

Article

Cold Atmospheric Plasma (CAP) Treatment of In Vitro Cultivated Plum Plantlets—A Possible Way to Improve Growth and Inactivate Plum Pox Virus (PPV)

Lilyana Nacheva ^{1,*}, Snezhana Milusheva ¹, Plamena Marinova ^{2,3}, Nataliya Dimitrova ¹
and Evgenia Benova ³

¹ Fruit Growing Institute, Agricultural Academy, 12 Ostromila Str., 4004 Plovdiv, Bulgaria; snezhanamilusheva@gmail.com (S.M.); natali.dimitrova@yahoo.com (N.D.)

² Faculty of Forest Industry, University of Forestry, 1756 Sofia, Bulgaria; plamena_dragozova@ltu.bg

³ Plasma Technology Laboratory, Clean & Circle CoC, Sofia University, 1164 Sofia, Bulgaria; ebenova@uni-sofia.bg

* Correspondence: lilyn@abv.bg

Abstract: Plasma technology, relatively new in the fields of biomedicine, agriculture, and ecology, is the subject of intensive research as a prospective means of decontamination of various microorganisms (bacteria, viruses, and fungi). The objectives of the present study were to follow the effect of cold atmospheric plasma (CAP) treatment on in vitro grown plum plants (*Prunus domestica* L. ‘Kyustendilska sinya’ cv.) and the possibility of eradicating or inactivating plum pox virus (PPV) causing Sharka disease by CAP. The source tree is naturally co-infected by PPV (both M and D strains). In the experiments, two different plasma sources were used. First, a surface-wave-sustained Argon plasma torch and second, an underwater diaphragm discharge. For the treatments, nodal segments (10 mm in length) from in vitro cultured plum plants with or without one leaf were prepared. Apical shoots from treated plants (PPV-positive and negative clones as well non-treated controls) were cultivated in vitro for four passages. Then they were rooted and acclimatized to ex vitro conditions, and their virus status was observed periodically for more than 3 years after treatment for the appearance of Sharka symptoms. All plants, acclimatized to ex vitro conditions, were tested for PPV by immune capture–reverse transcription–polymerase chain reaction (IC-RT-PCR). As a first step in understanding the plasma treatment of living plants, a plasma treatment variant causing no damage must be established; this has been done in our previous works. Treatment of plants by plasma with parameters that have been carefully selected leads to better development than the non-treated plants. In the treated in vitro plants, no significant differences were found in the number and length of shoots compared to the control plantlets. In ex vitro acclimated plants, greater stem length was reported, but no differences in leaf number were observed. No significant differences in growth were recorded between the control and plants that were treated twice or three times. At this stage, 3 years after ex vitro cultivation in a greenhouse, Sharka symptoms were not registered on treated in vitro negative PPV plants, and the virus was not detected by IC-RT-PCR. Very mild symptoms were showing in CAP-treated PPV-positive plants. Development of typical Sharka symptoms on non-treated controls were observed.

Keywords: plasma agriculture; cold atmospheric plasma; micropropagation; Sharka; virus eradication; microwave plasma torch; underwater diaphragm discharge



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1. Introduction

Plasma technology, relatively new in the fields of biomedicine, agriculture, and ecology, is the subject of intensive investigation as a promising tool and green alternative to conventional chemicals for the treatment of biological systems for the elimination of

bacteria, viruses, and fungi. More information about plasma-assisted agriculture can be found in [1].

For the treatment of vital plants, the plasma source used must provide low temperatures and high concentrations of active species and should be easy to operate at low cost in order to provide fast and efficient treatment. The appropriate choice of the plasma source and its operational conditions can provide plasma with temperatures lower than 40 °C, which is called cold atmospheric plasma (CAP). CAP affects the treated objects by a combination of electrons, ions, radicals, UV radiation, and an electromagnetic field.

Sharka disease caused by the plum pox virus (PPV) is widespread in stone fruit trees and is one of the major problems in plum production worldwide.

The movement of PPV-infected propagating material is considered the main pathway for its long-distance spread. Therefore, the production of PPV-free planting material is one of the main approaches to limit the spread of the virus. It is sometimes impossible to find PPV-free trees of various important varieties of stone fruits. This requires the application of various techniques, alone or in combination, to eliminate the virus and accordingly produce PPV-free planting material.

The most commonly used methods for eradicating phytoviruses from infected plants are thermotherapy and chemotherapy. These two techniques are expensive and time consuming.

The idea of applying CAP to inactivate plant viruses is based on data in the literature on the effects of CAP on some viruses that cause diseases in humans and animals [2] and on viruses, bacteriophages, air pollutants, and water [3]. The cited studies were carried out under laboratory conditions in aqueous solutions or cell cultures. At the start of the experiments in this work, there was only one report of attempts to eliminate the virus by inductively coupled plasma reactors with oxygen plasma in E-mode treatment of the necrotic strain of potato virus Y (PVY) in a suspension of strained plant material infected with PVY [4]. Later [5], PVY was successfully inactivated by plasma treatment in an aqueous medium. There was no information on the direct impact of CAP on infected live vegetative plants before our work; therefore, in the research process, a large number of options were carried out in terms of type of plant material treated, duration of treatment, environment, source of discharge, etc.

In searching for an efficient, rapid, and inexpensive approach to virus elimination in fruit trees, we initiated a study on the possibility of applying CAP to in vitro propagated PPV-infected plum plants. The results from the mentioned research were promising [6], and the study has been continued on the next stage by observing and testing the CAP-treated plants that were acclimatized to ex vitro conditions.

The plum cultivar ‘Kyustendilska sinya’ is highly sensitive to PPV, and that plum genotype was the first host of PPV observed and described by Atanasoff [7].

In the current research, we reported the results from that second stage of research on the durable effects of CAP application for PPV inactivation.

The aims of our research were to track:

- the effect of cold atmospheric plasma (CAP) produced by two different plasma sources (a surface-wave-sustained discharge (SWD) in open air plasma torch and an underwater diaphragm discharge) on in vitro cultivated plum (*Prunus domestica* L. ‘Kyustendilska sinya’) plantlets;
- the possibility of eliminating or inactivating plum pox virus (PPV) by the application of CAP.

2. Materials and Methods

2.1. Plant Material

In vitro cultured plum (*Prunus domestica* L.) cultivar ‘Kyustendilska sinya’ was used as an experimental model biological system. The source tree was grown in the orchard of the Fruit Growing Institute in Plovdiv, Bulgaria. The virological analyses carried out

before establishment of the *in vitro* culture showed that the mother tree was co-infected with PPV-M and PPV-D strains [6].

2.2. Establishment of *In Vitro* Shoot Culture

Shoots were collected from the mother tree and stripped of their leaves. They were surface disinfected by 70% ethanol for 30 s followed by 10 min immersion in a 5% calcium hypochlorite with 2–3 drops of Tween. The shoots were then treated four times (10 min each) in sterile distilled water. Nodal segments (10 mm) were cut from the shoots and inoculated on the solid MS medium [8], supplemented with 5 μ M 6-benzylaminopurine (BAP), 0.01 μ M indol-3-butyric acid (IBA), 30 g/L sucrose, and 6.5 g/L Phyto agar (Duchefa, The Netherlands). The medium was adjusted to pH 5.6 before autoclaving at 121 °C for 20 min. The cultures were cultivated at (22 \pm 2) °C under a 16 h photoperiod with (87 \pm 7.5) μ mol m⁻² s⁻¹ of photosynthetic photon flux density (PPFD).

