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Research article

In vitro propagation of the endemic ornamental tree, Persian ironwood (*Parrotia persica* (DC.) C.A. Mey)

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Parrotia persica (DC.) C.A. Mey is an ornamental tree native to the Hyrcanian forests in northern Iran. It displays stunning, colorful leaves in autumn and mosaic cream-grey trunks that exfoliate in winter. Because traditional horticulture methods have limited success in propagating Persian ironwood, using plant biotechnology tools to conserve and develop this endangered species is essential. The aim of the current study was, therefore, to optimize the micropropagation process of this plant. The excised shoot segments with one axillary or apical bud were dipped into 70% ethanol for 0, 30, 60, 90, 120, or 150 s, followed by disinfecting the explants in sodium hypochlorite at different concentrations (0, 0.5, 0.75, or 1%) for 10, 15, or 20 min. Moreover, the disinfecting effect of silver nanoparticles at0, 100, 200, or 400 mg L^{-1} was also examined. The established shoots were cultured on MS or WPM media containing Benzyl adenine (BA) (0, 0.3, 0.5, 0.7 or 1 mg L^{-1}), Thidiazuron (TDZ) (0, 0.022, 0.044, 0.088, 0.176 or 0.352 mg L^{-1}) or combinations of BA (0, 0.3, 0.5, 0.7 or 1 mg L^{-1}) with 0.01 mg L^{-1} Indole-3-butyric acid (IBA) were used for shoot proliferation. Rooting was studied using treatments of IBA (0, 0.25, 0.5, 1, 2, or 4 mg L^{-1}) or Naphthaleneacetic acid (NAA) (0, 0.25, 0.5, 1, 2, or 4 mg L^{-1}). The best explant sterilization procedure, with the highest survival rate ($p < 0.05$), used 70% ethanol (120 s), 0.75% sodium hypochlorite (20 min), and 200 mg L^{-1} silver nanoparticles (90 min). The WPM medium supplemented with 0.088 mg L^{-1} TDZ produced the highest mean shoot number (3.85; $p < 0.05$). Using either 1 mg L^{-1} IBA or 0.5 mg L^{-1} NAA produced the best rooting performance. Finally, the rooted plantlets were moved to greenhouse conditions and successfully acclimatized in pots containing vermiculite:perlite (1v:1v), with a 100% survival rate. This study's achievements can pave the way for future restoration plans and breeding programs for *P. persica*.

Keywords: Micropropagation, *Parrotia persica*, Persian Ironwood, Rooting, Shoot proliferation, Tissue culture.

Introduction

Parrotia persica, commonly known as Persian ironwood, is an endemic tree native to northern Iran. It is distributed across the hills and mountains of the Hyrcanian forest, particularly on steep and disturbed terrain. In hilly conditions, the root strength and density of *P. persica* reinforce soil layers on slopes. Persian ironwood's natural ability to develop suckers can strengthen bare soils, improve slope stability near forest roads, and reduce the risk of shallow landslides (Abdi *et al*., 2010).

Recent investigations revealed that *Parrotia persica* is a rich source of secondary metabolites such as flavonoids and terpenoids. It contains a significant amount of naringenin (200 mg), which has various biological activities, including anti-diabetic, immunomodulatory, antidepressant, antioxidant, anti-inflammatory, and memory-improving effects. The chemical constituent quercetin extracted from leaves and stems of *P. persica*, along with three derivatized compounds of naringenin (i.e., 7-O-propargylnaringenin, 4′,6′,4″-O-propargylchalcone, and 4′,4″-O-propargylchalcone), showed high potential for the inhibition of dementia and Alzheimer's disease (Ali *et al*., 2020). Ethnobotanical research has shown traditional use of this plant to treat various fevers and respiratory infections (Rezadoost *et al*., 2023). It possesses various biological activities such as antiperspirant, antifungal, antidiabetic, anticancer, antioxidant, and antimicrobial activities, as well as wound-healing effects

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(Ahanjan *et al*., 2007; Ahanjan *et al*., 2009; Azadbakht *et al*., 2004; Moghadam *et al*., 2017; Mohammad *et al*., 2007; Nabavi *et al*., 2008; Rezadoost *et al*., 2023).

Every attempt to propagate Persian ironwood has been futile because of numerous obstacles. The plant shows alternate bearing, and the resulting few seeds appear to exhibit multiple physical, morphological, and physiological dormancies. As the tree's common name implies, the hardwood is difficult to cut, and stem cuttings are very hard to root. Its natural multiplication in the Hyrcanian forests by a few suckers has not prevented its accelerated deterioration by extensive human exploitation.

