro* conditions. The completed estimation of this approach for obtaining of PPV-free plum plants will be made after more prolonged observation and testing of the *ex vitro* plants.

INTRODUCTION

As it is well known unlike other phytopathogens, the application of pesticides against plant viruses in the field is impossible. Only virus elimination by physical methods and a few chemical agents as well as the biotechnological

approaches like meristem culture can be used with different effectiveness in laboratory conditions.

Plum pox virus (PPV), the causal agent of Sharka disease, is one of the major limiting factors of the plum production. Scholthof et al. (2011) listed PPV to a group of 'Top10' plant viruses in molecular plant pathology due to its scientific and economic impact. To date, ten PPV strains - D, M, Rec, C, EA, W, T, CR, An (James et al. 2013) and CV (Chirkov et al. 2018) are recognized on the base of differences in complete genome sequences and phylogenetic analyses. Currently, in Bulgaria the PPV-M, PPV-D and PPV-Rec strains have been identified on stone fruits and in particular in plum (Kamenova et al. 2015).

PPV is widespread in stone fruit trees and the movement of PPV-infected propagating material is considered the main pathway for its long-distance distribution. In that respect production of PPV-free planting material is one of the most important measures for the virus containment. Sometimes, it is impossible to be found PPV uninfected trees of any stone fruit cultivars that require being applied sanitation in order to be obtained PPV-free propagating material. Some researchers reported about PPV elimination from plum and apricot cultivars through combining of tissue culture techniques and thermotherapy (Gabova 1989, Koubouris et al. 2007) or chemotherapy (Hauptmanova and Polak 2011, Paunovic et al. 2007) with different effectiveness. In our earlier experiment on PPV elimination, it was studied possibility to obtain PPV free clones from Sharka infected plum cultivars 'Kyustendilska sinya' and 'Valevka' through tissue culture using apex buds with 2-3 unfolded leaves and as result after eight subcultures 88% of subclones of 'Kyustendilska sinya' and 100% of 'Valjevka' were PPV negative (Nacheva et al. 2002).

The both most frequent applied approaches for sanitation of plants from phytoviruses thermotherapy and chemotherapy are time and costs consuming methods.

Recently, the low temperature, non-equilibrium atmospheric pressure plasma called cold atmospheric plasma (CAP) is under investigation for possibility to be applied for inactivation of pathogens in medicine (Woedtke et al. 2019), food technologies (Pankaj et al. 2018), water cleaning technologies and agriculture (Stryczewska et al. 2016). Using common gases such as air or argon, one can produce reactive oxygen and nitrogen species as well as ozone oxides, peroxides and monoxides, and even in a short treatment time of a few seconds there may have strong impact on pathogens. Both proteins and nucleic acids molecules can be destroyed during the plasma applying.

Searching for efficient, fast and cheap approach for sanitation of the fruit trees from viruses we have begun investigation on possibility to apply CAP for virus inactivation. The idea is based on some data for virucidal effect of CAP on *Feline calicivirus* (Abaubakr et al. 2016) and a few bacteriophages (Guo et al. 2018, Wu et al. 2015). More recently, Filipic et al. (2019) reported for inactivation of *Potato virus Y* (PVY) in water samples by CAP treatment while the previous attempts for elimination of necrotic isolates of PVY from potato nodes in tissue culture and infected plant extract were unsuccessful (Dobnik et al. 2016).

The plasma sources used in this work for biological systems treatment are two types: a surface-wave-sustained Argon plasma torch and an underwater diaphragm discharge. In contrary to the widely used plasma jets where the plasma treating area is in the afterglow region, in the surface-wave-sustained plasma torch the treatment is in the active discharge zone called from here on "plasma torch tip". This results in high concentrations of short-lived active particles together with electromagnetic field and UV radiation at plasma-liquid interface (Krcma et al. 2018). The underwater diaphragm discharge is the same type plasma source as the one used in Kozakova et al. 2015, Vyhnankova et al. 2015.

The aim of the study is to investigate the effect of cold atmospheric plasma on PPV infected plum microplants and in that respect the possibility for application of CAP for virus inactivation.

