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Optimizing *In Vitro* Micrografting Technique for Kalamata Olive (*Olea europaea* L.) and assessment their genetic fidelity

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Abstract: This study evaluates the extent of micro grafting success of the Kalamata cultivar (*Olea europaea* L.) onto two rootstocks, Kronaki and Coratina, as well as examining the influence of various factors on micrografting outcomes. Additionally, the research investigates the impact of mineral composition in the nutrient medium on the in vitro development of these rootstocks. The findings demonstrate that, cultivars and growth medium significantly impact germination performance, the Kronaki cultivar exhibiting a higher germination rate (74%) compared to Coratina (62.5%). Moreover, the Murashige and Skoog (MS) medium outperformed the Olive Medium (OM) in terms of survival rates and shoot elongation. Specifically, MS medium facilitated greater shoot growth (4.85 cm on average) compared to OM medium (4.2 cm). Kronaki showed higher shoot lengths (5.20 cm) than Coratina cultivar (4.5 cm) on MS that superiority than OM medium. During acclimatization, Kronaki also exhibited higher survival rates (72.5%) compared to Coratina (59%). Genetic fidelity analysis using Inter-Simple Sequence Repeat (ISSR) markers indicated a high level of genetic stability, with 96% monomorphic bands and only 4% polymorphism observed. These results highlight the critical role of genotype and growth medium in optimizing micrografting success and maintaining genetic integrity in olive rootstock propagation.

Key words: Olive, Micrografting, Rootstocks, Genetic fidelity.

Future Science Association

Available online free at
www.futurejournals.org

Print ISSN: 2692-5826

Online ISSN: 2692-5834

DOI:
10.37229/fsa.fjh.2024.06.05

Received: 22 April 2024
Accepted: 26 May 2024
Published: 5 June 2024

Publisher's Note: FA stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Kalamata olive (*Olea europaea* L.) is one of the most important cultivars worldwide, being known for premium table olives and oil. Since the demand for Kalamata olive-based products has risen considerably, adequate and reliable propagation methods have to be implemented to ensure its sustainable production. Conventional propagation plans through cuttings and grafting in situ often face issues, such as critical problems associated with low success rates, vulnerability to pests and diseases, and prolonged maturation times

Bardolino *et al.* (2017). These challenges highlight the need to develop advanced propagation techniques that are time-efficient and excellence in physical and genetic integrity. In vitro micrografting has emerged as an alternative method to overcome these limitations and holds the promise of combining the benefits of tissue culture and traditional grafting techniques **Franclet (1983)**. In vitro micrografting reduces the adverse impacts of environmental stresses, enhances compatibility, and accelerates the development of uniform and pathogen-free plants by mediating the fusion between scions and rootstocks under controlled conditions. Being a promising method though, this technique is in its infant stage for Kalamata olive, and further exploration in a systematic way would be needed to determine all parameters contributing to success.

Navarro *et al.* (1975) later improved the in vitro shoot-tip grafting technique, as an alternative for obtaining virus-free plants, the rejuvenation of mature shoot materials can be used. **(Hackett and Murray, 1993**). Moreover, in vitro shoot-tip grafting techniques are potentially useful, particularly for propagating hard-to-root cultivars or multiplying in vitro clones. An appropriate micrografting technique could offer a solution to the challenging or limited root regeneration commonly observed in in vitro olive tissues and explants. (**Rugini, 1984**). In plant tissue culture, identifying off-types and genetically true-to-type mother plants early in development is thought to be highly helpful for quality control. Therefore, using molecular approaches to verify the genetic homogeneity of micropropagated plants is required. Additionally, ISSR is a very easy, quick, economical, highly discriminative, and dependable approach **(Reddy *et al.*, 2002**). Numerous plants' micropropagated material has been effectively subjected to ISSR markers in order to identify genetic similarities or differences (**Venkatachalam *et al.*, 2006, Joshi and Dhawan, 2007, and Huang *et al.*, 2009).** Tissue browning is a frequent challenge during the initiation of in vitro cultures. (**Jones and Saxena, 2013 and Singh and Patel, 2016**). Similarly, in IVM, tissue wounding during scion/rootstock preparation can lead to browning and oxidation, ultimately reducing grafting success. (**Jonard *et al.*, 1983**, **Dobránszki *et al.*, 2005 and Teixeira *et al.*, 2019**). In olives, the negative effects of tissue browning can be reduced by presoaking scions in antioxidant solutions. **(Thimmappaiah *et al.*, 2002, Davoudi *et al.*, 2019 and Naddaf *et al.*, 2021**). Somatic embryogenesis can be initiated through multiple pathways, including direct embryogenesis, which relies on seed or embryo identity factors. **(Fehér *et al.*, 2016).** Somatic embryogenesis present multiple applications, as: (1) Large – scale propagation of selected genotypes; (2) Germplasm conservation and (3) production of synthetic seeds.

The aim of this study is to develop a comprehensive in vitro micrografting protocol of Kalamata olives to enhance survival and ensure the integrity of graft unions. This research aims to bring about methodologies for the improved propagation of olives through critical issues such as compatibility between rootstock and scion and post-grafting acclimatization and thus offers wider implications for sustainable agricultural practices and commercial viability.

2. Material and Methods

This study was conducted in the laboratory of National Gene Bank (NGB), ARC, Giza. Egypt.

2.1. Plant Material

The plant sources of olive cultivars involved Horticultural Research Institute (HRI) nursery. Three olive cultivars were used in this study namely: Kalamata (was employed as shoot –tip explant donor) onto Kronaki and Coratina as a rootstock to choose the more desirable rootstock for Kalamata cultivar.

Scion and rootstock tissues were collected from healthy, mature mother plant trees. Scions had excised as apical shoots and rootstocks were prepared from in vitro-grown olive seedlings.

2.2. Preparation of Rootstocks

Source Material

Fruits of Kronaki and Coratina olive cultivars were harvested when the color started to change from yellow-green to violet. According to **Sotomayor–Leon and Caballero (1990)**. The fruit pulp was removed to extract the seed.

Surface sterilization of olive seeds

The stony endocarps were broken in order to remove the seeds, the seeds were then surface sterilized under sterile conditions using a 20% commercial sodium hypochlorite solution for 15 minutes., after that, it transferred to 0.1% $HgCl_2$ for 5 mint. Finally, the seeds were rinsed three times with sterile water. The embryo axis from each seed, along with a cotyledonary tissue cube (15×15×8 mm), was then cultured on MS and OM medium containing 30 g/L sucrose.