Since the knowledge about horticultural practices and propagation of *P. persica* is scarce, *de novo* micropropagation of Persian ironwood is investigated in this research as a promising way and a preliminary step to preserve this valuable genetic resource of Iran and help future restoration and breeding programs, and possibly facilitate its widespread use in Persian gardens and landscapes.

Materials and Methods

Explant preparation

Three-year-old suckers were obtained from seventy-year-old *P. persica* (DC.) C.A. Mey trees (natural accessions), already cultivated in the National Botanic Garden of Iran, originating from Noor Jungle (Hyrcanian forests, North of Iran). Explants were taken in spring from single nodes of shoots with one axillary bud. The experiment was conducted in the tissue culture laboratory of the Department of Horticultural Science at Shiraz University.

Surface sterilization

After rinsing the shoot explants with water and a few drops of Tween-20, the nodes were disinfected with 70% ethanol for 0, 30, 60, 90, 120, and 150 seconds and subsequently sterilized with 0, 0.5, 0.75, and 1% sodium hypochlorite for 10, 15, and 20 min. We also investigated sterilization with silver nanoparticles (Nanocid, Pars Nano Nasb Co., Tehran, Iran) (0, 100, 200, and 400 mg L^{-1}) by immersing explants in liquid solution for 90 min, following Mahna et al. (2013). The nodal explants were later washed three times with sterile water and placed in the medium.

Proliferation treatments

Various concentrations of TDZ (0, 0.022, 0.044, 0.088, 0.176 and 0.352 mg L^{-1}) or combinations of BA (0, 0.3, 0.5, 0.7 and 1 mg L^{-1}) plus 0.01 mg L^{-1} IBA were used to increase the shoots in MS (Murashige & Skoog, 1962) or WPM medium (Lloyd & McCown, 1980). Every four weeks, we took new shoots for subculture and repeated this for 4 months. Tissue cultures were maintained at 25±1°C under a 16 h photoperiod (4000 lux). After 4 weeks, we cultured the proliferated shoots for rooting.

Rooting treatments

We placed new shoots in rooting medium containing different concentrations of IBA (0, 0.25, 0.5, 1, 2, and 4 mg L^{-1}) or NAA (0, 0.25, 0.5, 1, 2, and 4 mg L^{-1}).

Acclimatization treatments

Young plantlets were moved to greenhouse conditions, and their survival was examined in a variety of potting media: loam, loam-vermiculite (1:1), loam-peat moss (3:1), loam-sand-perlite (1:1:1), vermiculite-perlite (1:1), and peat moss-perlite (1:1).

Experimental design & Statistical analysis

The treatments had four replicates, each containing four explants. The study used a completely randomized design (CRD). Data were analyzed using SAS 9.4. We analyzed means using ANOVA and assessed significant differences between means with the LSD test ($p \leq 0.05$).

Results and Discussion

Ethanol sterilization

The surface sterilization parameters were significantly affected by 70% EtOH exposure time, with the highest disinfection percentage when explants were treated with detergent for 150s (100%), followed by 120s (87.5%). However, immersion beyond 120s in EtOH significantly reduced survival from 87.5% (120s) to 31.25% (150s), suggesting 120s as the best immersion time for strong sterilization and survival potential in Persian ironwood specimens (Table 1).

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Table 1. Nodal disinfection of *P. persica* by ethanol sterilization.

Sterilization time (s)	Disinfection (%)	Survival (%)
0	0	0
30	18.75^{d*}	18.75^{de}
60	50^{c}	50^{bc}
90	62.5^{bc}	62.5^{b}
120	87.5^{ab}	87.5^{a}
150	100^{a}	31.25^{cd}

* Means followed by the same letters are not significantly different according to the LSD test ($P \leq 0.05$).

Fungal and bacterial infections are among the most severe contaminations of the culture media. They are a significant challenge for *in vitro* culture and propagation of plants, especially woody plants, using tissue and organ culture. These organisms grow quickly and earlier than plant material, altering the defined conditions of the culture medium and causing poor growth or death of the specimens (Salehi *et al.*, 2016). When explants grown under natural conditions are used for in vitro cultivation, controlling internal microbial contamination becomes especially important. If these diseases are not properly controlled, they can cause fungal and bacterial infections and destroy the explants (Qaderi *et al*., 2021). Bacterial contamination is often difficult to detect because bacteria remain hidden inside the explant (Kim *et al*., 2017; Kumawat *et al*., 2022). Infected plants may reduce root numbers without visible symptoms or eventually die (Kim *et al.*, 2017).