2.3. Plasma Sources

In the present work, two types of plasma sources have been used for biological system treatment: a surface-wave-sustained Argon plasma torch and an underwater diaphragm discharge. This enabled several variants of plasma treatment to be performed.

A Surfatron-type electromagnetic wave launcher [9–12] together with a solid-state microwave generator (Sairem, GMS 200 W, SAIREM—FRANCE, 82 rue Elisée Reclus, Décines-Charpieu, France) have been used for sustaining surface-wave-sustained discharge (SWD) at an operational frequency of 2.45 GHz (Figure 1a). The plasma in the quartz tube is produced by the electromagnetic wave travelling along the tube inside the Surfatron, and outside of the Surfatron, it continues its propagation along the plasma–air boundary sustaining the plasma torch in the open air. The working gas in the discharge tube is Argon with a gas flow rate varying from 2 to 6 L/min, depending on the microwave power, which is from 12 to 20 W. Varying these two parameters, the plasma properties, like plasma density (concentration of electrons), concentrations of ions and other reactive species, electron and gas temperature, can be changed and controlled. This is especially important for the gas temperature of the plasma torch in order to keep it low enough and to prevent thermal damage in the *in vivo* treated samples. The temperature of the treated samples is controlled by infrared (IR) camera, and during the plasma treatment, it is not higher than 30 °C (Figure 1b). Thus, the microwave plasma torch at such a discharge condition is a CAP source and can be used for thermosensitive materials and for *in vitro* treatment. A detailed characterization of the surface-wave-sustained discharge in Argon at 2.45 GHz wave frequency and operational CAP regimes for biomedical applications can be found in [13–15].

The underwater diaphragm discharge set-up is shown in Figure 2 [16,17]. The dielectric discharge camera (polycarbonate in our case) is divided into two containers by a dielectric membrane called a diaphragm. The volume of the two containers is 50 mL water or water solutions. The membrane with a 1 mm thickness has in its center a pinhole with a 0.6 mm diameter. That is why this discharge is called “pinhole discharge” [17]. Two high voltage (HV) electrodes in planar configuration are mounted at fixed positions on both sides of the discharge chamber immersed in the water. A 5 kV voltage at 15 kHz high frequency is applied to one electrode. The other electrode is grounded.

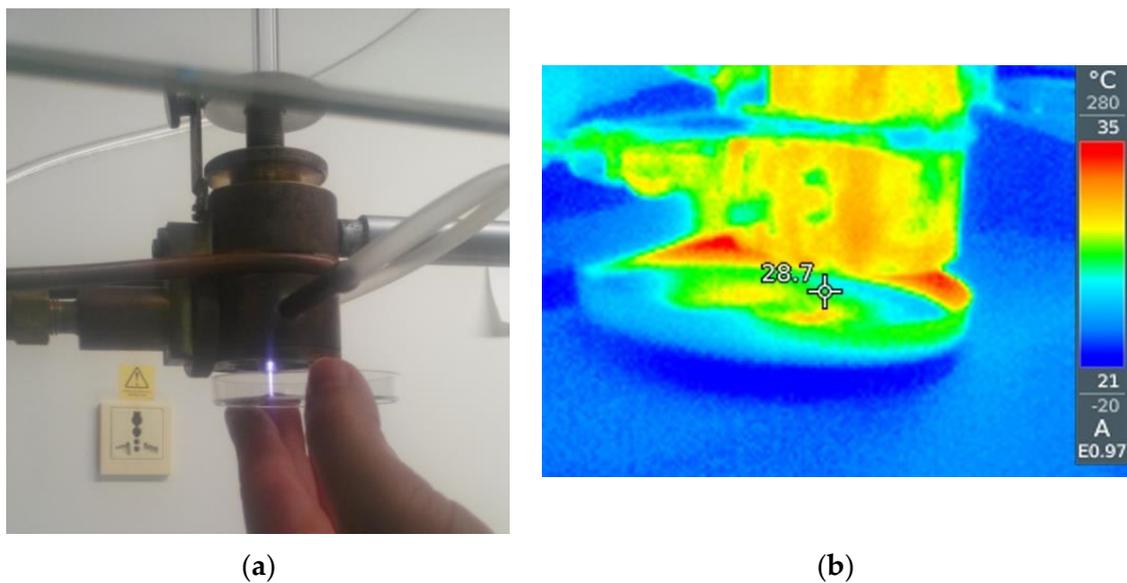


Figure 1. Microwave plasma torch (a) and IR camera image demonstrating the low temperature of the sample during the treatment (b).

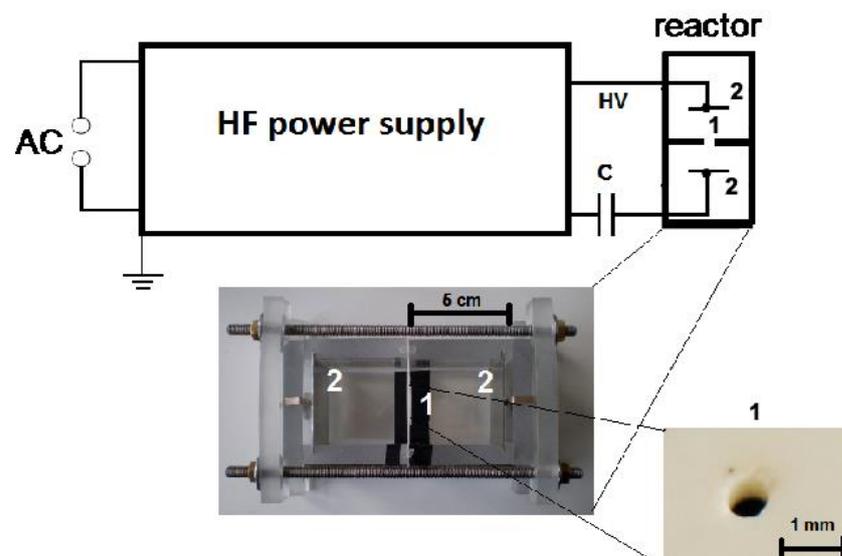


Figure 2. Underwater diaphragm discharge set-up. Diaphragm with pinhole—1; planar HV electrodes—2.

2.4. Cold Atmospheric Plasma (CAP) Treatment Procedure

Depending on the plasma source used, the treatment procedure and treatment time were different. Initially, the samples were treated by the microwave plasma torch. Reiterated treatment was performed for a part of the samples by the plasma torch and for another part by the underwater diaphragm discharge.

For the treatments, nodal segments (10 mm in length) from in vitro cultured plum plants with or without one leaf were prepared.

Two approaches were applied for CAP treating of the micro-propagated plants [6]:

- (i) CAP treatment allowed the plasma torch tip to get in contact with the explants for 5 s;

- (ii) CAP treatment in which the plasma torch tip was in contact only with the leaf of the explants for 5 s.

