

Original Article

EFFECT OF CHITOSAN AND PUTRESCINE-ANCHORED NANOCARBON ON THE *in vitro* PROPAGATION OF THE APPLE ROOTSTOCK MM106

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ABSTRACT

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Apple is one of the most important fruit crops cultivated in temperate regions worldwide. This study aimed to develop an efficient protocol for shoot multiplication and root induction during the *in vitro* propagation of the apple rootstock MM106. The results showed that the MM106 rootstock responded variably to chitosan (CS), polyvinylpyrrolidone (PVP), and thidiazuron (TDZ) concentrations during micropropagation. The highest shoot multiplication rate and shoot quality were obtained on MS medium supplemented with 0.1 mg L⁻¹ TDZ, 10 mg L⁻¹ CS, and 0.3 g L⁻¹ PVP during the long phase of shoot multiplication on the MS-medium. The addition of kinetin to the TDZ-CS-PVP combinations reduced the shoot multiplication rate. 1 mg/L IBA with 0.3 g/L PVP was the most effective concentration in the rooting phase, and 83.3% of shoots were rooted by adding carbon nanotubes (CNTs), putrescine (Put), or phloroglucinol (PG) separately at varying concentrations (with IBA + PVP), significantly increasing rooting percentage, root number per shoot, root length, rooting speed, and lateral roots.

Keywords: Apple, Micropropagation, Chitosan, Carbone nanotube, Putrescine, Phloroglucinol.

1. INTRODUCTION

Apple (*Malus Domestica* Borkh.) is one of the vital fruit crops in temperate regions worldwide. It is extensively farmed in Northern America, Asia and Europe (Kellerhals, 2009). The apple belongs to the *Malus* genus, and *Rosaceae* family, which includes 25–30 species. Although there are over 10 thousand known apple cultivars, commercial apple farming is limited to a small number of cultivars (Zhang *et al.*, 2025).

Apples are propagated sexually by seeds. Asexual methods are often used to propagate apple rootstocks (hardwood and semi-hardwood cutting, Layering, also, budding and grafting). These methods are not more active and protracted, sometimes linked to challenges such as stock plant constraints and prolonged production times (Castillo *et al.*, 2015). Also, fail to ensure disease-free plants, are seasonal, and yield low multiplication rates (Dobránszki & Teixeira da Silva., 2010).

Micropropagation rapidly multiplies plants and produces healthy stock, offering high commercial value (Vettori *et al.*, 2010). The choice of PGRs used in the medium is the most critical component that regulates apple shoots' *in vitro* formation and expansion (Dobránszki & Teixeira da Silva, 2010). A cytokinin-rich medium is employed to inoculate nodal explants for multiple shoot growth (Bhatia & Sharma, 2015). Thidiazuron (TDZ) is particularly effective in forming adventitious shoots in various woody plants (Sarwar & Skirvin, 1997). Other cytokinins, including Kinetin, have also been studied and shown to be less active in most cases (Lizárraga *et al.*, 2017).

Chitosan (Cs) is a natural polymer from crustacean exoskeletons, insect cuticles, fungal cell walls, and algal cells (Sharif *et al.*, 2018; Adiletta *et al.*, 2021) that can be obtained from various species, particularly from the exoskeletons of crustaceans. It is also found in insects'

cuticles, fungi cell walls, and some algae cell walls (Uthairatanakij *et al.*, 2007). Chitosan has also been used to culture crops and other economically important species *in vitro*, with encouraging results. Chitosan is a widely utilized growth enhancer in the agricultural industry, and it has been documented to be employed in plant species such as *Oryza sativa* (Malaysiana *et al.*, 2021). In cell suspensions of several species, chitosan was found to increase secondary metabolite production. When used in micropropagation, chitosan improved plantlet quality *in vitro* (Asgari-Targhi *et al.*, 2018; Nge *et al.*, 2006).