Moreover, the concentration and duration of disinfection treatments are critical factors in explant sterilization and have been shown to ensure explant survival with minimal toxicity (Farooq *et al*., 2021). Studies have shown that 70% ethanol removes the waxy layer of the cuticle surface and, as a primary disinfectant, has a better effect on explant tissue (Ghanbari *et al*., 2021). Additionally, although excessive chemical treatment can destroy microorganisms, it can also severely damage plant tissues (Ashrafuzzaman *et al*., 2012). Therefore, choosing optimal conditions for disinfecting various plant explants is important (Gammoudi *et al*., 2022). Similarly, in the current investigation, 70% ethanol treatment for 120 seconds showed strong sterilization and survival potential for the Persian ironwood specimens. Excessive chemical treatment can destroy microorganisms, so selecting optimal conditions for disinfecting various plant samples is important (Ghanbari Jahromi *et al*., 2022).

Sodium hypochlorite sterilization

The effect of various concentrations of NaOCl (0-1%) at different immersion times (10-20 min) on surface disinfection of ironwood was studied. The results showed an increasing trend in both disinfection and survival percentages with increasing time and concentration, with the highest survival rate (93.75%) when nodal segments were treated with 0.75% NaOCl for 20 min (Table 2).

Successful micropropagation depends on the condition of the plant material at the time of explant collection from field-grown trees and on removing exogenous and endogenous contaminating microorganisms (Da Silva *et al*., 2016; Hesami *et al*., 2018). Establishing a sterile culture under laboratory conditions is the first and most important step in successful commercial plant propagation (Hesami *et al*., 2019). Many surface disinfectants, such as ethanol, hydrogen peroxide, bromine water, mercuric chloride, silver nitrate, and antibiotics, are used for surface sterilization (Mihaljević *et al*., 2013; Nongalleima *et al*., 2014). However, sodium hypochlorite is widely used because its oxidizing properties react strongly with amino acids, nucleic acids, amines, and amides (Mihaljević *et al*., 2013). In the current study, household bleach strongly suppressed infections in *Parrotia* explants.

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Table 2. Disinfection of *P. persica* explants by sodium hypochlorite.

Sodium hypochlorite (%)	Sterilization time (min)					
	10	15	20	10	15	20
	Fungal disinfection (%)			Survival (%)		
0	6.25^{d*}	6.25^{d}	6.25^{d}	6.25^{f}	6.25^{f}	6.25^{f}
0.5	31.25^{c}	43.75^{c}	62.5^{b}	31.25^{de}	43.75^{cd}	62.5^{bc}
0.75	68.75^{b}	75^{b}	93.75^{a}	68.75^{b}	81.25^{ab}	93.75^{a}
1	100^{a}	100^{a}	100^{a}	43.75^{cd}	12.5^{ef}	6.25^{f}

* Means followed by the same letters are not significantly different according to the LSD test ($P \leq 0.05$).

Silver nanoparticles sterilization

The highest disinfection level (100%) was observed when explants were placed in 400 mg L^{-1} nanosilver for 90 min. However, the highest survival percentage (93.75%) was obtained at 200 mg L^{-1} (Table 3).

Silver nanoparticles are non-toxic materials with a high ability to destroy microorganisms (Arab *et al*., 2014). This ability stems from the small size of free silver particles (Yan *et al*., 2021). They can destroy not only bacteria and fungi but also viruses (Mikhailova *et al*., 2020). Silver nanoparticles dramatically increase contact surface area with microorganisms, strengthening their antimicrobial effects (Deshmukh *et al*., 2019). The mode of action of silver nanoparticles on microbes is thought to include cell lysis and growth inhibition (Ghanbari *et al*., 2021; Mervat, 2024; Parzymies, 2021). By binding to the (SH) group of metabolic enzymes in the electron transport chain, it prevents respiration (Wang *et al*., 2021).

Also, by opening the DNA helix, silver particles inhibit cell division and damage the bacterial cell membrane, disrupting hydrogen bonds and stopping cell membrane synthesis. A similar effect is seen in fungi, where growth deteriorates after electrolyte leakage (Mousavi & Naghsh, 2021). Consistent with the aforementioned studies, silver nanoparticles also successfully controlled contamination in the micropropagation of *P. persica*.