MATERIALS AND METHODS

Plant material

The model biological system used in the study was established from *in vitro* cultured plum (*Prunus domestica* L.) cultivar 'Kyustendilska sinya', naturally co-infected by M and D strains of PPV. The plum cultivar 'Kyustendilska sinya' is highly susceptible to PPV and for the first time the virus was observed and described on that plum cultivar by Atanasoff (1933).

Methods

In vitro propagation

The shoot tip culture was established from the mother tree and was maintained (28–day multiplication cycle) on a solidified MS (Murashige and Skoog 1962) nutrient medium,

supplemented with 2.5 µM N^6-benzyladenine (BA), 0.05 µM indole-3-butiric acid (IBA), 30 g l^{-1} sucrose, 6.5 g l^{-1} Phyto agar (Duchefa, The Netherlands). The cultures were incubated in a growth chamber under temperature of 22±2 °C, photoperiod 16/8 hours supplied by cool-white fluorescent lamps (OSRAM 40 W; 40 µmol $m^{-2}s^{-1}$ PPFD). For the CAP treatment nodal segments (10 mm in length) with or without one leaf were prepared. The shoot clumps obtained after CAP treatment were transferred to the above mentioned fresh culture medium every three weeks. After four passages apical shoots from treated plants and non-treated control were placed on rooting media based on MS with 50% reduced macro salts, 100% micro salts and vitamins, 1.5 µM IBA, 20 g L^{-1} sucrose, 6.5 g l^{-1} Phyto agar. The rooted plants have been acclimatized to *ex vitro* conditions and currently, six months after acclimatization, they are being under observation for arising of Sharka symptoms and molecular testing for presence/absence of PPV.

Cold atmospheric plasma (CAP) treatment

Two approaches were applied for CAP treating of the micropropagated plants:

First - CAP treatment allowing the plasma torch tip to get in contact with the explants for 5 s.

All of explants were treated individually at the torch tip on agar. The discharge is created in an Ar (purity of 99.99999%) flow at atmospheric pressure in open space at constant mass flow 2 l/min controlled by Omega FMA-A2408 mass flow controller. The gas temperature (i.e. the temperature of the heavy particles) in the plasma does not exceed 40 °C while the electron temperature is about 1 eV. The microwave Argon plasma torch tip is presented in Figure 1a.

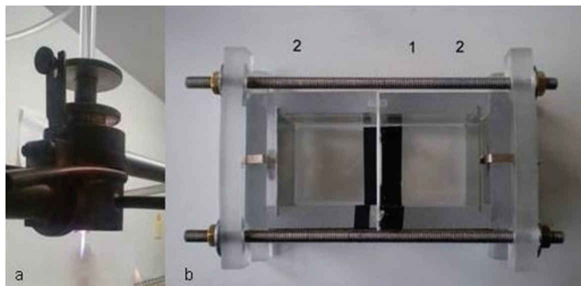

Figure 1. a) Microwave Argon plasma torch, b) Two-chambers tray of underwater diaphragm discharge. 1 – diaphragm with pin-hole; 2 – electrodes

Second - treatment with electrical discharges in liquid media (Figure 1b) for 5 s after plunging the plants in 50-60 ml water in a two-chamber tray for electrical discharges as the first lead was connected to alternating voltage and the second lead was ground connected.

The following variants of treatment were carried out:

a) Single time treatment by plasma torch tip to nodal segments without leaves, placed in petri dish on agar media;

b) Single time treatment by plasma torch tip to leaflets, placed in petri dish on agar media;

c) Reiterated treatment by plasma torch tip to nodal segments without leaves, prepared from shoots obtained on the fourth subculture after the first plasma torch tip treatment;

d) Reiterated treatment with electrical discharges in water media to nodal segments without leaves prepared from shoots obtained on the fourth subculture after the first plasma torch tip treatment.

Each one of the treated plants was labelled with unique number allowing tracing of the CAP effect plant by plant.