Selection and preparation rootstocks the seedling

Healthy seedlings approximately 4–6 weeks old, with a well-developed root system and a stem diameter of 1–2 mm, had selected as rootstocks. Uniformity in size and vigor had prioritized to ensure compatibility with the scions. The rootstocks were pruned to a height of 2–3 cm above the media level, leaving a clean vertical cut at the top to prepare for grafting. The prepared rootstocks remain in their culture vessels until the grafting process to maintain sterility and hydration.

2.3. Preparation of scions

Type of scion material

The scions had trimmed to approximately 1.5–2 cm in length, the apical meristem and shoot tip taken from newly emerged laterals on branches in March. Ensuring a clean, diagonal cut at the basal end to facilitate grafting. The prepared scions had stored in sterile Petri dishes moistened with sterile filter paper until use to prevent desiccation.

Surface Sterilization

The scions have been rinsed under running tap water for 30 minutes to remove surface debris. Then surface-sterilized by immersion in 70% ethanol for 30 seconds, followed by treatment with a 1% sodium hypochlorite solution containing a few drops of Tween-20 for 10–15 minutes. Finally, the scions had rinsed three times with sterile distilled water to eliminate traces of sterilizing agents.

Reduced tissue browning

The cut edge of the scions was soaked in antioxidant solutions at 0.01 % ascorbic acid and 0.015% citric acid (1:1) prior to in vitro grafting. According to **(Thimmappaiah *et al.*, 2002).**

1- Grafting experiments procedure and maintenance of in vitro micro grafts (IVM).

The process of micrografting involved the attachment of micro-scions to seedlings that had been germinated in vitro. The rootstock comprised seedlings that were two weeks old and had been cultivated under in vitro conditions. After decapitating the seedlings, a vertical incision of 2 mm was made at the top of each rootstock. The micro-scions were prepared by cutting the micro-shoots into lengths of 1.5 to 2.0 cm, with the lower end shaped into a wedge ('V'). This grafting was carried out in a sterile environment. Following the grafting, the micro-grafted seedlings were placed in MS and OM medium containing 1.0 mg l^{-1} BAP and IBA, while a control group was maintained without any plant growth regulators (PGRs).

2- Media and culture conditions

For in vitro culture, whole embryos from each sampling date were individually placed in sterile test tubes (21×150 mm) containing 10 ml of full-strength MS medium. (**Murashige and Skoog, 1962**)

and olive media (OM) (**Rugini, 1984**) and addition to the media 1.0 mg/L^{-1} BAP, IBA and the sucrose was added at 30 g per liter. The pH was adjusted at 5.8. The media was solidified by adding 6% agar and autoclaved. The culture tubes were covered with plastic caps and sealed with parafilm and placed in a growth chamber at 23 °C and a 16 h photoperiod (light intensity 30 µ mol $m^{-2}S^{-1}$). The development of in vitro embryos was monitored through continuous visual observation and photography, and the selected in vitro plant material was grafted. The success of testing various culture conditions to optimize micro-graft regrowth depends on the plant species and the source of the plant material used. For successful rootstock seedling growth, seeds are typically kept in continuous darkness for a period of 1 to 6 weeks. **(Jonard *et al.*, 1983 and Davoudi *et al.*, 2019).** 100 jars were used for each cultivar. The following parameters were recorded.

Germination percentage

The emergence of roots and opening of cotyledons was the sign of germination, the emerged platelets were counted and germination percentage was calculated.

Mean germination time

The time in days between the first emergence and complete germination.

Seedling growth

Growth measurements were performed for each plant; length of shoots/micro-grafting were measured.

Micrografting parameters

After 6 weeks of micro grafting on different rootstocks the success and growth of scion of (Kalamata cv.) was calculated as shoots number of micro-grafts, successful micro-grafts (survival %), graft union (%), micro-graft length (cm) and Micrografting rooting %.

3- Acclimatization

Successfully grafted plantlets were removed from the culture containers and rinsed with tap water to remove any residual agar from their root systems. They were then transplanted into individual commercial plastic pots filled with an autoclaved mixture of sand, perlite, and peat moss in a 1:1:1 (v/v) ratio. To maintain a relative humidity of 90±5%, the pots were covered with transparent bags. Over the first two weeks, the bags were gradually removed to facilitate air exchange and aid in plant acclimatization.

2.4. Statistical analysis.

This study was conducted using a randomized complete design with five replicates, each consisting of 20 jars. The data were analyzed according to **Snedecor and Cochran (1980)**. The treatment means were compared using the Least Significant Difference (L.S.D.) method according to (**Duncan, 1955**) at significance level of 0.05.

2.5. Molecular Marker

DNA extraction

Healthy shoot-tip leaves from each sample were collected for DNA extraction. Genomic DNA was isolated using the DNeasy Plant Mini Kit (QIAGEN, Chatsworth, CA). DNA concentration was measured at a wavelength of 260 nm using a Biophotometer (Eppendorf), and its quality was assessed by electrophoresis on a 0.8% agarose gel.

PCR reactions

PCR reactions were carried out using ten ISSR primers **(Table 1)** in a total volume of 25 µl. Each reaction contained 2X ready mix (EmeraldAmp Max PCR master mix), 20 pM oligonucleotide

primer, and 50 ng of genomic DNA. The amplification was performed on an Eppendorf Master Cycler programmed for 35 cycles with the following conditions: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at the respective temperature (Ta) for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. A total of 20 ISSR primers (Table 1) were used in this study, synthesized by HVD Corporation, Germany. Thermo 100 bp plus DNA ladder was used as a molecular size marker. The amplification products were visualized under an ultraviolet transilluminator, separated on a 2% agarose gel, and documented using a gel documentation system (Alpha Innotech).

Table (1). ISSR primer code names, sequence, and annealing temperature (Ta)

Serial number	Primer code Name	Primer Sequence	Ta (ºC)
1	ISSR-11	(AC)8YC	**40 ºC**
2	ISSR-15	(CT)8RG	**42 ºC**
3	UBC815	(CT)8G	**50 ºC**
4	UBC816	(CA)8T	**54 ºC**
5	UBC823	(TC)8C	**50 ºC**
6	UBC861	(ACC)6	**50 ºC**
7	UBC862	(AGC)6	**42 ºC**
8	K25	(GA)8A	**50 ºC**
9	K24A	(GA)8T	**53 ºC**
10	**HB 15**	**(GTG)3GC**	**45 ºC**

Analysis of molecular data

ISSR bands were scored, each band was assumed to represent a unique genetic locus. Molecular results were analyzed visually with Phoretix 1D Pro software from non-linear dynamics.

