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The effects of zinc oxide nanoparticles loaded with IAA and IBA on in vitro rooting of apple microcuttings

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Abstract: In the micropropagation of difficult-to-root plants, the low rates of rooting cause high economic losses. The local apple (*Malus domestica* Borkh.) cultivar #67, which is highly resistant to fire blight disease, has been shown to have low rooting success. Nanoparticles loaded with auxins could increase the rooting success with their physicochemical properties. To increase rooting percentage, the effects of zinc oxide nanoparticles (nZnO), nZnO loaded with indole-3-acetic acid (IAA-nZnO) and indole-3-butyric acid (IBA-nZnO) at different concentrations (1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg L⁻¹) on in vitro rooting of microcuttings were investigated. The concentrations of auxin carried by the nanoparticles were 2.8, 5.7, 8.0, 10.8, 13.7, 16.0 µM IAA or 2.5, 4.4, 6.9, 9.3, 11.3, 13.8 µM IBA. The various concentrations of IAA (0.0, 2.8, 5.7, 11.4, 17.1 µM) and IBA (0.0, 2.5, 4.9, 9.8, 14.8 µM) were also examined. All substances as described previously were incorporated to half-strength Murashige and Skoog (½ MS) medium containing 2% sucrose at pH 5.8, and solidified with 0.7% agar. The microcuttings were cultured on these media for 5 days in the dark before being transferred to a 16 h photoperiod conditions at 25 ± 1 °C for 6 weeks.

It was found that rooting did not occur at 0.0 µM IAA and IBA concentration and nZnO treatments. IBA-nZnO and IBA were more effective on the rooting of microcuttings. In the comparison of IBA-nZnO and IBA, the percentage of rooting was higher in IBA-nZnO (58.4%) than IBA (47.5%). In addition, 4.4 µM or 4.9 µM IBA, particularly, induced many favorable properties with high rooting percentage (62.7%) and root length (16.5 ± 3.5 mm) and a small callus diameter (5.3 ± 0.2 mm). In conclusion, IBA-nZnO loaded with 4.4 µM IBA could be a promising treatment for in vitro rooting of difficult-to-root apple cultivar #67.

Key words: Auxin, *Malus*, micropropagation, nanotechnology, tissue culture

1. Introduction

The main propagation methods of apple genotypes on their own roots without genetic expansion breakthrough are stooling, layering, cutting or micropropagation (Wertheim and Webster, 2003). Propagation by stooling, layering and cutting are methods in which the plant proliferation coefficient is low due to the fact that it can be done at certain times of the year, the propagation material is limited and have rooting problems as well. However, in micropropagation, an unlimited number of propagation material can be obtained with subcultures throughout the year, rooting can be provided under controlled conditions, and rapid and mass plant propagation can be performed. In this propagation method, rhizogenesis is one of the most critical stages of micropropagation, particularly in plants which are difficult-to-root. Low rates or failure in rooting can cause very high economic losses. In addition to genotype, physiological condition of microcuttings, type,

structure and content of rooting media, and environmental factors such as light, temperature, humidity, and air composition, the plant rooting stimulators and applications such as wounding, etiolation, etc., are effective on rooting success in micropropagation (Hartmann et al., 2011). Studies on micropropagation have shown that auxins are effective inducers for the formation of adventitious roots for apples as well as for many woody plant species (Dobranszki and Teixeira da Silva, 2010; Amirchakhmaghi et al., 2019; Teixeira da Silva et al., 2019; Kim et al., 2020). The most commonly used auxins are indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA). IAA, which can be synthesized naturally in plants, is the first auxin used for the rooting of cuttings. Afterwards, IBA was discovered which was even more effective on rooting (Pop et al., 2011). As a further step, investigating the possibilities of using nanotechnology in auxin applications to increase rooting success is a relatively

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new approach. Currently, there are very few sources in the literature on this subject (Thangavelu et al., 2018; Karakeçili et al., 2019).

Nanotechnology is the science that deals with substances at the scale of one billionth of a meter. Metallic nanoparticles have different physical and chemical properties than bulk metals. Properties such as lower melting points, higher specific surface areas, specific optical properties, mechanical strength and specific magnetizations make nanoparticles attractive in various industrial applications (Horikoshi and Serpone, 2013). Nanoparticles are colloidal polymeric particulate systems that release the dissolved, entrapped or adsorbed active substance in a controlled manner (Sengel-Turk and Hascicek, 2009). The effect of nanoparticles on plants depends on the composition, concentration, size, physical and chemical properties of the nanoparticles as well as the plant genotype (Siddiqui et al., 2015). Nanoparticles stabilized with auxins in genotypes with low rooting abilities have the potential to increase rooting success with their unique physicochemical properties (Thangavelu et al., 2018; Karakeçili et al., 2019).

In recent years, researchers have carried out various studies on the effects of nanoparticles on the growth and development of different plant species. Zinc oxide nanoparticles (nZnO) are receiving a great deal of attention in a number of recent studies. The effects of nZnO were investigated for entomotoxic efficacy against *Sitophilus oryzae* (L.) (Das et al., 2019), the content of bioactive compounds in habanero peppers (Garcia-Lopez et al., 2019), plant physiology in coffee (Rossi et al., 2019), antibacterial activity against *Xanthomonas oryzae* pv. *Oryzae* (Ogunyemi et al., 2019), rhizogenesis in *Pyrus* spp. (Karakeçili et al., 2019) and the initial development of corn seedlings after seed priming (Neto et al., 2020) in plants and agricultural crops.

Zinc is a micro nutrient element necessary for the metabolic activities in humans, animals and plants. This element has important functions in the biosynthesis pathway of IAA from tryptophan. Various studies have demonstrated how zinc plays a vital role in plant growth, propagation and yield. In its insufficiency, it creates physiological imbalances within the plant and affects enzyme activities and other metabolic processes (Takaki and Kushizaki, 1977; Mousavi et al., 2013; Hassan et al., 2020). Zinc oxide is a multifunctional substance with its unique physical and chemical properties; consequently, it is used in a wide variety of fields, including agriculture (Kolodziejczak-Radzimska and Jesionowski, 2014).