Therefore, *Malus* plants contain high levels of polyphenols (Silva *et al.*, 2019). It is a well-known problem linked with the micropropagation culture establishment stage, where phenolic compounds oxidize and drain out of the explant, causing browning and, finally death the tissue (Dobránszki & Teixeira da Silva, 2010). Polyvinylpyrrolidone (PVP), a polymer soluble in water, is produced from the N-vinylpyrrolidone monomer (Haaf *et al.*, 1985). Hence, adding to the culture media can help to reduce tissue browning in apple cultivars (Teixeira da Silva *et al.*, 2019). *in vitro* culture studies of apple, as reported in the literature, indicate that the most commonly used auxin for rooting is IBA at concentrations ranging from 0.3 to 3.0 mg/L. Also, IBA is a frequently used auxin for rooting in various genotypes of apple (Teixeira da Silva *et al.*, 2019). Further, exogenous polyamines Putrescine (Put) has similar influences on plants as auxins, which are directly engaged in not only the cell division but also cell elongation; and could be utilized as a beneficial substitute for auxin treatment in some circumstances. On cuts and hypocotyls of *Phaseolus* as well as *Vigna* treated with IBA, endogenous polyamine synthesis increased before the development of root primordial and during root growth (George *et al.*, 2008).

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Phloroglucinol (PG), a phenol derivative, can successfully improve the rooting of some plants in *in vitro* culture, including apples, which are not easy to root (Teixeira da Silva *et al.*, 2019). To improve the rooting process, systematic investigations were undertaken on the influence of PG on *in vitro* rooting. In some cultivars, PG enhanced the rooting percentage (Sharma *et al.*, 2000).

Mustafa *et al.*, (2013) found carbon nanotubes (CNTs) are being investigated *in vitro* regarding callus formation, embryogenesis, both embryo germination, and elongation, in addition to the rooting phase of the date palm. CNTs increased shoot length, leaf number, and root parameters.

This study aims to develop a reliable *in vitro* protocol for shoot multiplication and rooting in MM106 apple, optimizing medium compositions.

2. METHODS AND MATERIALS

in vitro cultures of apple rootstock Mallington Merton 106 (MM106) were established using shoots obtained from meristem-derived plantlets maintained in the Plant Tissue Culture and Plant Organogenesis Laboratory, Faculty of Agriculture, University of Tabriz. The explants had been maintained under *in vitro* conditions for approximately 8–12 months before the initiation of the experiments. Shoots were routinely subcultured every 4–6 weeks on a modified Murashige and Skoog (MS) medium supplemented with 0.5 mg L⁻¹ indole-3-butyric acid (IBA), 1.0 mg L⁻¹ gibberellic acid (GAs), and 1.0 mg L⁻¹ 6-benzylaminopurine (BAP). After four weeks of culture, healthy and uniform single shoots were selected for the multiplication and rooting experiments.

For the shoot multiplication experiment, single shoots were cultured on MS medium supplemented with three concentrations of chitosan (CS; 10, 20, and 40 mg L⁻¹), three concentrations of thidiazuron (TDZ; 0.1, 1.0, and 2.0 mg L⁻¹), and polyvinylpyrrolidone (PVP; 0.3, 0.6, and 0.9 g L⁻¹). To evaluate the effect of cytokinin interaction on shoot proliferation, a second set of treatments was prepared using the same concentrations of CS, TDZ, and PVP with the addition of kinetin (Kin) at 0.5, 1.0, and 2.0 mg L⁻¹. Shoot multiplication rate, shoot length, and leaf number were recorded after 40 days of culture.

