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PLANT TISSUE CULTURE

In vitro propagation and assessment of genetic stability in *Eriobotrya japonica* (Thunb.) Lindl. as affected by zinc oxide nanoparticles (ZnO-NPs)

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Abstract

Eriobotrya japonica (Thunb.) Lindl. is an evergreen tree that holds economic significance. It is cultivated for its fruits, leaves and ornamental properties. Its propagation by seeds and cuttings is inefficient due to high heterozygosity, lengthy juvenile phase and reproductive cycle. This study establishes a direct *in vitro* propagation technique for the plant from the mother tree. The impact of different zinc oxide nanoparticle (ZnO-NP) concentrations on *E. japonica*'s *in vitro* propagation was assessed. Genetic stability of the micropropagated plantlets was also conducted using start codon targeted (SCoT) and sequence-related amplified polymorphism (SRAP) markers to evaluate the protocols' efficiency. Successful shoot establishment involved 1.0 mg L^{-1} 6-benzyladenine (BA) and 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA) supplemented to Murashige and Skoog (MS) medium, yielding 80% growth induction, the highest shoot number (3.72) and length (4.84 cm). MS medium with 1.0 mg L^{-1} BA and 1.0 mg L^{-1} kinetin (Kin) achieved 95% multiplication and optimal shoot number (5.70). Adding 50.0 mg L^{-1} ZnO-NPs significantly influenced shoot formation, leading to 100% growth induction, highest shoot number (7.67) and highest shoot length (4.0 cm). Rooting using 1.5 mg L^{-1} indole-3-butyric acid (IBA) in half MS medium achieved 100% rooting response and 85% survival for post-greenhouse acclimatization. Genetic stability analysis showed low degree of polymorphism (3.3%) and high percentage of monomorphic bands (96.7%) between donor and micropropagated plants. Dice's similarity coefficient displayed high similarity (0.992 to 1.000) between sub-cultured and mother plants. An effective method for *in vitro* propagation of *E. japonica* was successfully established. ZnO-NPs significantly influence shoot formation and facilitate the mass production of *E. japonica* plants with desired characteristics.

Keywords Loquat tree · Micropropagation · ZnO-NPs · Genetic stability · SCoT · SRAP

Introduction

Eriobotrya japonica (Thunb.) Lindl. (loquat tree), also known as Japanese medlar, belong to the Rosaceae family, originating naturally in southeastern China. It can grow up to 10 m in height and has broad, evergreen leaves that are used in classical Chinese herbal therapy. The fruit of the loquat is round to oblong in shape, with a thin, yellow-orange skin and juicy, flavourful flesh. It is rich in vitamins A and C, as well as dietary fiber, and is commonly consumed fresh or processed into jams, jellies and other food products. The loquat is a popular plant in many parts of the world. The tree is also grown for ornamental purposes in gardens and parks. It has been naturalized in the Mediterranean region and southern United States (Deng *et al.* 2021). Loquat is a challenging plant to improve using traditional breeding methods due to its extensive heterozygosity, prolonged juvenile phase and reproductive cycle. However, advances in biotechnology have provided new tools for improving fruit plants, including loquat.

Plant tissue culture is a crucial technique used in various areas of research and practical applications. It involves cultivating and growing plant cells, tissues or organs in a

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controlled environment with an artificial nutrient medium. This method has fundamentally changed plant biotechnology, playing a key role in producing high-quality plants. Overall, plant tissue culture is a vital method with a wide range of applications and benefits. It allows for the production of genetically identical and disease-free plant materials, ensures the reliable production of bioactive compounds and facilitates the study of plant biology. Despite some limitations, tissue culture has brought about a revolution in plant biotechnology and continues to be an essential tool for researchers and practitioners (Simsek *et al.* 2024). Micropropagation technique can be applied to various fruit plants for mass production (Hasnain *et al.* 2022). The regenerated plants showed identical morphology and genetic profiles to the mother plant, making this technique valuable for genetic transformation studies in loquat.

Previous studies have explored the *in vitro* propagation methods for loquat through indirect approaches such as callus formation. Adventitious regeneration and somatic embryogenesis are known to carry a risk of somaclonal variation. Deng *et al.* (2021) developed a regeneration method for *E. japonica* cv. Dawuxing using young leaves from 2-yr-old grafted seedlings. They achieved an 89.2% callus induction rate on MS medium with 0.5 mg L^{-1} 6-benzyladenine (BA) and 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Adventitious buds formed on MS medium with 0.8 mg L^{-1} thidiazuron (TDZ), 0.3 mg L^{-1} NAA and 0.2 mg L^{-1} $AgNO_3$, with a 30.6% shoot induction rate. Rooting was achieved at 68.9% on ½ MS medium containing 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} IBA. Similarly, Abbasi *et al.* (2013) established an *in vitro* culture method for loquat cultivar Mardan using shoot apices, achieving a 70% survival rate through callus formation using 10.0% NaOCl for surface sterilization. Optimal shoot morphogenesis was achieved with a combination of BAP, kinetin and 2iP, while higher cytokinin levels led to callogenesis and vitrification. For rooting, the best results were obtained using half-strength MS medium supplemented with NAA, IBA and paclobutrazol, enhancing the rhizogenesis of micro-shoots. Lomtatidze *et al.* (2009) also described the optimal conditions for mass production of somatic clones from *E. japonica*, examining the effects of various phytohormones on shoot rooting. Low concentrations of benzylaminopurine (BAP) stimulated apical morphogenesis, while higher BAP levels increased callogenesis, particularly when combined with 1-naphthaleneacetic acid (NAA). The most favourable condition for morphogenesis involved 1 µM NAA. For shoot rooting, indolebutyric acid (IBA) at 5 µM was most effective, achieving an 80 to 90% rooting rate, making this approach valuable for efficient loquat propagation. Li *et al.* (2008) developed an embryogenesis and plant regeneration from anther culture in loquat. Given these findings, there is a need to refine a protocol for direct *in vitro* propagation of loquat using vegetative shoots from the loquat tree.

Nanoparticles are small particles with a size of less than 100 nm that have unique physicochemical properties, such as high surface area, reactivity and stability. Nanoparticles can be incorporated into the culture medium used for *in vitro* propagation to improve growth and foster plant maturation, and to improve the efficiency of the tissue culture process (Awad *et al.* 2020). Zinc, a vital plant micronutrient, is pivotal for growth and development. It aids enzymes, protein synthesis, carbohydrate metabolism and growth hormone control. Zinc deficiency in soil can negatively impact plant growth. Additionally, zinc helps plants combat oxidative stress by activating antioxidant enzymes. Zinc oxide nanoparticles (ZnO-NPs) are extensively employed across diverse industries due to their exceptional antimicrobial properties. In plants, ZnO-NPs play an active role in regulating different mechanisms that respond to abiotic stresses. Zinc oxide nanoparticles, due to their small size, can enhance plant growth by being absorbed and dispersed within plant cells. Factors such as concentration, solubility, exposure time and plant anatomy affect nanoparticle absorption and translocation (Bedi and Kaur 2015). ZnO nanoparticles are crucial in plant tissue culture. Al-Mayahi (2021) found that adding specific levels of ZnO NPs to the culture media improved the *in vitro* propagation of date palms (*Phoenix dactylifera* L.) cv. Quntar. Similarly, Ochatt *et al.* (2023) demonstrated that using 70 nm ZnO NPs in the *in vitro* multiplication of *Agave salmiana* var. Ayoteco enhanced organogenesis and modified the structures of shoots and seedlings, especially the stomata.

While ZnO-NPs were chosen for their known benefits in enhancing plant growth, nutrient uptake and stress resistance, other nanoparticles have also been explored in tissue culture. For instance, silver nanoparticles (Ag-NPs) are widely used for their antimicrobial properties, improving the sterilization of explants and reducing contamination rates. Gold nanoparticles (Au-NPs) have been employed to enhance genetic transformation efficiency and support plant regeneration. Similarly, iron oxide nanoparticles (Fe_3O_4-NPs) have been reported to improve root development and overall plant health by facilitating iron availability. The inclusion of ZnO-NPs, compared to other NPs, was based on their specific benefits of promoting cell division and elongation, enhancing photosynthetic activity and providing essential micronutrients, making them particularly suitable for the current study's objectives in tissue culture (Bhattacharjee *et al.* 2022).

