DOI: 10.22620/agrisci.2024.43.002

MICROPROPAGATION OF THE OLIVE (*OLEA EUROPAEA* L.) – A REVIEW OF CURRENT ACHIEVEMENTS AND CRITICAL ISSUES

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Abstract

This review summarizes the most relevant results for the olive (*Olea europaea* L.) in vitro propagation obtained from studies done by different scientists worldwide, discussing the achievements and elucidating some critical aspects influencing the micropropagation efficiency. Such crucial factors could be the problematic establishment of sterile cultures using explants originating from field or greenhouse plants due to significant contamination; tissue oxidation when using meristems and nodal cuttings in some varieties; the effect of the growth regulators regarding the hyperhidricity appearing in developing explants during the early or later subcultures; and finally, the strong genotype dependence of proliferation and rooting ability.

Although many protocols for the intensive propagation of olives are documented, the most competitive and economical methods would be beneficial for initiating in vitro research in olives. Therefore, this publication's findings would help the development of specific protocols for available olive germplasm and be useful for the commercial propagation and obtaining of the pathogen-free olive plants.

Keywords: explant, *in vitro*, medium, *Olea europaea* L., olive, plant growth regulators

INTRODUCTION

The olive (*Olea europaea* L.) is one of the most ancient crops that accompanied the development of Mediterranean civilizations. Ancient history is full of legends about the olive origin, cultivation, and significance, demonstrated by the enormous diversity of olive varieties and their names (El-Kholi et al., 2012). As many as 1250 different cultivars of olive trees have been cultivated globally in 54 countries, and this material has been conserved in over 100 collections (Bartolini, 2008; Abuzayed et al., 2018). The interest in expanding the area of olive plantations is increasing, mainly for economic and cultural reasons, including the growing consumer demand and the use of olive oil in the cosmetic industry and various healthy diets.

The Olive Propagation Manual by Fabbri et al. (2004) is a well-documented work that deals with all issues concerning olive propagation, overviewing several available traditional techniques for olive propagation by cuttings in mist cultivation tunnels or grafting on seedbeds and the main aspects of in vitro technique, and summarizing the rooting ability of all olive cultivars of which scientific literature has been available. The reported average results after the IBA treatment of the olive cuttings classify 116 genotypes with a high rooting ability (66 to 100%), 164 genotypes with medium rooting ability (33 to 66%), and 125 genotypes with low rooting ability (0 to 33%) known to be difficult to root or recalcitrant (Bartolini et al., 1998; Fabbri et al. 2004).

Considering the average rooting power of the majority of olive cultivars under natural conditions and the difficulties of their

propagation through cuttings, on the one hand, and the economic importance of olives in the canning and olive oil industry, on the other hand, using tissue culture methods for more efficient olive propagation is necessary (Ghane Golmohamadi et al., 2019).

Despite these facts, only 1% of the production of the olive trees in Italy is obtained by applying in vitro propagation techniques, mainly from stimulation of axillary buds of nodal explants taken from the selected mother plants (Petruccelli et al. 2012).

However, the commercial micro-propagation of the olive trees is currently not enough because of the production costs, and it is mainly used for research and development on various aspects of the olive culture (Haq et al., 2021). Additionally, the advantage of the technique to be applied is the production of high-quality, pathogen-free, and uniform plants of *Olea europaea* L. (Bayraktar et al., 2020).

The presented information determines the significance of the olive culture and the necessity to analyze the biotechnological studies for propagation as a prerequisite for quality olive growing. It seems essential to take in advance all the achievements and complications concerning optimizing the methods and developing an effective micropropagation system in the olives (Micheli & da Silva, 2020).

OLIVE MICROPROPAGATION ASPECTS

As a result of increased information, Kane (2005) defines five main stages (0–IV) for successful micropropagation.

Stage 0: Donor plant selection and explant preparation

Stage I: Establishment of aseptic cultures

Stage II: Proliferation of axillary shoots

Stage III: Pretransplant (rooting)

Stage IV: Transfer to the natural environment (acclimatization)

Most commercial and research laboratories have adopted such a system to simplify production scheduling, accounting, and cost analysis (Kurtz et al., 1991).