For the rooting experiment, well-developed shoots obtained from the multiplication stage were transferred to MS rooting medium and cultured for four weeks. All rooting media contained IBA at three concentrations (0.3, 1.0, and 2.0 mg L⁻¹) together with 0.3 g L⁻¹ PVP. Four independent treatment groups were evaluated. The first group served as the control and consisted only of IBA and PVP. The second group was supplemented with a nanocomposite formulation composed of carbon nanotubes (CNTs), putrescine (Put), and phloroglucinol (PG), which was applied at concentrations of 20, 40, and 80 mg L⁻¹ in combination with the corresponding IBA concentrations. To distinguish the effect of the nanocomposite from those of its individual components, two additional treatment groups were included. In the third group, putrescine alone (without CNTs or phloroglucinol) was applied at concentrations of 50, 100, and 150 mg L⁻¹, whereas in the fourth group, phloroglucinol alone (without CNTs or putrescine) was applied at concentrations of 40, 80, and 160 mg L⁻¹. In all rooting treatments, the corresponding concentration of IBA and 0.3 g L⁻¹

PVP was maintained in the MS medium. Rooting percentage, rooting time, root number per shoot, root length, and lateral root formation were evaluated after 40 days of culture.

Trait Measurement and Statistical Analysis:

Forty days after the cultured 1.5 cm shoots in the multiplication stage, number of shoots, shoot length, number of leaves, and multiplication rate were recorded, and single multiplicities shoot cultured in Free MS-media for four weeks. Forty days after being transferred to the rooting medium, the percentage of our treatment was recorded. The data were statistically evaluated using analysis of variance (ANOVA) as a factorial experiment in a completely randomized design (CRD) with four replicates per treatment. SPSS was used to conduct all statistical analyses. Duncan's multiple range test was used to compare means of main effects and interactions at $p \leq 0.05$.

3. RESULTS

Multiplication

This study confirms *in vitro* propagation of apple rootstock MM106. Treatments produced statistically significant differences in multiplication parameters (Table 1).

TDZ hormone, which is effective in developing multiplication shoots in various woody plants, significantly affected shoot multiplication in the current study. Cs 10 mg/L, PVP 0.3 g/L, and TDZ 0.1 mg/L were the most effective concentrations in the MS medium for MM106 apple rootstock and produced largest number of shoots (26.92). The treatment with the smallest number of shoots was observed in the highest concentrations of CS, PVP, and TDZ, respectively (40, 900, and 2 mg/L) (11.25). (13.00) Shots of the average concentration of Cs 20 mg/L, PVP 0.6 g/L, and TDZ 1 mg/L were observed (Table 1).

Kinetin (Kin) has also been studied and shown to be less active in most cases for multiplication in various woody plants (Lizárraga *et al.*, 2017). In this study, the result showed the lowest concentrations of Cs-PVP-TDZ with Kin, showing the lowest number of shoots (6.25). The highest number of shoots was gained at the highest concentration of Cs-PVP-TDZ with Kin (11.08). (9.33) shoots were observed at a moderate concentration of Cs-PVP-TDZ and Kin treatment.

Comparing all treatments in our experiments, we observed that when Cs and PVP were combined with TDZ, the lowest concentration produced the best effect on multiplication rate, number of leaves, and the shortest shoots of apple rootstock MM106. In contrast, at higher concentrations of Cs, PVP, and TDZ, the lowest shoot number, longest shoots, and reduced leaf number were obtained (Table 1). High levels of Cs and PVP, as well as TDZ with high concentrations of Kin, gave opposite results compared with TDZ concentration. In the lowest concentration of Kin with Cs, PVP, and TDZ, the lowest number of branches, short shoots, and the low number of leaves were gotten. And the highest concentration of Cs, PVP, TDZ, and Kin gave the highest number of branches, longest shots, and the high number of leaves. Low TDZ levels optimize multiplication for MM106, while high TDZ + Kin favours elongation over proliferation. Chitosan polymer influenced shoot elongation; at low concentrations, the shortest shoots were obtained, whereas at higher concentrations, the longest shoots were produced.

Table 1: Effects of media on shoot multiplication and quality in micropropagated apple rootstock 'MM106' after 40 days.