Somaclonal variations play a crucial role in enhancing key horticultural crops, providing valuable natural diversity for breeders. Similar to chemically and physically induced mutations, these variations from plant tissue culture can be utilized in crop enhancement programs (Rajan and Singh 2021).

However, somaclonal variation can disrupt the propagation of elite germplasm, making it essential to maintain genetic uniformity in cultured plants to preserve desired traits and ensure quality, making the assessment of genetic fidelity in regenerated plants vital (Ioannidis *et al.* 2022).

Diverse DNA-based molecular methods were employed to identify genetic differences in micropropagated plants. Start codon targeted (SCoT) and sequence-related amplified polymorphism (SRAP) markers effectively ensure genetic fidelity, as shown in various plant studies (EL-Shaer and Ibrahim 2021; Ioannidis *et al.* 2022; Ismail *et al.* 2022).

SCoT markers are based on the conserved regions around the start codon (ATG) in plant genomes. They are highly reproducible, simple and efficient, making them ideal for assessing genetic variation, polymorphism and gene expression changes in plants subjected to tissue culture conditions (Erişen *et al.* 2020). SCoT markers are particularly useful in monitoring somaclonal variation, ensuring the genetic stability of *in vitro* propagated plants as reported successfully in different plants such as *Digitalis purpurea* (Bansal *et al.* 2022) and *Carthamus tinctorius* (Ejaz *et al.* 2022).

SRAP markers target open reading frames (ORFs) in the genome, focusing on gene-rich regions, which makes them effective in studying functional genetic variation. SRAP is useful for evaluating genetic diversity, identifying polymorphisms and comparing genetic similarities between tissue culture–derived plants and their parent plants (Borsai *et al.* 2020). This technique is especially beneficial in tissue culture for optimizing regeneration protocols and assessing the genetic integrity of micropropagated plants (Lakshmanan *et al.* 2007; Senapati *et al.* 2013; Borsai *et al.* 2020).

Both SCoT and SRAP markers provide valuable insights into the genetic changes that occur during tissue culture, supporting the development of efficient and genetically stable propagation methods.

This study represents the first effort to develop a direct *in vitro* propagation method for *E. japonica* using vegetative shoots from the mother tree which has not been achieved so far. Also, it aims to evaluate the effectiveness of ZnO nanoparticles at various concentrations on the *in vitro* multiplication of the plant and assess the genetic stability of micropropagated plantlets using SCoT and SRAP markers to determine the protocol's effectiveness.

Materials and Methods

***In vitro* culture of *E. japonica* - plant material collection and sterilization** Terminal actively growing shoots of a 6-yr-old *E. japonica* plant were obtained from a private orchard in Wadi El-molak Ismailia Governorate, Egypt. They underwent a thorough washing using running tap water and a detergent for a duration of 5 min. Shoots were initially exposed to 70% ethanol for 1 min, then sanitized with 0.1% mercuric chloride ($HgCl_2$) for 2 min. Following the previous treatment, shoots were soaked in 20% Clorox (5.25% sodium hypochlorite) for 30 min and washed four to five times with sterile distilled water.

Culture establishment Under sterile conditions, *E. japonica* stem nodal segments were cultured on Murashige and Skoog (MS) (Murashige and Skoog 1962) containing 3.0% sucrose, 0.01% meso-inositol, 0.3% phytagel and 0.1 mg L^{-1} β-naphthalene acetic acid (NAA) combined with 6-benzyladenine (BA) or kinetin (Kin) (0.5, 1.0, 2.0 mg L^{-1}) (Sigma Cell Culture, min. 90%, St. Louis, MO). A control lacking growth regulators was employed. The medium pH was set at 5.7 ± 0.1, autoclaved, and cultures incubated at 25 ± 1 °C, 16/8-hr light/dark with 2500 to 3000 lx. After 8 wk, survival percentage (%), shoot growth percentage (%), axillary shoot count and average length of shoots (cm) were assessed.

Culture multiplication To enhance the proliferation and growth of *in vitro* shoots of *E. japonica*, axillary shoots obtained during the establishment stage were divided into two to three segments. These segments were then multiplied using the two most successful treatments identified in the previous experiments: (1) MS medium containing 1.0 mg L^{-1} Kin and (2) MS medium containing 1.0 mg L^{-1} BA. Additionally, a third treatment consisting of MS medium containing 1.0 mg L^{-1} Kin and1.0 mg L^{-1} BA was used for further multiplication of the shoots. A control lacking growth regulators was employed. Growth parameters were recorded after 8 wk.

Nanoparticle treatment ZnO nanoparticles (20 nm, Sigma-Aldrich, Darmstadt, Germany) were tested to enhance *E. japonica in vitro* multiplication. Using MS medium, three treatments (1.0 mg L^{-1} Kin, 1.0 mg L^{-1} BA, 1.0 mg L^{-1} Kin plus 1.0 mg L^{-1} BA) were coupled with ZnO-NPs (50.0, 100.0, 200.0 mg L^{-1}). MS medium lacking PGRs was used as control. Medium pH was adjusted at 5.7, then medium was autoclaved at 121 °C, 1.04 kg cm^{-2} for 15 min, and cultures were incubated at 27 ± 2 °C, 16/8-hr light/dark with fluorescent lamps. Regular subculturing occurred every 4 wk. After 4 wk, growth through culture response (%), axillary shoot number and length (cm) were evaluated.

Rooting of axillary shoots and acclimatization stages *E. japonica*'s axillary shoots (two to three nodes) were cultured on medium for root induction. The medium included ½ MS salts, 3.0% sucrose, 0.01% meso-inositol, 0.3% phytagel and varying IBA concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1}). After 4 weeks, rooting parameters (rooting percentage (%), root number and root length (cm)) were recorded. Rooted plantlets were transplanted to 1:1:1(v/v/v)

sand-soil-peat moss mix in pots. Pots were covered initially with clear plastic for keeping humidity, later making pores to reduce excess water (after 2 d). The covered pots were moved to a shaded greenhouse and the plastic sheets were completely removed 7 to 12 d after transfer.

Experimental design and statistical analysis In each experiment, 40 explants were used per treatment and the entire experiment was replicated four times. A fully random design was used. Data underwent analysis of variance (ANOVA), and for significance, Duncan's test (Duncan 1955) modified by Snedecor and Cochran (1990) was applied. Shared letters in means indicate no significant difference ($P \leq 0.05$).

Assessment of genetic homogeneity by SCoT and SRAP To investigate the genetic stability of the *in vitro* multiplied plants, two types of markers, SCoT and SRAP, were employed. Cultured plants on the most favourable multiplication medium, with and without ZnO-NPs, underwent seven consecutive subcultures (each subculture was every 4 wk) which were used to assess any potential alterations in the genetic makeup of the plants during the course of subculturing.

Plant material for genomic DNA isolation Approximately 2 g of fresh, healthy green leaves from the middle section of the *in vitro* plantlets were taken. Five samples (taken from five plants) were taken from plant material that had undergone seven subcultures (each subculture was every 4 wk) on MS medium with 1.0 mg L^{-1} BA and Kin. Another five samples (taken from five plants) were collected from plant material that had undergone the same process of subculturing but with the addition of 50.0 mg L^{-1} ZnO-NPs. Additionally, two samples were obtained from field-grown mother plant. Samples were carefully wrapped in aluminium foil, labelled and stored at − 20 °C until further analysis. DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from leaves as per instructions. DNA purity and quantity were assessed with NanoDrop 2000 Spectrophotometer (Thermo Scientific, Dreieich, Germany).

SCoT and SRAP analysis For SCoT analysis, 12 primers were used for polymorphism identification (Table 1), following Ibrahim *et al.* (2019). The PCR reaction (20.0 µL) contained 10.0 µL Master Mix (Sigma, Burlington, MA), 2.5 µL primer (10.0 pcmol), 2.5 µL template DNA (10.0 ng) and 5.0 µL dH2O. Amplification was performed using a Perkin-Elmer/GeneAmp® PCR System 9700 with an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 50 s and elongation at 72°C for 1 min, ending with a final extension at 72°C for 7 min.