Molecular methods for PPV detection

The detection of PPV in the plum tree source of the explants for micropropagation, microplants before treatment, CAP-treated plants, non-treated controls and adapted plants was carried out by immunocapture–reverse transcription-polymerase chain reaction (IC-RT-PCR), performed as described by Wetzel et al. (1992), using primer pair P1/P2, targeting 3’-coat protein (CP) genomic region (Wetzel et al. 1991). PPV polyclonal antibodies from Agritest S.r.l (Italy) were used in immunocapture step. The clones obtained from CAP-treated plants were tested on the third subculture after treatment. Each sample was composed from leaves of the microshoots from one cultivation vessel - usually 3 plants.

RNA based two steps RT-PCR was carried out for identification of PPV strains in the mother tree, CAP-treated microplants and acclimatized to *ex vitro* conditions plants. Strain specific RT-PCR tests were performed with primer pairs distinguish PPV-M and PPV-D strains in 3’- terminus of the CP gene using P1/PM and P1/PD primer pairs (Olmos et al. 1997) and genomic region corresponding to C-terminus of the viral replicase and N-terminus of CP using mM5/mM3, mD5/mD3 primer pairs (Subr et al. 2004). Initially, the plum tree source of the explants was also tested with primer pair mD5/mM3 identifying PPV-Rec strain isolates (Subr et al. 2004). Non-treated controls from each variant and

microplants propagated from the mother tree were included as controls.

Total RNA was extracted by using commercial kit (Jena Bioscience) according to the instruction of the manufacturer. The reverse transcription (RT) step for synthesis of complementary (c) DNA was performed with random hexamer, using AMV reverse transcriptase (New England, BioLabs Inc.) following the protocol of the supplier.

PCR products were analysed by electrophoresis on 1.5% agarose gel in 1x TBE buffer and stained with ethidium bromide.

RESULTS

Physiological effect of the CAP on treated nodal segments

After CAP torch tip treatment of the nodal segments with leaves necrotic lesions were observed on the leaves (Figure 2a) but later on the normal growth recovered and new leaves expanded (Figure 2b). No visual damages were registered on

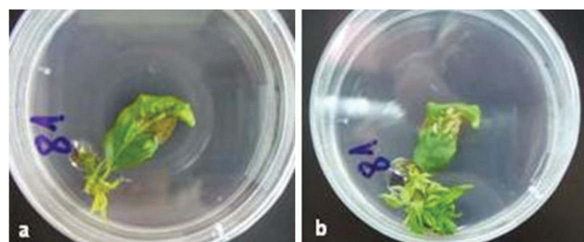

Figure 2. Developing microplants from cold atmospheric plasma (CAP)-treated stems with leaves after treatment. 2a – treated stems with leaves on the 10th day after treatment; 2b – treated stems with leaves on the 33rd day after treatment

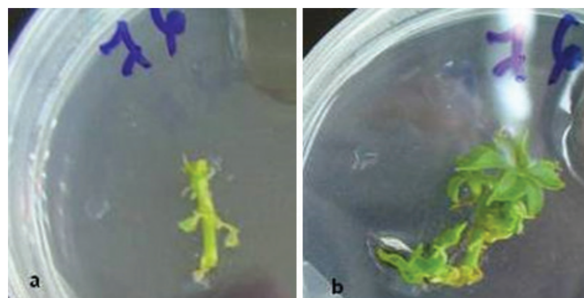

Figure 3. Developing microplants from cold atmospheric plasma (CAP)-treated stems without leaves. 3a – treated stems without leaves on the 10th day after treatment; 3b – treated stems without leaves on the 33rd day after treatment

the CAP-treated nodal segments without leaves (Figure 3a and 3b) as well as plants treated in water media.

IC-RT-PCR and strain specific RT-PCR

Based on the data of IC-RT-PCR tests of the microplants on the third subculture after treatment, it was found that the most effective variant was the reiterated plasma torch tip treatment of nodal segments without leaves in gas media on agar (Table 1). Single plasma torch treatments and treatment in water media of the plasma torch treated plants were the less effective manner of treatments. Comparison between the both variants of single torch treatments showed a little bit higher effectiveness in variant with nodal segments without leaves.

The results from the strain specific RT-PCR tests of the PPV positive CAP-treated microplants performed using mentioned above primer pairs, distinguished PPV-M and PPV-D in two viral genomic regions, showed that only PPV-M was identified in analysed samples (Table 1) although the starting material was co-infected by M and D strains of PPV. Both strains were detected in the non-treated control microplants.