3. Results and Discussions

Data in Table (2) showed effect of growing medium on germination Percentage. The results indicated that, the higher germination percentages of seeds in OM medium (71%) compared to MS (65%). 'Kronaki' demonstrated the highest germination rate, with (74%) than Coratina cultivar that attained the lower germination rate (62.5%). Similarly, the highest seed germination was appearing in koronaki rootstock (77%) in OM medium, that may be due that olive medium provided a more favorable environment for seed germination. These findings align with previous studies that emphasize the role of organic substrates in enhancing seed germination by improving aeration, moisture retention, and microbial activity beneficial for root development (**Ramakrishna *et al.*, 2019**). Studies on olive propagation have also suggested that different rootstocks may respond differently to various substrates due to genetic variability affecting water uptake and nutrient absorption (**Bartolini *et al.*, 2017**). The observed differences between 'Kronaki' and 'Coratina' suggest a genetic component influencing germination success. 'Kronaki' consistently outperformed 'Coratina,' which may be due to inherent differences in seed viability, dormancy-breaking requirements, or tolerance to different media compositions. Similar results were reported by **Trigui *et al.* (2020)**, who found that certain olive cultivars exhibit higher germination percentages due to differences in seed coat permeability and endogenous hormone levels.

Table (2). Effect of MS and OM medium on in vitro seed germination percentage of two rootstocks

	Seed germination %		Mean
Rootstock	MS	OM	
Kronaki	71 b	77 a	**74 A**
Coratina	60 d	65 c	**62.5 B**
Mean	**65.5 B**	**71 A**	

Mean followed by the same letter(s) in each column are not significantly different at P≤0.05 level

Table (3) demonstrated the mean Germination Time (MGT) is a crucial parameter in seed biology, as it indicates the average time required for a seed population to germinate. This parameter helps assess the vigor and viability of different rootstocks under varying conditions. In the present study, the germination performance of two olive (*Olea europaea* L.) rootstocks, Kronaki and Coratina, was evaluated under two different growth media Murashige and Skoog and Olive Medium. The results indicate a significant difference in MGT between the two rootstocks. Kronaki exhibited a lower MGT (9.5 days on average) compared to Coratina (18 days). This suggests that Kronaki seeds germinate faster and may have higher vigor under the tested conditions. Faster germination is often associated with better seed quality and improved seedling establishment (**Bewley *et al.*, 2013).** The type of growth medium also influenced germination time. Seeds in the OM medium showed a consistently lower MGT (11.5 days) compared to those in the MS medium (16 days). This could be attribute to differences in nutrient composition, water retention capacity, or microbial interactions in olive media (OM), which may enhance seed germination (**Basra, 2006**). Studies have shown that organic substrates can improve seed germination rates by providing a more favorable microenvironment (**Kaur *et al.*, 2020).** The delayed germination observed in Coratina may be due to factors such as seed coat dormancy, hormonal regulation, or lower enzymatic activity associated with germination (**Finch-Savage and Leubner-Metzger, 2006**). Additionally, variations in seed genetic makeup influence germination rates, as different cultivars exhibit different physiological responses to environmental conditions (**Górski *et al.*, 2018**). From an agricultural perspective, the faster germination observed in Kronaki under both media suggests that it may be a preferable rootstock in nurseries where rapid establishment is critical. Moreover, the enhanced germination in the olive medium suggests that sustainable growing conditions could be favorable for olive seedling production.

Micrografting is a valuable technique in plant tissue culture, particularly for woody species such as olives (*Olea europaea* L.). The success of this method depends on several factors, including the type of growth medium used, which influences both survival rates and shoot elongation.

Table (3). Influence of the mineral composition of the nutrient medium on the in vitro development of Kronaki and Coratina rootstocks

	Mean germination Time (MGT)/day		Mean
Rootstock	MS	OM	
Kronaki	11 c	8 d	**9.5 B**
Coratina	21 a	15 b	**18 A**
Mean	**16 A**	**11.5 B**	

Mean followed by the same letter(s) in each column are not significantly different at P≤0.05 level.

Effect of Medium Type on Survival Rates

Data Table (4) and Fig. (1) indicated that, the Murashige and Skoog (MS) medium resulted in higher survival rates (75.5%) compared to the olive medium (OM), which had a lower mean survival rate (59. 5%). Among the cultivars tested, 'Kronaki' showed consistently higher survival (74%) than 'Coratina' (61%).As the interaction effect, the 'Kronaki' cultivar on MS medium gave the highest survival percentage (83%). These findings suggest that MS medium provides a more favorable environment for micrografting success, likely due to its balanced supply of essential macronutrients and micronutrients. Previous studies have demonstrated that MS medium is one of the most widely used formulations in plant tissue culture due to its rich composition of nitrogen, phosphorus, and potassium (**Murashige and Skoog, 1962**). Similar studies on olives (**Rugini, 1984**) found that MS-based media improved survival rates during in vitro propagation due to its optimal nutrient balance. The observed lower survival rates in OM medium suggest that organic-based formulations may not provide the necessary nutrients for effective micrograft establishment.

Table (4). Effect of medium type on some grafting characteristics of Kalamata micro-grafts on two rootstocks

Rootstocks	Survival (%)		Mean	Length of shoots (cm)/micrografting		Mean
	MS	OM		MS	OM	
Kronaki	83 a	65 c	**74 A**	5.2 a	4.8 b	**5.00 A**
Coratina	68 b	54 d	**61 B**	4.5 c	3.6 d	**4.05 B**
Mean	**75.5 A**	**59.5 B**		**4.85 A**	**4.20 B**	

Mean followed by the same letter(s) in each column are not significantly different at P≤0.05 level.