In this study, we investigated the effects of zinc oxide nanoparticles (nZnO) loaded with IAA and IBA on in vitro rooting of apple microcuttings. To the best of our

knowledge, this is the first study evaluating the effects of auxin loaded ZnO nanoparticles in the basal medium on in vitro rooting of microcuttings.

2. Materials and methods

2.1. In vitro microcutting preparation

The microcuttings of the local apple genotype #67 (*Malus domestica* Borkh.) with a high level of resistance to fire blight disease (*Erwinia amylovora* (Burr.) Winslow et al.) were used as plant materials. These microcuttings (approximately 2 cm long with apex and two leaves) were prepared from microshoots from fifth and seventh subcultures under aseptic conditions according to the protocol reported by Dumanoglu et al. (2009). For subcultures, Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1.0 mg L⁻¹ BAP (6-benzylaminopurine) and 0.25 mg L⁻¹ GA₃ (gibberellic acid) and 3% sucrose was used. The medium was adjusted to pH 5.8 and solidified with 0.7% agar. The media (50 mL) were dispensed into 250-mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min. Eight microshoots were planted in each flask. Cultures were incubated in a growth chamber at 25 ± 1 °C under a 16 h photoperiod (35 µmol m⁻² s⁻¹) for 4–5 weeks.

2.2. Auxins (IAA and IBA) and auxin loaded nanoparticles (IAA-nZnO and IBA-nZnO) affecting rooting of microcuttings

For the rooting of microcuttings, auxins (IAA and IBA) (Sigma-Aldrich) and auxin loaded zinc oxide nanoparticles (IAA-nZnO and IBA-nZnO) experiments were designed. In the latter case, zinc oxide nanoparticles (nZnO) was also performed. The nZnO (Sigma-Aldrich) used in the study is in nanopowder form, <50 nm particle size, with a purity of >97%. IAA-nZnO and IBA-nZnO nanoparticles were produced according to the protocol reported by Karakeçili et al. (2019), and characterized by Fourier transform infrared spectroscopy (FTIR-ATR), zeta potential, a scanning electron microscope (SEM), energy dispersive X-ray spectroscopy (EDX), a transmission electron microscope (TEM) and thermal analysis (DTA). The loading efficiency was 87% for IAA-nZnO and 89% for IBA-nZnO. In the nanoparticle experiments, nZnO, IAA-nZnO and IBA-nZnO were used in amounts of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg L⁻¹. According to the loading efficiency of IAA-nZnO and IBA-nZnO, the auxin concentrations in nanoparticles were 0.5, 1.0, 1.4, 1.9, 2.4, 2.8 mg L⁻¹ (2.8, 5.7, 8.0, 10.8, 13.7, 16.0 µm) IAA and 0.5, 0.9, 1.4, 1.9, 2.3, 2.8 mg L⁻¹ (2.5, 4.4, 6.9, 9.3, 11.3, 13.8 µm) IBA. In the auxin experiments, IAA concentrations were 0.0, 0.5, 1.0, 2.0 and 3.0 mg L⁻¹ (0.0, 2.8, 5.7, 11.4 and 17.1 µm) and IBA concentrations were 0.0, 0.5, 1.0, 2.0 and 3.0 mg L⁻¹

Table 1. The substances in the experiments.

Substance	Substance amount (mg L ⁻¹)	Concentration of auxin contained in substance	
		(mg L ⁻¹)	(μ M)
IBA-nZnO	1.0	0.5	2.5
	2.0	0.9	4.4
	3.0	1.4	6.9
	4.0	1.9	9.3
	5.0	2.3	11.3
	6.0	2.8	13.8
IAA-nZnO	1.0	0.5	2.8
	2.0	1.0	5.7
	3.0	1.4	8.0
	4.0	1.9	10.8
	5.0	2.4	13.7
	6.0	2.8	16.0
IBA	0.5	0.5	2.5
	1.0	1.0	4.9
	2.0	2.0	9.8
	3.0	3.0	14.8
IAA	0.5	0.5	2.8
	1.0	1.0	5.7
	2.0	2.0	11.4
	3.0	3.0	17.1

(0.0, 2.5, 4.9, 9.8 and 14.8 μ m) (Dobranszki and Teixeira da Silva, 2010) (Table 1).

The half-strength Murashige and Skoog medium (1/2 MS) with 2% sucrose and 0.7% agar was used as the basal medium. For all experiments including auxins (IAA and IBA) and auxin loaded nanoparticles (nZnO, IAA-nZnO, IBA-nZnO), various concentrations of auxin which were prepared as described previously were added to the basal medium and pH was adjusted to 5.8 before the addition of 0.7% agar. Then, seven microcuttings were cultured on 250-mL jars each containing 50 mL of medium. After planting the microcuttings, the cultures were incubated in dark conditions for 5 days then under a 16 h photoperiod ($35 \mu\text{mol m}^{-2} \text{s}^{-1}$) in a growth chamber at 25 ± 1 °C for a total of 6 weeks. At the end of the experiment, the rooting percentage, rooting level of rooted microcuttings on 1–4 scale (1 = weak, 2 = medium, 3 = good, 4 = very good), root number per microcutting, average root length and callus diameter (mm) were determined. In the study, rooting level visually rated. Level 1 was defined by the presence of a few thin and short roots on one side of the

bottom of the microcutting. At other rooting levels, the root thickness and length was fine to medium, and root formation at the bottom of the microcutting was on both sides at level 2, on three sides at level 3, and on all sides at level 4. Average root length was determined by measuring the length of each root in rooted cuttings and dividing by the number of roots. In addition, the callus diameter formed at the bottom of the rooted cuttings was measured using a small ruler.

