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# Eradication of PPV and PNRSV Viruses from Three Peach Cultivars Using Thermotherapy *In Vitro*, Including Optimization of Microshoots' Multiplication and Rooting Medium

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**Abstract:** Peach cultivars ('Elberta', 'Red Top', and 'Dixie Red') were studied for their *in vitro* adoptability and performance in producing virus-free plantlets. The thermotherapy method with increasing temperatures (25 °C to 37 °C) was applied for the elimination of famous peach infecting plum pox viruses (PPVs) and prunus necrotic ringspot virus (PNRSV), and the DS-ELISA test and RT-PCR technique were performed to confirm the production of virus-free microshoots. The application of 30 mg $L^{-1}$ of Fe-EDDHA treatment resulted in the best performance for culture establishment of all cultivars, where the best subsequent morphological performance in terms of branch and leaf numbers was recorded for the 'Dixie Red' cultivar in MS medium, supplemented with 0.5 mg$L^{-1}$ of gibberellic acid ($GA_3$) and 0.5 mg $L^{-1}$ of 6-Benzylaminopurine (BAP). At the regeneration stage, the highest (26.96 mm) and lowest (18.43 mm) shoot lengths were obtained from the 'Dixie Red' cultivar treated with $GA_3$ (2 mg $L^{-1}$) + thidiazuron (TDZ) (2.5 mg $L^{-1}$) and the 'Red Top' cultivar treated with $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$), respectively. The leaf numbers were affected by the application of growth regulators, where the 'Elberta' cultivar under $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) treatment showed the highest numbers and the 'Red Top' cultivar under $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) showed the lowest mean values. The thermotherapy treatment and micropropagation of shoot tips resulted in 100% virus-free plantlets, as confirmed by both applied diagnostic methods. The result of the application of the rooting stage with growth regulators on 'Elberta' plantlets showed the best performance (90%) in $\frac{1}{2}$ MS medium supplemented with 0.5–1 mg $L^{-1}$ of IBA, which was significantly higher than the same treatment in MS medium. The obtained results should constitute the basis for further optimization of the multiplication and rooting of virus-free peach plantlets to be served for nurseries and planation orchards.

**Keywords:** plum pox virus; prunus necrotic ringspot virus; *Prunus persica*; virus eradication



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## 1. Introduction

Prunus necrotic ringspot virus—a member of the genus Ilarvirus of the family Bromoviridae—and plum pox virus—a member of the genus Potyvirus of the family Potyviridae (ICTV taxonomy available at https://ictv.global/taxonomy, accessed on 3 October 2022)—commonly infect important stone fruit trees such as peach (*Prunus persica* (L.) Batsch) and nectarine (*P. persica* var. *nucipersica*), causing severe loss of productivity due to the consequent poor fruit quality and shortened tree life [1–3]. The severity of the host

reaction and the consequent extent of interaction depend on the virus types and strains (persistent, semi-persistent, and persistent), which may range from largely latent (with no visible symptoms) to substantial damages with enormous economic impacts [1].

The presence of virus-infected fruit trees is remarkably high in orchards worldwide, as confirmed in an Australian study, with more that 70% of the investigated stone fruit trees being positive for one or more types of viruses [4]. The same virus infection incidence rate was found in a Czech Republic germplasm collection orchard in 2020 [5]. In another recent study in Bosnia and Herzegovina, more that 50% of the studied plum trees were positive for PPV [6]. So far, no natural form of PPV resistance has been reported or confidently identified for peach trees [7]. Therefore, the production and cultivation of virus-free plants is indispensable and the most realistic approach to establish sustainable orchards with optimized yield, to import/export novel cultivars from/to other countries, to safely exchange breeding materials between countries or regions, and to preserve plant germplasm [8].