Table 1. Name and sequence of SCoT and SRAP primers employed for evaluating genetic stability in *Eriobotrya japonica* (Thunb.) Lindl. *in vitro* cultured plants

Primer name	Sequence
SCoT primers used	
SCoT-1	5′-ACGACATGGCGACCACGC-3′
SCoT-2	5′-ACCATGGCTACCACCGGC-3′
SCoT-3	5′-ACGACATGGCGACCCACA-3′
SCoT-4	5′-ACCATGGCTACCACCGCA-3′
SCoT-5	5′-CAATGGCTACCACTAGCG-3′
SCoT-6	5′-CAATGGCTACCACTACAG-3′
SCoT-7	5′-ACAATGGCTACCACTGAC-3′
SCoT-8	5′-ACAATGGCTACCACTGCC-3′
SCoT-9	5′-ACAATGGCTACCACCAGC-3′
SCoT-10	5′-ACAATGGCTACCACTACC-3′
SCoT-11	5′-CAACAATGGCTACCACCG-3′
SCoT-12	5′-ACCATGGCTACCACGGCA-3′
SRAP primers used	
SRAP-01	Forward: me1-5′-TGAGTCCAAACCGGATA-3′ Reverse: em1-5′-GACTGCGTACGAATTAAT-3
SRAP-02	Forward: me1-5′-TGAGTCCAAACCGGATA-3′ Reverse: em2-5′-GACTGCGTACGAATTTGC-3′
SRAP-03	Forward: me1-5′-TGAGTCCAAACCGGATA-3′ Reverse: em3-5′-GACTGCGTACGAATTGAC-3′
SRAP-04	Forward: me2-5′-TGAGTCCAAACCGGAGC-3′ Reverse: em1-5′-GACTGCGTACGAATTAAT-3
SRAP-05	Forward: me2-5′-TGAGTCCAAACCGGAGC-3′ Reverse: em2-5′-GACTGCGTACGAATTTGC-3′
SRAP-06	Forward: me2-5′-TGAGTCCAAACCGGAGC-3′ Reverse: em3-5′-GACTGCGTACGAATTGAC-3′
SRAP-07	Forward: me3-5′-TGAGTCCAAACCGGAAT-3′ Reverse: em3-5′-GACTGCGTACGAATTGAC-3′
SRAP-08	Forward: me3-5′-TGAGTCCAAACCGGAAT-3′ Reverse: em4-5′-GACTGCGTACGAATTTGA-3′
SRAP-09	Forward: me4-5′-TGAGTCCAAACCGGACC-3′ Reverse: em3-5′-GACTGCGTACGAATTGAC-3′
SRAP-10	Forward: me4-5′-TGAGTCCAAACCGGACC-3′ Reverse: m4-5′-GACTGCGTACGAATTTGA-3′

SCoT start codon targeted, *SRAP* sequence-related amplified polymorphism.

For SRAP analysis, ten primer combinations from Macrogen Company were used to assess polymorphism (Table 1), following the methods of Dhutmal *et al.* (2018) and Ibrahim *et al.* (2019). The PCR reaction was conducted in a 20 µL volume containing 10.0 µL Master Mix, 1.5 µL of both forward and reverse primers (10.0 pcmol each), 2.0 µL of template DNA (10.0 ng) and 5.0 µL of dH2O. PCR was performed using a Perkin-Elmer/GeneAmp® PCR System 9700, with the following conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 50 s, annealing at 40°C for 50 s and elongation

at 72°C for 1 min. A final extension step was carried out at 72°C for 7 min.

PCR product detection and data analysis PCR results were analyzed using 1.5% agarose gel by electrophoresis, ethidium bromide (0.5 ug mL^{-1}) and 1 × TBE buffer at 95 V. DNA bands were observed under UV light and captured with a BIO-RAD 2000 Gel Documentation System. For SCoT and SRAP examination, distinct bands were assessed visually as present (1) or absent (0) for all samples. Data encompassed polymorphic and monomorphic bands. A binary matrix was formed. Dice's coefficients (Dice 1945) were calculated between genotypes using unweighted pair group method with arithmetic averages (UPGMA). This matrix generated a phylogenetic tree (dendrogram) using PAST software Version 1.91 (Hammer *et al.* 2001).

Results

***In vitro* culture of E. japonica - culture establishment from stem nodal segments** The sterilization process of *E. japonica* stem nodal segments resulted in a survival rate ranging from 80 to 100%. When these segments were cultured in MS media supplemented with higher concentrations of cytokinins (1.0 and 2.0 mg L^{-1} of BA or Kin), shoots exhibited a 100% survival rate. According to the data presented in Table 2, the percentage of growth induction (percentage of explants that show induction of axillary shoots) ranged from 60 to 80%. The highest concentrations of cytokinins (1.0 and 2.0 mg L^{-1} BA or Kin) exhibited 80% of growth induction. In terms of mean number and length of new shoots, the best outcome (3.72 shoots per explant, 4.84 cm length) occurred with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA on basal MS medium (Fig. 1*a*). Interestingly, there were no significant differences between the aforementioned optimal



Figure 1. *In vitro* propagation stages of *Eriobotrya japonica* (Thunb.) Lindl. (loquat). (*a*) *In vitro* establishment of stem nodal segments on Murashige and Skoog (MS) medium with 1.0 mgL^{-1} 6-benzyladenine (BA) and 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA). (*b*) Multiplication of axillary shoots on MS medium with 1.0 mg L^{-1} BA and 1.0 mg L^{-1} kinetin (Kin). (*c*) Multiplication of axillary shoots on MS medium with 1.0 mg L^{-1} BA, 1.0 mg L^{-1} Kin and 50 mg L^{-1} zinc oxide nanoparticles (ZnO-NPs). (*d*) Rooted plantlet after culture in half-strength MS medium containing 1.5 mg L^{-1} indole-3-butyric acid (IBA). (*e*) Acclimatized plantlets growing in the greenhouse. (*f*) Plants grown outdoors following greenhouse acclimatization.

Table 2. Influence of 0.1 mg L^{-1} NAA with varying BA or Kin levels in Murashige and Skoog medium on *Eriobotrya japonica* (Thunb.) Lindl. nodal segment establishment *in vitro* after 8 wk

PGR (mg L^{-1})			Survival (%) ± SE	Growth induction (%) ± SE	Average no. of axillary shoots explant^{-1} ± SE	Average length of axillary shoots (cm) ± SE
BA	Kin	NAA				
0.0	0.0	0.0	80 ± 1.15^{c}	60 ± 1.70^{c}	1.16 ± 0.02^{c}	4.56 ± 0.20^{ab}
0.5	0.0	0.1	80 ± 0.57^{c}	70 ± 0.56^{b}	2.25 ± 0.01^{b}	2.47 ± 0.01^{c}
1.0	0.0	0.1	100 ± 0.00^{a}	80 ± 1.13^{a}	3.72 ± 0.0^{ab}	4.84 ± 0.00^{a}
2.0	0.0	0.1	100 ± 0.00^{a}	80 ± 0.75^{a}	2.16 ± 0.02^{b}	2.97 ± 0.01^{abc}
0.0	0.5	0.1	90 ± 1.73^{b}	70 ± 1.01^{b}	2.25 ± 0.01^{b}	3.19 ± 0.01^{abc}
0.0	1.0	0.1	100 ± 0.00^{a}	80 ± 1.13^{a}	3.25 ± 0.01^{ab}	3.50 ± 0.02^{abc}
0.0	2.0	0.1	100 ± 0.00^{a}	80 ± 0.01^{a}	2.00 ± 0.05^{b}	1.83 ± 0.01^{c}

PGR plant growth regulators, *BA* 6-benzyladenine, *Kin* kinetin, *NAA* α-naphthaleneacetic acid, *SE* standard error.