Cs mg/L	PVP g/l	TDZ mg/L	Kin mg/L	Multiplication rate	Shoot length (mm)	Number of Leaves
10	0.3	0.1	-	26.92 a	11.63 c	135.25 a
20	0.6	1	-	13.00 b	13.00 bc	78.17 b
40	0.9	2	-	11.25 bc	14.83 b	52.91 cd
10	0.3	0.1	0.5	6.25 d	12.67 bc	27.00 e
20	0.6	1	1	9.33 c	14.56 b	48.30 d
40	0.9	2	2	11.08 bc	17.83 a	58.91 c
p-value				.526	0.434	0.675

- Table note: The first three treatments consisted of chitosan (CS), thidiazuron (TDZ), and polyvinylpyrrolidone (PVP), whereas the second three treatments contained the same components supplemented with kinetin (Kin). Different letters indicate significant differences among treatments according to Duncan's multiple range test ($P \leq 0.05$).

- Means with the same letter per column are not significantly different (Duncan's MRT, $p \leq 0.05$; ANOVA).

- Mean does not exist

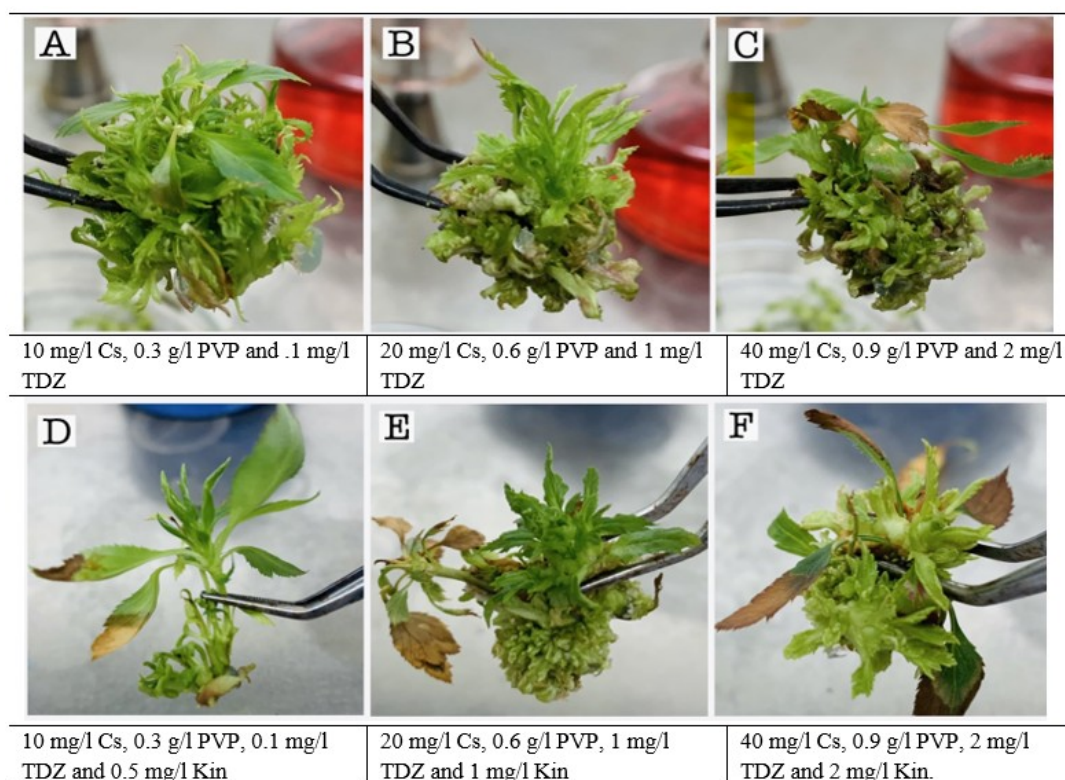


Figure 1: Effect of treatments on shoot multiplication of MM106 after 40 days.