These stages illustrate the procedural steps in the micropropagation process and represent the altered cultural environment (Figure 1).

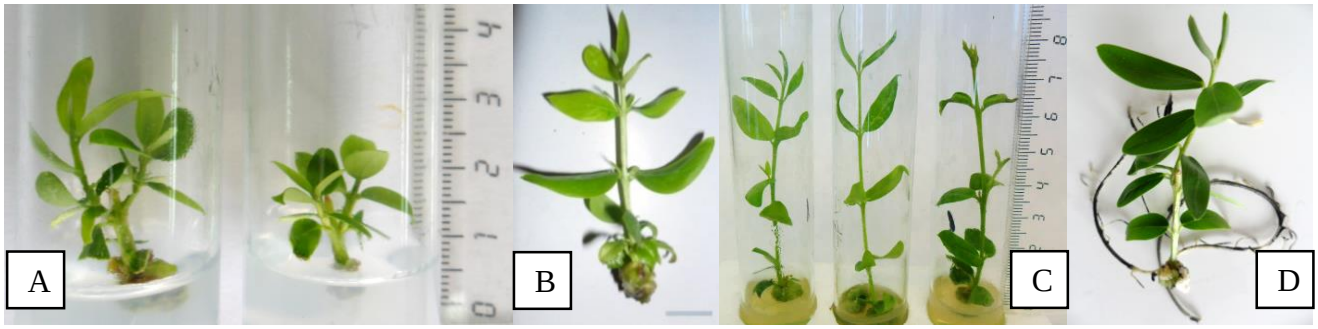

Figure 1. Stages in micropropagation of the olive cultivar Chondrolia Chalkidikis: A. Effect of the explant size on the initial development of uninodal (right) and binodal (left) segments, B. Propagation stage of the olive after five-week culture (scale bar = 1 cm), C. Elongation of olive shoots, D. Rooted olive plants (Yancheva and Kondakova, 2018).

The olive micropropagation, reported initially by Rugini & Frontanazza (1981), traced the development of the tissue and cell culture techniques in recent decades and has drawn the attention of scientists and growers to the possibility of mass propagation and the production of certified planting material (Rugini & Lavee, 1992). Despite the numerous studies done on different olives grown worldwide, micropropagation is not always successful, and there are still several problems in the individual stages of the process - introduction into a sterile

culture, multiplication, rooting, and hardening of the plantlets. Most olive cultivars' multiplication rate remains unsatisfactory and highly depends on the genotype. Therefore, the present review attempts to elucidate some critical factors influencing micropropagation efficiency, summarizing the knowledge with various olive varieties.

Donor plant selection and explant preparation

The selection of healthy stock plants is the foremost step in *in vitro* propagation. Stock plants can be grown in fields or greenhouse conditions. Before the collection of explants, a fungicide spray applied to the donor plants is a helpful approach to control high contamination (Haq et al., 2021). The donor plant's origin and age are crucial for preparing the initial olive explants. Fabbri et al. (2004) noted that potted olive stock plants, grown under greenhouse conditions, are the best material to initiate in vitro propagation.

Among most plant species, the apical meristems and explants, such as the shoot tips, are preferred explants for establishing virus-free cultures (Hassan & Zayed, 2018). The early reports of olive micropropagation described procedures using zygotic embryos and seed explants (Bao et al., 1980; Cañas et al., 1988; García-Berenguer & Durán González, 1990), but this method is not preferred due to the segregation and a long period of juvenility (Lambardi & Rugini, 2003), and finally the lack of clonal fidelity.

It was found that the youngest tissues, such as apical meristem and shoot tips, are inappropriate in olive. Nodal explants collection in winter or early spring and subsequent axillary meristem development is mainly associated with actively growing shoots from young trees. Single-node and two-node cuttings (Figure 1A), isolated from actively growing shoots or suckers, were the preferred explant source for in vitro culture initiation (Table 1).