In the same *column*, identical *letters* indicate that there is no significant difference ($P \leq 0.05$).

combination and the combination of 1.0 mg L^{-1} Kin with 0.1 mg L^{-1} NAA. The latter combination resulted in an average of 3.25 shoots per explant, with an average length of 3.5 cm.

Culture multiplication In this experiment, the goal was to enhance the proliferation and growth of *in vitro* established shoots of *E. japonica*. MS medium containing the most successful levels of Kin or BA without NAA, as well as a combination of both, was tested. The results from Table 3 indicate that the percentage of growth induction ranged from 65 to 95%. The highest growth induction percentage (95%) was obtained when MS medium was incorporated with 1.0 mg L^{-1} Kin and 1.0 mg L^{-1} BA.

With the exception of the control group, there were no significant distinctions observed in mean shoot numbers among the examined media. Nevertheless, the most prominent result emerged with the MS medium enriched by 1.0 mg L^{-1} Kin and 1.0 mg L^{-1} BA, yielding the greatest percentage of axillary shoot growth and mean shoot number per explant (5.70 shoots on average, Fig. 1*b*). Also, the shoots' average length with this blend was 2.88 cm. These findings suggest that the combination of 1.0 mg L^{-1} Kin and 1.0 mg L^{-1} BA is the most effective treatment for enhancing shoot multiplication in *E. japonica*.

Effect of zinc oxide nanoparticles (ZnO-NPs) on *E. japonica* multiplication In order to promote plant's *in vitro* growth, three concentrations of ZnO-NPs (50.0, 100.0 and 200.0 mg L^{-1}) were added to the most successful multiplication media (MS containing 1.0 mg L^{-1} BA, MS containing 1.0 mg L^{-1} Kin and MS containing 1.0 mg L^{-1} BA and 1.0 mg L^{-1} Kin) (Table 4). ZnO-NPs significantly impacted shoot formation in cultured buds, causing noticeable differences.

The best growth induction percentages (90 and 100%), along with the highest numbers of shoots per explant, were resulted when 50.0 mg L^{-1} ZnO-NPs were used. However, when examining all treatments supplemented with 50.0 mg L^{-1} of ZnO-NPs, it was observed that the highest average shoot count (7.67) with 4.0-cm length occurred consistently with 1.0 mg L^{-1} from each Kin and BA along with 0.0 mg L^{-1} ZnO-NPs in MS medium (Fig. 1*c*). Interestingly, higher ZnO-NPs concentrations led to reduced growth induction percentage and mean shoot numbers.

Root development and successful plantlet acclimatization Microshoots from favourable multiplication medium (MS containing 1.0 mg L^{-1} BA, 1.0 mg L^{-1} Kin and 50 mg L^{-1} ZnO-NPs) were moved to MS rooting media for rooting induction of *E. japonica*. IBA-supplemented media for root induction was examined. The percentage of root

Table 3. Impact of MS medium containing 1.0 mg L^{-1} BA or Kin, separately or combined, on *Eriobotrya japonica* (Thunb.) Lindl. nodal segment multiplication after 8 wk

Cytokinins (mg L^{-1})		Growth induction (%) ± SE	Average number of axillary shoots explant^{-1} ± SE	Average length of axillary shoots (cm) ± SE
BA	Kin			
0.0	0.0	65 ± 0.75^{c}	1.12 ± 0.01^{b}	4.91 ± 0.01^{a}
1.0	0.0	85 ± 1.40^{b}	5.32 ± 0.01^{a}	2.80 ± 0.03^{b}
0.0	1.0	83 ± 1.13^{b}	4.80 ± 0.02^{a}	2.52 ± 0.02^{b}
1.0	1.0	95 ± 0.17^{a}	5.70 ± 0.03^{a}	2.88 ± 0.00^{b}

BA 6-benzyladenine, *Kin* kinetin, *SE* standard error.

In the same *column*, identical *letters* indicate that there is no significant difference ($P \leq 0.05$).

Table 4. Impact of varied ZnO-NP concentrations in MS medium with 1.0 mg L^{-1} BA or Kin, or their combination, on *Eriobotrya japonica* (Thunb.) Lindl. *in vitro* multiplication after 4 wk

ZnO nanoparticles (mg L^{-1})	PGR (mg L^{-1})		Growth induction (%) ± SE	Average number of axillary shoots explant^{-1} ± SE	Average length of axillary shoots (cm) ± SE
	BA	Kin			
0	0.0	0.0	63 ± 0.57^{e}	1.50 ± 0.03^{d}	3.90 ± 0.01^{a}
50	1.0	0.0	90 ± 1.11^{b}	5.67 ± 0.03^{ab}	1.88 ± 0.04^{cd}
50	0.0	1.0	90 ± 1.80^{b}	6.00 ± 0.11^{ab}	1.52 ± 0.01^{d}
50	1.0	1.0	100 ± 00^{a}	7.67 ± 0.04^{a}	4.00 ± 0.05^{a}
100	1.0	0.0	80 ± 2.88^{c}	3.00 ± 0.05^{c}	2.83 ± 0.02^{abc}
100	0.0	1.0	80 ± 1.70^{c}	5.00 ± 0.20^{bc}	1.50 ± 0.05^{d}
100	1.0	1.0	82 ± 1.15^{c}	5.44 ± 0.02^{b}	1.67 ± 0.01^{cd}
200	1.0	0.0	70 ± 0.57^{d}	3.00 ± 0.02^{c}	2.33 ± 0.2^{bcd}
200	0.0	1.0	78 ± 0.57^{c}	4.33 ± 0.07^{bc}	2.17 ± 0.04^{bcd}
200	1.0	1.0	79 ± 0.57^{c}	5.33 ± 0.01^{b}	3.33 ± 0.02^{ab}

PGR plant growth regulators, *BA* 6-benzyladenine, *Kin* kinetin, *SE* standard error.

In the same *column*, identical *letters* indicate that there is no significant difference ($P \leq 0.05$).

Table 5. Effect of different IBA concentrations in ½ Murashige and Skoog medium on *Eriobotrya japonica* (Thunb.) Lindl. root development after 4 wk

IBA (mg L^{-1})	Rooting induction (%) ± SE	Mean number of roots explant^{-1} ± SE	Mean length of roots (cm) ± SE	Mean length of shoots (cm) ± SE
0.0	50 ± 5.77^{c}	0.75 ± 0.03^{b}	0.25 ± 0.01^{c}	1.5 ± 0.06^{b}
0.5	50 ± 11.54^{c}	2.25 ± 0.02^{b}	2.25 ± 0.02^{abc}	3.5 ± 0.05^{ab}
1.0	60 ± 11.54^{b}	6.00 ± 0.28^{b}	0.79 ± 0.02^{bc}	7.13 ± 0.02^{a}
1.5	100 ± 00.00^{a}	13.25 ± 0.14^{a}	3.21 ± 0.03^{a}	5.75 ± 0.11^{a}
2.0	100 ± 00.00^{a}	7.25 ± 0.14^{ab}	2.62 ± 0.06^{ab}	6.50 ± 0.17^{a}
2.5	100 ± 00.00^{a}	6.50 ± 0.28^{ab}	2.29 ± 0.02^{abc}	6.50 ± 0.28^{a}

IBA indole 3-butyric acid, *SE* standard error.

In the same *column*, identical *letters* indicate that there is no significant difference ($P \leq 0.05$).

induction ranged from 50 to 100% (Table 5). IBA at 1.5, 2.0 and 2.5 mg L^{-1} led to 100% rooting, yet more IBA lowered mean root count. Notably, ½ MS containing 1.5 mg L^{-1} IBA exhibited the highest average roots (13.25) and root length (3.21), along with longest shoot length (Fig. 1*d*). In the greenhouse environment, the plantlets that were generated through *in vitro* techniques and had strong root systems that were effectively acclimatized in the greenhouse conditions (Fig. 1*e*). The transplants exhibited positive progress and displayed consistent development and uniformity. Regenerated plantlets displayed an 85% survival rate.