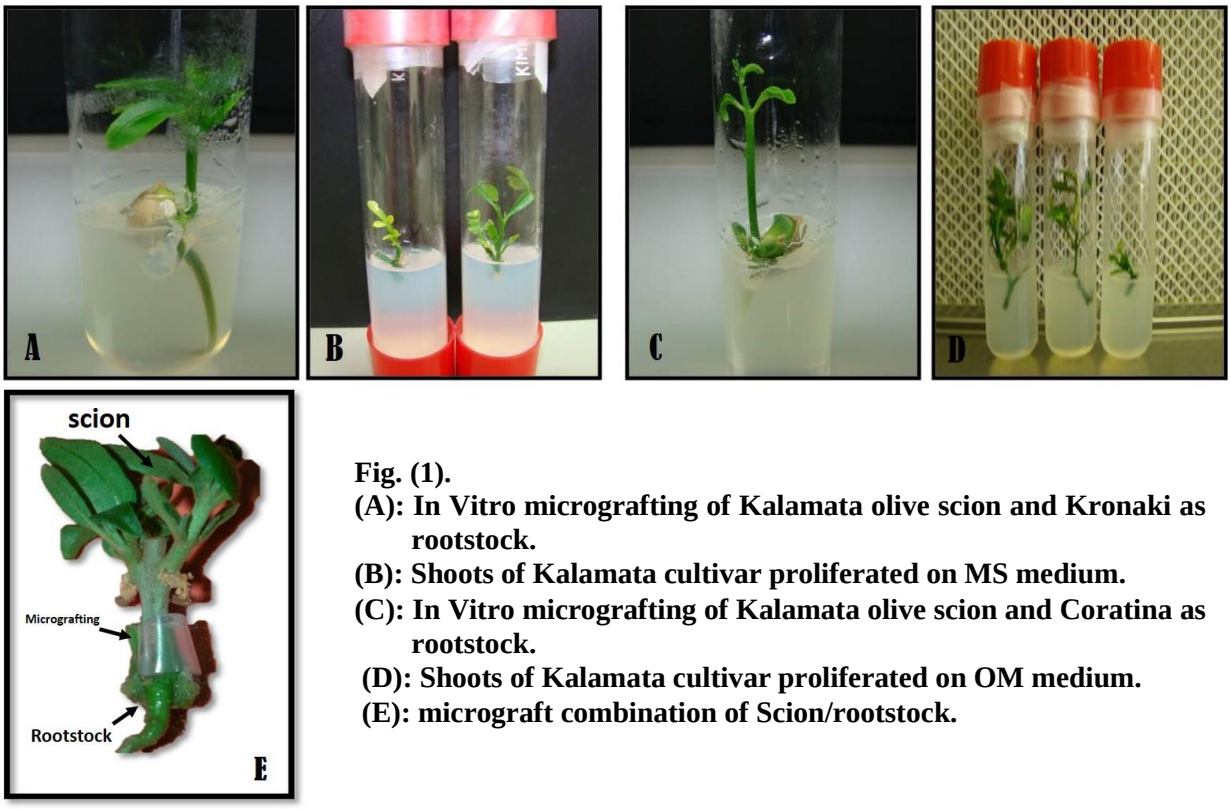

Fig. (1).
(A): In Vitro micrografting of Kalamata olive scion and Kronaki as rootstock.
(B): Shoots of Kalamata cultivar proliferated on MS medium.
(C): In Vitro micrografting of Kalamata olive scion and Coratina as rootstock.
(D): Shoots of Kalamata cultivar proliferated on OM medium.
(E): micrograft combination of Scion/rootstock.

Effect of Medium Type on Shoot Growth

Shoot length is another critical parameter for assessing micrografting success. Data in (Table 4) demonstrated that, MS medium resulted in greater shoot elongation, with a mean of 4.85 cm compared to 4.2 cm in olive medium OM. Moreover, Kronaki' cultivar was superiority than 'Coratina'. As the interaction effect between medium type and cultivars, the highest shoot length achieved by 'Kronaki' with the average 5.2 cm on MS medium otherwise, 'Coratina' displayed shortest o 3.6 cm on OM. This variation in shoot growth can be attribute to the availability of essential nutrients and growth regulators in the MS medium. Previous research suggests that higher nitrogen content in MS medium plays a crucial role in promoting shoot elongation in woody plants (**Bonga and von Aderkas, 1992**). Additionally, studies on olive micro propagation (**Fabbri *et al.*, 1994**) have reported that MS-based media enhance shoot growth due to the presence of ammonium and nitrate in optimal proportions. The relatively lower shoot elongation in OM medium suggests that nutrient limitations or imbalanced mineral composition may restrict shoot development.

Cultivar-Specific Responses

It is also notable that 'Kronaki' consistently performed better than 'Coratina' in both survival and shoot elongation across both media types. This suggests that 'Kronaki' may have a higher inherent capacity for micrograft establishment and growth under in vitro conditions. Genotypic differences in tissue culture responses are well documented in olives, where some cultivars exhibit higher adaptability to in vitro conditions than others (**Brito *et al.*, 2009**). This could be attribute to differences in endogenous hormone levels, nutrient uptake efficiency, or overall physiological adaptability to micropropagation techniques.

The study presents data on graft union success and micrograft rooting percentages for two olive cultivars, Kronaki and Coratina, under different medium types: Murashige and Skoog (MS) and Olive Medium (OM). The results indicate variations in both parameters depending on the medium type used.

Graft Union Success

The percentage of successful graft unions was generally high across in both of cultivars and media. Kronaki cv. observed slightly higher success rates than Coratina **(**Table 5). The MS medium resulted in a higher mean graft union success (92.5%) compared to OM (89%). Meantime, Kronaki rootstocks appears the highest graft union% (100%) on MS medium. This may be due that MS formulation provides a more favorable environment for graft healing and vascular connection establishment. Previous studies have shown that the composition of the culture medium plays a crucial role in graft success. MS medium, which is rich in macronutrients and micronutrients, has been widely used in olive micro propagation (**Rugini, 1984**). The higher success rate observed in MS could be attribute to its balanced nutrient composition, which supports cell division and callus formation at the graft interface (**Gonçalves *et al.*, 2006**).

Table (5). Effect of medium type on some grafting characteristics of Kalamata micrograft on two rootstocks

	Graft union %		Mean	Micrograft rooting %		Mean
Rootstocks	**MS**	**OM**		**MS**	**OM**	
Kronaki	100 a	95 b	**97.5 A**	84 a	76 b	**80 A**
Coratina	85 c	83 d	**84 B**	65 c	55 d	**60 B**
Mean	**92.5 A**	**89 B**		**74.5 A**	**65.5 B**	

Mean followed by the same letter(s) in each column are not significantly different at P≤0.05 level.