2.3. Statistical analysis

Two experiments were designed according to the completely randomized design with factorial combinations. The nanoparticle experiment had two-factors with nanoparticle type (ZnO, IBA-nZnO and IAA-nZnO) and amount (1, 2, 3, 4, 5 and 6 mg L⁻¹). The auxin experiment also had two-factors with auxin type (IBA and IAA) and concentration (0.0, 0.5, 1.0, 2.0 and 3.0 mg L⁻¹). The experiments consisted of three replications, with each jar being a replication. Thus, three jars were used for each treatment. The number of microcuttings in each replication (jar) was seven. The treatments were repeated twice. In both experiments the data were subjected to analyses of variance (ANOVA) by the Minitab Package Program (MINITAB Inc., trial version). Significant differences among means were determined using Duncan's multiple range test at $p \leq 0.05$. Data in percentage were statistically evaluated after arcsine transformations.

3. Results

In the experiments, the interaction effects of nanoparticle type $\times$ amount and auxin type $\times$ concentration on rooting percentage, rooting level, root length and callus diameter were not statistically significant (Tables 2–5).

In the nanoparticle experiment, the differences between the nanoparticle types were significant ($p = 0.000$), but the differences in the nanoparticle amounts were not deemed significant in terms of the mean rooting percentage. Rooting in microcuttings did not occur in nZnO treatments. The highest rooting percentage of 56.3% was obtained in IBA-nZnO (Table 2, Figures 1A–1F and 2). In this experiment, the differences between nanoparticle types and amounts were insignificant in terms of the mean rooting level and the mean root number per microcutting (Tables 2 and 3). However, the mean root length was significantly higher in IAA-nZnO (18.4 ± 2.7 mm). This parameter was also significantly higher at 1–3 mg L⁻¹ nanoparticle amounts containing 0.5–1.4 mg L⁻¹ IBA or IAA and the means ranged from 22.1 ± 4.8 mm to 18.2 ± 5.0 mm. The mean callus diameter was lower in IAA-nZnO (4.2 ± 0.3 mm) compared to IBA-nZnO (5.6 ± 0.2 mm) (Table 3).

Table 2. The effects of zinc oxide nanoparticles loaded with IAA and IBA on in vitro rooting percentage and rooting level of apple genotype #67 microcuttings¹.

Effects	Rooting (%)	p	Rooting level (1–4)	p
Nanoparticle type		0.000***		0.931^{ns}
nZnO	0.0 ± 0.0 C ²		-	
IBA-nZnO	56.3 ± 2.9 A		2.0 ± 0.2	
IAA-nZnO	34.6 ± 5.4 B		1.9 ± 0.2	
Nanoparticle amount		0.722^{ns}		0.605^{ns}
1.0 mg L ⁻¹	22.1 ± 6.6		2.0 ± 0.3	
2.0 mg L ⁻¹	32.1 ± 11.1		2.0 ± 0.2	
3.0 mg L ⁻¹	29.5 ± 9.0		1.8 ± 0.4	
4.0 mg L ⁻¹	31.7 ± 10.3		1.6 ± 0.2	
5.0 mg L ⁻¹	31.2 ± 10.0		2.4 ± 0.3	
6.0 mg L ⁻¹	35.2 ± 9.8		1.9 ± 0.3	
Nanoparticle type × amount		0.578^{ns}		0.405^{ns}
nZnO × 1.0 mg L ⁻¹	0.0 ± 0.0		-	
nZnO × 2.0 mg L ⁻¹	0.0 ± 0.0		-	
nZnO × 3.0 mg L ⁻¹	0.0 ± 0.0		-	
nZnO × 4.0 mg L ⁻¹	0.0 ± 0.0		-	
nZnO × 5.0 mg L ⁻¹	0.0 ± 0.0		-	
nZnO × 6.0 mg L ⁻¹	0.0 ± 0.0		-	
IBA-nZnO × 1.0 mg L ⁻¹	40.3 ± 3.7		2.0 ± 0.4	
IBA-nZnO × 2.0 mg L ⁻¹	70.4 ± 4.9		2.3 ± 0.4	
IBA-nZnO × 3.0 mg L ⁻¹	51.5 ± 2.4		2.2 ± 0.7	
IBA-nZnO × 4.0 mg L ⁻¹	65.4 ± 5.3		1.7 ± 0.2	
IBA-nZnO × 5.0 mg L ⁻¹	53.0 ± 6.0		2.0 ± 0.3	
IBA-nZnO × 6.0 mg L ⁻¹	57.6 ± 5.0		1.6 ± 0.4	
IAA-nZnO × 1.0 mg L ⁻¹	25.9 ± 9.8		1.9 ± 0.5	
IAA-nZnO × 2.0 mg L ⁻¹	25.9 ± 13.4		1.8 ± 0.3	
IAA-nZnO × 3.0 mg L ⁻¹	37.0 ± 16.1		1.4 ± 0.4	
IAA-nZnO × 4.0 mg L ⁻¹	29.6 ± 13.4		1.6 ± 0.3	
IAA-nZnO × 5.0 mg L ⁻¹	40.7 ± 19.6		2.8 ± 0.6	
IAA-nZnO × 6.0 mg L ⁻¹	48.1 ± 13.4		2.3 ± 0.4	

¹Cultures were incubated at dark during first 5 days.²According to Duncan's multiple range test ($p \leq 0.05$), means separation within columns followed by the same letter do not involve statistically significant differences.*** $p \leq 0.001$ statistically significant, ^{ns} statistically not significant.

In the auxin experiment, the differences between the auxin types and auxin concentrations for rooting percentage were highly significant ($p = 0.000$). Rooting did not occur in either auxin types at 0.0 mg L⁻¹ concentration. IBA was more effective than IAA on the mean rooting percentage and the mean rooting level (Figure 3). The

mean rooting percentage was 38.0% in IBA and 17.8% in IAA. The mean rooting level was also higher in IBA (2.1 ± 0.1) than IAA (1.5 ± 0.2) (Table 4). Auxin types and concentrations had statistically similar effects on the mean root number per microcutting, ranging from 1.7 ± 0.4 to 2.5 ± 0.7 . However, the mean callus diameter (5.8 ± 0.4

Table 3. The effects of zinc oxide nanoparticles loaded with IAA and IBA on in vitro root number, root length and callus diameter of apple genotype #67 microcuttings¹.