Each explant was treated individually at the torch tip. The discharge was created in an Ar (purity of 99.99999%) flow at atmospheric pressure in open space at a constant gas flow of 2 L/min, controlled by an Omega FMA-A2408 mass flow controller (Omega Engineering Inc., 800 Connecticut Ave. Suite 5N01, Norwalk, CT 06854, USA). The gas temperature (i.e., the temperature of the heavy particles) in the plasma did not exceed 40 °C (Figure 1b), while the electron temperature was about 1 eV.

Explants of plasma-treated plantlets were similarly prepared and treated with CAP two and three times, respectively.

The following variants of treatment were carried out:

- (a) One leaf nodal segment treated one time by plasma torch tip to leaflets;
- (b) Nodal segment without leaves, treated one time;
- (c) Nodal segment without leaves reiterated treated by plasma torch tip. The shoots were prepared from one treated shoot clump;
- (d) Third treatment of twice-plasma-treated plantlets, obtained from variant (c);
- (e) Second treatment with electric discharges in an aqueous medium of leafless stem segments obtained from plants of variant (b).

Each treated plant was labelled with a unique number and then cloned. This allowed the biological response of each clone to be tracked.

The microplants treated by the described approaches and growing under in vitro conditions were tested by immune capture–reverse transcription–polymerase chain reaction (IC-RT-PCR), and the results of the first stage were reported in [6].

At 40 days after CAP treatment, the shoot clumps obtained were transferred to the fresh culture medium in glass jars with transparent Magenta B-Cap lids with 25 mL nutrient medium per vessel. After 4 weeks of cultivation, the shoots were divided and placed on fresh nutrient medium (5 explants per jar). In this way, they were transferred every 4 weeks.

Four passages after the treatment, some physiological parameters were recorded: number of shoots, length of the stem, and fresh and dry biomass of one plant. Apical cuttings were taken from treated plants and untreated controls and placed for rooting. The rooting was achieved on media based on MS (50% reduced macro salts, 100% micro salts and vitamins, 1.5 µM IBA, 20 g/L sucrose, 6.5 g/L Phyto agar).

Ex vitro acclimatization of the rooted plants was done in pots with peat-perlite (2:1) substrate and grown and maintained under insect-proof conditions. Their virus status was observed periodically for 3 years after treatment for the appearance of Sharka symptoms. Non-treated controls were grown using the same method.

For a more detailed evaluation of the physiological state of the CAP-treated plants, an analysis of the chlorophyll fluorescence of the plants was performed. The HandyPEA Fluorimeter (Hansatech Instruments Ltd., King's Lynn, UK) was used to analyze the structure and functional state of the photosynthetic apparatus to detect early symptoms of stress and various disorders [18,19]. The method is non-destructive and is applied without damaging or destroying the analyzed plants. Chlorophyll *a* fluorescence induction curves (OJIP) were recorded after dark adaptation of a spot on the analyzed leaves for 40 min. The measurement was carried out on the first fully developed leaf from the shoot tip of five representative plants. The induction OJIP curves were recorded after illumination with 3000 µmol m⁻² s⁻¹ PPFD for 1 s. The primary data from the measurement were processed with PEA Plus Software (V1.10, Hansatech Instruments Ltd., UK). The parameters of the OJIP test were presented according to Strasser and Strasser [18] and Goltsev [19].

Chlorophyll content (Chl) in the leaves was measured (in relative units) with a chlorophyll meter CL-01 (Hansatech Instruments Ltd., UK). For each clone (treatment), five plants were used.

Following, the growth parameters representing the development of plants were measured: fresh (FW) and dry biomass weight (DW), length and number of shoots, and number of leaves. The base of the *in vitro* plants was washed with tap water to remove traces of the agar medium, then dried with filter paper, and the fresh weight (FW) of the plants was measured. After drying at $(105 \pm 5) ^\circ\text{C}$ to constant mass, the plant dry weight (DW) was determined. The FW and DW of the *ex vitro* acclimatized plants were measured in the same way.

2.5. Statistical Analysis

The obtained data were statistical analyses by one-way analysis of variance (ANOVA), followed by Tukey's b test, with the program SPSS ver. 26.0 (IBM, Armonk, NY, USA).

2.6. PPV Detection

The virological study included visual observations for the appearance of PPV symptoms on CAP-treated plants, acclimatized to *ex vitro* conditions, as well laboratory tests for detection of the virus.

The detection of PPV and its strains M and D in the treated and non-treated plants was carried out by IC-RT-PCR, performed as described in [20], using primer pair P1/P2 [21] for general detection of the virus, and primer pairs mM5/mM3 and mD5/mD3 [22] that distinguish PPV-M and PPV-D strains, respectively. In the immunocapture step, PPV polyclonal antibodies from Agritest S.r.l. (Valenzano, Italy) were used.

The PCR products were separated electrophoretically on 1% agarose gel in $1 \times$ TBE buffer and stained with ethidium bromide.

3. Results and Discussion

3.1. Treatment by Plasma Torch Tip

Treatment performed on nodal segments of *in vitro* cultivated plum plants demonstrated that although *in vitro* plants are very delicate, they could be treated with cold atmospheric plasma without being damaged (Figure 3).

Plants treated by plasma with appropriately selected plasma parameters did not suffer any damage from heating, electromagnetic fields, or other plasma components. They not only survived the treatment but also developed better than the non-treated plants (Figure 3, Table 1). The *in vitro* treated plants showed a trend for greater stem length, and fresh and dry biomass, but no statistically significant differences were found compared to the control (untreated) plants.

(a) First group—treatment on the explant (b) Second group—treatment on the leaf

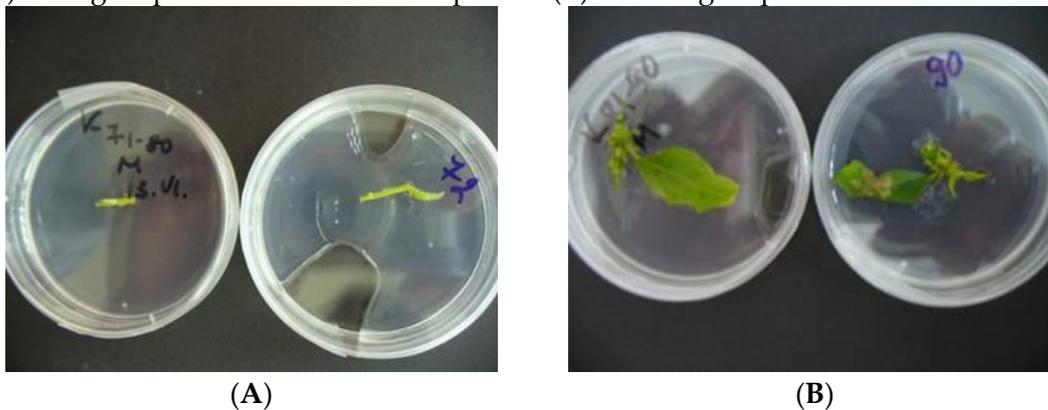


Figure 3. Cont.