Genetic homogeneity analysis with SCoT and SRAP SCoT and SRAP markers were successfully assessed genetic similarities between *E. japonica* mother plant and sub-cultured plants. Sub-cultured plants underwent seven rounds on favorable medium (MS containing 1.0 mg L^{-1} BA and 1.0 mg L^{-1} Kin), with/without ZnO-NPs. Tables 6 and 7 and Figs. 2 and 3 depict 12 samples: 1 and 2 (mother plant), 3–7 (*in vitro* raised plants on the best multiplication medium (MS containing1.0 mg L^{-1} BA and 1.0 mg L^{-1} Kin) without ZnO-NPs) and 8–12 (*in vitro* raised plants on the best multiplication medium with the best concentration of ZnO-NPs (50 mg L^{-1})).

SCoT analysis Using 12 SCoT primers, amplification of SCoT fragments occurred. The number of bands produced by each primer differed, with a minimum of 8 bands observed for SCoT-05 and a maximum of 20 bands observed for SCoT-07 (Table 6). Overall, 160 scorable, reproducible bands resulted, averaging 13.3 bands per primer (Table 7). Amplicons ranged from 110 to 1600 bp (Tables 6 and 7). Most bands (154 out of 160) were monomorphic, indicating a high level of genetic stability. Six primers (1, 2, 4, 6, 7, 10) had one polymorphic band each. Representative gel profiles for all primers are shown in Fig. 2.

SRAP analysis Ten SRAP primers were employed, yielding resolvable, reproducible and scorable SRAP fragments. Scorable bands per primer varied between 6 (SRAP-04) and 14 (SRAP-02). DNA profile by SRAPs is shown in Fig. 3 and summarized in Table 6. In total, SRAP markers generated 108 bands, averaging 10.8 bands per primer. Primers produced distinct sets of amplified fragments, sizes ranging from 150 to 1650 bp (Table 7).

Comparing SRAP primer bands among *in vitro* plants and the mother plant, it was noted that the majority of bands were monomorphic, indicating genetic similarity. However, three SRAP primers, namely SRAP-01, SRAP-02 and SRAP-05, displayed one polymorphic band each. The polymorphic bands were observed at positions 960 bp, 1300 bp and 1150 bp for SRAP-01, SRAP-02 and SRAP-05, respectively. Figure 3 displays gel images depicting these specific results.

Overall, the evaluation of genetic stability through SCoT analysis and SRAP markers revealed a low degree of polymorphism (3.3%) and a high percentage of monomorphic bands (96.7%) between the mother plant and sub-cultured regenerated plants of *E. japonica* raised with the most favorable multiplication medium, with and without ZnO-NPs.

Dendrogram The study utilized Dice's similarity coefficient from the combined data of SCoT and SRAP to estimate the similarity indices between the *in vitro* produced plants and their mother plant, which ranged from 0.992 to 1.000. UPGMA analysis was then performed, which divided the 12 samples into two main groups at a similarity coefficient of 0.992, indicating relatively minor genetic differences between the mother plant and *in vitro* regenerants. Group 1 consisted of mother plant (1 and 2) and *in vitro* plants (3 and 4) that were cultivated on the best multiplication medium without ZnO-NPs. Conversely, the second group encompassed a combination of *in vitro* propagated plants (5, 6 and 7) on the best multiplication medium without ZnO-NPs and *in vitro* propagated plants (8, 9, 10, 11 and 12) on the best multiplication medium with ZnO-NPs (Fig. 4). Notably, samples 3 and 4 displayed the highest genetic similarity with the mother plant, boasting a similarity coefficient of 1.00,

Table 6. Total number and size range of amplified fragments produced by SCoT and SRAP analysis in *Eriobotrya japonica* (Thunb.) Lindl

Primers code	Number of amplicons	Number of monomorphic bands	Number of polymorphic bands	Monomorphic bands (%)	Polymorphic bands (%)	Size of amplicons (bps)
SCoT-01	11	10	1	91	9	130–1200
SCoT-02	14	13	1	93	7	210–1500
SCoT-03	12	12	0	100	0	150–1400
SCoT-04	12	11	1	92	8	130–1150
SCoT-05	8	8	0	100	0	110–890
SCoT-06	11	10	1	91	9	150–1250
SCoT-07	20	19	1	95	5	140–1150
SCoT-08	13	13	0	100	0	190–820
SCoT-09	13	13	0	100	0	220–1600
SCoT-10	17	16	1	94	6	140–1300
SCoT-11	13	13	0	100	0	160–660
SCoT-12	16	16	0	100	0	190–790
SRAP-01	12	11	1	92	8	290–1650
SRAP-02	14	13	1	93	7	220–1300
SRAP-03	11	11	0	100	0	190–1050
SRAP-04	6	6	0	100	0	200–780
SRAP-05	12	11	1	92	8	240–1150
SRAP-06	11	11	0	100	0	160–570
SRAP-07	10	10	0	100	0	200–530
SRAP-08	9	9	0	100	0	200–750
SRAP-09	11	11	0	100	0	150–1300
SRAP-10	12	12	0	100	0	200–1250
Total	268	259	9	-	-	
Average	11.95	11.5	0.45	96.7	3.3	

SCoT start codon targeted, *SRAP* sequence-related amplified polymorphism.

Table 7. Summary of SCoT and SRAP amplified products from twelve samples of *Eriobotrya japonica* (Thunb.) Lindl

Description	SCoT	SRAP	SRAP and SCoT
Total bands scored	160	108	268
Number of monomorphic bands	154	105	259
Number of polymorphic bands	6	3	9
Number of primers used	12	10	22
Average number of fragments per primer	13.3	10.8	24.1
Size range of amplified fragments (bp)	110–1600	150–1650	110–1650

SCoT start codon targeted, *SRAP* sequence-related amplified polymorphism.

while the similarity coefficient between the mother plant and all other *in vitro* produced plants was 0.99 (Table 8). This suggests a strong genetic resemblance between the mother plant and most of the *in vitro* regenerants, indicating successful propagation and limited genetic variation among the studied samples (Fig. 4).

Dice's similarity indices were used to measure the genetic variance among tested samples. Samples 1 and 2 (mother plant) showed a close genetic relationship, with very low distances of 0.004 between samples 3 and 4 to 1 and 2, and 0.002 between samples 5 and 6 to 1 and 2. Sample 7 displayed a slightly higher distance of 0.004 from the mother plant (1 and 2). Regarding the *in vitro* propagated plants with the addition of ZnO-NPs, samples 8 and 11 exhibited a distance of 0.002 to 1 and 2, while samples 9 and 10 had a distance of 0.003 to 1 and 2. Sample 10 had a distance of 0.002 to 1 and 2, and sample 12 had a distance of 0.004 to 1 and 2. The UPGMA analysis validated the genetic consistency and uniformity in the mother plant and