Effects	Roots per microcutting (no.)	p	Root length (mm)	p	Callus diameter (mm)	p
Nanoparticle type		0.408^{ns}		0.014[*]		0.001^{***}
nZnO	-		-			
IBA-nZnO	2.7 ± 0.3		12.1 ± 1.6 B		5.6 ± 0.2 A	
IAA-nZnO	2.4 ± 0.2		18.4 ± 2.7 A		4.2 ± 0.3 B	
Nanoparticle amount		0.960^{ns}		0.004^{**}		0.792^{ns}
1.0 mg L ⁻¹	2.5 ± 0.5		22.1 ± 4.8 A		4.7 ± 0.3	
2.0 mg L ⁻¹	2.2 ± 0.3		21.8 ± 2.5 A		4.4 ± 0.4	
3.0 mg L ⁻¹	2.6 ± 0.8		18.2 ± 5.0 AB		4.9 ± 0.7	
4.0 mg L ⁻¹	2.4 ± 0.1		12.4 ± 3.4 BC		4.9 ± 0.9	
5.0 mg L ⁻¹	2.8 ± 0.4		9.8 ± 1.8 BC		5.2 ± 0.4	
6.0 mg L ⁻¹	2.7 ± 0.5		7.2 ± 1.2 C		5.4 ± 0.5	
Nanoparticle type × amount		0.225^{ns}		0.110^{ns}		0.868^{ns}
IBA-nZnO × 1.0 mg L ⁻¹	2.6 ± 0.7		20.4 ± 1.6		5.1 ± 0.3	
IBA-nZnO × 2.0 mg L ⁻¹	2.5 ± 0.6		20.2 ± 3.4		5.2 ± 0.3	
IBA-nZnO × 3.0 mg L ⁻¹	3.5 ± 1.4		7.2 ± 1.7		6.1 ± 0.3	
IBA-nZnO × 4.0 mg L ⁻¹	2.4 ± 0.2		7.7 ± 1.3		5.7 ± 0.3	
IBA-nZnO × 5.0 mg L ⁻¹	3.2 ± 0.7		8.9 ± 2.1		5.9 ± 0.2	
IBA-nZnO × 6.0 mg L ⁻¹	1.9 ± 0.5		7.9 ± 1.9		5.8 ± 1.0	
IAA-nZnO × 1.0 mg L ⁻¹	2.4 ± 0.8		32.4 ± 10.9		4.3 ± 0.6	
IAA-nZnO × 2.0 mg L ⁻¹	2.0 ± 0.4		23.5 ± 7.2		3.7 ± 0.0	
IAA-nZnO × 3.0 mg L ⁻¹	1.6 ± 0.6		29.2 ± 2.0		3.7 ± 0.9	
IAA-nZnO × 4.0 mg L ⁻¹	2.4 ± 0.2		22.1 ± 5.1		4.0 ± 1.7	
IAA-nZnO × 5.0 mg L ⁻¹	2.4 ± 0.2		10.6 ± 3.3		4.5 ± 0.3	
IAA-nZnO × 6.0 mg L ⁻¹	3.4 ± 0.5		6.5 ± 1.8		4.9 ± 0.5	

¹Cultures were incubated at dark during first 5 days.

²According to Duncan's multiple range test ($p \leq 0.05$), means separation within columns followed by the same letter do not involve statistically significant differences.

^{*}, ^{**}, ^{***} $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ statistically significant, ^{ns} statistically not significant.

mm) was significantly larger, but the mean root length (11.1 ± 2.1 mm) was shorter in IBA compared to IAA. The differences between auxin concentrations were significant in terms of the mean rooting percentage but insignificant for the mean rooting level, the mean root length and the mean callus diameter (Tables 4 and 5). The rooting percentages as means of auxin types ranged from 23.5% to 40.5% at auxin concentrations of 0.5–3.0 mg L⁻¹. But, there were no statistical differences between these values (Table 4).

This study also examined the differences between IBA-nZnO and IBA in all rooting parameters based on four approximately overlapping concentrations of IBA and IBA-

nZnO. The effects of 0.5, 1.0, 2.0, 3.0 mg L⁻¹ IBA and 0.5, 0.9, 1.9, 2.8 mg L⁻¹ IBA carried by 1.0, 2.0, 4.0, 6.0 mg L⁻¹ IBA-nZnO were compared (Figure 4). The interaction effects between substance (IBA or IBA-nZnO) × concentration in all parameters were statistically insignificant (Tables 6 and 7). However, differences between substances and IBA concentrations in terms of the rooting percentage were statistically significant. IBA-nZnO (58.4%) had a significantly higher rooting percentage compared to IBA (47.5%). The rooting percentage was higher at 1.0 mg L⁻¹ (62.7%), 2.0 mg L⁻¹ (54.5%) and 3.0 mg L⁻¹ (54.5%) IBA concentrations than at 0.5 mg L⁻¹ IBA (40%) (Table 6). The differences between IBA-nZnO and IBA and IBA

Table 4. The effects of IAA and IBA on in vitro rooting percentage and rooting level of apple genotype #67 microcuttings¹.