Table 2: Effects of CNTs, putrescine (Put), phloroglucinol (PG), and other additives with IBA and PVP on rooting of MM106 apple rootstock shoots after 40 days.

CNTs-Put-Flu (mg/L)	IBA (mg/L)	PVP (g/L)	Speed of rooting (days)	Rooted shoots (%)	Root number per shoot	Length of root (cm)
20	0.3	0.3	15.5 ab	41.7 b-d	6.42 bc	0.68 ef
40	1	0.3	14.5 a	100 a	5.08 bc	1.04 d-f
80	2	0.3	18.5 cd	25.0 cd	4.00 bc	0.30 f
<i>Put mg/L</i>						
50	0.3	0.3	20.5 e	83.3 ab	11.42 a	0.61 b-d
100	1	0.3	17 bc	100 a	12.25 a	3.60 a
150	2	0.3	24 f	41.7 b-d	5.33 bc	0.31 f
<i>PG mg/L</i>						
40	0.3	0.3	24 f	58.3 a-c	4.75 bc	2.08 b
80	1	0.3	19.5 de	91.7 a	7.25 b	0.90 d-f
160	2	0.3	24 f	25.0 cd	4.00 bc	0.65 ef
-	0.3	0.3	23 f	66.7 a-c	3.35 bc	1.85 bc
-	1	0.3	21 e	83.3 ab	3.50 bc	1.27 c-e
-	2	0.3	27 g	8.3 d	3.00 c	0.93 d-f
<i>p-value</i>			0.325	0.076	0.273	0.250

- Table note: The first treatment group consisted of a CNTs–Put–PG nanocomposite applied at 20, 40, and 80 mg L⁻¹ in combination with the corresponding IBA concentrations (0.3, 1.0, and 2.0 mg L⁻¹) and 0.3 g L⁻¹ PVP. The second treatment group contained putrescine alone at 50, 100, and 150 mg L⁻¹, whereas the third treatment group contained phloroglucinol (PG) alone at 40, 80, and 160 mg L⁻¹. The final treatment group served as the control and consisted of IBA (0.3, 1.0, and 2.0 mg L⁻¹) and 0.3 g L⁻¹ PVP without the addition of the nanocomposite, putrescine, or phloroglucinol. Different letters indicate significant differences among treatments according to Duncan's multiple range test ($P \leq 0.05$).

- Mean does not exist

Rooting of Micro Shoots:

IBA, a potent rooting auxin, was used in several different concentrations in this study. The most rooting shoots in the treatment had 1 mg/L IBA, and 0.3 mg/L PVP; the rooting percentage was 83%. When the IBA concentration increased to 2 mg/L combined with 0.3 g/l PVP, the rooting percentage decreased to 8%. 0.3 mg/L IBA with

0.3 g/l PVP was the active concentration and significantly affected the rooting of 1.5 cm branches that were planted for 40 days and 67% of the shoots rooted.

PG, combined with auxins, enhances rooting in some apple cultivars. Also, there was an increased rooting percentage in MM106 apple rootstock in this study. When 80 mg/L of PG with 1 mg/L IBA and 0.3 g/l PVP, it was able to increase the rooting percentage to 8.3%, as well