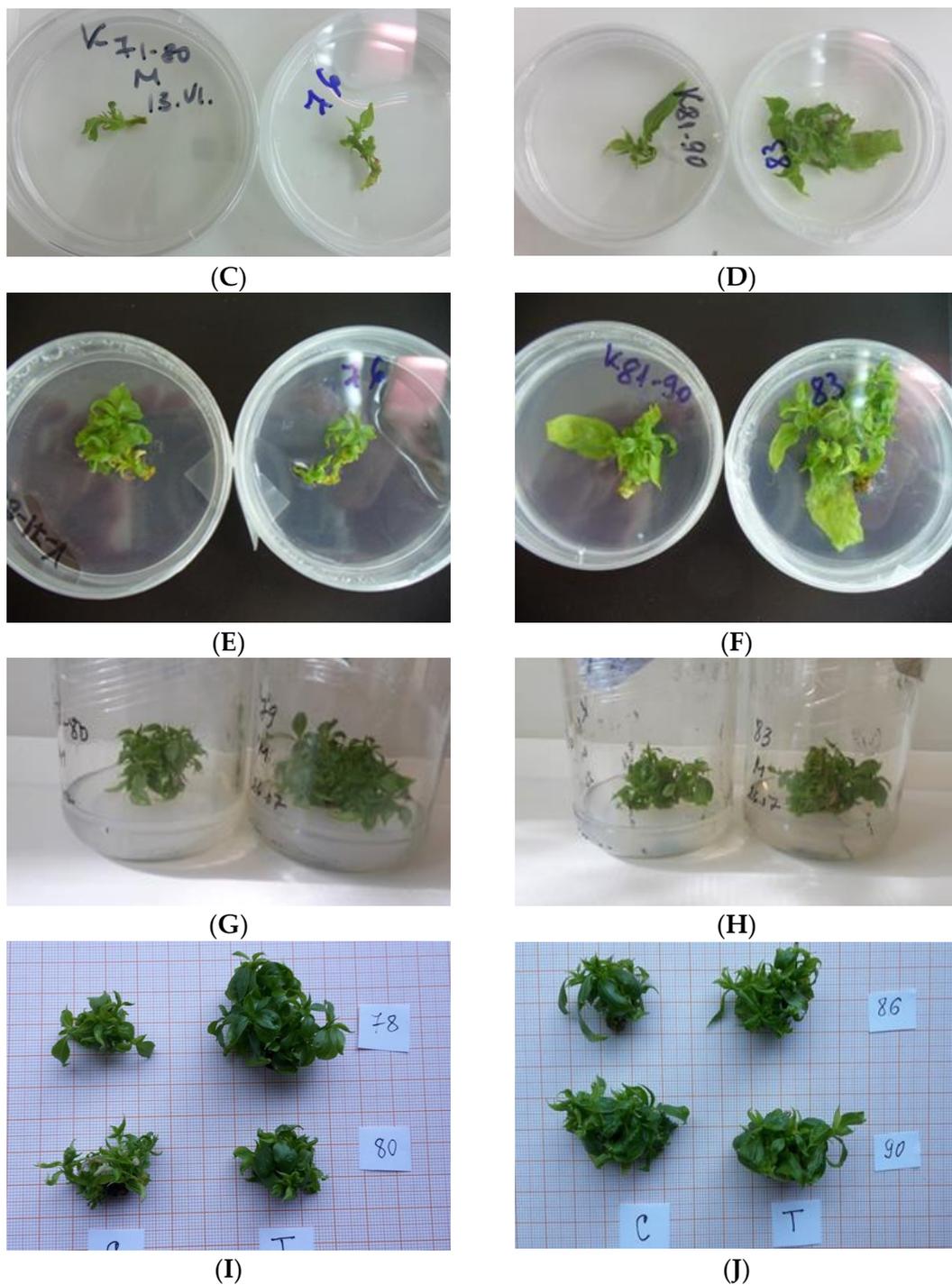


Figure 3. The development of the in vitro plum (*Prunus domestica* L.) plants after CAP treatment; (A,B) 10 days after treatment (on every picture: left, control; right, treated); (C,D) 20 days after treatment; (E,F) 33 days after treatment; (G–J) 80 days after treatment.

In the ex vitro acclimated plants from the first group, greater stem length and number of leaves than the control plants were reported (Table 2, Figure 4). In the plants from the second group, no significant difference between the treated and control (non-treated) plants were observed.

Table 1. Growth parameters of the in vitro plum plants 80 days after CAP treatment. FW—Fresh weight of plantlet; DW—Dry weight of plantlet.

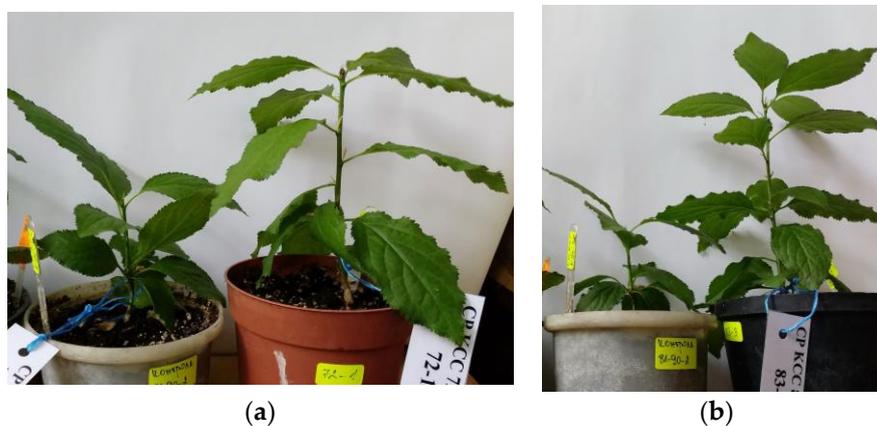
Treatment	Length of the Stem (mm)	Number of Shoots	FW (g)	DW (g)
First group—treatment on the explant (i)				
Control	9.66 ± 0.87 a*	9.00 ± 1.15 a	0.68 ± 0.04 a	0.05 ± 0.01 a
CAP Treated	10.31 ± 1.55 a	8.33 ± 2.33 a	0.83 ± 0.36 a	0.07 ± 0.02 a
Second group—treatment on the leaf (ii)				
Control	8.68 ± 0.95 a	12.00 ± 5.50 a	0.78 ± 0.25 a	0.07 ± 0.01 a
CAP Treated	8.80 ± 0.19 a	14.66 ± 3.28 a	0.92 ± 0.16 a	0.08 ± 0.01 a

* Means ± SD in each column followed by the same letter were not different at $p \leq 0.05$.

Table 2. Growth parameters of the ex vitro plum plants acclimatized in the greenhouse (60 days after transplantation to ex vitro conditions), treated in vitro with CAP.

Treatment	Length of the Stem, mm	Number of Leaves
(a) First group—treatment on the explant		
Control	50.03 ± 2.37 b*	9.50 ± 2.50 b
CAP Treated	82.50 ± 7.55 a	12.90 ± 0.30 a
(b) Second group—treatment on the leaf		
Control	54.40 ± 1.95 a	10.30 ± 2.50 a
CAP Treated	62.50 ± 12.9 a	13.63 ± 1.70 a

* In each column, means ± SD followed by the same letter were not different at $p \leq 0.05$.