Effects	Rooting (%)	p	Rooting level (1–4)	p
Auxin type		0.000***		0.037*
IBA	38.0 ± 5.4 A		2.1 ± 0.1 A	
IAA	17.8 ± 4.6 B		1.5 ± 0.2 B	
Auxin concentration		0.000***		0.852^{ns}
0.0 mg L ⁻¹	0.0 ± 0.0 B		-	
0.5 mg L ⁻¹	23.5 ± 7.6 A		1.8 ± 0.2	
1.0 mg L ⁻¹	38.7 ± 8.1 A		1.6 ± 0.2	
2.0 mg L ⁻¹	36.7 ± 4.7 A		2.0 ± 0.4	
3.0 mg L ⁻¹	40.5 ± 8.9 A		1.9 ± 0.4	
Auxin type × concentration		0.188^{ns}		0.621^{ns}
IBA × 0.0 mg L ⁻¹	0.0 ± 0.0		-	
IBA × 0.5 mg L ⁻¹	39.7 ± 3.8		2.2 ± 0.2	
IBA × 1.0 mg L ⁻¹	51.4 ± 2.1		1.8 ± 0.3	
IBA × 2.0 mg L ⁻¹	43.7 ± 2.6		2.1 ± 0.1	
IBA × 3.0 mg L ⁻¹	51.4 ± 4.5		2.5 ± 0.5	
IAA × 0.0 mg L ⁻¹	0.0 ± 0.0		-	
IAA × 0.5 mg L ⁻¹	7.4 ± 3.7		1.5 ± 0.3	
IAA × 1.0 mg L ⁻¹	22.2 ± 6.4		1.4 ± 0.3	
IAA × 2.0 mg L ⁻¹	29.6 ± 7.4		1.8 ± 0.8	
IAA × 3.0 mg L ⁻¹	29.6 ± 16.1		1.2 ± 0.1	

¹Cultures were incubated at dark during first 5 days.

²According to Duncan's multiple range test ($p \leq 0.05$), means separation within columns followed by the same letter do not involve statistically significant differences.

*, *** $p \leq 0.05$ and $p \leq 0.001$ statistically significant, ^{ns} statistically not significant.

concentrations were not significant for rooting level and root number (Tables 6 and 7). In terms of root length and callus diameter, the differences between the IBA-nZnO and IBA were not deemed significant, but the differences between concentrations of IBA were significant. As the mean data of IBA-nZnO and IBA, root length decreased and callus diameter increased at high IBA concentrations compared to low concentrations. The mean root length was longer at 0.5 mg L⁻¹ (16.6 ± 2.9 mm) and 1.0 mg L⁻¹ (16.5 ± 3.5 mm) IBA compared to the higher concentrations (Table 7). In contrast, the mean callus diameter was smaller at lower IBA concentrations of 0.5 mg L⁻¹ (4.8 ± 0.4 mm) and 1.0 mg L⁻¹ (5.3 ± 0.2 mm) than the higher concentrations of 2.0 mg L⁻¹ (6.2 ± 0.4 mm) and 3.0 mg L⁻¹ IBA (6.7 ± 0.4 mm) (Table 7). Thus, 1.0 mg L⁻¹ IBA carried by 2.0 mg L⁻¹ IBA-nZnO would be suggested for in vitro rooting of apple genotype #67 microcuttings which had a high rooting percentage (Table 6, Figure 4), high root length and small callus diameter (Table 7, Figure 1B).

4. Discussion

Agricultural studies have previously discussed the effects of zinc oxide nanoparticles in areas such as plant growth and development, germination, plant nutrition, and disease and pest control (Laware and Raskar, 2014; Abobatta, 2018). In this study with a new perspective, the effects of zinc oxide nanoparticles loaded with IBA and IAA on in vitro rooting of microcuttings of apple cultivar #67 were investigated. The mean rooting percentage was 1.5 times higher in IBA-nZnO (56.3%) compared to IBA (38.0%) and 1.9 times higher in IAA-nZnO (34.6%) compared to IAA (17.8%). Our findings coincide with Karakeçili et al. (2019) who found that IAA-nZnO and IBA-nZnO were effective on the rooting of microcuttings in a difficult-to-root wild pear genotype (*Pyrus elaeagnifolia* Pall.), and the rooting percentage was significantly higher in IBA-nZnO (50.0%) and IAA-nZnO (41.7%) than IBA (25.0%) and IAA (25.0%). Indeed, nanoparticles are targeted and slow release carriers for auxins (Thangavelu et al., 2018). In our

Table 5. The effects of IAA and IBA on in vitro root number, root length and callus diameter of apple genotype #67 microcuttings¹.

Effects	Roots per microcutting (no.)	p	Root length (mm)	p	Callus diameter (mm)	p
Auxin type		0.097^{ns}		0.048[*]		0.001^{***}
IBA	2.5 ± 0.2		11.1 ± 2.1 B		5.8 ± 0.4 A	
IAA	1.7 ± 0.4		18.3 ± 3.3 A		3.5 ± 0.4 B	
Auxin concentration		0.730^{ns}		0.056^{ns}		0.238^{ns}
0.5 mg L ⁻¹	2.3 ± 0.4		14.9 ± 3.1		3.7 ± 0.7	
1.0 mg L ⁻¹	1.9 ± 0.3		22.6 ± 6.3		4.9 ± 0.4	
2.0 mg L ⁻¹	2.5 ± 0.7		12.8 ± 2.5		5.3 ± 0.8	
3.0 mg L ⁻¹	1.9 ± 0.4		8.4 ± 1.0		4.9 ± 0.9	
Auxin type × concentration		0.550^{ns}		0.246^{ns}		0.275^{ns}
IBA × 0.5 mg L ⁻¹	3.1 ± 0.5		12.9 ± 4.9		4.4 ± 0.9	
IBA × 1.0 mg L ⁻¹	2.0 ± 0.3		12.8 ± 5.9		5.4 ± 0.3	
IBA × 2.0 mg L ⁻¹	2.5 ± 0.4		11.9 ± 5.1		6.6 ± 0.7	
IBA × 3.0 mg L ⁻¹	2.6 ± 0.5		6.8 ± 1.4		6.8 ± 0.4	
IAA × 0.5 mg L ⁻¹	1.5 ± 0.3		17.0 ± 8.0		3.0 ± 1.1	
IAA × 1.0 mg L ⁻¹	1.8 ± 0.6		32.3 ± 8.2		4.3 ± 0.7	
IAA × 2.0 mg L ⁻¹	2.5 ± 1.5		13.7 ± 1.8		3.9 ± 1.1	
IAA × 3.0 mg L ⁻¹	1.2 ± 0.1		10.0 ± 0.7		2.9 ± 0.4	

¹Cultures were incubated at dark during first 5 days.