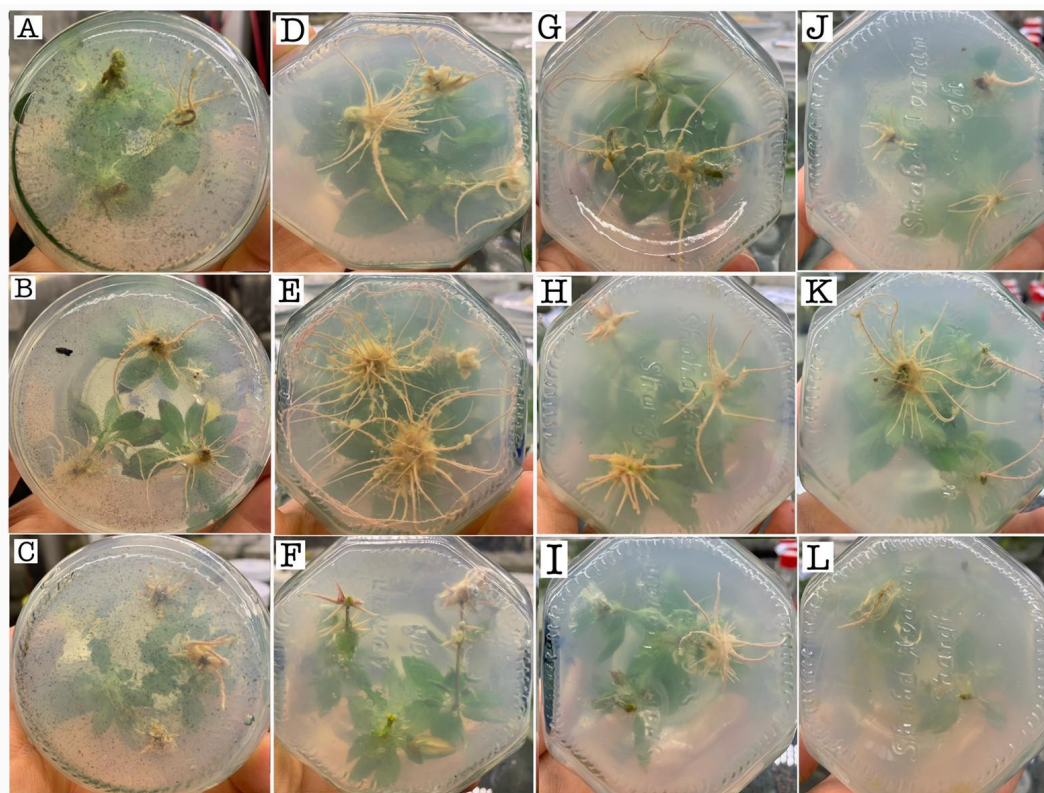


Figure 2: Effect of treatments on shoot rooting of MM106 after 40 days; A-B-C) treated by CNTs-Put-Flu + IBA, respectively (20 + 0.3, 40 + 1 and 80 + 2 mg/L) and PVP 0.3 g/l for our treatments. D-E-F) treated by Put + IBA respectively (50 + 0.3, 100 + 1 and 150 + 2 mg/L) and PVP 0.3 g/l for our treatments. G-H-I) treated by PG + IBA respectively (40 + 0.3, 80 + 1 and 160 + 2 mg/L) and PVP 0.3 g/l for our treatments. J-K-L) treated by IBA respectively (0.3, 1 and 2 mg/L) and PVP 0.3 g/l for all treatments.

4. DISCUSSION

In the present study, the highest shoot multiplication rate was obtained on MS medium supplemented with 0.1 mg L^{-1} TDZ, whereas increasing the TDZ concentration or combining TDZ with kinetin (Kin) reduced shoot multiplication. This finding suggests that low concentrations of TDZ are sufficient to stimulate axillary bud proliferation, while higher concentrations may disturb the endogenous hormonal balance required for normal shoot development. High TDZ concentrations have also been associated with reduced shoot elongation and physiological disorders, which may explain the lower multiplication rate observed at higher TDZ levels (Tashmatova *et al.*, 2021). Similarly, Van Nieuwkerk *et al.* (1986) reported that the lowest TDZ concentration produced the highest number of shoots in the apple cultivar 'Gala', whereas increasing TDZ concentration reduced shoot proliferation. In agreement with these findings, the present study showed that 0.1 mg L^{-1} TDZ produced the highest shoot multiplication rate. In the second experiment, the highest shoot multiplication among the TDZ-Kin treatments was obtained with 2 mg L^{-1} Kin combined with 2 mg L^{-1} TDZ, although the multiplication rate remained lower than that obtained with TDZ alone. Overall, increasing TDZ concentration, particularly when combined with lower Kin levels, consistently reduced the shoot multiplication rate (Table 1). In the present study, the addition of chitosan (CS) significantly improved shoot multiplication and shoot growth, although the response varied according to its concentration. The highest shoot multiplication rate was obtained with 10 mg L^{-1} CS combined with TDZ, whereas the longest shoots were observed at the higher CS concentration (40 mg L^{-1}). The beneficial effects of chitosan may be attributed to its role as a biostimulant and elicitor, promoting cell division, improving nutrient uptake, enhancing endogenous hormone balance, and stimulating antioxidant enzyme activity, all of which contribute to enhanced shoot