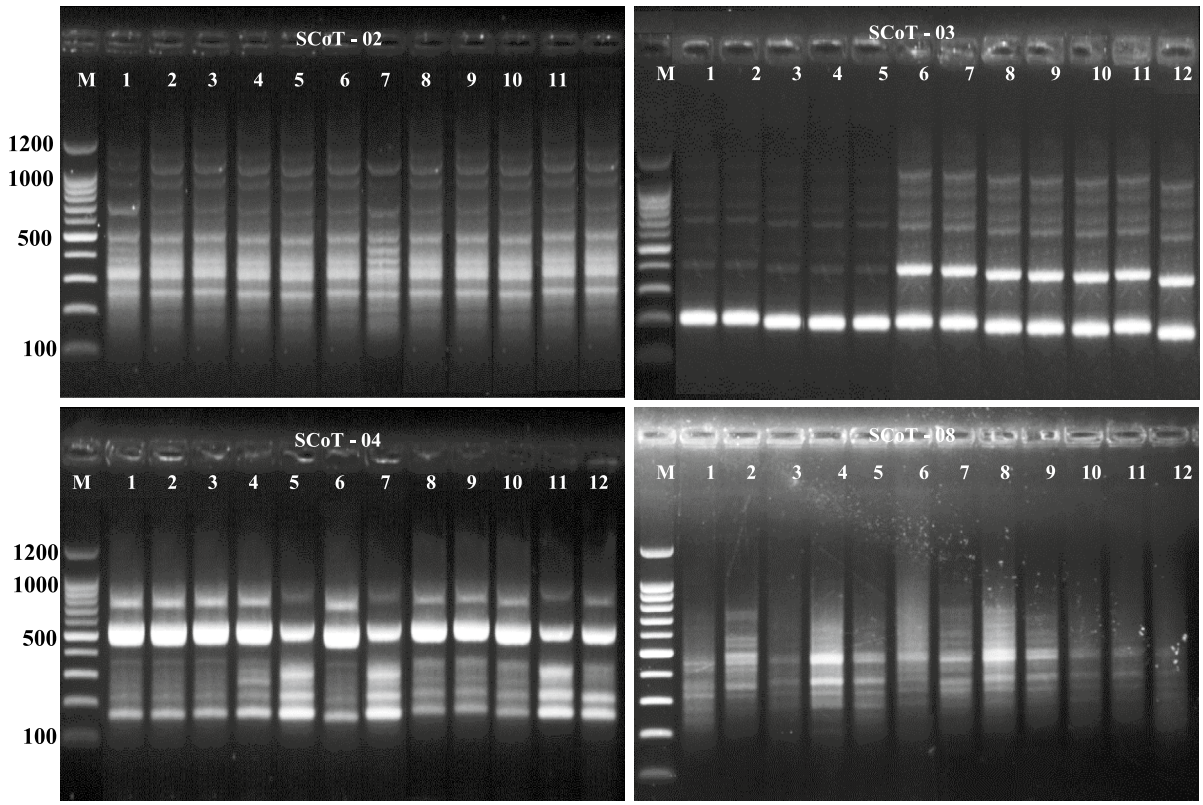


Figure 2. Start codon targeted (SCoT) results profiles generated by PCR amplification of *Eriobotrya japonica* (Thunb.) Lindl. from primers 2, 3, 4 and 8. *Lane M*: Molecular marker (100 to 1200 bp); *lanes 1 and 2*: mother plant; *lanes 3 to 7*: *In vitro* propagated plantlets after seven subcultures on Murashige and Skoog (MS) medium containing 1.0 mg L^{-1} 6-benzyladenine (BA) and 1.0 mg L^{-1} kinetin (Kin); *lanes 8 to 12*: *In vitro* propagated plantlets after seven subcultures on MS medium containing 1.0 mg L^{-1} BA, 1.0 mg L^{-1} Kin and 50.0 mg L^{-1} zinc oxide nanoparticles (ZnO-NPs).

in vitro propagated plants, both with and without ZnO-NPs, with very low percentage variations ranging from 0.002 to 0.004% (Fig. 4). These results indicate a high level of similarity and low genetic variation between the studied samples.

Discussion

In vitro plant propagation is an essential horticultural technique for vegetative reproduction, particularly in fruit species. The main benefit of this method is its ability to rapidly produce large numbers of plants that are genetically identical, high in quality and free of pathogens. Tissue culture is recognized as an efficient approach for large-scale clonal propagation and genetic modification of plant germplasm (Cai *et al.* 2015).

Previous studies on loquat (*E. japonica*) by Stamp *et al.* (1990), Li *et al.* (2008), Lomtatidze *et al.* (2009), Abbasi *et al.* (2013) and Deng *et al.* (2021) primarily focused on regeneration through indirect organogenesis pathways. This method, while successful, tends to be complex and time-consuming. In contrast, the present research utilizes direct shoot organogenesis from stem nodal segments of *E. japonica*, offering a more efficient and straightforward alternative for plant regeneration. This approach reduces the time and complexity involved, making it a preferable method for *in vitro* propagation of loquat.

Plant growth regulators (PGRs) play a crucial role in the culture media as they guide the development of plant cells and tissues (Romo-Paz *et al.* 2021). In this study, various combinations and concentrations of plant growth regulators (PGRs) alongside a basal MS medium were tested. The nodal segment explants demonstrated a successful sterilization process, with survival rates ranging from 80to 100%. Moreover, culturing these segments in MS medium with 0.1 mg L^{-1} NAA plus 1.0 or 2.0 mg L^{-1} BA or Kin resulted in a 100% survival rate of shoots, highlighting the significant role of cytokinins in shoot viability. This approach not only improves efficiency by passing the callus phase but also ensures greater genetic stability and rapid plant development.

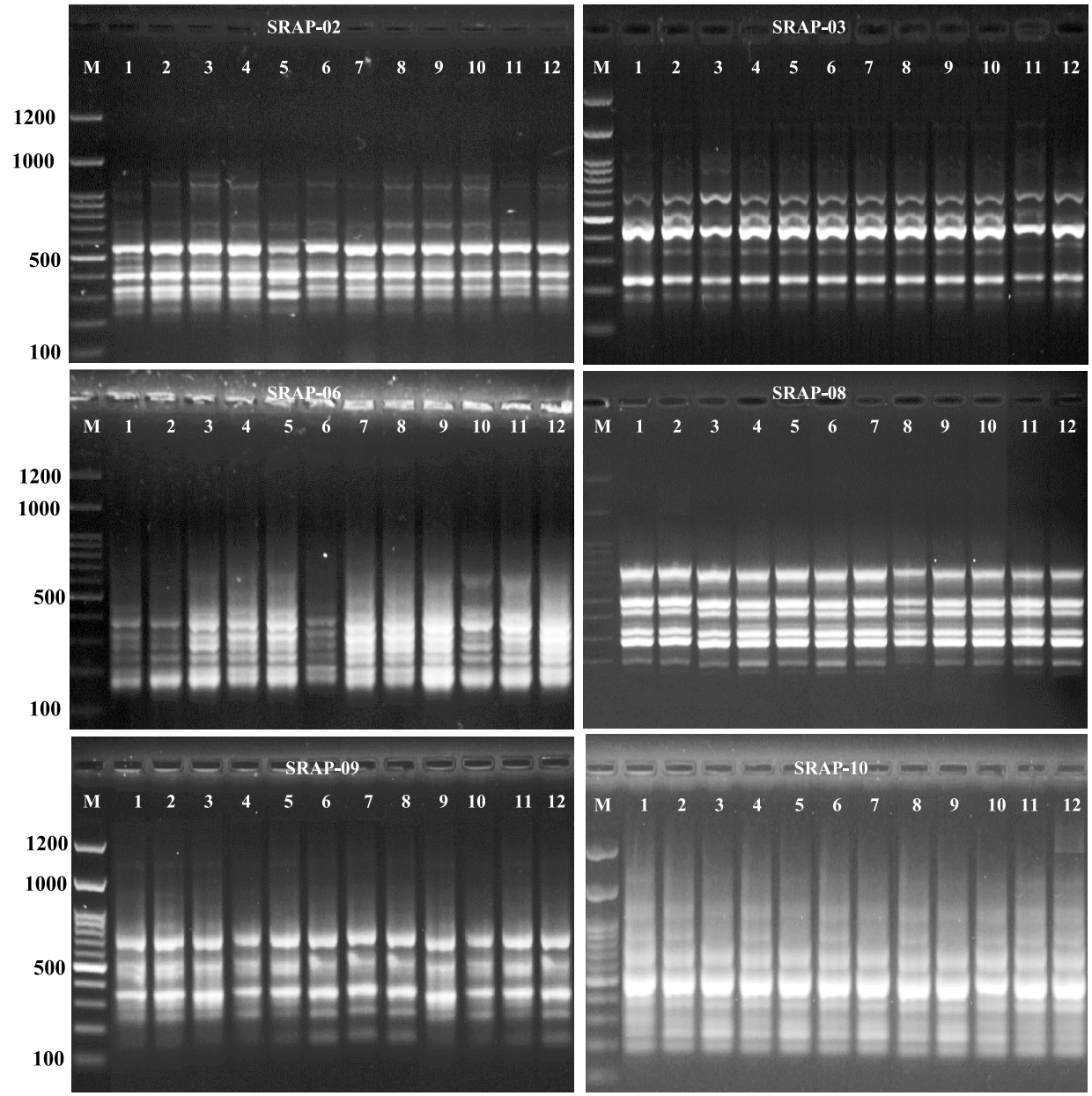


Figure 3. Sequence-related amplified polymorphism (SRAP) results profiles generated by PCR amplification of *Eriobotrya japonica* (Thunb.) Lindl. from primers 2, 3, 6, 8, 9 and 10. *Lane M*: Molecular marker (100 to 1500 bp); *lanes 1 and 2*: mother plant; *lanes 3 to 7*: *In vitro* propagated plantlets after seven subcultures on Murashige and Skoog (MS) medium containing 1.0 mg L^{-1} 6-benzyladenine (BA) and 1.0 mg L^{-1} kinetin (Kin); *lanes 8 to 12*: *In vitro* propagated plantlets after seven subcultures on MS medium containing 1.0 mg L^{-1} BA, 1.0 mg L^{-1} Kin and 50.0 mg L^{-1} zinc oxide nanoparticles (ZnO-NPs).