Spheroblasts (tissue hyperplasia) are formations that naturally occur at the stem base of adult olive plants, which are rich in adventitious buds. Rama and Pontikis (1990) reported the spheroblasts (ovules) as initial explants for micropropagation. Later, Cañas et al. (1992) described a practical procedure with single-node segments isolated from shoots originating from spheroblasts as a suitable explant source.

IN VITRO CULTURE ESTABLISHMENT

The following factors may affect the successful establishment of an in vitro culture: explantation time, explant position on the stem, explant size, sterilization system, choice of disinfectant, and polyphenol oxidation.

Sterilization procedure

The presence of an effective sterilization system is of primary importance in the development of micropropagation protocol. The tissue disinfection protocol has to be tailored to the level of contamination and the origin, juvenility, and physiology of the plant material. Pioneer procedures for surface disinfection included ethanol treatments with different concentrations (Rugini et al., 1981), but later works indicated a tendency to avoid its application due to tissue dehydration. Various substances have been tested for the sterilization of olive explants, typically chlorine-containing compounds such as sodium hypochlorite and calcium hypochlorite, peroxide, and sulfuric acid (for seeds) (Rugini et al., 1986; Martino et al., 1999; Grigoriadu et al., 2002; Sghir et al., 2005). Mercury chloride ($HgCl_2$) is shown to be more effective, but its use is restricted because of its toxicity and harm to human health. Nevertheless, due to intense contamination found on olive starting material, the application of $HgCl_2$ (0.1-0.3%) has been established as a usual sterilization method (Zacchini & De Agazio, 2004; Sghir et al., 2005; Peixe et al., 2007; Fazeli-Nasab et al., 2021).

Compounds such as detergents, adhesives, antioxidants, antibiotics, and fungicides have also been tested for pre- and post-sterilization washing to enhance disinfection efficiency and prevent tissue oxidation. Ahmed et al. (2016) stated that mancozeb treatment prevented fungal contamination up to 90% and 20% chlorox maximized sterilization. The addition of antibiotics in culture media also allowed the problem to be overcome (Zacchini & De Agazio, 2004).

First, Rostami & Shahsavar (2009) considered that adding silver nano-particles (4 mg/l) in the nutrient medium had a positive effect by suppressing the explant's infection without affecting their development and growth. The finding aligns with a study done by Hegazi et al. (2021), who recommended low concentrations (5 mg/l) of nano-silver particles in olive plant tissue cultures.

Tissue oxidation

Earlier studies reported that when introducing explants from field or cultivation facilities grown olive trees, several problems related to tissue browning and microbial contamination were observed (Bao et al., 1980; García-Berenguer & Durán González, 1990; Cañas et al., 1992).

The darkening of plant tissues is caused by the oxidation of various phenolic compounds called exudates released from the injured cells. The polyphenol oxidase (PPO) and peroxidase (POD) enzymes oxidize phenolic substances. Roussos & Pontikis (2001) stated that their accumulation in the nutrient medium inhibited growth, causing tissue necrosis and death of olive explants. The degree of browning is related to the concentration of phenolic substances and enzymatic activity of the tissues, especially PPO. Reduced PPO and POD activity increases the ability of tissues to initiate growth in vitro.

The high total phenolic content in olives (Mitsopoulos et al., 2010; Kontogianni & Gerothanassis, 2012) adversely affects the growth and survival of the explants *in vitro*. In recent decades, scientists have tried to optimize different steps in olive micropropagation and overcome these problems by using chemical, enzymatic, or physical methods. The immersion of olive explants in water inhibits the synthesis of phenolic compounds or their precursors (Rugini, 1984; Briccoli-Bati et al., 1999), and recommend explant washing with a sterile antioxidant solution for at least 30 minutes and subsequent culture medium supplemented with antioxidants such as ascorbic acid (10-20 mg/l). The explant prevention from browning and phenolic compound oxidation is also recorded by adding activated charcoal, citric acid, or sodium chloride to the culture medium, and subsequent cultivation in the darkness (Rostami & Shahsavar, 2012).