Micrograft Rooting Success

The ability of micrograft to form roots varied significantly between cultivars and medium types. Kronaki consistently exhibited higher rooting percentages (80%) than Coratina (60%)in both medium types, with MS yielding better rooting percent (74.5%) than OM (65.5%). As the interaction effect, the highest micro graft rooting success was attained by Kronaki rootstock on OM medium. This aligns with previous findings suggesting that olive cultivars differ in their rooting ability due to genetic factors affecting auxin metabolism and root initiation (**Bati *et al.*, 2006).** The lower rooting percentages observed in OM could be due to differences in nitrogen and carbohydrate availability, which are crucial for root development. Research has shown that media with a balanced nitrogen-to-carbon ratio promote better root induction in woody species (**Ruffoni *et al.*, 2010**).

Cultivar-Specific Responses

Between the two cultivars, Kronaki consistently outperformed Coratina in both graft union and rooting percentages, regardless of the medium used. This suggests that Kronaki may possess intrinsic physiological traits that enhance graft compatibility and rooting potential. Similar trends have been reported in other olive studies, where cultivar-specific differences were attributed to variations in endogenous hormone levels and tissue differentiation capabilities (**Grigoriadou *et al.*, 2002**).

Table (6). Effect of rootstock type on the survival percentage during acclimatization stage after 4 weeks

Rootstock	survival %		Mean
	MS	OM	
Kronaki	80 a	65 b	**72.5 A**
Coratina	63 c	55 d	**59 B**
Mean	**71.5 A**	**60 B**	

Mean followed by the same letter(s) in each column are not significantly different at P≤0.05 level.

The survival percentage of two rootstocks (Kronaki and Coratina) was evaluated under two conditions (MS and OM) after four weeks of acclimatization. The results indicate that, the MS medium gave the highest value (71.5%) than OM (60%).Additionally, Kronaki exhibited higher survival rates (72.5%) compared to Coratina (59%). As the interaction effect between cultivars and medium type, Kronaki on MS had the overall mean survival rate (80%) survival rate, while Coratina had (63%). Otherwise, in OM medium, Kronaki had 65% survival, whereas Coratina had 55% which appears the lowest value. The findings suggest that Kronaki is a more robust choice for improving survival rates in olive nurseries, especially in MS conditions. Future research should explore physiological responses such as root architecture, biochemical stress markers, and water-use efficiency to determine the underlying mechanisms of Kronaki's superior survival. Additionally, optimizing OM formulations could enhance Coratina's viability, making it a more competitive option for growers.

Rootstock plays a critical role in plant survival, growth, and adaptability to environmental stresses (**Hartmann *et al.*, 2018**). Previous studies have shown that genetic differences among rootstocks affect their physiological and biochemical responses during micropropagation and acclimatization stages (**Almeida *et al.*, 2020**). In this study, Kronaki had a higher survival rate than Coratina, which could be attributed to inherent differences in rootstock vigor, stress tolerance, or rooting efficiency. A study by **Navarro *et al.*, (2019)** on olive rootstocks reported that genotype selection significantly influences survival during acclimatization, as certain cultivars exhibit greater

resistance to water stress and nutrient limitations. This aligns with our findings, where Kronaki demonstrated better survival, possibly due to superior adaptation to the acclimatization conditions. The results also indicate that survival rates were higher in MS medium (71.5%) compared to OM medium (60%), suggesting that MS medium provided more favorable conditions for rootstock establishment. MS medium is widely recognized for its balanced nutrient composition, particularly its nitrogen and cytokinin content, which promotes shoot and root development (**Murashige and Skoog, 1962**). Several studies have demonstrated that MS-based media enhance survival rates in micropropagated plants, particularly during the acclimatization stage when plantlets transition from in vitro to ex vitro conditions (**Rai *et al.*, 2021**). In contrast, OM medium resulted in lower survival rates, which may be due to differences in nutrient availability or water retention properties. Similar findings were reported by **Garcia-Sogo *et al.*, (2018)**, who observed that plant survival during acclimatization is strongly influenced by the choice of substrate and nutrient composition. Thus, the lower survival rates observed in OM could be linked to suboptimal nutrient uptake or increased stress during the acclimatization phase. Studies have shown that rootstock vigor and water-use efficiency play a significant role in survival (**Trentacoste *et al.*, 2018**). It is possible that Kronaki exhibits better root system development or higher water retention capacity, which may have contributed to its slightly higher survival rate.

Maintaining genetic Fidelity in *In Vitro* micrografting

The assessment of genetic fidelity in *in vitro* micrografting of olive (*Olea europaea* L.) 'Kalamata' cultivar is critical in understanding the maintenance of genetic stability during tissue culture practices. Inter-Simple Sequence Repeat (ISSR) markers provide a robust tool for analyzing genetic differences between *in vitro* propagated plants and their donor plant. Genetic fidelity in this context is primarily determined by the number of monomorphic and polymorphic bands observed in ISSR analysis.

Table (7) and Fig. (2) provides data on the genetic fidelity of in vitro micro grafted olive (*Olea europaea* L.) Kalamata cv. using ISSR primers. The analysis includes the number of scorable bands per primer, their range of amplification, and the classification into monomorphic and polymorphic bands, with the resulting polymorphism percentage.

Table (7). The assessment of genetic fidelity in in vitro micrografting of Kalamata cv. In different rootstock using ISSR marker

Primers Name	Primer Sequence 5′-3′	NO. of Scorble Band per Primer	Range of Amplification (bp)	No. of Monomorphic Bands	No. of polymorphic Bands	Polymorphism (%)
ISSR-11	(AC)8YC	8	200-1500	8	0	0
ISSR-15	(CT)8RG	5	250-700	5	0	0
UBC815	(CT)8G	3	300-900	3	0	0
UBC816	(CA)8T	5	400-800	5	0	0
UBC823	(TC)8C	2	500-1200	2	0	0
UBC861	(ACC)6	5	300-1000	5	0	0
UBC862	(AGC)6	3	350-700	3	0	0
K25	(GA)8A	8	300-1500	6	2	25
K24A	(GA)8T	5	250-1000	5	0	0
HB 15	(GTG)3GC	6	400-1250	6	0	0
Total		**50**		**48**	**2**	**4**