The application of healthy, free-from pathogens, and uniform plants is the core requisite of a productive horticultural system. Among the approaches in plant virus eradication, *in vitro* techniques proved to be relevant and a successful strategy [8]. The historic development of *in vitro* plant cell and tissue culture has undoubtedly been a major factor in the advancement of our knowledge in cell biology and physiology. Micropropagation and plant tissue culture techniques are widely used for virus-free germplasm production from several vegetatively propagated plant species.

Thermotherapy is the term used to refer to the application of a high temperature that generates unfavorable conditions for virus multiplication in the cells and effectively decreases the virus load in the shoot tips [9–12]. Among the established thermotherapy-based methods typically used for virus elimination, combined *in vitro* thermotherapy with shoot tip culture, micrografting, electrotherapy, chemotherapy (especially using ribavirin), and shoot tip cryotherapy has been reported to be efficient for the eradication of different viruses from plants [8,13–16]. The efficiency of thermotherapy-mediated elimination of viruses from plant materials is very diverse and ranges from 20 to 100% [11,15,17–20].

In the current research, we investigated the effect of thermotherapy and optimized the *in vitro* conditions for the eradication of PPV and PNRSV from *in vitro* cultured shoots of three important peach cultivars of Iranian orchards. We believe that the presented methodology can be adopted and utilized in plant research institutes and laboratories to obtain and deliver pathogen-free plant materials for distribution in nurseries.

## 2. Materials and Methods

The study was performed on peach cultivars infected with an important peach virus (PPV and PNRSV) in a factorial experiment with a completely randomized design. Three peach cultivars ('Elberta' (known as 'Alberta' in Iran), Red Tob, and 'Dixie Red') were selected to obtain virus-free plantlets.

### *2.1. In Vitro Culture Establishment and Thermotherapy*

The spring shoot tips (1.5 cm) of infected plants with both PPV and PNRSV were used for *in vitro* culture initiation after their infection status was confirmed by double antibody sandwich-ELISA kit (Bioreba, Basel, Switzerland) and RT-PCR methods. The plant materials were placed *in vitro* on autoclaved MS medium supplemented with $GA_3$ (0.5 mg $L^{-1}$) and BAP (0.5 mg $L^{-1}$) for culture establishment. According to the fact that the peach plants are highly sensitive to iron content and iron type deficiency, treatments included the supplementation of different concentrations of ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA) at three levels (0, 15, and 30 mg $L^{-1}$) for the three cultivars.

After one week of *in vitro* culture initiation, the non-contaminated cultures were subjected to thermotherapy, where the light room temperature was gradually raised from 25 °C to 37 °Cover one week (2 °C/day) and then kept at 37 °C for 4 weeks until newly grown *in vitro* shoots were distinguishable.

### 2.2. Establishment of Regeneration Culture

At the end of the thermotherapy period, the explants (microshoots of 2.5–3 cm) were excised and collected from tolerant (to high temperature) branches. At this stage, MS medium (with 30 g $L^{-1}$ sucrose and 7 g $L^{-1}$ agar) was used with different concentrations of growth regulators' supplementation for micropropagation.

In two different setups, a two-factorial experiment was designed for the determination of the best plant growth regulators' application.

MS medium was supplemented with the following:

Exp 1: $GA_3$ (1 mg $L^{-1}$) + BAP (1.5 mg $L^{-1}$) or $GA_3$ (2 mg $L^{-1}$) + BAP (2 mg $L^{-1}$)

Exp 2: $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$), or $GA_3$ (1 mg $L^{-1}$) + TDZ (1.5 mg $L^{-1}$), or $GA_3$ (2 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$), or $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$).

The cultures were transferred to growth chamber at 22 ± 2 °C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 µmol $m^{-2}$ $s^{-1}$ at culture level, provided by cool-white-fluorescent lamps. At this period, subcultures were conducted in 4-week intervals in the same culture medium.