Rugini et al. (2011) concluded that establishing sterile cultures and further growth of shoots are crucial in olive micropropagation. Rapid oxidation may occur even when using high doses of active antioxidants when the explants are collected from field or greenhouse-grown plants. Recently, Khatoon et al. (2022) reported that pre-cooling treatments for 48 hours had a beneficial effect on in vitro culture initiation and subsequent morphological, physiological, and biochemical traits of different olive cultivars. The success of different approaches for overcoming such a problem depends on the complex of factors, but the olive genotype is determinative.

PROLIFERATION OF AXILLARY SHOOTS

Basal medium composition

Developing a specific nutrient medium for olive (OM) by Rugini et al. (1984) that stimulates axillary proliferation and subsequent shoot growth is an essential step for further optimizing the micropropagation protocols. The composition of the medium is based on the analysis of the main mineral elements of olive

shoots actively growing in the field. Compared to the most commonly used MS composition, it is characterized by:

(i) OM medium is richer in Ca, Mg, S, P, B, Cu, and Zn,

(ii) it has a slightly different Ca/N ratio (1:11), and

(iii) it is also supplemented with glutamine (2.19 mg/l) as a nitrogen source.

The Ca/N ratio, in particular, proved to be an important factor in olive micropropagation, as it can interfere with the maintenance of healthy shoots (Fiorino & Leva, 1986).

Table 1. Proliferation stage - summary of the best culture conditions in the olive cultivars provided by Fabri et al. (2004) and Yancheva and Kondakova (2018) with supplements

Genotype	Initial explant	Basal medium*	Growth regulators for multiplication	Reference
Aglandau, Tanche	Uninodal explants	mOM	Zeatin 4 mg/l	Binet et al., 2007
Arbequina	Young shoots from grafted plants	DKW	BA 4,4µM + IBA 0,05µM	Revilla et al., 1996
Arbequina	Uninodal explants	OM	Zeatin 10 mg/l	Otero and Docampo, 1998
Arbequina Empeltre Picual	Uninodal explants	OM	BA 1µM+ TDZ 1µM	Garcia-Fèrriz et al., 2002
Chondrolia Chalkidikis	Uninodal explants	WPM	Zeatin 20µM	Grigoriadou et al., 2002
Carolea	Uninodal explants	½ OM	Zeatin 4 mg/l	Briccoli Bati et al., 1994
Dolce Agogia	Uninodal explants from suckers	½ MS	Zeatin 10 mg/l + IBA 0,5 mg/ l+ GA_3 0,5 mg/l	Rugini and Frontanazza, 1981
Ecotype 05300	Uninodal explants	WPM	Zeatin 0,1 mg/l	Binet et al., 2007
Frantoio	Uninodal explants	OM	Zeatin 4mg/l or 2iP 4mg/l	Rugini, 1984
Frontio	Uninodal explants	MS	IBA 1 mg/l + kinetin 1 mg/l	Mangal et al., 2014
FS-17	Uninodal explants	mOM	Zeatin 2 mg/l or 2iP 4 mg/l	Mencuccini, 1998
Galega vulgar	Uninodal explants	OM	Coconut water 50 ml/l + BAP 2,22µM	Peixe et al., 2007
Gemlik	Nodal explants	WPM	BA 4 mg/l or Zeatin 0,5 mg/l	Bayraktar, et al., 2020.
Kalamon	Uninodal explants	mOM	Zeatin 10 mg/l + crude extract	Rama and Pontikis, 1990
Kalamon	Uninodal explants	WPM[(e)]	BA 1 mg/l + IBA 1 mg/l + GA_3 0,1 mg/l	Dimassi-Theriou, 1994
Koroneiki Kalamon	Uninodal explants	MS	IBA 0,5 mg/ l+ BAP 10 mg/l	Ozkaya et al., 2003