Table 3. Disinfection of *P. persica* explants by silver nanoparticles.

Silver Nanoparticles (mg L^{-1})	Disinfestation (%)	Survival (%)
0	50 ^{bc*}	50 ^{c}
100	75 ^{ab}	75 ^{b}
200	93.75 ^{a}	93.75 ^{a}
400	100 ^{a}	37.5 ^{c}

* Means followed by the same letters are not significantly different according to the LSD test ($P \leq 0.05$).

In contrast, the use of nanosilvers for fungal and bacterial disinfection in plant tissue culture studies has been extensively studied and continues to rise (Sarmast & Salehi, 2016; Hürkan & Hürkan, 2025). Among culture media contaminants, fungi and bacteria are the most common microorganisms. These organisms grow faster than plant material, change the growth conditions for the plant sample, and cause the plant material to weaken or die. These harmful effects occur when contaminating microorganisms consume nutrients in the culture medium, excrete metabolites into the growth medium, or affect plant tissue (Ahmad *et al*., 2022).

Shoot proliferation by BA

The greatest shoot proliferation (i.e., 2.1 shoots) was obtained using WPM medium supplemented with 0.7 mg L^{-1} BA. It also produced the highest shoot length (1.14 cm) and leaf number (11.5), respectively. This result was significantly higher than that obtained with MS medium and other BA concentrations (Table 4).

The number of nutrients, vitamins, the type and concentration of plant growth regulators, and organic

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additives in the culture medium affect the success of in vitro culture. Because each genus, species, and variety responds differently to cultivation-environment factors, it is necessary to optimize and standardize cultivation conditions for each plant (Erfani *et al*., 2017). The modified MS culture medium and WPM have different macro-element concentrations. Overall, the modified MS culture medium contains more salts than the WPM culture medium. Although MS medium is widely used, it is sometimes replaced by other media, especially those with a lower nitrogen concentration in the form of ammonium nitrate. The amount of ammonium nitrate in the MS culture medium is 1105 mg L^{-1}, and in the WPM culture medium it is 455 mg L^{-1}. Most plants prefer nitrate to ammonium (Zhou *et al*., 2022) because nitrate can accumulate in vacuoles in excess amounts, but too much ammonium can be toxic (Krämer *et al*., 2022).

The chlorosis and premature aging of the *in vitro* plantlets are believed to result from high concentrations of salts, mainly nitrate and ammonium, in the culture medium, which cause ethylene release (Khan & Anis, 2012). In contrast, WPM culture medium contains more calcium than MS culture medium, and calcium plays an essential role in signaling between plant cells (Fallahpour *et al*., 2015). In addition, calcium may directly affect cell and organ growth, elongation, and division. Calcium affects intracellular pH and acts as a regulatory ion that determines the source, destination, and transfer of carbohydrates, thereby mediating effects on cell membranes and cell walls (Schumann *et al*., 2022). The culture medium can be very effective in the growth of *in vitro* plants. The type and amount of macro elements in the WPM culture medium differ from the modified MS culture medium. Microelements such as iodine and cobalt, as well as the types of vitamins, also differ between the two cultures. Previous research on *Liquidamber formosana* (Xu *et al*., 2007) and *L. styraciflua* (Sutter & Barker, 1985) showed that all shoots proliferated successfully in WPM medium. Similarly, in the current research, WPM medium produced the highest shoot length, number of shoots, and number of leaves (Table 4).

Table 4. Shoot proliferation of *P. persica* using BA in the WPM or MS medium.

BA (mg L^{-1})	Shoots No.		Shoot length (cm)		Leaves No.	
	WPM	MS	WPM	MS	WPM	MS
0	0.45^{g*}	0.45^{g}	0.38^{d}	0.38^{d}	6.25^{d}	6.25^{d}
0.3	0.95^{ef}	0.68^{fg}	0.82^{b}	0.49^{cd}	8.25^{bc}	7.75^{cd}
0.5	1.47^{bc}	1.25^{cd}	0.93^{b}	0.62^{c}	9.75^{ab}	9.25^{bc}
0.7	2.1^{a}	1.55^{b}	1.14^{a}	0.89^{b}	11.5^{a}	9.25^{bc}
1	1.05^{de}	0.77^{f}	0.92^{b}	0.57^{c}	9.25^{bc}	9^{bc}

* Means followed by the same letters are not significantly different according to the LSD test ($p \leq 0.05$).