²According to Duncan's multiple range test ($p \leq 0.05$), means separation within columns followed by the same letter do not involve statistically significant differences.

^{*}, ^{***} $p \leq 0.05$ and $p \leq 0.001$ statistically significant, ^{ns} statistically not significant.

study, microcuttings of apple genotype #67 treated with nZnO alone at concentrations of 1–6 mg L⁻¹ did not root. However, Helaly et al. (2014) reported that the percentage of rooting in banana microshoots increased above 70% on media containing nZnO at concentrations of 50, 100 and 200 mg L⁻¹. This result does not agree with our nZnO findings. The difference between the findings might be due to the fact that 50–200 mg L⁻¹ nZnO concentrations are much higher than the 1–6 mg L⁻¹ nZnO concentrations used in our study. This result indicates the effectiveness of high concentrations (50–200 mg L⁻¹) of nZnO on rooting. Pandey et al. (2010) report that due to oxygen vacancies, the oxygen deficient, i.e. zinc-rich ZnO nanoparticles increased the level of indole acetic acid. IAA is a natural auxin effective on rooting (Pop et al., 2011).

According to the findings of auxin treatments, in vitro rooting percentages of apple genotype #67 microcuttings were found to be higher in IBA compared to IAA. The result is compatible with the finding of other studies that IBA is a more effective auxin than IAA on rooting in plants (Zimmerman and Fordham, 1985; Stefancic et al., 2005; Ciccotti et al., 2008). Indeed, IBA maintains its

existence in rooting environment for longer than IAA, as it is a more stable auxin than IAA. The actual level of IBA in the tissue does not decrease due to the presence of IBA in the environment even if a significant part of IBA is converted to IAA or attached (Nordstrom et al., 1991; Van der Krieken et al., 1993; Stefancic et al., 2005; Frick and Strader, 2018). In addition, Van der Krieken et al. (1993) suggest that IAA deteriorates faster than IBA in apple shoots. Many studies revealed the positive effect of IBA on rooting of microcuttings in different apple genotypes (Boudabous et al., 2010; Ghanbari, 2014; Meneguzzi et al., 2017). In different studies, researchers have determined that IBA concentrations varying between 0.5 mg L⁻¹ and 3.0 mg L⁻¹ were effective on rooting apple microcuttings. These results are highly consistent with the findings that we obtained from 0.5 mg L⁻¹ and 3.0 mg L⁻¹ IBA concentrations for in vitro rooting of microcuttings of apple cultivar #67 in our study. In general, IBA has a strong influence on the formation and development of root apical meristem, rootlets, and adventitious roots (Frick and Strader, 2018). Although the percentage of callused microcuttings was not investigated in the current study, it