**Figure 4.** CAP-treated and non-treated plum plants (*Prunus domestica* L.) 60 days after transplantation to ex vitro conditions in a greenhouse. (a) Plants from first group; (b) plants from second group. On each picture, the left plant is the non-treated control; the right plant is the treated plant.

3.2. Reiterated Treatment by Plasma Torch Tip

The results after the reiterated and third treatment are presented in Table 3 and Figure 5, following the notations used in Section 2.4, namely:

- (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) Third treatment by plasma torch tip to nodal segments without leaves;
- (e) 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.

Table 3. Growth parameters of the in vitro plum plants, treated once (c) and twice with CAP (d and e).

Treatment	Shoot Length, mm	Number of Shoots
c (clone 79)	10.29 a*	5.6 a
d (clone 79–306)	10.40 a	7.0 a
e (clone 79–307)	10.74 a	6.0 a

* Means followed by the same letter in each column were not different at $p \leq 0.05$.

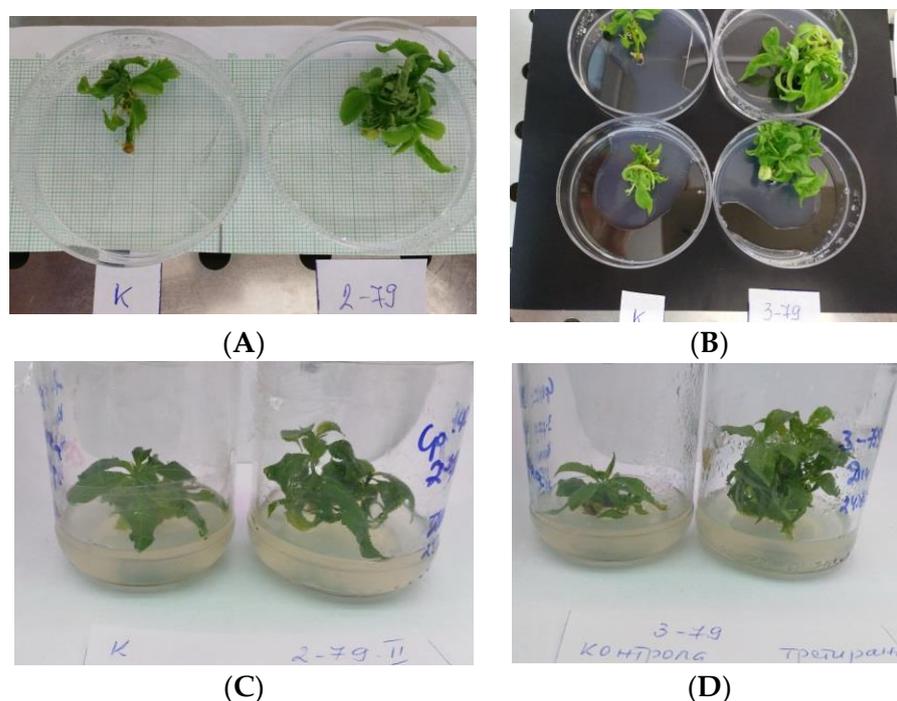


Figure 5. In vitro plum plants one month (A,B) and three months (C,D) after treatment with plasma torch tip. (A,C): left plant, once treated (control); right plant, twice treated with plasma torch tip. (B,D): left plant, once treated (control); right plant, treated three times with plasma torch tip.

No significant differences in growth were recorded between once (control) and twice-treated plants (Table 3, Figure 5).

The plants were transferred monthly to a fresh nutrient medium, and 3 months after the treatment, growth and physiological analyses were performed (Table 4, Figure 5C,D).

No significant differences were found in terms of fresh weight (FW) and dry weight (DW), as well as chlorophyll content (in relative units) in single, double, and triple treatment of explants with CAP (Table 4).

There was some tendency for a greater number of shoots and a greater number of leaves in the plants obtained from the two- and three-times plasma-treated explants, but due to the high variability, these differences were not statistically significant (Table 4, Figure 5).

Table 4. Growth parameters of the in vitro plum plants, treated once and twice with CAP three months after treatment. FW and DW—fresh and dry mass of one plantlet; Chl—chlorophyll content (SPAD method, in relative units).

Treatment	FW, g	DW, g	Chl	Number of Shoots	Shoot Length, mm	Number of Leaves
Clone 79	1.676 ± 0.223 a*	0.162 ± 0.023 b	4.6 ± 2.7 a	2.11 ± 1.10 b	35.55 ± 4.36 a	31.3 ± 3.2 b
Clone 2–79	2.187 ± 0.516 a	0.246 ± 0.018 a	5.2 ± 2.3 a	5.67 ± 2.14 a	37.62 ± 2.18 a	68.2 ± 4.7 a
Clone 3–79	1.359 ± 0.744 a	0.168 ± 0.051 b	6.4 ± 2.1 a	4.13 ± 1.30 a	23.47 ± 3.43 b	61.7 ± 3.8 a

* Means ± SD followed by the same letter in each column were not different at $p \leq 0.05$.

In all three studied plants, the fluorescence curves have a typical OJIP shape from the initial (F_0) to the maximum (F_M) fluorescence, with clearly defined J and I phases (Figure 6), showing that the plum plants included in the experiment are photosynthetically active [23]. Regardless of the fluctuations in initial (F_0), maximum (F_M), and variable fluorescence (F_v), the quantum yield (Yield, $Y = F_v/F_M$) reflecting the potential photochemical activity of photosystem II (PS II) was within very close limits (Table 5), from 0.801 (at clone 3–79) to 0.821 (at clone 79) and corresponded to the normal (0.750–0.830) in healthy, unstressed leaves [24]. This showed that a normally developed photosynthetic apparatus was functioning in the observed plants. This was also confirmed by the close values in the chlorophyll content (Table 5).

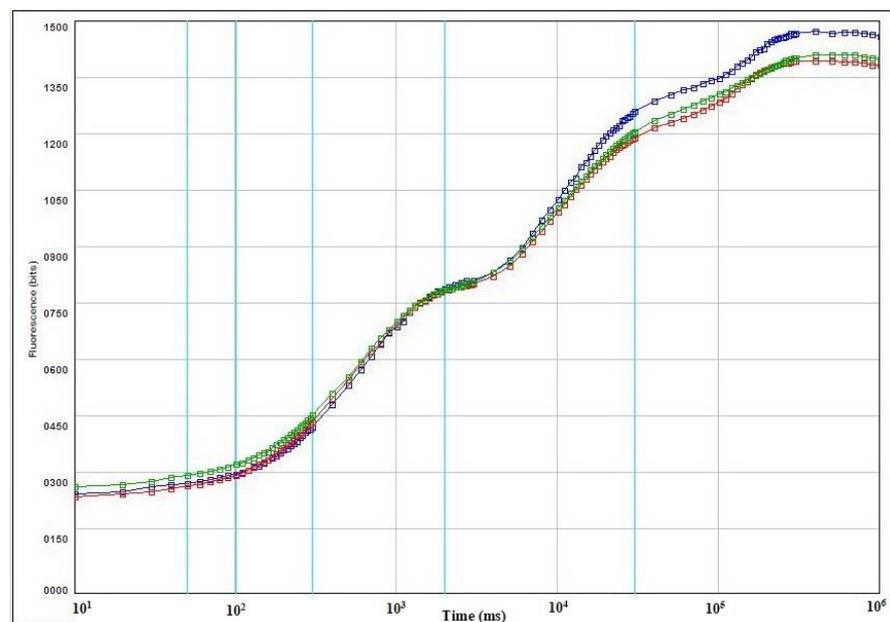


Figure 6. Induction curves of fast chlorophyll fluorescence (OJIP test) of plum plants treated with CAP (torch) once (79, blue), twice (2–79, red), and three times (3–79, green).