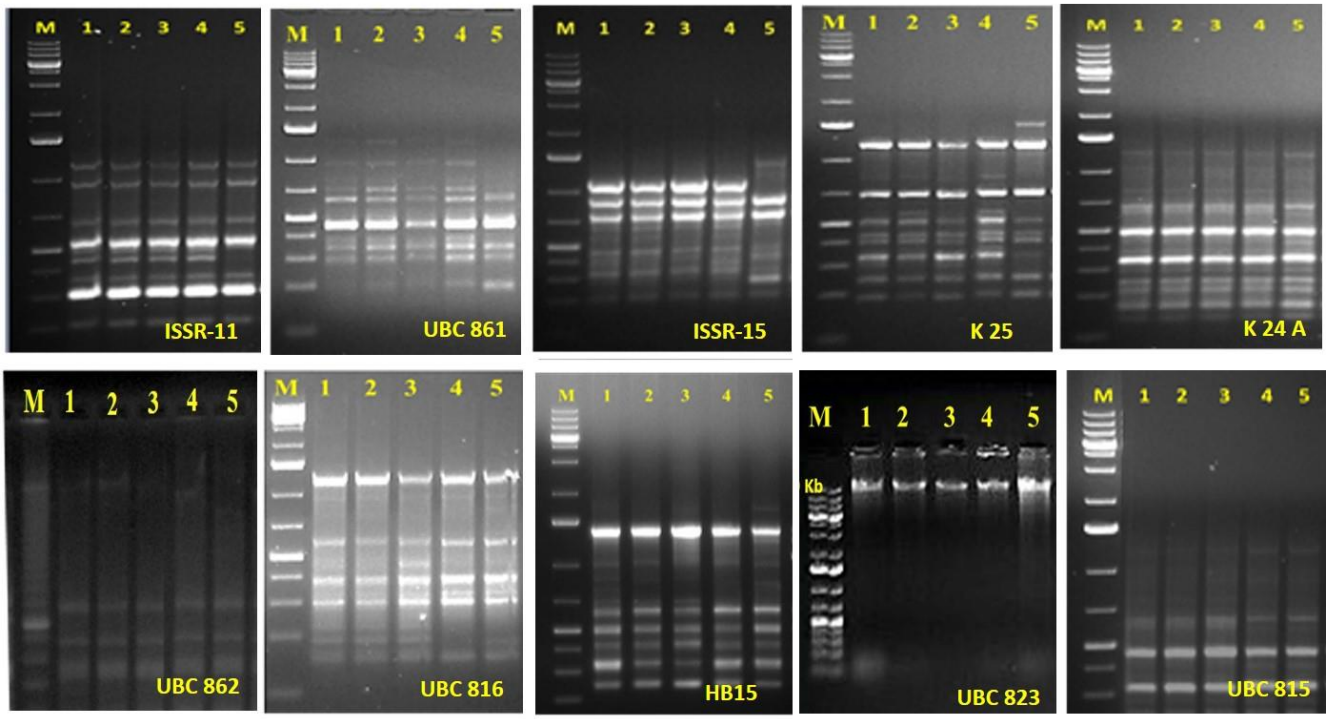

Fig. (2). Electrophoretic separation patterns of ISSR-PCR products (as revealed on 1.8% agarose gel) using 10 primers. Lane M: 1Kb plus DNA ladder marker. Lanes 1 to 5 represent Kalamata mother plant, Kalamata grafted on Kronaki on MS media, Kalamata grafted on Kronaki on OM media, Kalamata grafted on Coratina on MS media and Kalamata grafted on Coratina on OM media, respectively

Interpretation of the Data

Monomorphic Bands (Genetic Fidelity)

Out of the 50 total scorable bands across 10 primers, 48 bands were monomorphic (96%). This indicates a high level of genetic fidelity in the *in vitro* micro grafted plants, as monomorphic bands represent genetic similarity to the donor plant. This result aligns with previous findings in *Olea europaea* micropropagation studies, where stringent protocols maintained genetic stability (**Bracci *et al.*, 2009**).

Polymorphic Bands (Genetic Variation)

Only 2 polymorphic bands were observed, corresponding to a polymorphism rate of 4%. These polymorphic bands were generated by the primer K25, which showed a polymorphism rate of 25% for its scorable bands. This primer's high sensitivity to genetic variation suggests that minor genetic changes occurred in specific regions of the genome. This could be due to slight stress during *in vitro* micrografting, as discussed in studies like **Poljuha *et al.* (2008).**

Primer Efficacy and Range of Amplification

The primers used in the study showed variable efficiency in generating scorable bands, ranging from 2 bands (UBC823) to 8 bands (ISSR-11 and K25). The wide amplification range (200–1500 bp) indicates that ISSR markers were effective in targeting diverse genomic regions.

Polymorphism Percentage Across Primers

The overall polymorphism percentage across all primers is low (4%), further confirming the genetic stability of the *in vitro* propagated plants. This low rate of polymorphism matches earlier studies, such as those by **Ghimire *et al.*, (2012)** and **Benelli *et al.*, (2012**), which demonstrated that controlled propagation conditions limit somaclonal variation. The findings are consistent with earlier

research on *in vitro* propagation of perennial crops. Studies like **Bhatia *et al*., (2015), Rai *et al*., (2013)** and **Poljuha *et al*., (2008)** reported similar results, with polymorphism percentages ranging from 0% to 5% when optimized protocols were used. This highlights the reliability of ISSR markers for assessing genetic fidelity.

4. Recommendations

1- Ensuring genetic fidelity in propagation, regular genetic monitoring implement routine molecular marker analyses (e.g., ISSR, SSR) during large-scale propagation to ensure genetic stability and early detection of somaclonal variations.

2- Develop customized acclimatization protocols based on rootstock genotype, focusing on nutrient and hormonal adjustments to improve the survival of sensitive cultivars like “Coratina”

5. Conclusion

The study demonstrates that, the olive medium (OM) supports higher germination rates for both olive rootstocks, with ‘Kronaki’ showing superior germination performance compared to ‘Coratina.’ These findings highlight the importance of selecting an appropriate substrate and rootstock to optimize seedling establishment. Further research could explore the biochemical and physiological mechanisms underlying these differences to enhance olive propagation techniques.

Additionally, the study indicates that, Kronaki rootstock showed higher survival rates than Coratina, but the difference was not statistically significant. MS medium provided better survival outcomes than OM medium, supporting previous findings on the importance of nutrient composition in plant acclimatization. While rootstock choice appears to influence survival, further research is needed to establish the physiological and biochemical mechanisms underlying these differences. Understanding these factors will help improve acclimatization protocols and enhance survival rates in micropropagated plants.