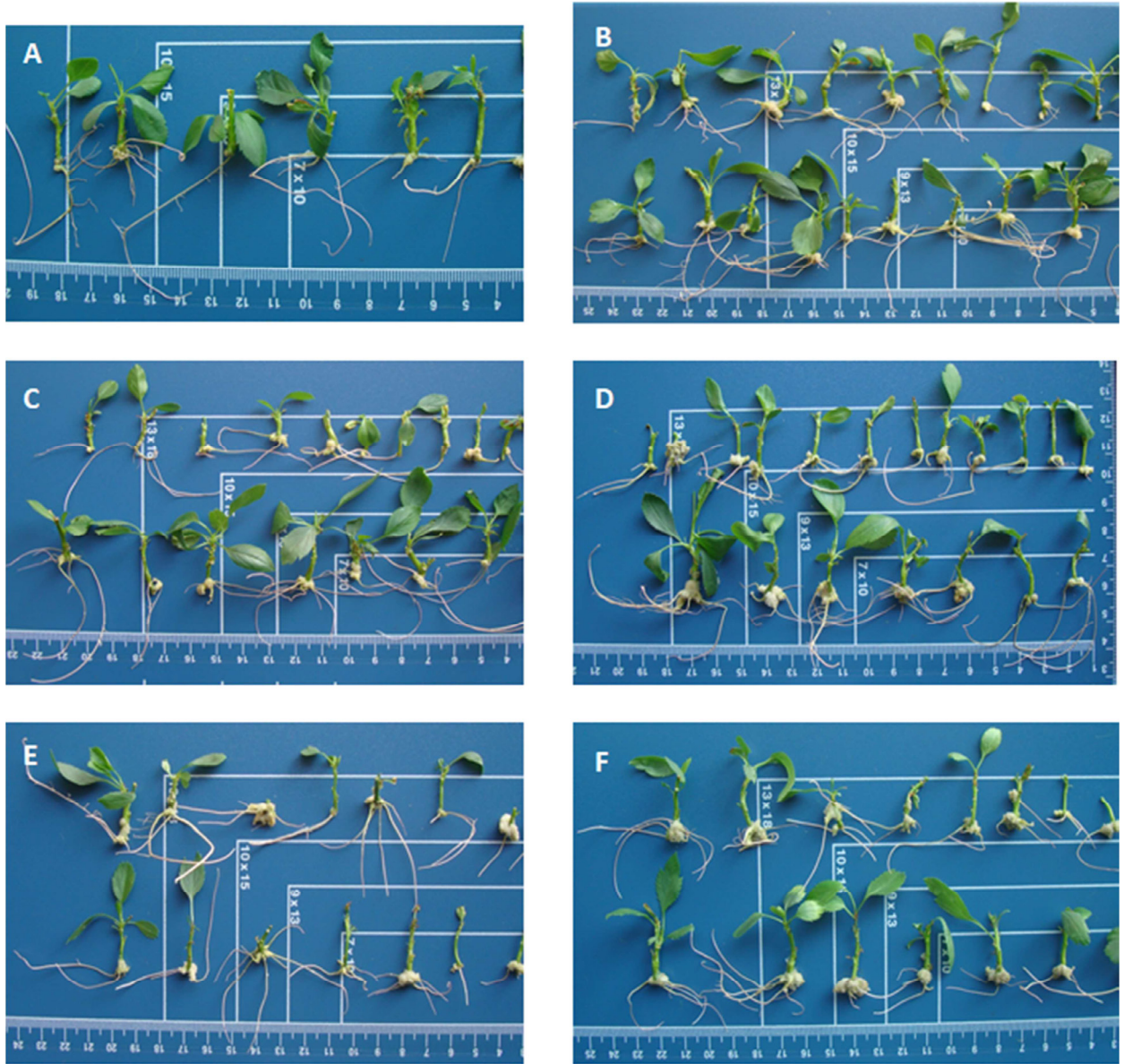


Figure 1. Rooted microcuttings in apple genotype #67 on basal $\frac{1}{2}$ MS medium supplemented with IBA-nZnO particles. **A)** 1.0 mg L⁻¹ IBA-nZnO (containing 2.5 μ m IBA), **B)** 2.0 mg L⁻¹ IBA-nZnO (containing 4.4 μ m IBA), **C)** 3.0 mg L⁻¹ IBA-nZnO (containing 6.9 μ m IBA), **D)** 4.0 mg L⁻¹ IBA-nZnO (containing 9.3 μ m IBA), **E)** 5.0 mg L⁻¹ IBA-nZnO (containing 11.3 μ m IBA), **F)** 6.0 mg L⁻¹ IBA-nZnO (containing 13.8 μ m IBA).

was determined that callus diameter increased undesirably at IBA concentrations above 1.0 mg L⁻¹ and the percentage of increase reached 40% at 3.0 mg L⁻¹ compared to 0.5 mg L⁻¹ (Table 7). Meneguzzi et al. (2017) reported that 1.5 mg L⁻¹ IBA was best on the rooting parameters of microcuttings of Geneva series new apple rootstock G.814, however the percentage of explants with callus was 60% which reached to 100% at 2.5 mg L⁻¹ IBA concentration. High IBA concentrations during the rooting stage

increased callus formation, which weakened or killed the plant at a high rate during the acclimatization stage, and the callus impairs the vascular connection between roots and microshoots, and inhibits the absorption of water and nutrients (Arab et al., 2018).

5. Conclusion

The individual findings from nanoparticle and auxin experiments indicated that IBA-nZnO (56.3%)

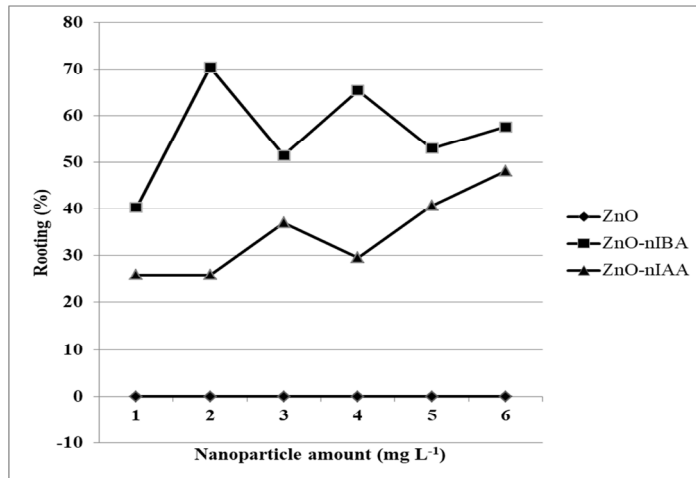


Figure 2. Rooting percentages of microcuttings in apple genotype #67 on basal ½ MS medium supplemented with nanoparticles. Auxin concentrations were 2.5, 4.4, 6.9, 9.3, 11.3 and 13.8 μM IBA or 2.8, 5.7, 8.0, 10.8, 13.7 and 16.0 μM IAA at 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg L^{-1} of loaded nZnO.

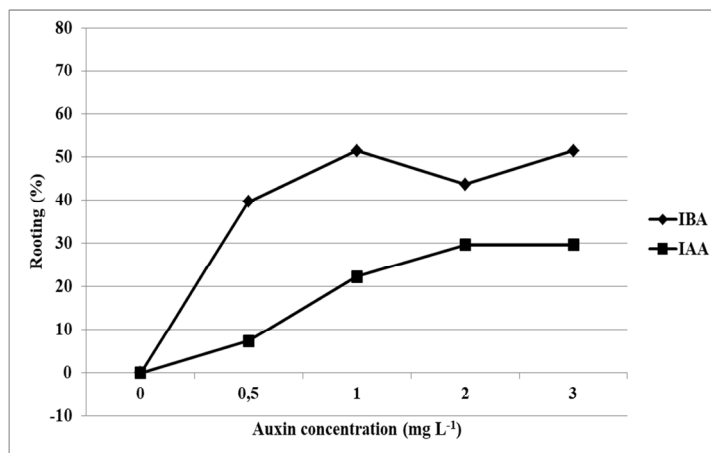


Figure 3. Rooting percentages of microcuttings in apple genotype #67 on basal ½ MS medium supplemented with auxins. Auxin concentrations of 0.0, 0.5, 1.0, 2.0 and 3.0 mg L^{-1} were 0.0, 2.5, 4.9, 9.8 and 14.8 μM IBA or 0.0, 2.8, 5.7, 11.4 and 17.1 μM IAA.