Table 5. Parameters of fast chlorophyll fluorescence (OJIP test) of plum plants treated with CAP (torch) once (clone 79), twice (clone 2–79), and three times (clone 3–79).

Clone	F_0	F_M	F_v	F_v/F_M	PI Abs	PI Total
79	264 ± 6 b*	1471 ± 32 a	1207 ± 22 b	0.821 ± 0.002 a	4.32 ± 0.62 a	1.89 ± 0.43 a
2–79	251 ± 9 b	1397 ± 51 ab	1146 ± 45 a	0.820 ± 0.010 a	3.39 ± 0.68 a	1.74 ± 0.59 a
3–79	281 ± 8 a	1413 ± 22 b	1132 ± 23 a	0.801 ± 0.006 b	3.19 ± 0.57 a	1.54 ± 0.11 a

* Means ± SD followed by the same letter in each column were not different at $p \leq 0.05$.

The total performance index (PI_{total}) reflects the functional activity of photosystem II (PS II), photosystem I (PS I), and the electron transport chain between them [18,19]. The PI_{total} indicator is closely related to the total growth and survival of plants under stress conditions and is considered one of the most sensitive parameters of the OJIP test [19]. There was a tendency for PI_{total} to decrease in twice- (1.74) and three-times-treated (1.54) plants compared to once treated (1.89) (Table 5), but these differences were not statistically proven.

Although in vitro plants were very delicate, in most of the single, double, and triple plasma treatments, they survived and developed normally.

3.3. PPV Inactivation

The results from visual observations and IC-RT-PCR of the ex vitro acclimatized plum plants are consistent with data from the tests of the in vitro plants [6]. So far, three years after acclimatization of the plants, Sharka symptoms were not registered on treated in vitro negative PPV plants, and the virus was not detected by IC-RT-PCR (Figures 7–9). Very mild symptoms were observed on the CAP-treated PPV-positive plants. Typical symptoms of Sharka disease have been observed on the control plants. According to the data from the strain-specific IC-RT-PCR tests, only PPV-M was identified in the CAP-treated PPV symptomatic plants in spite of co-infection by both M and D strains in the starting material.

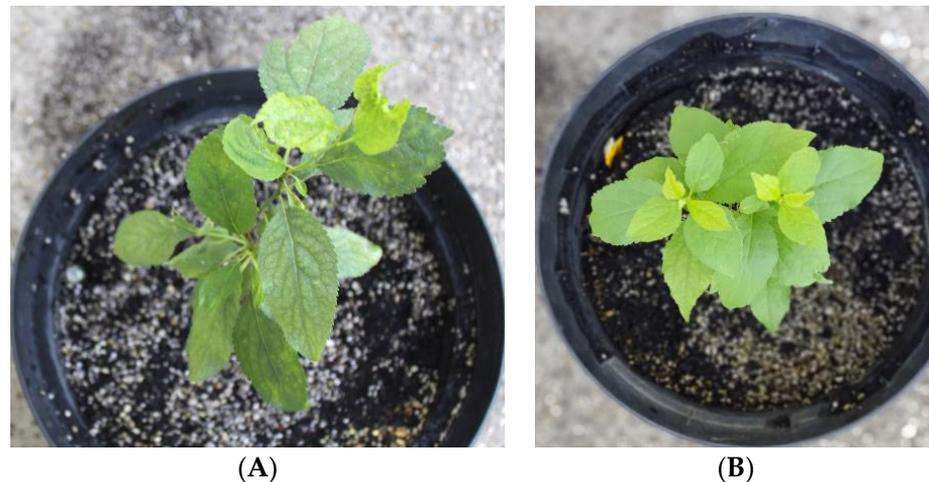


Figure 7. Ex vitro acclimatized plum plants (*Prunus domestica* L. ‘Kyustendilska sinya’) grown for 18 months in a greenhouse. (A) Non-treated plant with visual Sharka symptoms; (B) three times CAP-treated plant (clone 3–79)—no visual symptoms were observed.

Retreatments of nodal segments without leaves by torch tip proved to be more efficient (Table 6). The highest percent (87.5%) of PPV-free clones from ‘Kyustendilska sinya’ cv. was obtained after three treatments with CAP. In all variants, the virus-negative clones remained PPV free three years after their acclimatization to ex vitro conditions (Figures 8 and 9).

Most of the research on plants is related to the plasma treatment of seeds and monitoring the long-term effect on the development of the resulting plants [25,26]. Many authors have reported the growth stimulation of different species by different plasma treatments of seeds [27–32]. However, limited information is available in the literature [4] about the plasma treatment on the living plant parts and tissues (except for the sanitation of fruits and vegetables for food).



Figure 8. Plum plants (*Prunus domestica* L. 'Kyustendilska sinja') grown for 3 years in a greenhouse. (1) Non-treated plant with typical Sharka symptoms; (2) once CAP-treated asymptomatic plant; (3) twice CAP-treated asymptomatic plant (clone 2-79); (4) three times CAP-treated asymptomatic plant (clone 3-79).

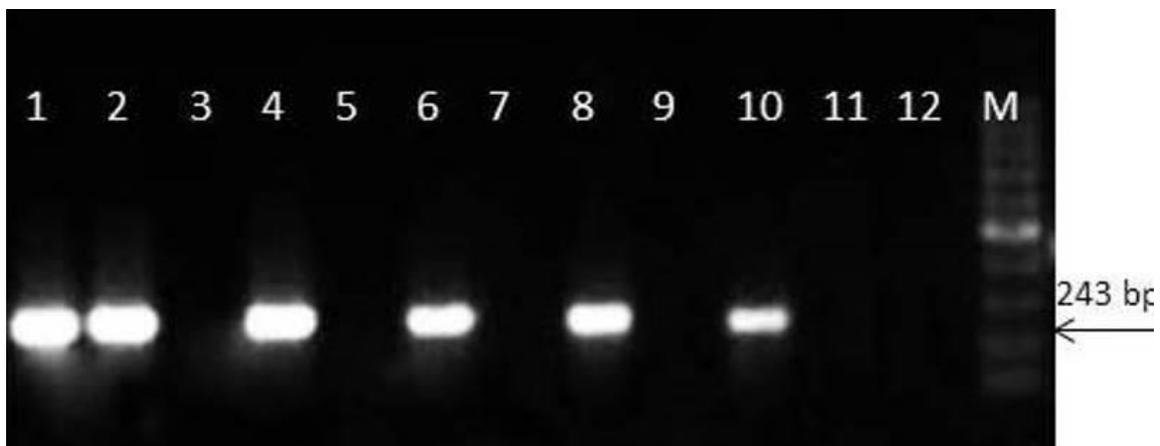


Figure 9. Electrophoresis of IC-RT-PCR products with P1/P2 primers of PPV positive and negative CAP-treated three-year-old ex vitro plants. Lane 1—non-treated control; lane 2—symptomatic plant from variant (a); lane 3—ex vitro asymptomatic plant from variant (a); lane 4—symptomatic plant obtained from variant (b); lane 5—asymptomatic plant obtained from variant (b); lane 6—symptomatic plant obtained from variant (c); lane 7—asymptomatic plant obtained from variant (c); lane 8—symptomatic plant from variant (e); lane 9—asymptomatic plant from variant (e); lane 10—symptomatic plants from three-times-treated plant (variant (d)); lane 11—asymptomatic plants from three-times treated (variant (d)); lane 12—PPV-free plum tree; lane 13—non-template control; M 100 bp DNA Ladder (Jena Bioscience, Germany).