The ISSR analysis demonstrates that in vitro micrografting of olive Kalamata cv. maintains a high degree of genetic fidelity, as evidenced by 96% monomorphic bands and only 4% polymorphism. These results reinforce the suitability of micrografting for large-scale propagation of olive trees while preserving their genetic identity. Further research could investigate the specific causes of the few polymorphic bands and refine protocols to eliminate even minor variations.

References

Almeida, R., Costa, J. and Silva, P. (2020). Rootstock influence on micropropagated olive plant acclimatization. Journal of Horticultural Science, 55(3): 245-258.

Bardolino, G., Petruccelli, R. and Leva, A. (2017). Olive propagation and rootstock influence on seedling growth. Journal of Horticultural Science, 45(3): 215-228.

Bartolini, L., Feroldi, F., Weda, J. J. A., Slaman, M., De Boer, J. F. and Iannuzzi, D. (2017). Multimodal probe for optical coherence tomography epidetection and micron-scale indentation. Journal of Innovative Optical Health Sciences, 10(6).

Basra, S. M. A. (2006). Handbook of Seed Science and Technology. Haworth Press.

Benelli, C., De Carlo, A. and Pezzotti, M. (2012). Somaclonal variation in olive plants regenerated from somatic embryos. Scientia Horticulturae, 144: 42-48.

Bewley, J. D., Bradford, K., Hilhorst, H., and Nonogaki, H. (2013). Seeds: Physiology of Development, Germination and Dormancy. Springer.

Bhatia, S., Bera, T. and Sharma, K. (2015). Assessment of genetic fidelity of micropropagated banana plants using ISSR markers. 3 Biotech, 5(4), 737-744.

Bonga, J. M. and Von Aderkas, P. (1992). *In vitro* culture of trees. Springer Science and Business Media.

Bracci, T., Sebastiani, L., Natali, L. and Busconi, M. (2009). Molecular markers for cultivar identification and genetic stability in olive tree. Plant Cell, Tissue and Organ Culture, 99(3): 307-315.

Bati, C. B., Godino, G., Monardo, D. and Nuzzo, V. (2006). Influence of propagation techniques on growth and yield of olive trees cultivars 'Carolea'and 'Nocellara Etnea'. Scientia Horticulturae, 109: 173-182

Brito, G., Loureiro, J., Lopes, T., Rodriguez, E. and Santos, C. (2009). Genetic characterisation of olive trees from Madeira Archipelago using flow cytometry and microsatellite markers. Genetic Resources and Crop Evolution 55(5): 657-664.

Davoudi Pahnekolayi, M.; Tehranifar, A.; Samiei, L.; Shoor, M. (2019) Optimizing culture medium ingredients and micrografting devices can promote in vitro micrografting of cut roses on different rootstocks. Plant Cell Tiss. Organ Cult., 137, 265–274.

Dobránszki, J., Jámbor-Benczúr, E., Hudák, I., Magyar-Tábori, K. (2005). Model experiments for establishment of in vitro culture by micrografting in apple. Int. J. Hortic. Sci., 11: 47–49.

Duncan, D. B. (1955). "Multiple Range and Multiple F-Tests. Biometrics, 11: 1-42.

Fabbri, A., Bartolini, G., Lambardi, M., and Kailis, S. (1994). Olive propagation by tissue culture. Advances in Horticultural Science, 8(3): 77-89.

Fehér, A., Bernula, D. and Gémes, K. (2016). The many ways of somatic embryo Initiation. In Somatic Embryogenesis: Fundamental Aspects and Applications; Loyola-Vargas, V.M., Ochoa-Alejo, N., Eds.; Springer International Publishing: Cham, Switzerland, pp. 23–37.

Finch-Savage, W. E. and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. New Phytologist, 171(3): 501-523.

Franclet A. (1983). Rejuvenation: theory and practical experiences in clonal silviculture. In: L. Zsuffa, R.M. Rauter y C.W. Yeatman (eds.) Clonal forestry: Its Impact on Tree Improvement and Our Future Forests. Proc. Can Tree Improv. Assoc, 19th meeting, Part II, pp. 96-134.

Garcia-Sogo, B., Pérez, P. and Moreno, R. (2018). Effects of growth media on in vitro and ex vitro survival of olive rootstocks. Plant Cell, Tissue and Organ Culture, 132(1): 79-89.

Ghimire, B. K., Yu, C. Y., and Chung, I. M. (2012). ISSR marker-based analysis of genetic stability and somaclonal variation in micropropagated plants of Momordica charantia L. Plant Omics Journal, 5(6): 473-480.

Gonçalves, B., Moutinho-Pereira, J., Santos, A., Silva, A. P., Bacelar, E. and Correia‹ C. (2006). Scion–rootstock interaction affects the physiology and fruit quality of sweet cherry. Tree Physiol. 26, 93–104.

Górski, T., Bujarska-Borkowska, B. and Słomka, A. (2018). Genetic and environmental control of seed dormancy and germination. Journal of Plant Growth Regulation, 37(1): 1-18.

Grigoriadou, K., Vasilakakis, M. and Eleftheriou, EP. (2002). In vitro propagation of the Greek olive cultivar 'Chondrolia Chalkidikis.' Plant Cell Tissue Org. Cult 71: 47–54.

Hackett, W. and Murray, J. (1993). Maturation and rejuvenation in woody species. M.R. Ahuja[Ed.], Micropropagation of woody plants,93-105.

Hartmann, H. T., Kester, D. E., and Davies, F. T. (2018). Plant Propagation: Principles and Practices. 9th ed. Prentice Hall.

Huang, J., Huen, MS., Kim. H., Leung. CC., Glover. JN., Yu, X. and Chen, J. (2009). AD18 transmits DNA damage signalling to elicit homologous recombination repair. Nat Cell Biol 11(5):592-603.

Jonard, R., Hugard, J., Macheix, J.J., Martinez, J., Mosella-Chancel, L., Poessel, J.L. and Villemur, P. (1983). In Vitro micrografting and its applications to fruit science. Sci. Hortic-Amst., 20,:147–159.

Jones, A.M. and Saxena, P.K. (2013). Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L. A novel approach to reduce oxidative browning in plant tissue culture. PLoS ONE 2013, 8, e76802.