Optimizing the levels of auxins and cytokinins in the culture medium is essential for promoting shoot formation and overall plant growth. The right balance and concentration of these growth regulators can influence the quantity and quality of shoots produced from explants (North *et al.* 2010). For the *in vitro* establishment of *E. japonica*, the concentrations of 1.0 or 2.0 mg L^{-1} BA or Kin plus 0.1 mg L^{-1} NAA led to 80% growth and development of new axillary shoots. However, the combination of 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA in the basal MS medium showed the most favourable outcome in terms of the mean number of new shoots. On average, this combination yielded 3.72 new shoots per explant, with an average length of 4.84 cm. Interestingly, there were no significant differences between the optimal combination of BA and NAA and the combination of 1.0 mg L^{-1} Kin with 0.1 mg L^{-1} NAA. Both combinations resulted in similar average numbers of shoots per explant, with the BA and NAA combination producing slightly more shoots.

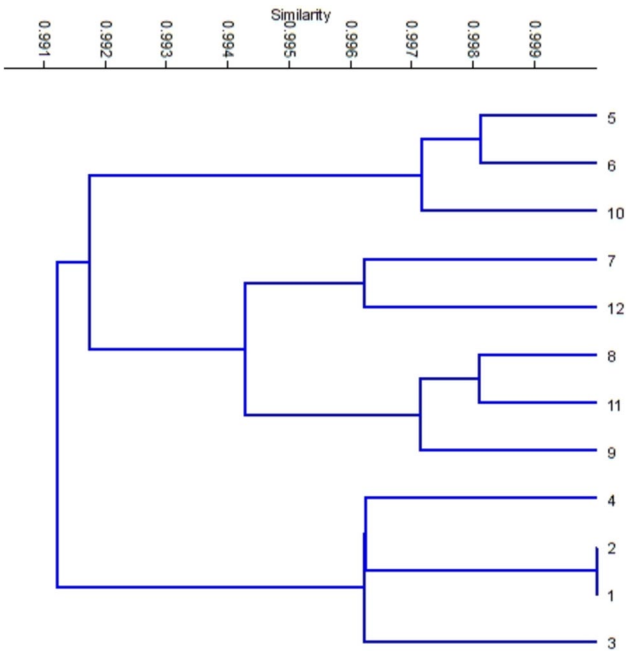


Figure 4. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram illustrating genetic relationship between micropropagated *Eriobotrya japonica* (Thunb.) Lindl. plants and their mother plant, using Dice's similarity indices from start codon targeted (SCoT) and sequence-related amplified polymorphism (SRAP) markers.

The average shoot length was also slightly higher in the BA and NAA combination.

These findings indicate that both BA and Kin, when combined with NAA, effectively stimulated shoot formation in *E. japonica*. As reported by Datta *et al.* (2022), NAA plays a key role in cytokinin metabolism and stability. It helps achieve higher shoot regeneration frequency in explants, promoting increased shoot proliferation and elongation. This is supported by Ngomuo *et al.* (2013), who reported on tissue culture of banana that the optimum combination of cytokinins and auxins in a medium is necessary for efficient *in vitro* propagation for a specific cultivar. The findings of Hieu *et al.* (2022) are consistent with the previous statement, as they also observed the highest shoot regeneration when culturing stem explants of passion fruit on MS medium supplemented with 0.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA. Also, the study conducted by Datta *et al.* (2022) with *Eclipta alba* demonstrated that the addition of NAA to BA resulted in an improved explant regeneration frequency. This enhancement was observed in terms of increased shoot proliferation and elongation per explant when compared to using BA alone in MS medium. These findings highlight the positive impact of combining NAA and BA on the successful micropropagation of plants.

The multiplication rate, length and number of shoots in plant micropropagation are affected by factors like plant variety, type and concentration of plant growth regulators (PGRs). In the present study on *E. japonica*, the combination of 1.0 mg L^{-1} BA and 1.0 mg L^{-1} Kin proved to be optimal for shoot multiplication, leading to increased shoot numbers and improved growth compared to using either cytokinin individually. This combination effectively promoted axillary bud growth, reducing apical dominance and enhancing shoot proliferation (Baghdady *et al.* 2018). In contrast, Abbasi *et al.* (2013) used a more complex combination of BAP, kinetin and 2iP for loquat cv. Mardan, with a 70% survival rate through callus formation. However, the higher concentrations of cytokinins in their study led to callus formation, vitrification and stunted growth, highlighting the importance of optimizing PGR concentrations to avoid unwanted tissue development. The simpler and more effective cytokinin combination used in this study suggests a more efficient approach for shoot multiplication in *E. japonica* without the

Table 8. Similarity matrices of the 10 micropropagated plants of *Eriobotrya japonica* (Thunb.) Lindl. and their corresponding mother plant based on Dice's similarity coefficient from SCoT and SRAP markers

	M-1	M-2	3	4	5	6	7	8	9	10	11	12
M-1	1.00											
M-2	1.00	1.00										
3	1.00	1.00	1.00									
4	1.00	1.00	1.00	1.00								
5	0.99	0.99	0.99	0.99	1.00							
6	0.99	0.99	0.99	0.99	1.00	1.00						
7	0.99	0.99	0.99	0.99	0.99	0.99	1.00					
8	0.99	0.99	0.99	0.99	0.99	0.99	0.99	1.00				
9	0.99	0.99	0.99	0.99	0.99	0.99	0.99	1.00	1.00			
10	0.99	0.99	0.99	0.99	1.00	1.00	0.99	0.99	0.99	1.00		
11	0.99	0.99	0.99	0.99	0.99	0.99	1.00	1.00	1.00	0.99	1.00	
12	0.99	0.99	0.99	0.99	0.99	0.99	1.00	0.99	0.99	0.99	1.00	1.00

SCoT start codon targeted, *SRAP* sequence-related amplified polymorphism.

negative effects observed in Abbasi *et al.*'s method. In general, the efficiency of pairing BA and Kin to achieve highest shoot numbers during *in vitro* propagation in different fruit plant species and cultivars was affirmed by Bekheet (2013) in date palm and Kaviani *et al.* (2023) in *Pyrus communis*.

In this study, the effect of ZnO-NPs on the multiplication of *E. japonica via* tissue culture was investigated, marking the first application of ZnO-NPs for this purpose in *E. japonica*. Zinc, as a co-factor in key enzymes like superoxide dismutase and catalase, plays a crucial role in reducing reactive oxygen species (ROS) stress in plant cells (Alenezi *et al.* 2022). ZnO-NPs are particularly beneficial in enhancing mineral nutrient uptake and transport, which supports physiological processes and promotes bud growth (Alharby *et al.* 2016). This study investigated the effects of different ZnO-NP concentrations (50.0 mg L^{-1}, 100 mg L^{-1} and 200.0 mg L^{-1}) in *E. japonica* multiplication media to identify the optimal concentration for plant propagation. Interestingly, the lowest concentration (50.0 mg L^{-1}) resulted in better growth compared to the control and higher concentrations. This aligns with findings from Al-Mayahi (2010) and Khan *et al.* (2019), which indicated that high ZnO-NP concentrations can stimulate elevated ethylene production, inhibiting growth by suppressing certain genes. Numerous studies have confirmed that lower concentrations of nanoparticles promote plant growth, while higher concentrations can lead to phytotoxicity (Fouda *et al.* 2021). In this study, the best results for growth induction and shoot number were obtained with a combination of 1.0 mg L^{-1} BA, 1.0 mg L^{-1} Kin and 50 mg L^{-1} ZnO-NPs in MS medium. This is consistent with Al-Mayahi (2021), who also reported that 50 mg L^{-1} ZnO-NPs positively impacted most growth parameters during the *in vitro* regeneration of date palm (*Phoenix dactylifera* L.). The enhanced growth observed at 50 mg L^{-1} ZnO-NPs highlights its optimal concentration for promoting shoot proliferation.