Shoot proliferation by combination of BA with IBA

Adding auxin (0.01 mg L^{-1} IBA) to different concentrations of BA in the WPM medium increased axillary shoot growth in *Parrotia*. Higher shoots per explant (2.92 shoots) were obtained when 0.7 mg L^{-1} BA was combined with 0.0.1 mg L^{-1} IBA. This concentration also produced the longest shoot (1.53 cm) and the highest number of leaves (13.75) in Persian ironwood plantlets, significantly higher than other concentrations (Table 5).

Cytokinins are a group of plant growth regulators with growth-stimulating effects and are essential for regulating various growth and development processes (Gupta *et al*., 2022). Cytokinins in the culture medium are necessary for plant regeneration because this plant growth regulator is required for the expression of genes that drive stem bud differentiation (Bull *et al*., 2022). Different cytokinins can induce branching because of factors such as stability, mobility, and the rate of synthesis and oxidation. BA has been found to enhance tissue synthesis of natural hormones such as zeatin. Plant tissues absorb it faster than other artificial growth regulators (Lindiro *et al*., 2013; Eisa *et al*., 2022). Therefore, hormonal conditions determine the successful increase in adventitious bud formation (Sjahril *et al*., 2015). Because endogenous cytokinin levels vary, the percentage of shoot stimulation and shoot buds differs among explants (Hussein & Jawad, 2021). Cytokinins increase nutrient absorption and chlorophyll content and promote cell division and leaf development (Li *et al*., 2021). Among cytokinins, benzyladenine (BA) is the most widely used (Iiyama *et al*., 2022). Compared with

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cytokinins used alone, cytokinins combined with auxin are most potent for inducing shoot regeneration. Moreover, they increase lateral shoot growth and promote vegetative branch formation (Alsoufi *et al*., 2021). It also enlarges phloem and xylem vessels, prevents chlorophyll degradation, promotes cell division, and improves nucleic acid production (Al-Jobouri *et al*., 2020). In a previous study by Yildirim *et al*. (2012), BA use with IBA increased the shoot length of *Pistacia lentiscus*. Shoot growth and proliferation also increased as BA concentration rose from 0.1 mg L^{-1} to 0.7 mg L^{-1} in WPM medium. In the current research on Persian ironwood, we observed the highest shoot growth and number of leaves when 0.7 mg L^{-1} BA and 0.01 mg L^{-1} IBA were used.

However, higher concentrations of plant growth regulators may cause toxicity and negatively affect plant growth processes. Reducing the BA concentration in the culture medium can decrease the vitrification problem seen in plantlets at high BA concentrations. High concentrations of this plant growth regulator can stop shoot growth.

Table 5. Shoot proliferation of Persian ironwood in WPM medium containing BA in combination with 0.01 mg L^{-1} IBA.

BA (mg L^{-1})	Shoots No.	Shoot length (cm)	Leaves No.
0	0.45^{e*}	0.38^{e}	6.25^{e}
0.3	1.22^{c}	1.11^{b}	9.5^{b-d}
0.5	1.73^{b}	1.22^{b}	11.75^{ab}
0.7	2.92^{a}	1.53^{a}	13.75^{a}
1	1.58^{b}	1.21^{b}	11.25^{a-c}

* Means followed by the same letters are not significantly different according to the LSD test ($p \leq 0.05$).

Shoot proliferation by TDZ

Supplementing 0.088 mg L^{-1} TDZ to the WPM medium increased proliferation to 3.85 shoots per explant and shoot elongation to 1.36 cm. Furthermore, 12.25 leaves were produced in plantlets when 0.022 mg L^{-1} TDZ was used in the medium, with no significant difference from 0.088 mg L^{-1} TDZ (Table 6) (Figure 1).

TDZ is one of the most active cytokinins for woody plant tissue culture and promotes woody plant growth (Rohela *et al*., 2020; Naaz *et al*., 2021). A low concentration of 1 micromolar TDZ, compared to other cytokinin concentrations, causes more stimulation of shoot proliferation (Kodad *et al*., 2021). Cytokinin's high activity and TDZ's positive response in woody species have made it one of the active cytokinins for in vitro proliferation of many woody species (Ahmad *et al*., 2022). Reports indicate that TDZ may act by directly modulating endogenous plant growth regulators, acting through adenine-type cytokinin activity, stimulating endogenous cytokinins, or binding to cytokinin receptors. In some plants, TDZ has been shown to inhibit cytokinin oxidase activity (Nisler *et al*., 2018; Li *et al*., 2022). In TDZ-treated tissues, mineral ions and other stress-related metabolites accumulate simultaneously, including proline, abscisic acid, and 4-aminobutyrate (Murch *et al*., 1997). Consistent with previous findings, TDZ showed stronger shoot proliferation potential for Persian ironwood than BA.