### 2.3. Selection of Virus-Free In Vitro Shoots

Leaf samples from microshoots were collected for virus detection by the ELISA and RT-PCR test after 4 weeks of thermotherapy. The sanitary status of in vivo collected samples regenerated from therapies and controls was determined using DAS-ELISA, as described by Manganaris et al. [17]. Plant extracts were prepared by homogenizing the leaf tissues in phosphate buffered saline–Tween (1:20 *w*/*v*) containing 2% polyvinylpyrrolidone-40 (PVP-40) and 0.2% egg albumin. The enzyme conjugated-IgG concentration was 1 mg $mL^{-1}$ and the coating immunoglobulin G (IgG) concentration for PNRSV and PPV was 0.5 and 1.0 mg $mL^{-1}$, respectively. Five individual samples from each treatment were tested for maximum absorbance at 405 nm with a plate reader (Sunrise Model, TECAN, Austria) after 2 h of incubation.

RT-PCRs were performed on cDNA samples from total RNAs isolated [21] from the leaf tissues (200 mg) of infected mother plants with both viruses, leaves from *in vitro* cultured plants before the thermotherapy application, the microshoots obtained after the *in vitro* propagation, and the young peach rooted plantlets of 'Elberta' (five months old). The cDNAs were synthesized from 1 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA, K1622) according to the manufacturer's description. The oligonucleotide sequences of applied primers are listed in Table 1. The PCR amplifications were carried out in a Veriti 96-well thermal cycler (Applied Biosystem, Waltham, MA, USA) and the presence or absence of products was visualized by ethidium bromide staining on 1.5% agarose gel in TAE buffer.

**Table 1.** Primers used for partial amplification of viruses' cDNA for RT-PCR.

| Primer Name | Sequence (5′→3′) | Amplicon Size (bp) | Reference |
|---|---|---|---|
| PNRSV-F3 | GCCGAATTTGCAATCATACCC | 599 | Naderpour and Shahbazi [22] |
| PNRSV-R3 | ACTTCGGTCTTGAATTCGAT | | |
| PPV-RR | CTCTTCTTGTGTTCCGACGTTTC | 345 | Varga and James [23] |
| PPV-F3 | GGAATGTGGGTGATGATGG | | |

### 2.4. Establishment of Rooting Culture

The virus-free plantlets were subjected to the optimization of the rooting condition. Only the 'Elberta' cultures under $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) were considered for this stage because of their best and significantly better performance in the case of the studied morphological traits. Three different conditions were set up (Table 2), including an intermediate sub-culturing for 6 days (in MS medium supplemented or not with IBA (4 mg

$L^{-1}$) at dark) followed by transferring the microshoots to the rooting media for 6 weeks. Finally, microshoots were transferred to hardening containers after adequate amounts of root growth and kept in a greenhouse for 6 months for the final examination of their sanitary status.

**Table 2.** Growth regulators' composition of the rooting culture applied for 'Elberta' peach cultivar.

| Treatment | Rooting Condition (a) * | Rooting Condition (b) ** | Rooting Condition (c) *** |
|---|---|---|---|
| 1 | MS + IBA (0.5 mg $L^{-1}$) | MS + IBA (0.5 mg $L^{-1}$) | $\frac{1}{2}$MS + IBA (0.5 mg/l) |
| 2 | MS + IBA (1 mg $L^{-1}$) | MS + IBA (1 mg $L^{-1}$) | $\frac{1}{2}$MS+ IBA (1 mg $L^{-1}$) |
| 3 | MS + IBA (2 mg $L^{-1}$) | MS + IBA (2 mg $L^{-1}$) | $\frac{1}{2}$MS + IBA (0.5 mg $L^{-1}$) + NAA (0.25 mg $L^{-1}$) |
| 4 | MS + IBA (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) | IBA (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) | $\frac{1}{2}$MS + IBA (1 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) |
| 5 | MS + IBA (0.5 mg $L^{-1}$)+ NAA (0.25 mg $L^{-1}$) | IBA (0.5 mg $L^{-1}$) + NAA (0.25 mg $L^{-1}$) | - |

*: transferred to 1–5, after 1 week in MS medium; **: transferred to 1–5, after 1 week in MS medium containing IBA (4 mg $L^{-1}$); ***: transferred to 1–4, after 1 week in MS medium.