Figure 4. Electrophoresis of the products from strain specific RT-PCR with mD5/mD3 (lines 2-6) and mM5/mM3 (lines 7-11) primer pairs of PPV positive CAP-treated *ex vitro* plants. Line 1 non-infected plum - negative control; lines 2 and 7 mother tree; lines 3 and 8 *ex vitro* plant obtained from treated nodal segments with leaves; lines 4 and 9 *ex vitro* plant obtained from treated nodal segments without leaves; lines 5 and 10 non-treated *in vitro* propagated plant; lines 6 and 11 non-template controls; line 12 1kb DNA Ladder (Bioneer Inc. 500, 1000, 1600, 2000, 2961, 4000, 5007, 5991, 8000, 10200 bps)

A part of treated PPV negative and positive microplants from two variants and non-treated controls have been acclimatized to *ex vitro* conditions. They are under observations for

Table 1. Results of *Plum pox virus* tests by IC-RT-PCR and strain specific RT-PCR of cold atmospheric plasma (CAP)–treated plants on the third subculture after treatment

Variant	IC-RT-PCR tested samples	IC-RT-PCR positive samples	Strain specific RT-PCR of positive samples
a) Single time treatment by plasma torch tip to nodal segments without leaves	15	5	PPV-M
b) Single time treatment by plasma torch tip to the leaflets	10	5	PPV-M
c) Reiterated treatment by plasma torch tip to nodal segments without leaves, prepared from shoots obtained on the fourth subculture after the first torch tip treatment	10	1	PPV-M
d) Reiterated treatment with electrical discharges in water media to nodal segments without leaves prepared from shoots obtained on the fourth subculture after the first torch tip treatment	10	5	PPV-M

Table 2. Results of visual observation and *Plum pox virus* tests by IC-RT-PCR and strain specific RT-PCR of acclimatized to *ex vitro* conditions cold atmospheric plasma (CAP)–treated plants

Variant	Number of PPV negative clones	Number of PPV positive clones	Number of symptomatic plants	Symptoms	IC-RT-PCR positive plants/ tested plants	Strains identified by RT-PCR
a) Single treatment by plasma torch tip to nodal segments with leaves	12	3	3	Single chlorotic spots	3/15	PPV-M
b) Single treatment by plasma torch tip to nodal segments without leaves	12	3	3	Single chlorotic spots	3/15	PPV-M
Non-treated control microplants	0	7	7	Chlorotic spots or rings	7/7	PPV-M, PPV-D

arising of PPV symptoms. At this stage, six months after *ex vitro* acclimatization, Sharka symptoms are not registered on CAP-treated PPV negative plants. Very mild symptoms are showing CAP-treated PPV positive plants. Developing of typical Sharka symptoms on non-treated controls have been observed.

The results obtained from IC-RT-PCR of all acclimatized to *ex vitro* conditions plants (Table 2) are in agreement with data from molecular analyses of the microplants tested. PPV was identified only in symptomatic plants and PPV positive plants were not found among asymptomatic *ex vitro* plums. According to data obtained from the strain specific RT-PCR tests of the acclimatized to *ex vitro* conditions plants, only PPV-M was detected the CAP-treated PPV positive plants (Table 2, Figure 4).

More recently, rooted plantlets from the other two CAP variants of treatment were potted in peat and after successful acclimatization to *ex vitro* conditions they will be also screened.

DISCUSSION

At this stage, the results from current study have given initial

information about effect of CAP treatment on the used biological model. Torch tip treatment of nodal segments without leaves proved to be more efficient variant and likely one of the reasons for that result is due to removing of the leaves leads to decrease the virus content in plant tissue. The most effective approach was the reiterated CAP torch tip treatment of nodal segments without leaves as that result gives opportunity for improving of the experimental protocol by multiplying the number of treatments and shortening the interval between treatments.