Table 6. Shoot proliferation of *P. persica* in WPM medium containing TDZ.

TDZ (mg L^{-1})	Shoots No.	Shoot length (cm)	Leaves No.
0	0.44^{e*}	0.58^{d}	5.5^{b}
0.022	1.03^{cd}	1.06^{bc}	12.25^{a}
0.044	1.37^{b}	0.96^{bc}	7.75^{b}
0.088	3.85^{a}	1.36^{a}	11.75^{a}
0.176	1.12^{bc}	1.07^{b}	8^{b}
0.352	0.71^{de}	0.82^{cd}	6.75^{b}

* Means followed by the same letters are not significantly different according to the LSD test ($p \leq 0.05$).

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Figure 1. Microscope 1X view of shoot induction after one month in *P. persica* explants in WPM medium containing 0.088 mg L^{-1} TDZ.

Root induction by IBA

The highest rooting percentage (100%) was recorded when 2 or 4 mg L^{-1} IBA were added to the medium, with no significant difference from 93.75% rooting with 1 mg L^{-1} IBA. Using 1 mg L^{-1} IBA produced a maximum of 3.54 roots per shoot and a root length of 1.71 cm. The best concentration for rooting was 1 mg L^{-1} IBA (Table 7). Treatment with 2 mg L^{-1} IBA produced orange roots on the stem and petiole (Figure 2).

Poor rooting is a major problem in horticultural plants and causes significant losses in ornamental plants. Auxin, a plant growth regulator, stimulates rooting, and its use is the best method to achieve rapid rooting (Thangavelu *et al*., 2018). Auxin is a plant growth regulator that influences multiple physiological processes, including cell differentiation, organ development, and overall growth. (Kaviani & Negahdar, 2017b). Auxin stimulates most roots. Lower concentrations of IBA alone, or in combination with other plant growth regulators, yield better results than higher concentrations of the same regulator (Akhtar *et al*., 2015). This study also found that IBA was very effective during the rooting stage, where it promotes root meristem formation and adventitious root development. Adventitious root formation is an important step in woody plant growth (Nadal *et al*., 2022).

Inhibition of root growth at auxin concentrations above the optimum is mainly attributed to auxin-induced ethylene biosynthesis. It is difficult to prove auxin's controlling role in root growth, probably because auxin stimulates ethylene production and ethylene inhibits root growth (Taiz & Zeiger, 2006). In the present study, high auxin concentrations reduced root number and length. However, when ethylene biosynthesis is blocked, low auxin concentrations (10-9 to 10-10 mol) stimulate root growth, while higher concentrations (10-6 mol) inhibit it. Therefore, roots may need low auxin concentrations for growth. Low auxin concentrations initiate primary lateral roots and adventitious roots. Lateral roots usually appear above the elongation zone of the primary root and in root filaments (Alarcón *et al*., 2019). Auxin concentration regulates the number of wood vessels and phloem formed. High auxin concentration stimulates wood and phloem tissue differentiation, but only phloem can differentiate at low auxin concentrations. Cutting the leaves prevents restoration of vascular tissue, though exogenous auxin can mimic this role (Taiz & Zeiger, 2006).

When considering the effect of plant growth regulator treatments on rooting, carefully consider the type, concentration, duration, and application method (Zarei *et al*., 2019). To induce rooting, researchers use various auxins, especially IBA, IAA, or NAA, alone or in combination (Mohammed & Vidaver, 1990). However, in previous research on the endangered *Picea chihuahuana* Martinez shoots, 14.8 or 24.6 μM IBA as a short-term treatment (48 hrs) resulted in the best rooting percentage (López-Escamilla *et al*., 2000). In the current investigation, a much lower IBA concentration induced rooting in *Parrotia*.

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Table 7. Root induction of Persian ironwood plantlets in WPM medium containing different concentrations of IBA.