Maurino	Three-nodal explants	mMS	Zeatin 0,5 mg/l or TIBA 4 mg/l	Bartolini et al., 1990
Memecik Domat	Nodal explants	mOM	BA 1 mg/l	Seyhan and Özzambak, 1994
Meski Chemlali de Sfax Chetoui Sig de Sfax	Uni- and binodal explants	mMS	Zeatin 2 mg/l + kinetin 1-2 mg/l (or BA 2 mg/l)	Chaari-Rkhis et al., 1999
Meski	Uninodal explants	mOM	Zeatin 1 mg/l	Chaari-Rkhis et al., 2002
Mission	Uninodal explants	½ MS	BA 2,1 mg/l + GA_3 1,26 mg/ l+ NAA 0,6 mg/l	Rostami and Shahsavar, 2009
Mission	Nodal explants	½ MS	BA 2,1 mg/l + GA_3 2.8 mg/l + NAA 0,6 mg/l	Rostami and Shahsavar, 2012
Moraiolo	Shoot tips	OM	Zeatin 4 mg/l or 2iP 4 mg/l	Rugini, 1984
Moraiolo	Shoot tips	OM	Zeatin 3,0 mg/l + BAP 0,5 mg/l	Ali et al., 2009
Nebbiara	Nodal explants	OM MS	Zeatin 13.68 μM + GA3 4.33 μM+ IBA 0.49 μM BAP 8.88 μM + GA3 4.33 μM+ IBA 0.49 μM	Zacchini and Agazio, 2004
Nocellara Etnea	Uninodal explants	mOM	Zeatin 4 mg/l	Briccoli Bati and Lombardo, 1995
Oueslati	Uninodal explants	mOM	Zeatin 1 or 2 mg/l	Chaari- Rkhis et al., 2011
Picual	Nodal explants	mMS, OM, mOM	Zeatin 2-4 mg/l or BAP 2-6 mg/l	Hegazi et al., 2021
Rowghani	Uninodal explants	DKW	2iP 4 mg/l	Peyvandi et al., 2009
ZDH4, Lucques, Haouzia, Dahbia, Amwllau, Salonenque Picholine marocaine Picholine du Landuedoc	Uninodal explants	OM	Zeatin Riboside 13,6 μM	Sghir et al., 2005

*m-modified medium, media: DKW (Driver and Kuniyuki, 1984), MS (Murashige and Skoog, 1962), OM (Rugini, 1984), WPM (McCown and Lloyd, 1981)

Of all trace elements, boron (B) plays an important role, but its effect in olives is contradictory and genotype-dependent. According to Leva et al. (1992), the presence of B significantly increased proliferation in the cultivars Maurino and Nocellara etnea, while Dimasi et al. (1999) reported a shoot number doubling in micropropagation of Kalamon when B was absent.

Considering the carbohydrate source used, Leva et al. (1994) compared the effect of sucrose and mannitol as the main carbohydrates of olive metabolism. Later studies proved that the mannitol significantly increased the proliferation, improving the overall crop quality and the uniformity and reducing callus formation at the base of the cuttings (Zacchini & De Agazio, 2004; Rejšková et al., 2007; Vidoy-Mercado et al., 2012; Leva et al., 2013; Bayraktar et al., 2020).

The effectiveness of the basal medium OM has been proven for many olive cultivars, as shown in Table 1, and most of the developed protocols are based on the original composition or with some modifications.

Plant growth regulators

The olive is a recalcitrant species characterized by apical dominance; therefore, depending on the genotype, the type and concentration of growth regulators differ widely. Commonly, a lower concentration of growth regulators is preferential in the medium for culture establishment.

The multiplication stage (Figure 1B) is based on axillary shoot proliferation, and the cytokinins significantly regulate this phenomenon (Table 1).

Zeatin is the most efficient and preferred cytokinin in olive micropropagation, providing maximum cell division stimulation and resulting in a high proliferation rate in olive explants. It was used solely in various concentrations as well, with the addition of other growth regulators. Zeatin was efficient at concentrations from 0.5 mg/l in Maurino (Bartolini et al., 1990) to 10 mg/l in Arbequina (Otero & Docampo, 1998), while for Chondrolia Halkidikis the optimal concentration was 20 μM, providing the highest number of the new micro shoots/explant (1.68), with 3.0 cm shoot height and 4.2 proliferation rate (Grigoriadou et al., 2002).

Haq et al. (2021) stated that most of the studies with olive cultivars recorded the maximum shoot proliferation with zeatin because it does not quickly degrade and remains in a nutrient medium, but also pointed out that its high cost could be an obstacle to commercial employment.