ZnO-NPs, due to their minute size, can penetrate plant explants and potentially induce genetic reprogramming. This may stimulate rapid zinc assimilation, triggering the production of proteins essential for chloroplast development (Kouhi and Lahouti 2018). Zayed *et al.* (2020) also suggest that the positive effect of ZnO on shoot regeneration in date palm stems from zinc's role in enhancing protein synthesis and energy production. However, as ZnO-NP concentrations increase, their effects become adverse, leading to negative outcomes. Higher concentrations can damage cell walls and membranes, inhibit various growth processes and interfere with electron transport in mitochondria and chloroplasts, causing oxidative stress, increased reactive oxygen species (ROS) levels and ultimately cell death (Shafique *et al.* 2020). This explains the reduced shoot formation and multiplication in *E. japonica* at higher ZnO-NP concentrations. Therefore, careful optimization of ZnO-NP levels is critical for achieving positive outcomes in *E. japonica* tissue culture. In tissue culture, inducing root formation in explants from the multiplication stage is a vital yet challenging step, especially for fruit trees. Successful root development relies on the appropriate type and concentration of auxins, with indole-3-butyric acid (IBA) being the most commonly used, followed by naphthaleneacetic acid (NAA), indole acetic acid (IAA) and 2,4-D (Kaviani *et al.* 2023). Root induction also depends on the genotype and the culture medium's composition, including light, salts and sugars (Aghdam *et al.* 2021). For instance, low-salt media are favourable for rooting, as they reduce the nitrogen requirement for root formation (Silveira *et al.* 2022). Previous studies on fruit species like Japanese cherry (*Prunus* sp.) and gooseberry (*Ribes grossularia*) have shown that half-strength MS medium enhances root development (Linh *et al.* 2018; Wójcik *et al.* 2021). In this study, the addition of 1.5 to 2.5 mg L^{-1} IBA to half-strength MS medium significantly improved root induction, achieving 100% rooting with an average of 13.25 roots per shoot and a root length of 3.21 cm, without callus formation at the shoot base. Comparatively, earlier studies on *E. japonica* reported lower rooting rates. Abbasi *et al.* (2013) achieved 74.93% root induction with 12.06 roots per shoot using ½ MS medium supplemented with 1.00 mg L^{-1} NAA, 2.00 mg L^{-1} IBA and 1.00 mg L^{-1} paclobutrazol (PBZ), but observed callus formation that inhibited root development. Deng *et al.* (2021) reported a 68.9% rooting rate using ½ MS medium with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} IBA. Lomtatidze *et al.* (2009) achieved 80 to 90% rooting with ½ MS medium containing 5 µM IBA, and 70 to 80% survival after acclimatization. The effectiveness of IBA in promoting root formation in various plants is well documented. Aygun and Dumanoglu (2015) found that 5 µM IBA in half-strength MS medium produced the best rooting in *Pyrus elaeagrifolia*, while Wójcik *et al.* (2021) obtained similar results with 1.0 mg L^{-1} IBA in *Ribes grossularia*. IBA's effectiveness is largely due to its slow oxidation rate in plant tissues, making it a reliable choice for root induction (Hausman 2003).

To study the genetic stability of the micropropagated *E. japonica* plantlets, both the regenerants produced through tissue culture and the donor plants undergo fingerprinting analysis using molecular markers. This comparison allows us to study if the plantlets retain the same genetic characteristics as the original donors, ensuring they are genetically stable or minor genetic variations were emerged. DNA-based markers are chosen for evaluating genetic fidelity due to their reliability and efficiency. SCoT and SRAP markers are favoured due to their simplicity, cost-effectiveness and significant polymorphism rate, as emphasized by Jedrzejczyk (2020). By amplifying various regions of the genome, these markers allow a comprehensive analysis of genetic stability and variation in the produced plantlets. Moreover, Palombi and Damiano (2002) recommended using multiple

DNA amplification techniques as an advantageous approach for assessing somaclonal variation. SCoT and SRAP markers have been utilized for detecting somaclonal variation in various micropropagated plants in several plants, such as banana (El-Shahed *et al.* 2017), *Lycopersicon esculentum* (Abd El-Fattah and Haridy 2019) and *Withania somnifera* (Ismail *et al.* 2022).

In this study, the genetic integrity of *in vitro* propagated *E. japonica* plants, both with or without the inclusion of ZnO-NPs in the growth medium, exhibited minimal variations and substantial genetic resemblance to the mother plant under the given conditions even after undergoing seven subcultures (each subculture was every 4 wk). Sultana *et al.* (2022) reported that various factors influence the emergence of minor genetic variations in tissue culture-derived plants. These include the age of the culture, the number of subcultures performed, natural variations and environmental factors. Additionally, accumulated mutations can arise due to factors such as treatment duration, *in vitro* stress, hormonal balance, presence of added biochemicals and specific nutritional conditions. All these elements collectively contribute to the occurrence of genetic variations during the tissue culture process. In this study, the observed limited genetic variations could possibly be attributed to factors like cytokinin concentrations, the increased number of subcultures or the presence of ZnO-NPs. The genetic variation observed in *in vitro* propagated *E. japonica* plantlets in the presence of ZnO-NPs may be attributed to their interaction with DNA and nuclear proteins after diffusing into the cell. This interaction could influence the cell cycle or result from oxidative stress caused by reactive oxygen species, which in turn affects DNA repair mechanisms. Similar findings were reported by Fouda *et al.* (2021) in *Salvadora persica* callus. This lower level of somaclonal variation may offer an additional source of valuable and unique variability that can be harnessed for the genetic enhancement of *E. japonica*. Interestingly, certain plant species, such as *Ribes grossularia* (Wójcik *et al.* 2021), banana cv. Ney Poovan (Bhalang *et al.* 2018) and *Thunbergia coccinea* (Sultana *et al.* 2022), have reported somaclonal variations during *in vitro* micropropagation. However, other species like *Citrus maxima*, *strawberry*, *Elettaria cardamomum* and *date palm*, as reported by Kaur *et al.* (2019), Naing *et al.* (2019), Malhotra *et al.* (2021) and Al-Mayahi (2022), respectively, exhibited no genetic variation between micropropagated plants and the mother plant.

Conclusions

This study presents an effective direct micropropagation method for *Eriobotrya japonica* using stem nodal segments. The optimal conditions for shoot establishment were found using MS medium with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA, while maximum shoot multiplication was achieved with 1.0 mg L^{-1} Kin and BA, enhanced by the addition of ZnO-NPs. Rooting was most successful with half-strength MS medium containing 1.5 mg L^{-1} IBA, and rooted plantlets were successfully acclimatized. Genetic analysis using SCoT and SRAP markers revealed slight variations but demonstrated the utility of these markers for assessing genetic homogeneity. These findings contribute valuable insights for optimizing direct micropropagation techniques, enhancing the efficiency and reliability of mass-producing *E. japonica*. Future studies will focus on optimizing ZnO-NP concentrations and reducing subcultures to minimize genetic variation, with a view toward potential commercial applications.

Author contribution The planning and designing of the experiments were done by Heba Ghareb. Heba Ghareb and Sabha Mustafa performed the experiment of the *in vitro* propagation section and analyzed the data. Shafik Ibrahim performed the experiment and analyzed the data of the genetic stability section. Heba Ghareb wrote the paper and the corresponding publication. Mohamed Abd Alhady and Mahdia Gabr revised the paper. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests The authors declare that no competing interests.

Ethics approval Not applicable.

Consent to publish Not applicable.

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