IBA (mg L^{-1})	Rooting (%)	Roots No.	Root length (cm)
0	0	0	0
0.25	31.25^{c*}	1^{d}	0.56^{c}
0.5	68.75^{b}	1.97^{c}	1.02^{b}
1	93.75^{a}	3.54^{a}	1.71^{a}
2	100^{a}	2.43^{b}	0.96^{b}
4	100^{a}	1.03^{d}	0.46^{c}

* Means followed by the same letters are not significantly different according to the LSD test ($p \leq 0.05$).

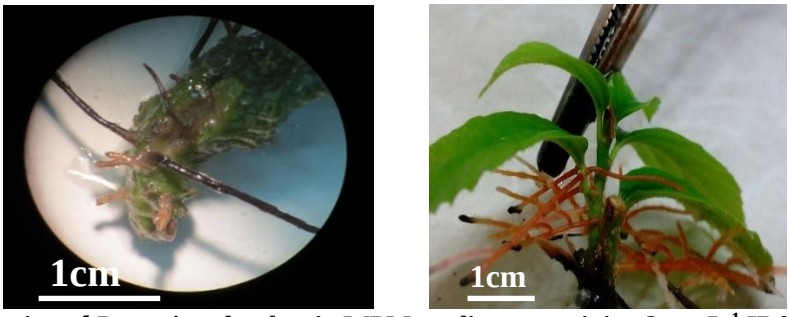

Figure 2. Root induction of *P. persica* plantlets in WPM medium containing 2 mg L^{-1} IBA. Roots were also produced on the petioles.

Root induction by NAA

The highest rooting percentage (100%) was observed with 4 mg L^{-1} NAA, although it did not differ significantly from 0.5, 1, and 2 mg L^{-1} concentrations. After auxin application, 0.5 mg L^{-1} NAA produced the highest number of roots per shoot (4.75). The latter treatment also produced the longest roots, with a length of 2.07 cm (Table 8). Aerial roots were formed above the medium, originating from the stem and petiole when 2 and 4 mg L^{-1} NAA were used (Figure 3).

Rooting is the most critical factor in successful plant propagation. Plant growth regulators, especially auxins, are vital in rooting studies. Auxins stimulate adventitious root formation, especially in woody plants (Kaviani & Negahdar, 2017a). The most common auxins used for rooting are NAA and IBA (Singh *et al*., 2014). NAA is more efficient and effective in rooting different plants, such as blueberry (Braha *et al*., 2016). Similarly, in the current study, NAA also produced more roots than IBA (Tables 7-8).

Table 8. Root induction of *P. persica* plantlets in WPM medium containing NAA.

NAA (mg L^{-1})	Rooting (%)	Roots No.	Root length (cm)
0	0	0	0
0.25	31.25^{b*}	1.5^{cd}	1.27^{bc}
0.5	87.5^{a}	4.75^{a}	2.07^{a}
1	87.5^{a}	2.56^{b}	1.53^{b}
2	93.75^{a}	1.93^{c}	1.2^{bc}
4	100^{a}	0.97^{d}	0.88^{c}

* Means followed by the same letters are not significantly different according to the LSD test ($p \leq 0.05$).

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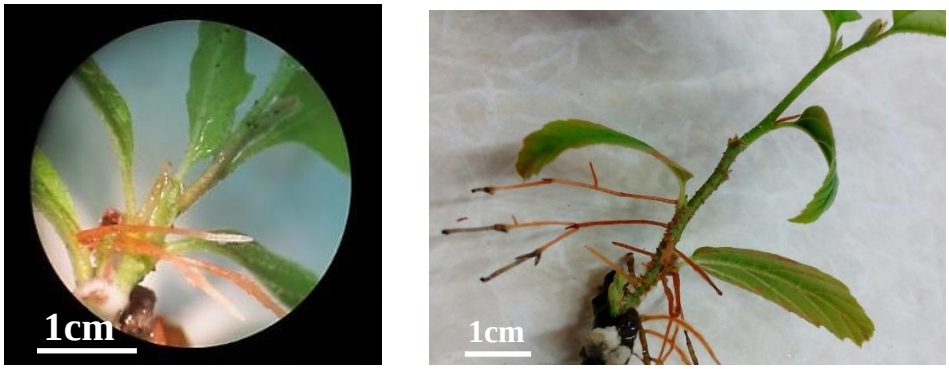

Figure 3. Root induction of *P. persica* plantlets after one month in WPM medium containing 4 mg L^{-1} NAA.