proliferation and growth. Chitosan has previously been reported to enhance root, shoot, and leaf growth in several plant species, including Gerbera and other horticultural crops (Nge *et al.*, 2006a). Similarly, orchid roots treated with low concentrations of chitosan exhibited improved vegetative growth, flowering, and increased resistance to fungal and viral diseases (Uthairatanakij *et al.*, 2007). In addition, chitosan has been successfully used as a growth stimulant to enhance intracellular productivity in plant tissue culture (S. Sadqpour *et al.*, 2014). Nge *et al.* (2006) also reported that protocorm multiplication in orchid tissue culture was highest at 15 and 20 ppm chitosan in liquid and agar media, respectively. These findings are consistent with the present study, where 10 mg L^{-1} CS in combination with TDZ produced the highest multiplication rate, while 40 mg L^{-1} CS promoted greater shoot elongation. Furthermore, in the second experiment, the combination of 40 mg L^{-1} CS with kinetin also enhanced shoot multiplication and produced longer shoots, indicating that the response to chitosan was influenced by its interaction with different plant growth regulators.

In the present study, the incorporation of 0.3 g L^{-1} PVP into the culture medium effectively supported shoot multiplication of MM106, particularly when combined with chitosan and TDZ. The beneficial effect of PVP is mainly attributed to its ability to adsorb oxidized phenolic compounds released from wounded explants, thereby reducing tissue browning, preventing the accumulation of toxic substances, and improving explant survival and growth. Mert and Soylu (2010) reported that the use of axillary or terminal shoot tips together with an appropriate culture initiation period reduced medium browning during *in vitro* regeneration of the apple rootstocks M9, MM106, and MM111, although the degree of browning varied among cultivars. Similarly, Kassahun Bante and Feyissa (2015) demonstrated that low concentrations of PVP resulted in 100% and 80% survival of explants in sugarcane genotypes C86-56 and C86-12, respectively, by minimizing

the adverse effects of phenolic oxidation during culture establishment. These findings support the results of the present study, in which the combination of 0.3 g L⁻¹ PVP with chitosan and TDZ provided favorable conditions for shoot multiplication by reducing oxidative browning and maintaining healthy explants throughout the culture period. Auxins like IBA, IAA, and NAA are commonly used for apple rooting (Dobránszki & Teixeira da Silva, 2010). Among these auxins, IBA is generally considered the most effective because of its greater stability and slower degradation than IAA, allowing it to maintain auxin activity for a longer period during root initiation. In addition, IBA stimulates cell division in the basal region of the shoots, promotes root primordia formation, and enhances auxin transport, thereby improving adventitious root development. Among the auxins, IBA has been reported as the most effective plant growth regulator for rooting in apple (Amiri & Elahinia, 2011). After two successive subcultures, medium of rooting stage contained MS supplemented with 0.5 mg/L IBA. For MM106, increase the rooting percentage (Mert & Soylu, 2010). Teixeira da Silva *et al.* (2019) investigated the rooting responses to IBA levels (1, 2, or 3 mg/L IBA) were genotype-dependent in MM106 rootstocks. Rooting was low in 'MM106' at all levels of IBA (33–46%), and IBA treatment did not influence root's number in 'MM106'. Teixeira da Silva *et al.* (2019) studied *in vitro* rooting of MM106 rootstocks, a high percentage of rooting at 1.0 mg/L IBA is gated. Three different levels of IBA in this study are used as a high effect on rooting percentage in 1 mg/L IBA with 0.3 g/l PVP.