### *2.5. Statistics*

Data obtained from measurements were statistically analyzed with the software IBM SPSS (v25) (IBM Corp, Armonk, NY, USA). The results are shown as mean values with standard deviations. The normal distribution of residuals was proved by Shapiro–Wilk's test ($p > 0.05$) and the homogeneity of variances was checked by Levene's test ($p > \alpha$) ($\alpha = 0.05$). The differences among the mean values were evaluated by one-way ANOVA and considered significant at $p < 0.05$.

## 3. Results

### *3.1. In Vitro Culture Establishment*

According to analysis of variances, the Fe-EDDHA treatment had significant effects ($p < 0.01$) on plant length, branch number, and leaf number (Table S1). An Fe-EDDHA -concentration-dependent and significant improvement was observed for all studied parameters in this stage. The results showed the highest mean of height (34 ± 2.1 mm) for 'Dixie Red' in response to 30 mg $L^{-1}$ Fe-EDDHA treatment, whereas the lowest mean with 15.97 ± 2.3 mm for 'Elberta' without Fe-EDDHA supplementation (Table 3). The Fe-EDDHA treatment led to an increase in branch and leaf numbers among all cultivars, but 'Dixie Red' showed the highest response to Fe-EDDHA application, where the highest mean of branch numbers (3.66) and leaf numbers (24.5) was obtained after 30 mg $L^{-1}$ Fe-EDDHA application (Table 4).

**Table 3.** Effect of MS medium supplementation with Fe-EDDHA on shoot multiplication of different peach cultivars.

| Cultivars | Fe * Treatments mg $L^{-1}$ | Shoot Length (mm) | Branch Number | Leaf Number |
|---|---|---|---|---|
| 'Elberta' | 0 | 15.97 ± 2.3 f | 1.00 ± 0.02 d | 1.33 ± 0.05 h |
| | 15 | 21.42 ± 3.1 d | 1.16 ± 0.03 d | 9.50 ± 0.26 e |
| | 30 | 24.00 ± 2.4 c | 2.33 ± 0.01 bc | 20.67 ± 0.37 b |
| 'Dixie Red' | 0 | 18.97 ± 1.9 e | 1.00 ± 0.01 d | 2.04 ± 0.03 h |
| | 15 | 26.87 ± 1.9 b | 3.21 ± 0.02 ab | 17.33 ± 0.9 c |
| | 30 | 34.00 ± 2.1 a | 3.66 ± 0.02 a | 24.5 ± 1.2 a |
| 'Red Top' | 0 | 18.97 ± 1.3 e | 1.16 ± 0.03 d | 2.04 ± 0.04 h |
| | 15 | 21.68 ± 1.5 d | 1.70 ± 0.014 cd | 11.49 ± 2.3 d |
| | 30 | 22.32 ± 1.9 cd | 1.44 ± 0.012 cd | 12.44 ± 2.5 d |

*: ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA). Values in columns with different letters are significantly different ($p < 0.05$; $n = 30$).

**Table 4.** Effect of $GA_3$ and BAP in MS medium on shoot multiplication of different peach cultivars.

| Cultivar | Growth Regulator | Shoot Length (mm) | Branch Number | Leaf Number |
|---|---|---|---|---|
| 'Elberta' | $GA_3$ (1 mg $L^{-1}$) + BAP (1.5 mg $L^{-1}$) | 23.17 ± 3.9 e | 1.33 ± 0.04 b | 12.33 ± 1.8 bc |
| | $GA_3$ (2 mg $L^{-1}$) + BAP (2 mg $L^{-1}$) | 29.53 ± 4.1 a | 2.66 ± 0.4 a | 13.67 ± 2.05 b |
| 'Dixie Red' | $GA_3$ (1 mg $L^{-1}$) + BAP (1.5 mg $L^{-1}$) | 28.89 ± 3.0 bc | 2.59 ± 0.8 a | 13.89 ± 3.0 b |
| | $GA_3$ (2 mg $L^{-1}$) + BAP (2 mg $L^{-1}$) | 28.37 ± 2.8 c | 1.733 ± 0.1 b | 9.36 ± 1.15 c |
| 'Red Top' | $GA_3$ (1 mg $L^{-1}$) + BAP (1.5 mg $L^{-1}$) | 26.55 ± 1.95 d | 1.66 ± 0.2 b | 15.67 ± 2.75 b |
| | $GA_3$ (2 mg $L^{-1}$) + BAP (2 mg $L^{-1}$) | 27.11 ± 2.3 d | 3.00 ± 0.6 a | 38.33 ± 5.4 a |