Joshi, P. and Dhawan, V. (2007) Assessment of Genetic Fidelity of Micropropagated Swertia Chirayita Plantlets by ISSR Marker Assay. Biologia Plantarum, 51, 22-26.

Kaur, P., Sharma, A., and Kumar, R. (2020). Influence of organic amendments on seed germination and seedling growth in agricultural crops. Journal of Sustainable Agriculture, 12(2): 45-57.

Reddy, M.P., Sarla, N. and Siddiq, E.A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding, Euphytica 128:9-17.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15(3): 473-497.

Naddaf, M.E., Rabiei, G., Moghadam, E.G. and Mohammadkhani, A. (2021). In vitro production of PPV-free Sweet cherry (*Prunus avium* cv. Siahe-Mashhad) by meristem culture and micro-grafting. J. Plant Bioinform. Biotechnol., 1:51–59.

Navarro, L., Roistacher, CN. and Murashige, T. (1975). Improvement of shoot-tip grafting in vitro for virus-free citrus. J. Amer. Soc. Hort. Sci.100:471-479.

Navarro, C., Bañón, S. and Sánchez-Blanco, M. J. (2019). Genotypic differences in acclimatization success among olive rootstocks. Scientia Horticulturae, 250: 67-75.

Poljuha, D., Sladonja, B., Sijacic-Nikolic, M. and Smole Mozina, S. (2008). Assessment of genetic fidelity in tissue-cultured olive (*Olea europaea* L.) plants by ISSR markers. Acta Botanica Croatica, 67(2), 127-135.

Rai, M., Jadhav, H., and Gaikwad, N. (2021). Micropropagation and acclimatization of fruit tree rootstocks: Challenges and solutions. Plant Biotechnology Reports, 15(2): 195-208.

Rai, M. K., Shekhawat, N. S., Harish, G., Jain, N., Tamta, S. and Pandey, S. (2013). Genetic fidelity of *in vitro*-raised plants: A critical review. Biotechnology Advances, 30(4), 524-537.

Ramakrishna, A., Ravindra, R., and Ganesan, M. (2019). Effect of organic substrates on seed germination and seedling growth. Agricultural Research, 8(2):102-109.

Ruffoni, B., Raffi, D., Rizzo, A., Oleszek, W., Giardi, M. T., Bertoli, A. and Pistelli, L. (2010). Establishment of in vitro Salvia cel biomass for the controlled production of antioxidant metabolites. Acta Horticulturae, 829: 423-427.

Rugini, E. (1984). In vitro propagation of some olive cultivars with different root-ability and medium development using ammonium nitrate. Scientia Horticulturae, 24(2): 123-134.

Singh, P. and Patel, R.M. (2016). Factors affecting in vitro degree of browning and culture establishment of pomegranate. Afr. J. Plant Sci. 10, 43–49.

Snedecor, G. W. and Chochran, W.G. (1980). Statistical Methods, Seventh Edition, Ames: Iowa State University Press, 507 p.

Sotomayor-Leon, E.M. and. Caballero, J.M. (1990). An easy method of breaking olive stone to remove mechanical dormancy Acta Hortic., 286: 113-116.

Teixeira da Silva, J.A., Gulyás, A., Magyar-Tábori, K., Wang, M.R., Wang, Q.C. and Dobránszki, J. (2019). In vitro tissue culture of apple and other Malus species: Recent advances and applications. Planta 2019, 249, 975–1006.

Thimmappaiah, Puthra, G.T. and Anil, S.R. (2002). In vitro grafting of cashew (Anacardium occidentale). Sci. Hortic., 92: 177–182.

Trentacoste, E. R., Puertas, C. M., and Villalobos, D. P. (2018). Growth and physiological responses of olive rootstocks under acclimatization conditions. Acta Horticulturae, 1222:77-85.

Trigui, A., Ben Ali, S., and Ferchichi, A. (2020). Germination responses of different olive cultivars to environmental conditions. Journal of Plant Physiology, 55(4): 332-341.

Venkatachalam, L., Thimmaraju, R., Sreedhar, R.V. and Bhagyalakshmi, N. (2006) Direct shoot and cormlet regeneration from leaf explants of ‘‘silk’’ banana (AAB). In Vitro Cell Develop. Biol. Plant, 42:262–269.

تحسين تقنية التطعيم المجهري لزيتون الكالاماتا وتقييم دقتها الوراثية

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الموجز

تقيم هذه الدراسة نجاح التطعيم المجهري لصنف كالاماتا (*Olea europaea* L.) على أصلين، كروناكي وكوراتينا، مع فحص تأثير العوامل المختلفة على نتائج التطعيم المجهري. بالإضافة إلى ذلك، يبحث البحث في تأثير التركيب المعدني في وسط المغذيات على نمو هذه الأصول في المختبر. توضح النتائج أن النمط الجيني ووسط النمو يؤثران بشكل كبير على أداء الإنبات، حيث أظهر كروناكي معدل إنبات أعلى (٧٤٪) مقارنة بكوراتينا (٦٢,٥٪). تفوق وسط موراشيج وسكوج (MS) على وسط الزيتون (OM) من حيث معدلات البقاء واستطالة البراعم. على وجه التحديد، سهّل وسط MS نمو البراعم بشكل أكبر (٤,٨٥ سم في المتوسط) مقارنة بوسط OM (٤,٢ سم). أظهر كروناكي أطوال براعم متفوقة (٥,٢٠ سم على MS و ٤,٨ سم على OM) مقارنة بكوراتينا (٤,٥ سم على MS و ٣,٦ سم على OM). خلال عملية التأقلم، أظهرت كروناكي أيضًا معدلات بقاء أعلى (٧٢,٥٪) مقارنة بكوراتينا (٥٩٪). أشار تحليل الدقة الجينية باستخدام علامات تكرار التسلسل البسيط (ISSR) إلى مستوى عالٍ من الاستقرار الجيني، مع ملاحظة ٩٦٪ من النطاقات أحادية الشكل و ٤٪ فقط من تعدد الأشكال. تسلط هذه النتائج الضوء على الدور الحاسم للنمط الجيني ووسط النمو في تحسين نجاح التطعيم الدقيق والحفاظ على السلامة الجينية في إكثار أصول الزيتون.

الكلمات المفتاحية: الزيتون ، التطعيم المجهري ، الأصول ، الدقة الوراثية.