Table 6. Number of observed and tested PPV-negative and PPV-positive plants (clones) acclimatized to ex vitro conditions.

Variant of Treatment	Number of PPV-Negative Clones	Number of PPV-Positive Clones	% PPV-Free Clones
(a) One leaf nodal segment treated one time by plasma torch tip to leaflets	23	10	69.6
(b) Nodal segment without leaves, treated one time	15	4	78.9
(c) Nodal segment without leaves, treatment reiterated by plasma torch tip	20	4	83.3
(d) Third treatment of twice-plasma-treated plantlets obtained from variant (c)	14	2	87.5
(e) Second treatment with electric discharges in an aqueous medium of leafless stem segments obtained from plants of variant (b)	9	6	60.0

The results presented in this study are one of the few for successful treatment of in vitro grown plants and the first for successful elimination/inactivation of PPV after plasma treatment in a woody (fruit) species.

To our knowledge, there are no published in-depth studies elucidating the cause of the stimulatory effect of cold plasma on the growth of seeds and young plants. Changes in the structure and function of biopolymers have been investigated as a possible mechanism explaining these effects [32,33].

The studies of some authors [2,25] on the mechanism of virus inactivation by exposure to CAP highlighted the main role of singlet oxygen among the plasma-generated reactive species for the degradation of viral proteins and/or nucleic acids.

We support the opinion of Filipic et al. [34] that the wide variety of plasma sources with different characteristics and different treated subjects make it difficult to directly compare these studies and determine mechanistic conclusions or any universal parameters of inactivation of viruses and other pathogens.

The results of the experiments carried out with this host–pathogen combination (PPV—‘Kyustendilska sinya’ cultivar) give reasons to assume that treatment with CAP leads to inactivation of PPV to a certain extent; therefore, it is necessary to deepen the research on the use of CAP for inactivation of phytoviruses.

4. Conclusions

This investigation gathers experimental results about the effect of plasma treatment on a wide range of biological systems. The cold atmospheric plasma (CAP) treatment has been applied to in vitro plants to study the effect during the growth of the plants. In vitro shoots were very delicate, but they could be treated with the used type of CAP without damages. The plants, treated with plasma with suitably selected plasma parameters, not only survived the treatment but also developed better than the non-treated controls.

Plasma torch treatment was a more effective approach for inactivating PPV in microplants compared to underwater discharge treatment.

Double and triple plasma torch treatment of micropropagated plants resulted in a higher percentage of PPV-free plum plants.

The duration of treatment is essential for the degree of viral inactivation. The results of the research demonstrated that CAP treatment combined with in vitro techniques could be applied successfully for obtaining a high percentage of PPV-free plum plants for the short term.

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Data Availability Statement: The data that support the presented results of this study are available from the corresponding author upon reasonable request.

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References

1. Simek, M.; Homola, T. Plasma-assisted agriculture: History, presence, and prospects—A review. *Eur. Phys. J. D* **2021**, *75*, 210. [[CrossRef](#)]
2. Aboubakr, H.A.; Gangal, U.; Youssef, M.M.; Goyal, S.M.; Bruggeman, P.J. Inactivation of virus in solution by cold atmospheric pressure plasma: Identification of chemical inactivation pathways. *J. Phys. D Appl. Phys.* **2016**, *49*, 204001. [[CrossRef](#)]
3. Wu, Y.; Liang, Y.D.; Wei, K.; Li, W.; Yao, M.S.; Zhang, J.; Grinshpun, S.A. MS2 virus inactivation by atmospheric-pressure cold plasma using different gas carriers and power levels. *Appl. Environ. Microb.* **2015**, *81*, 996–1002. [[CrossRef](#)] [[PubMed](#)]
4. Dobnik, D.; Junkar, I.; Primc, G.; Ravnikar, M. Plasma treatment of potato plants and plant extracts infected with PVYNTN. In Proceedings of the Workshop on Application of Advanced Plasma Technologies in Central Europe Agriculture, Ljubljana, Slovenia, 17–21 April 2016; Primc, G., Ed.; Slovenian Society for Vacuum Technique (DVTS): Ljubljana, Slovenia, 2016; 48p.
5. Filipić, A.; Primc, G.; Zaplotnik, R.; Mehle, N.; Gutierrez-Aguirre, I.; Ravnikar, M.; Mozetič, M.; Dobnik, D. Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus Y in Water Samples. *Food Environ. Virol.* **2019**, *11*, 220–228. [[CrossRef](#)] [[PubMed](#)]
6. Milusheva, S.; Nacheva, L.; Benova, E.; Marinova, P.; Dimitrova, N.; Georgieva-Hristeva, A. Experiments on Plum pox virus inactivation from micropropagated plum plants through non-thermal plasma treatment. *Plant Prot. Bull.* **2020**, *60*, 83–90. [[CrossRef](#)]
7. Atanasoff, D. Plum pox. In *A New Viral Disease*; Annuaire de L’ Universite de Sofia, Faculte d’Agronomie et de Silviculture, V: Sofia, Bulgaria, 1933; Volume 1932/33, pp. 49–70. (In Bulgarian, English Summary)
8. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays for tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [[CrossRef](#)]
9. Moisan, M.; Beaudry, C.; Leprince, P. A new HF device for the production of long plasma columns at a high electron density. *Phys. Lett.* **1974**, *55A*, 125–126. [[CrossRef](#)]
10. Moisan, M.; Beaudry, C.; Leprince, P. A small microwave plasma source for long column production without magnetic field. *IEEE Trans. Plasma Sci.* **1975**, *PS-3*, 55–59. [[CrossRef](#)]
11. Moisan, M.; Leprince, P.; Beaudry, C.; Bloyet, E. Devices and Methods of Using HF Wave to Energize a Column of Gas Enclosed in an Insulating Casing. U.S. Patent No. 4049940, 20 September 1977.
12. Moisan, M.; Nowakowska, H. Contribution of surface-wave (SW) sustained plasma columns to the modeling of RF and microwave discharges with new insight into some of their features. A survey of other types of SW discharges. *Plasma Sources Sci. Technol.* **2018**, *27*, 073001. [[CrossRef](#)]
13. Benova, E.; Marinova, P.; Hadjiolova, R.; Sabit, Z.; Bakalov, D.; Valchev, N.; Traikov, L.; Hikov, T.; Tsonev, I.; Bogdanov, T. Characteristics of 2.45 GHz Surface-Wave-Sustained Argon Discharge for Bio-Medical Applications. *Appl. Sci.* **2022**, *12*, 969. [[CrossRef](#)]
14. Krčma, F.; Tsonev, I.; Smejkalová, K.; Truchlá, D.; Kozáková, Z.; Zhekova, M.; Marinova, P.; Bogdanov, T.; Benova, E. Microwave micro torch generated in argon based mixtures for biomedical applications. *J. Phys. D Appl. Phys.* **2018**, *51*, 414001. [[CrossRef](#)]