Values in columns with different letters are significantly different ($p < 0.05$; $n = 30$).

The observed improvement in growth performance of the *in vitro* cultured plants could potentially contribute to the high temperature tolerance level during the thermotherapy. Therefore, only the cultures supplemented with the 30 mg $L^{-1}$ Fe-EDDHA were subjected for thermotherapy treatment and further sub culturing.

### *3.2. Optimization of Shoot Multiplication*

After the thermotherapy, the microshoots of tolerant and healthy plantlets (from the cultures supplemented with the 30 mg $L^{-1}$ Fe-EDDHA) were placed in MS medium supplemented with two different concentrations of $GA_3$ + BAP or with four different concentrations of $GA_3$ and TDZ for *in vitro* growth. Growth regulators' treatments, cultivars, and their interaction had significant effects ($p < 0.01$) on shoot length, branch number, and leaf number in both growth regulator combination experimental setups (Tables S2 and S3).

The 2 mg $L^{-1}$ $GA_3$ + BAP supplementation significantly increased the shoot length of 'Elberta' plants only (from 23.17 ± 3.9 mm to 29.53 ± 4.1 mm) in comparison with 1 mg $L^{-1}$ of the same growth regulator treatment (Table 4). The shoot lengths of 'Dixie Red' and 'Red Top' were not significantly altered under $GA_3$ (2 mg $L^{-1}$) + BAP (2 mg $L^{-1}$) treatment when compared with lower concentrations of $GA_3$ (1 mg $L^{-1}$) + BAP (1.5 mg $L^{-1}$). The branch numbers of 'Elberta' and 'Red Top' cultivars were significantly enhanced ($p < 0.05$) when increasing the concentration of $GA_3$ and BAP. The 2 mg $L^{-1}$ of $GA_3$ and BAP supplementation had a significant and negative impact on the branch number of 'Dixie Red' plants (Table 4).

Consequently, the number of leaves also followed the same trend as the branch number. The 'Red Top' microshoots showed a significantly higher number of leaves at higher concentrations of $GA_3$ and BAP (2 mg $L^{-1}$) than the treatment with lower concentrations.

The $GA_3$ and TDZ supplementation had also significant effects on shoot length, branch number, and leaf number of studied cultivars. Considering the effect of treatments on cultivars, the highest (26.96 ± 1.9 mm) and lowest (18.43 ± 1.3 mm) shoot length means were obtained among the 'Dixie Red' cultivar under $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) treatment and 'Red Top' cultivar under $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) treatment, respectively (Table 5). Among the treatments, the highest branch number (2.00 ± 0.1) belonged to the 'Elberta' cultivar under $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$). The highest number of leaves (15. 33 ± 2.8) was recorded among the 'Elberta' plants treated with $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) and the lowest values (5.66 ± 0.65) were counted for 'Red Top' plants under $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$).

### *3.3. Selection of Virus-Free Plantlets*

Samples of leaf tissues were subjected to the DAS-ELISA test and RT-PCR analysis prior to root induction culture. All plantlets of the three cultivars were 100% negative for PPV and PNRSV. A representative result of ELISA test (absorbance at 405 nm) is presented in Table 6. The same negative results of *in vitro* grown plantlets were confirmed by PCR where no amplification and PCR products of PPV and PNRSV were observed after agarose gel electrophoresis.