Scientists have also proposed the use of kinetin (Chaari-Rkhis et al., 1999; Mangal et al., 2014), thidiazuron (TDZ) (Garcia-Fèrriz et al., 2002), and TIBA (Bartolini et al., 1990) alone or in combination with zeatin. It was demonstrated that other frequently used cytokinins are less efficient and cannot replace zeatin as the primary growth regulator influencing the stimulation of olive axillary buds. A commonly used cytokinin, 6-benzylaminopurine (BAP), is less effective in most olive genotypes since its application is associated with excessive callus proliferation and weak shoot development. The lowest proliferation efficiency was reported for 2iP and BAP, respectively (Rugini, 1984; Dimassi-Theriou, 1994; Seyhan and Özzambak, 1994; Revilla et al., 1996; Mencuccini, 1998; Garcia-Fèrriz et al., 2002; Peyvandi et al., 2009; Rostami & Shahsavar, 2009) (Table 1).

Due to the high cost of zeatin, research efforts focused on modifying the nutrient media composition and cytokinin interactions, able to replace or at least reduce the concentration of zeatin while maintaining the proliferation rate. Combining zeatin with 2iP (Rugini, 1984; Mencuccini, 1998; Peyvandi et al., 2009) and TDZ (Garcia-Fèrriz et al., 2002) demonstrated promising results. An alternative approach towards optimizing micropropagation was the coconut milk and BAP combination, replacing

zeatin's effect and providing high proliferation in the cultivar Galega vulgar (Peixe et al., 2007).

Dikegulac was also shown to be efficient at low concentrations and, in combination with zeatin, promoting the formation of lateral shoots, improving the ability to reduce apical dominance (Mendoza-de Gyves et al., 2008). Additionally, the dikegulac application with white light cultivation helped to overcome the apical dominance and obtain a suitable micropropagation rate in cv. Arbequina (Moradnezhad et al., 2017). Although different responses of olive genotypes to dikegulac were expected, the use of both BA and dikegulac in Chondrolia Chalkidikis propagation resulted in increased proliferation and new shoots of good quality development, reducing vitrification symptoms (Antonopoulou et al., 2018). The authors concluded that if the dikegulac application has such an effect as producing more shoots, it could lower the costs of micropropagation by the replacement of expensive cytokinins with the much cheaper dikegulac (Antonopoulou et al., 2018).

Among the substances of natural origin that have beneficial effects in olive micropropagation, neem oil has also been reported to reduce zeatin use and optimize the rooting phase. It was noted that neem oil addition at a low concentration (0.1 ml/l) to the nutritive medium with zeatin (4 mg/l) improved the shoot length, the multiplication rate, and fresh and dry weights of the proliferated explants (Micheli et al., 2018; Regni et al., 2023).

Neem oil contains at least 100 biologically active compounds (Campos et al., 2016), and thanks to its complex chemical composition, may act in synergy with the nutrient components of the growth medium, improving its trophic function and simulating the effects of gibberellins and cytokinins such as a higher shoot length and the development of secondary adventitious shoots (Regni et al., 2023). However, according to the abovementioned, knowledge about the effects of neem oil in olive in vitro cultures is still scarce.

Most of the olive micropropagation media contain the addition of auxins, such as IBA (Revilla et al., 1996; Ozkaya et al., 2003; Mangal et al., 2014) or NAA (Rostami & Shahsavar, 2009). Moreover, a specific growth regulator combination established as efficient for one or several olive varieties could be less or ineffective for others. Shoot proliferation increases at higher cytokinin concentrations, but the developing shoots are usually smaller and may exhibit symptoms of hyperhydricity. In some olive cultivars (Table 1), auxin application in the proliferation medium resulted in the balancing cytokinin carryover effect and subsequent shoot elongation, thus increasing the number of usable shots of sufficient length for rooting. On the other hand, exogenous auxin may enhance cytokinin-induced axillary shoot proliferation depending on the particular genotype.

SHOOT ELONGATION

The elongation stage is an optional (not obligatory) step as a prerequisite for an efficient rooting procedure. It could be performed by transferring to a hormone-free subculture, modifying salt content, reducing growth regulators concentration, and applying gibberellic acid to the medium before the transfer to a proper rooting medium (Fig. 1C).