Plantlet acclimatization

Vermiculite-perlite showed the highest viability (100%) and did not differ significantly from loam-peat moss. Moreover, when plantlets were placed in the vermiculite-perlite medium, shoot length increased to 5.2 cm, significantly longer than in the other medium. Also, when vermiculite-perlite medium was chosen for acclimatization, a maximum of 6.2 leaves developed on each shoot compared with the other medium, although it did not differ significantly from the peat moss-perlite potting substance (Table 9) (Figure 4).

Plants grown in tissue culture have leaves with poor or no cuticular waxes. The seedling leaves are thin and have underdeveloped mesophyll with low chlorophyll and poorly developed chloroplasts (Chandra *et al*., 2010). Photosynthetic enzyme activity is low, reducing seedling photosynthetic efficiency. The root system is inadequate, and the hairy roots' development is weak. The vascular connection between the root and the stem is imperfect. As a result, nutrient and moisture absorption decreases, making the plant more vulnerable after pot transfer and leading to fragile seedling establishment. (Darwesh *et al*., 2015). For this reason, researchers use different methods or substrates to increase seedling survival during the adaptation period (Diaz *et al*., 2010). Cultivation media serve different functions during plant growth, including providing air and water, creating conditions for maximum root growth, and providing physical support to help the plant establish optimally. The substrate should contain many coarse particles and enough fine particles (Droudi & Safarnejad, 2017). It is also necessary to carefully choose the appropriate particle size, or combination of particle sizes, for a light medium with good drainage and aeration. Different organic and inorganic substrates allow plants to absorb nutrients and grow well by maintaining proper water and oxygen levels in the substrate (Droudi & Safarnejad, 2017). Adaptation is a vital step in micropropagation because a large percentage of plant losses occur during this stage (Droudi & Safarnejad, 2017). The plants are gradually adapted to lower relative humidity, more light, and delayed watering (Diaz *et al*., 2010). The last step, and one of the main factors in tissue culture success, is producing seedlings with proper roots and adapting rooted seedlings to ex vitro conditions (Abbaszadeh *et al*., 2018).

Maximum survival in a culture medium containing vermiculite and perlite creates suitable conditions for pot survival and root development, leading to complete plant adaptation. The lighter the substrate composition, the better tissue culture seedlings can adapt to natural conditions. That is why seedlings survive better in the vermiculite:perlite combination (Chauhan *et al*., 2020). Perlite's porous nature allows it to hold water. In addition, the small size of these materials in the culture bed improves aeration and drainage. Vermiculite is also a suitable culture medium for most plants due to its lightweight, high water-holding capacity, improved aeration and drainage, high cation exchange capacity, and low weight (Sriskanda *et al*., 2021). These results align with previous research.

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Table 9. Acclimatization of *P. persica* plantlets by different cultivation media.

Components	Viability (%)	Shoot length (cm)	Leaves No.
Loam	80^{bc*}	2.32^{b}	3.6^{cd}
Loam-vermiculite (1:1)	78^{bc}	2.28^{b}	2.4^{d}
Loam-peat moss (3:1)	94^{ab}	2.88^{b}	3.6^{cd}
Loam-sand-perlite (1:1:1)	72^{c}	2.9^{b}	4.4^{bc}
Vermiculite-perlite (1:1)	100^{a}	5.2^{a}	6.2^{a}
Peat moss-perlite (1:1)	76^{bc}	3.48^{b}	5.4^{ab}

* Means followed by the same letters are not significantly different according to the LSD test ($p \leq 0.05$).



Figure 4. Acclimatization and subsequent growth of *P. persica* plantlets.

Conclusions

Overall, the optimum explant sterilization of *P. persica* was achieved with 70% ethanol (120 seconds), 0.75% sodium hypochlorite (20 min), and 200 mg L^{-1} silver nanoparticles (90 min). WPM medium supplemented with 0.088 mg L^{-1} TDZ produced the greatest shoot proliferation, while treatment with either 1 mg L^{-1} IBA or 0.5 mg L^{-1} NAA produced the best rooting of plantlets. Acclimatization of *in vitro* plants with a mixture of vermiculite: perlite (1:1) (v/v) revealed a survival rate of 100%. These findings can inform future restoration plans and breeding programs for *P. persica*. However, future complementary studies are suggested using juvenile donor plants collected directly from the Hyrcanian forests to study the genes responsible for vibrant autumn leaf hues, natural shoot grafting, wood hardness, secondary metabolites, and their medicinal benefits.

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