When adding Phloroglucinol (PG) to the rooting medium, the rooting proportion increased to 60–70% and had better development (Dobránszki & Teixeira da Silva, 2010). The addition of PG to the LM improved root initiation as well (Modgil *et al.*, 1999). In this study, decreasing the rooting percentage in a low level of PG with IBA and a high concentration increases the rooting percentage (8.4–17%) and the roots number per shoot in all cases (Table 2).

Putrescine (Put), a naturally occurring polyamine, plays an important role in root development by regulating cell division, stabilizing cell membranes, delaying senescence, and interacting with endogenous auxin metabolism during adventitious root formation. These physiological functions may explain its positive effect on root initiation and subsequent root growth. Putrescine was reported to improve rooting and subsequent acclimatization of several apple cultivars, including Free Redstar, Gala Must, Pinova, and Redchief, when applied in combination with NAA and IBA. Podwyszyńska and Cieślńska (2018) reported that the application of IBA alone (2.5 µM) or in combination with 5 µM IAA and/or 50 µM Put significantly improved rooting efficiency in the cultivar 'Redchief', reaching 76.6%. Similarly, Najja *et al.* (2009) found that MM106 shoots rooted at 96.7% after auxin treatment followed by transfer to an auxin-free medium. In the absence of IBA, Put increased rooting from 0 to 42.22%, indicating that putrescine can partially compensate for auxin deficiency during the early stages of root induction. Consistent with these findings, putrescine supplementation in the present study increased rooting percentage (16.6–33.4%), root number per shoot, and root length, suggesting that Put enhanced root development through its synergistic interaction with endogenous auxin and its stimulatory effect on cell division.

Carbon nanotubes (CNTs) in date palm rooting media boosted root number, length, plantlet length, and hairy roots. The largest root number was obtained at the higher concentration, which was followed by the lower concentration insignificantly. Meanwhile, the lowest concentration of CNTs resulted in the longest root and plantlet length, which was significantly followed by the highest concentration of CNTs. Similarly, lesser concentrations of CNTs resulted in the hairiest roots, but greater concentrations showed no difference from the control (Taha *et al.*, 2016).

However, single-walled CNTs can inhibit root hair growth (Yan *et al.*, 2013). In this study, CNTs, putrescine, or phloroglucinol reduced rooting percentages and improved root number per shoot. High levels can increase rooting percentage, root number for any shoots, and secondary or lateral roots.

5. CONCLUSION

In this study, cytokinin types (TDZ and Kin) separately from Cs and PVP were examined. The results showed a low concentration of Cs while TDZ significantly improved apple growth rootstock MM106 in *in vitro* propagation. TDZ with Kin and Cs, as well as PVP, have a negative effect on the *in vitro* multiplication stage of apples compared with the first experiment, and the number of multiplicities shoots was low. The most effective IBA concentration in rooting of apple rootstock MM106 shoots is 1 mg/L. 40 mg/L CNTs-Put-Flu with 1 mg/L of IBA and 0.3 g/l PVP increase rooting %, and root numbers per each shoot. Adding 100 mg/L of Put with 1 mg/L IBA and 0.3 mg/L PVP improves root length and number per shoot and rooting percentage. 80 mg/L of PG can increase root's number per every shoot and rooting percentage.

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Ethical Statment:

Ethical approval has not been required for this manuscript because we did not work with animals or humans to need approvals.

Conflict of Interests:

The authors declare no competing interests.

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Author Contributions:

MR.D. designed the study; I.N.A. performed the experiments; S.M.S.Z. prepared hormone concentration and other lab work; I.N.A. analyzed the data; S.P. and MR.D. reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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