Depending on the number of subcultures, at the end of the multiplication stage, the obtained shoots are usually too short and weak to pass through a rooting medium, where they stop growing. Therefore, different approaches, such as explant transferring to a subculture with a half-strength medium or modified mineral composition and application of gibberellic acid (GA3), are prerequisites to overcoming the effect of growth regulators, internode elongation and subsequent better rooting. Bayractar et al. (2020) reported an adapted protocol for the cultivar Gemlik, applying modified OM with three times the

concentrations of KNO_3 and 2.0 mg/l Zeatine in the multiplication stage, resulting in sufficient shoot elongation. Additionally, by optimizing the proliferation medium and changing the gelling agent to agar, hyperhydricity development was prevented. Moreover, in Chondrolia Chalkidikis, Grigoriadou et al. (2002) noted that adding 10 μM GA3 to a zeatin-containing medium positively affected shoot proliferation and elongation, resulting in 3.0 cm shoot height. At the same time, the same concentration of GA3 in combination with BA reduced the number of the new microshoots per explant and the proliferation rate, as the shoot height remained nearly the same (2.7). The GA3 influence on the olive shoot elongation was also confirmed, but the results obtained were highly genotype-dependent (Rugini & Frontanazza, 1981; Dimassi-Theriou, 1994; Antonopoulou et al., 2006; Rostami & Shahsavar, 2009; 2012).

ROOTING

The olive genotype remains the dominant issue influencing the rooting efficiency. It is characterized by root-formation time, root number, and root length. In addition, the composition of the basal medium (OM, WPM, DKW, MS) and the auxin used are the crucial factors many scientists have tested and modified, aiming to enhance rooting efficiency (Table 2).

The most used auxins for olive rooting are IBA and NAA at 0.5 - 4 mg/l concentrations. Due to IBA's positives, non-toxic behavior, and high stability, it is the preferred auxin (Hartmann et al., 2007). Saghir et al. (2005) reported that IBA produced maximum roots in olives under dark culture conditions.

It was shown that in some genotypes such as Arbequina (Revilla et al., 1996), FS-17 (Mencuccini, 1998), and the Tunisian varieties Sig de Sfax, Chemlali de Sfax, Chetoui, and Meski (Chaari-Rkhis et al., 1999) root differentiation might occur on proliferation nutrient medium but when the macronutrient composition was half-reduced.

Substantial progress in the rhyzogenesis of micropropagated olives has been achieved, and some cultivars considered recalcitrant (difficult to root), such as Nocellara etnea, have been successfully rooted in vitro (Briccoli Bati & Lombardo, 1995). Maximum rooting efficiency of 100% has been achieved for cultivars FS-17 (Mencuccini, 1998), Meski (Chaari-Rkhis et al., 2002), Nocellara etnea (Briccoli Bati & Lombardo, 1995), and Tunisian cultivars such as Sig de Sfax, Chemlali de Sfax, Chetoui and Meski (Chaari-Rkhis et al., 1999), which confirms the strict genotype dependence of the rhizogenesis. Rooting rates between 70-93% have been reported in Chondrolia Chalkidikis using WPM basal medium with auxin combination of IBA and NAA and putrescine addition (Grigoriadou et al., 2002).

Alternative approaches towards optimizing rooting have been proposed for Galega vulgar (Peixe et al., 2007) and Oueslati (Chaari Rkhis et al., 2011) by briefly immersing the microplants in IBA solution (2-3 mg/l) and subsequent cultivation on hormone-free or medium with cytokinin. However, Allatif & Hmmam (2022) stated that the response of the shoots to the rooting method was genotype-dependent, and the root formation in vitro on OM medium containing IBA was more successful than dipping in IBA solution. Adding putrescine (Briccoli Bati et al., 1994; Grigoriadou et al., 2002; Ozkaya et al., 2003) or crude extract from ovules (Rama & Pontikis, 1990) observed better and faster root differentiation. Leva (2011) developed an *ex vitro* method for olive micro cuttings rooting, presenting a viable solution for lowering the production cost and time required to propagate intensively grown olive plants (Haq et al., 2021).