**Table 5.** Effect of $GA_3$ and TDZ in MS medium on shoot multiplication of different peach cultivars.

| Cultivar | Growth Regulator | Shoot Length (mm) | Branch Number | Leaf Number |
|---|---|---|---|---|
| 'Elberta' | $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) | 22.79 ± 2.8 c | 1.55 ± 0.15 ab | 11.58 ± 2.3 b |
| | $GA_3$ (1 mg $L^{-1}$) + TDZ (1.5 mg $L^{-1}$) | 23.70 ± 3.1 bc | 1.90 ± 0.23 a | 11.63 ± 1.5 b |
| | $GA_3$ (2 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) | 19.08 ± 2.3 d | 1.51 ± 0.25 ab | 9.00 ± 1.25 d |
| | $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) | 26.55 ± 3.1 a | 2.00 ± 0.1 a | 15.33 ± 2.8 a |
| 'Dixie Red' | $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) | 22.53 ± 1.1 c | 1.41 ± 0.2 ab | 7.26 ± 1.07 e |
| | $GA_3$ (1 mg $L^{-1}$) + TDZ (1.5 mg $L^{-1}$) | 20.00 ± 1.8 d | 1.00 ± 0.1 b | 6.00 ± 1.04 f |
| | $GA_3$ (2 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) | 23.52 ± 2.2 bc | 1.80 ± 0.15 ab | 10.76 ± 1.9 c |
| | $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) | 26.96 ± 1.9 a | 1.74 ± 0.2 ab | 10.78 ± 2.1 c |
| 'Red Top' | $GA_3$ (1 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) | 18.43 ± 1.3 d | 1.00 ± 0.3 b | 5.66 ± 0.65 f |
| | $GA_3$ (1 mg $L^{-1}$) + TDZ (1.5 mg $L^{-1}$) | 18.87 ± 2.1 e | 1.33 ± 0.4 ab | 8.66 ± 1.32 d |
| | $GA_3$ (2 mg $L^{-1}$) + TDZ (1 mg $L^{-1}$) | 24.67 ± 2.2 b | 1.00 ± 0.06 b | 8.66 ± 1.37 d |
| | $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) | 18.44 ± 2.3 d | 1.66 ± 0.15 ab | 6.133 ± 1.41 f |

Values in columns with different letters are significantly different ($p < 0.05$; $n = 30$).

**Table 6.** Result of thermotherapy analysis in the DAS-ELISA test.

| ELISA | 'Elberta' | 'Dixie Red' | 'Red Top' |
|---|---|---|---|
| PPV | 0.134 ± 0.043 | 0.139 ± 0.010 | 0.143 ± 0.025 |
| PPV Positive control * | 3.849 ± 0.046 | 3.849 ± 0.046 | 3.849 ± 0.046 |
| PNRSV | 0.182 ± 0.092 | 0.174 ± 0.039 | 0.112 ± 0.084 |
| PNRSV Positive control * | 3.809 ± 0.042 | 3.809 ± 0.042 | 3.809 ± 0.042 |
| Negative Control ** | 0.150 ± 0.044 | 0.150 ± 0.044 | 0.150 ± 0.044 |
| Results | Negative | Negative | Negative |

*: Samples from infected mother trees. **: Samples from virus-free certified plant extract. $n = 30$ (*in vitro* samples) and $n = 6$ (controls).