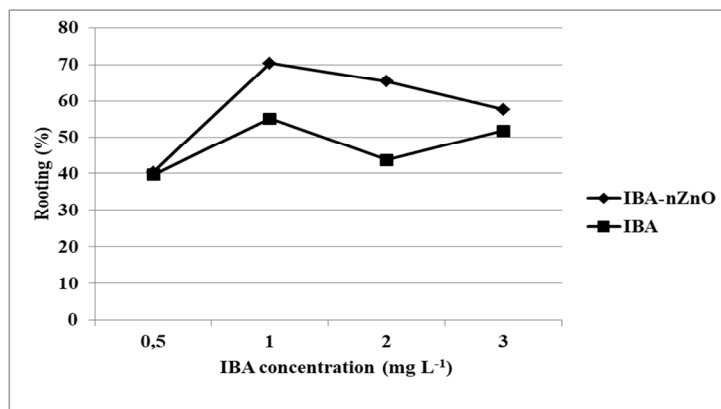


Figure 4. Rooting percentages of microcuttings in apple genotype #67 on basal ½ MS medium supplemented with IBA or IBA-nZnO. IBA concentrations of 0.5, 1.0, 2.0 and 3.0 mg L^{-1} were 2.5, 4.4, 9.3 and 13.8 μM for IBA-nZnO and 2.5, 4.9, 9.8 and 14.8 μM for IBA.

Table 6. The effects of IBA and zinc oxide nanoparticles loaded with IBA on in vitro rooting percentage and rooting level of apple genotype #67 microcuttings¹.

Effects	Rooting (%)	p	Rooting level (1–4)	p
Substance		0.002^{**}		0.346^{ns}
IBA-nZnO	58.4 ± 4.0 A		1.9 ± 0.2	
IBA	47.5 ± 2.4 B		2.1 ± 0.1	
IBA concentration		0.001^{***}		0.946^{ns}
0.5 mg L ⁻¹	40.0 ± 2.4 B		2.1 ± 0.2	
1.0 mg L ⁻¹	62.7 ± 4.4 A		2.1 ± 0.2	
2.0 mg L ⁻¹	54.5 ± 5.5 A		1.9 ± 0.1	
3.0 mg L ⁻¹	54.5 ± 3.3 A		2.0 ± 0.4	
Substance × IBA concentration		0.106^{ns}		0.232^{ns}
IBA-nZnO × 0.5 mg L ⁻¹	40.3 ± 3.7		2.0 ± 0.4	
IBA-nZnO × 1.0 mg L ⁻¹	70.4 ± 4.9		2.3 ± 0.4	
IBA-nZnO × 2.0 mg L ⁻¹	65.4 ± 5.3		1.7 ± 0.2	
IBA-nZnO × 3.0 mg L ⁻¹	57.6 ± 5.0		1.6 ± 0.4	
IBA × 0.5 mg L ⁻¹	39.7 ± 3.8		2.2 ± 0.2	
IBA × 1.0 mg L ⁻¹	55.1 ± 3.6		1.8 ± 0.3	
IBA × 2.0 mg L ⁻¹	43.7 ± 2.6		2.1 ± 0.1	
IBA × 3.0 mg L ⁻¹	51.7 ± 4.5		2.5 ± 0.5	

¹Cultures were incubated at dark during first 5 days.²According to Duncan's multiple range test ($p \leq 0.05$), means separation within columns followed by the same letter do not involve statistically significant differences.^{**}, ^{***} $p \leq 0.01$ and $p \leq 0.001$ statistically significant, ^{ns} statistically not significant.

significantly increased the in vitro rooting percentage of apple genotype #67 microcuttings compared to IAA-nZnO (34.6%). IBA (38.0%) was also significantly more effective than IAA (17.8%) on the rooting percentage. The IBA-nZnO (loaded with 4.4 μ M IBA) providing high rooting percentage and root length values as well as small callus diameter was recommended for in vitro rooting of apple microcuttings with a healthy root system.

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Table 7. The effects of IBA and zinc oxide nanoparticles loaded with IBA on in vitro root number, root length and callus diameter of apple genotype #67 microcuttings¹.

Effects	Roots per shoot (no.)	p	Root length (mm)	p	Callus diameter (mm)	p
Substance		0.554^{ns}		0.271^{ns}		0.702^{ns}
IBA-nZnO	2.4 ± 0.2		14.1 ± 2.1		5.6 ± 0.3	
IBA	2.5 ± 0.2		11.1 ± 2.1		5.8 ± 0.4	
IBA concentration		0.505^{ns}		0.047[*]		0.017[*]
0.5 mg L ⁻¹	2.9 ± 0.4		16.6 ± 2.9 A		4.8 ± 0.4 C	
1.0 mg L ⁻¹	2.3 ± 0.2		16.5 ± 3.5 A		5.3 ± 0.2 BC	
2.0 mg L ⁻¹	2.4 ± 0.2		9.8 ± 2.6 AB		6.2 ± 0.4 AB	
3.0 mg L ⁻¹	2.2 ± 0.3		7.3 ± 1.1 B		6.7 ± 0.4 A	
Substance × IBA concentration		0.640^{ns}		0.347^{ns}		0.584^{ns}
IBA-nZnO × 0.5 mg L ⁻¹	2.6 ± 0.7		20.4 ± 1.6		5.1 ± 0.3	
IBA-nZnO × 1.0 mg L ⁻¹	2.5 ± 0.4		20.2 ± 3.4		5.2 ± 0.3	
IBA-nZnO × 2.0 mg L ⁻¹	2.4 ± 0.2		7.7 ± 1.3		5.7 ± 0.3	
IBA-nZnO × 3.0 mg L ⁻¹	1.9 ± 0.5		7.9 ± 1.9		6.6 ± 1.1	
IBA × 0.5 mg L ⁻¹	3.1 ± 0.5		12.9 ± 4.9		4.4 ± 0.9	
IBA × 1.0 mg L ⁻¹	2.0 ± 0.3		12.8 ± 5.9		5.4 ± 0.3	
IBA × 2.0 mg L ⁻¹	2.5 ± 0.4		11.9 ± 5.1		6.6 ± 0.7	
IBA × 3.0 mg L ⁻¹	2.6 ± 0.5		6.8 ± 1.4		6.8 ± 0.4	

¹Cultures were incubated at dark during first 5 days.²According to Duncan's multiple range test ($p \leq 0.05$), means separation within columns followed by the same letter do not involve statistically significant differences.*, ***, $p \leq 0.05$ and $p \leq 0.001$ statistically significant, ^{ns} statistically not significant.

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