15. Bogdanov, T.; Tsonev, I.; Atanasova, M.; Marinova, P.; Topalova, Y.; Todorova, Y.; Yotinov, I.; Benova, E. Surface-wave-sustained plasma source for biomedical applications. In *Proc. X International Workshop Microwave Discharges: Fundamentals and Applications*; Lebedev, Y.A., Ed.; Yanus-K: Moscow, Russia, 2018; pp. 55–64.
16. Krcma, F.; Staraa, Z.; Prochazkova, J. Diaphragm discharge in liquids: Fundamentals and applications. *J. Phys. Conf. Ser.* **2010**, *207*, 012010. [[CrossRef](#)]
17. Stara, Z.; Krcma, F.; Prochazkova, J. Physical aspects of diaphragm discharge creation using constant DC high voltage in electrolyte solution. *Acta Tech. CSAV* **2008**, *53*, 277–286.
18. Strasser, R.; Strasser, B. Measuring fast fluorescence transients to address environmental questions: The JIP test. In *Photosynthesis: From Light to Biosphere*; Mathis, P., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp. 977–980.
19. Goltsev, V.; Kalaji, H.; Paunov, M.; Baba, W.; Horaczek, T.; Mojski, J.; Kociel, H.; Allakhverdiev, S. Variable chlorophyll fluorescence and its use for assessing physiological condition of plant photosynthetic apparatus. *Russ. J. Plant Physiol.* **2016**, *63*, 869–893. [[CrossRef](#)]
20. Wetzels, T.; Candresse, T.; Masquiere, M.; Ravelonandro, M.; Dunez, J. A highly sensitive immunocapture polymerase chain reaction for Plum pox virus detection. *J. Virol. Methods* **1992**, *39*, 27–37. [[CrossRef](#)]
21. Wetzels, T.; Candresse, T.; Ravelonandro, M.; Dunez, J. A polymerase chain reaction adapted to Plum pox potyvirus detection. *J. Virol. Methods* **1991**, *33*, 355–366. [[CrossRef](#)]
22. Subr, Z.; Pittnerova, S.; Glasa, M. A simplified RT-PCR-based detection of recombinant Plum pox virus isolates. *Acta Virologica* **2004**, *48*, 173–176.
23. Yusuf, M.; Kumar, D.; Rajwanshi, R.; Strasser, R.; Tsimilli-Michael, M.; Sarin, N. Overexpression of γ -tocopherol methyl transferase gene in transgenic Brassica juncea plants alleviates abiotic stress: Physiological and chlorophyll a fluorescence measurements. *Biochim. Biophys. Acta (BBA)-Bioenerg.* **2010**, *1797*, 1428–1438. [[CrossRef](#)] [[PubMed](#)]
24. Bolhar-Nordenkamp, H.; Oquist, G. Chlorophyll fluorescence as a tool in photosynthesis research. In *Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual*; Chapman & Hall: London, UK, 1993; pp. 193–206.
25. Adhikari, B.; Adhikari, M.; Park, G. The effects of plasma on plant growth, development, and sustainability. *Appl. Sci.* **2020**, *10*, 6045. [[CrossRef](#)]
26. Huijser, P.; Schmid, M. The control of developmental phase transitions in plants. *Development* **2011**, *138*, 4117–4129. [[CrossRef](#)]
27. Guo, Q.; Wang, Y.; Zhang, H.; Qu, G.; Wang, T.; Sun, Q.; Liang, D. Alleviation of Adverse Effects of Drought Stress on Wheat Seed Germination Using Atmospheric Dielectric Barrier Discharge Plasma Treatment. *Sci. Rep.* **2017**, *7*, 16680. [[CrossRef](#)] [[PubMed](#)]
28. Štěpánová, V.; Slavíček, P.; Kelar, J.; Prášil, J.; Smékal, M.; Stupavská, M.; Jurmanová, J.; Cernák, M. Atmospheric Pressure Plasma Treatment of Agricultural Seeds of Cucumber (*Cucumis sativus* L.) and Pepper (*Capsicum annuum* L.) with Effect on Reduction of Diseases and Germination Improvement. *Plasma Process. Polym.* **2018**, *15*, 1700076. [[CrossRef](#)]
29. Zahoranová, A.; Hoppanová, L.; Šimončicová, J.; Tučeková, Z.; Medvecká, V.; Hudecová, D.; Kalináková, B.; Kováčik, D.; Cernák, M. Effect of Cold Atmospheric Pressure Plasma on Maize Seeds: Enhancement of Seedlings Growth and Surface Microorganisms Inactivation. *Plasma Chem. Plasma Process* **2018**, *38*, 969–988. [[CrossRef](#)]
30. Abarghuei, F.M.; Etemadi, M.; Ramezani, A.; Esehaghbeygi, A.; Alizargar, J. An Application of Cold Atmospheric Plasma to Enhance Physiological and Biochemical Traits of Basil. *Plants* **2021**, *10*, 2088. [[CrossRef](#)] [[PubMed](#)]
31. Bozhanova, V.; Marinova, P.; Videva, M.; Nedjalkova, S.; Benova, E. Effect of Cold Plasma on the Germination and Seedling Growth of Durum Wheat Genotypes. *Processes* **2024**, *12*, 544. [[CrossRef](#)]
32. Motrescu, I.; Lungoci, C.; Calistru, A.E.; Luchian, C.E.; Gocan, T.M.; Rimbu, C.M.; Bulgariu, E.; Ciolan, M.A.; Jitareanu, G. Non-Thermal Plasma (NTP) Treatment of Alfalfa Seeds in Different Voltage Conditions Leads to Both Positive and Inhibitory Outcomes Related to Sprout Growth and Nutraceutical Properties. *Plants* **2024**, *13*, 1140. [[CrossRef](#)] [[PubMed](#)]
33. Motrescu, I.; Hara, T.; Ogino, A.; Tanaka, S.; Fujiwara, T.; Kawagishi, H.; Kodani, S.; Popa, G.; Nagatsu, M. Investigation of low temperature plasma capabilities to modify the structure and function of bio-polymers. *J. Autom. Mob. Robot. Intell. Syst.* **2009**, *3*, 150–152.
34. Filipić, A.; Ion Gutierrez-Aguirre, I.; Primc, G.; Mozetič, M.; Dobnik, D. Cold Plasma, a New Hope in the Field of Virus Inactivation. *Trends Biotechnol.* **2020**, *38*, 1278–1291. [[CrossRef](#)]

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