### *3.4. Effects of Different Growth Regulators on Rooting*

The 'Elberta' plants grown under $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) were tested for their rooting performance. According to the results presented in Tables 7 and 8, the 6-day dark incubation condition remarkably influenced the rooting performance of the plantlets in rooting medium. The dark incubation in 4 mg $L^{-1}$ IBA resulted in a longer root length (2.5 ± 0.6 cm) when compared with the incubation in MS medium only (1 ± 0.4 cm). It was evident that the lowest concentration of IBA resulted in better rooting performance (40%) and even better (70%) when the dark incubation of the plantlets took place in MS medium without any growth regulator (Table 7). The half concentration of MS medium in general was more suitable for the root induction (Table 9). The highest rooting induction rate (90%) was obtained in $\frac{1}{2}$ MS medium supplemented with 0.5 mg $L^{-1}$ of IBA and 0.5 mg $L^{-1}$ of IBA + 0.25 mg $L^{-1}$ NAA (Table 9).

**Table 7.** Effect of IBA and NAA in MS medium on rooting of 'Elberta' microshoots that were incubated for 6 days on medium free of growth regulators.

| Rooting Medium | No. of Samples | No. of Rooted Microshoots | Root Length cm | Rooting % |
|---|---|---|---|---|
| MS + IBA (0.5 mg $L^{-1}$) | 30 | 21 | 1 ± 0.4 | 70 |
| MS + IBA (1 mg $L^{-1}$) | 30 | 0 | 0 | 0 |
| MS + IBA (2 mg $L^{-1}$) | 30 | 0 | 0 | 0 |
| MS + IBA (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) | 30 | 0 | 0 | 0 |
| MS + IBA (0.5 mg $L^{-1}$) + NAA (0.25 mg $L^{-1}$) | 30 | 0 | 0 | 0 |

**Table 8.** Effect of IBA and NAA in MS medium on rooting of 'Elberta' microshoots that were incubated for 6 days on medium containing 4 mg $L^{-1}$ IBA.

| Rooting Medium | No. of Samples | No. of Rooted Microshoots | Root Length cm | Rooting % |
|---|---|---|---|---|
| MS + IBA (0.5 mg $L^{-1}$) | 30 | 12 | 2.5 ± 0.6 | 40 |
| MS + IBA (1 mg $L^{-1}$) | 30 | 0 | 0 | 0 |
| MS + IBA (2 mg $L^{-1}$) | 30 | 0 | 0 | 0 |
| IBA (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) | 30 | 0 | 0 | 0 |
| IBA (0.5 mg $L^{-1}$) + NAA (0.25 mg $L^{-1}$) | 30 | 0 | 0 | 0 |

**Table 9.** Effect of IBA and NAA in $\frac{1}{2}$ MS medium on rooting of 'Elberta' microshoots that were incubated for 6 days on MS medium free from growth regulators.

| Rooting Medium | No. of Samples | No. of Rooted Microshoots | Root Length cm | Rooting % |
|---|---|---|---|---|
| $\frac{1}{2}$MS + IBA (0.5 mg $L^{-1}$) | 30 | 27 | 2 ± 0.4 | 90 |
| $\frac{1}{2}$MS + IBA (1 mg $L^{-1}$) | 30 | 25 | 1.5 ± 0.4 | 83 |
| $\frac{1}{2}$MS + IBA (0.5 mg $L^{-1}$) + NAA (0.25 mg $L^{-1}$) | 30 | 27 | 2 ± 0.5 | 90 |
| $\frac{1}{2}$MS + IBA (1 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) | 30 | 22 | 1 ± 0.3 | 73 |