Filipic et al. (2019) and Wu et al. (2015), studying virus inactivation in liquid samples, have found correlation between the time of exposure to atmospheric pressure cold plasma and the degree of virus inactivation and, respectively, damages of the viral genes. More prolonged treatment can cause seriously degradation of the viral RNA and the genes coding virus proteins as coat protein, replicase protein, etc. Taking attention that in the present research the experimental model system includes living explants, the CAP treatments were carried out for very short time, only 5 seconds. Even if that short exposure after CAP torch tip treatment on the leaves of the nodal segments were observed necrotic lesions. Nevertheless that later on the normal growth recovered and new leaves expanded, prolonged treatment probably would be injured the plantlets. Some studies on mechanism of virus inactivation by CAP treatment reported (Abaubark et al. 2016, Guo et al. 2018) the primary role of the singlet oxygen among plasma-generated reactive species for viral nucleic acids and proteins degradation. Working with living plants, there exists risk the plant nucleic acids and proteins to be damaged simultaneously with the viral nucleic acids and proteins degradation so because of that reason the exposure time of CAP treatment there is critical importance. Experiments for optimization of the exposure time of CAP treatment could be made in variants with subject nodal segments without leaves, treated by plasma torch tip on agar media and with electrical discharges in water media.

In virological point of view, the most interesting result from the experiments is the "disappearance" of PPV-D strain from the treated PPV positive microplants as well from the treated PPV positive acclimatized *ex vitro* plants. One of possible explanations of that PPV-D has not been detected in PPV positive CAP treated plants is that after James (2017) some PPV-D isolates may not be aggressive and may not replicate efficiently resulting in low levels of inoculum that are not always detected reliably in routine diagnostic tests. PPV-M strain is more virulent and it replicates in susceptible host plants faster than PPV-D.

These are the first experiments on CAP ability for inactivation of PPV from tissue of living woody plants even if in *in vitro* conditions. The results obtained are promising and the experiments have to be continued by including of more variants and parameters of CAP treatments. The completed estimation of this approach for obtaining of PPV-free plum plants will be made after more prolonged observation and testing of the *ex vitro* plants.

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ÖZET

Son zamanlarda, soğuk atmosferik plazma (SAP)'ın tıp, gıda teknolojileri, su arıtma teknolojileri ve tarımda patojenlerin inaktivasyonu için uygulanabilme olasılığı araştırılmaktadır. Bu çalışmanın amacı SAP'ın, *Plum pox virus*'ünün (PPV) M ve D ırkları tarafından doğal olarak enfekte edilen erik ağacından, *in vitro* koşullar altında elde edilmiş bitkicikler üzerindeki etkisini ve bu bağlamda SAP'ın virüs inaktivasyonunda uygulanması olasılığını araştırmaktır. Çalışmada, biyolojik sistemlerin uygulanmasında "yüzey dalgası-sürekli Argon plazma torçu" ve "su altı diyafram deşarjı" olmak üzere iki tip plazma kaynağı kullanılarak plazma tedavisinin birkaç varyantının gerçekleştirilmesini sağlamıştır. Uygulamadan sonra üçüncü alt kültür üzerindeki bitkiciklerin IC-RT-PCR verilerine dayanarak, en etkili varyantın gaz ortamında yapraksız nodal segmentlere tekrarlanan plazma torç ucu uygulaması olduğu bulunmuştur. SAP ile muamele edilmiş, her iki PPV ırkı ile enfekteli bitkiciklere yapılan ırka spesifik RT-PCR analiz sonucunda, sadece PPV-M tespit edilmiştir. İklimlendirilmiş *ex vitro* bitkilerinin IC-RT-PCR ve ırka spesifik RT-PCR'dan elde edilen sonuçları, test edilmiş bitkiciklerin moleküler analizlerinden elde edilen verilerle uyumlu olduğu görülmüştür. Elde edilen bu veriler, *in vitro* koşullarda yapılmış olsa da canlı odunsu bitkilerin dokusundan PPV'nin inaktivasyonu için CAP yeteneği üzerinde yapılan ilk çalışma niteliğindedir. PPV'den ari erik bitkilerinin elde edilmesinde kullanılan bu yaklaşımın son değerlendirmesi, *ex vitro* bitkilerin daha uzun süre gözlemlenmesi ve test edilmesinden sonra yapılacaktır.

Anahtar kelimeler: *Plum pox virus*, soğuk atmosferik plazma, doku kültürü, virüs inaktivasyonu

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