## 4. Discussion

The elimination of viruses from plants is the most relevant priority to obtain healthy propagating materials for the establishment of pathogen-free orchards and to ensure the conservation of important germplasms [24]. Thermotherapy-based virus eradication has been successfully applied for almost all of the economically important fruit trees that are vegetatively propagated [8]. Several factors including temperature, duration of thermotherapy regime, and size of explants may affect the efficiency of virus eradication [14]. Although the rate of virus elimination from plant tissues was considerably high and reliable with this method, the upstream *in vitro* and in vivo processes to establish the rooted and stable plantlets were challenging and variable [16,25]. Even though we could obtain 100% virus-free microshoots after the applied thermotherapy methods, a variety of different responses to the same *in vitro* condition were observed among the selected cultivars of peach plants, which may be the indirect consequence of prolonged exposure to elevated temperature (thermotherapy) or the genetic makeups of the selected breeding lines. The numbers of leaves in response to the same growth regulator treatments were different between cultivars, for example, leaf numbers of 'Dixie Red' cultivar reduced with the increase in $GA_3$ and BAP dosage, but the leaf numbers of 'Red Top' cultivar increased at higher concentrations of the same growth regulators. A similar cultivar-specific response has been also reported by Gentile et al. [26] for *in vitro* shoot induction from *P. persica* leaves, where their best results were obtained from leaf tissues when BAP was used as cytokinin in the medium. Furthermore, Zong et al. [27], in their optimized *in vitro* peach leaf regeneration protocol for the "Hansen 536" cultivar, found the maximum shoot regeneration rate for explants cultured on WPM medium supplemented with BAP in IBA. The comparison of the applied growth regulators (concentration and composition) in peach *in vitro* organogenesis and regeneration points to the cultivar-specific nature of morphological development [28,29]. The application of TDZ increased the organogenesis and was found to be more effective in micropropagation, multiple shoot induction, somatic embryogenesis, callus induction, and shoot organogenesis as compared with other cytokinins [30]. In some systems, the synergistic effect of TDZ with other cytokinin/auxin was found to be more effective than using it alone. The optimum concentration of TDZ and other plant growth regulators varies based on the plant species, explants, and duration of exposure [30,31]. The obtained result of the current study also indicates the importance of the inclusion of a wide range of plant growth regulators and their concentrations to optimize the *in vitro* culture of every single

cultivar of interest. In the *in vitro* process to produce the healthy plantlets, the rooting of microshoots is usually one of the major obstacles for successful micropropagation [32]. In our trials, the sensitivity of 'Elberta' microshoots to IBA and NAA was evident. Only 0.5 mg $L^{-1}$ IBA in MS medium resulted in root formation. It was also evident that a decrease in MS salt concentration to $\frac{1}{2}$ was a key factor in rooting of 'Elberta' microshoots. A survey of the literature related to stone fruits micropropagation reveals the fact that almost every plant species and cultivar respond differently to treatment with growth regulators for organogenesis. This indeed justifies the need for more studies and experimental trials.

## 5. Conclusions

Trials were undertaken in order to develop a method to obtain peach plants free from PPV and PNRSV viruses using *in vitro* shoot cultures. The applied method of thermotherapy was effective for the elimination of both viruses from three cultivars. Experiments on finding the optimal medium for the propagation and rooting of microshoots have shown a large difference in the reaction of cultivars to the type and concentration of growth regulators. For all cultivars, the addition of 30 mg $L^{-1}$ Fe-EDDHA increased the efficiency of shoot regeneration. Thermotherapy at 25–37 °C resulted in 100% PPV- and PNRSV-free microshoots of three selected peach cultivars. The *in vitro* application of plant growth regulators revealed the variability in cultivars' response to any of the applied growth regulators. The best overall morphological performance was recorded for the 'Dixie Red' cultivar in MS medium supplemented with 0.5 mg $L^{-1}$ of $GA_3$ and 0.5 mg $L^{-1}$ BAP, and for the 'Elberta' cultivar under $GA_3$ (2 mg $L^{-1}$) + TDZ (2.5 mg $L^{-1}$) treatment. The optimal rooting medium was tested for the 'Elberta' cultivar with the best performance (90%) in $\frac{1}{2}$ MS medium supplemented with 0.5–1 mg $L^{-1}$ of IBA. Here, we presented an experimental *in vitro* protocol to establish virus-free peach plantlets that can be the foundation of further optimizations and adaptations for practical use in developing virus-free nurseries.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae8100929/s1, Table S1: Mean of square for morphological traits in response to Fe and cultivars interaction; Table S2: Mean of square for traits in response to $GA_3$ + BAP, and cultivars interaction; Table S3: Mean of square for traits in response to $GA_3$ + TDZ, and cultivars interaction.

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