As a recalcitrant plant, the olive rooting ability could be influenced additionally by optimizing the cultural conditions. Improved root differentiation was achieved by keeping the cultures in a dark environment or incorporating chemicals into media that have darkening

effects, painting vessel bases, adding activated charcoal (Figure 1D) and polycarbonate granules as darkening agents to the media (Rugini et al., 1993; Mencuccini, 1995; 2003; and Haq et al., 2009). The beneficial effect of activated charcoal and ascorbic acid application was also noted in the rooting of the cultivar Chondrolia Chalkidikis (Mavromatis & Yancheva, 2014).

Table 2. Summary of the rooting efficiency of olive cultivars in vitro by Fabri et al. (2004), Yancheva and Kondakova (2018) supplemented.

Genotype	**Rooting (%)**	**Basal medium***	**Growth regulators**	**Reference**
Aglandau Tanche	80 62	mOM	IBA 4 mg/l	Binet et al., 2007
Arbequina	57	½ DKW	IBA 0,5 µM	Revilla et al., 1996
Arbequina	NR	OM	IBA 3 mg/l or NAA 1mg/l	Otero & Docampo, 1998
Arbequina Empeltre Picual	75	Compost	IBA 15 µM + IAA 10 µM	Garcia-Fèrriz et al., 2002
Carolea	NR	½ OM	NAA 4 mg/l or IBA 2,5 mg/l + Putrescine 160 mg/l	Briccoli Bati et al., 1994
Chondrolia Chalkidikis	70-93	WPM	IBA 12µM + NAA 3µM + Putrescine 30µM	Grigoriadou et al., 2002
Dolce Agogia	84	½ Knop + Heller vitamins	NAA 4 mg/l	Rugini and Frontanazza, 1981
Ecotype 05300	NR	WPM	IBA 1 mg/l NAA 0,75 mg/l	Binet et al., 2007
Frantoio	80	½ MS, ½ Knop/Heller	NAA 1 mg/l	Rugini, 1984
Frontio	60	½ MS	IBA 0,2 mg/l + NAA 0,2 mg/l	Mangal et al., 2014
FS-17	100	½ MS	NAA 2 mg/l	Mencuccini, 1998
Galega vulgar	85	OM	Dipping for 10 sec. in IBA 3 mg/l and culture in OM + AC*	Peixe et al., 2007
Gemlik	50	OM	NAA 1.5 mg/l 160 mg/l Putrescine	Bayraktar et al., 2020
Kalamon	82	mOM	NAA 2 mg/l + ovules crude extract	Rama and Pontikis, 1990
Kalamon	80	WPM	IBA 2mg/l	Dimassi-Theriou, 1994
Koroneiki Kalamon	45 29	Knop-macro + Heller-micro salts	Putrescine 160 mg/l + NAA 1mg/l	Ozkaya et al., 2003
Maurino	60	mMS	IBA 2 mg/l (”dip method”)	Bartolini et al., 1990

Meski	100	mOM	IBA 1 mg/l + NAA 1 mg/l	Chaari-Rkhis et al., 2002
Meski Chemlali de Sfax Chetoui Sig de Sfax	100	½ mMS	NAA 2 mg/l	Chaari-Rkhis et al., 1999
Mission	80-93	½ MS	IBA 4 mg/l	Rostami and Shahsavar, 2012
Moraiolo	80	½ MS ½ Knop/Heller	NAA 1 mg/l	Rugini, 1984
Moraiolo	13-87	OM	IBA or NAA, 0,5-3,0 mg/l	Ali et al., 2009
Nebbiara	93-100	½ OM	NAA 3.22 μM	Zacchini and Agazio, 2004
Nocellara Etnea	100	mOM	NAA 1,5 mg/l	Briccoli Bati and Lombardo, 1995
Oueslati	45-50	mOM	Dipping in IBA 2 mg/l + Zeatin 1- 2 mg/l	Chaari Rkhis et al., 2011
Picual	15-58	mMS, OM, mOM	IBA 2-4 mg/l or NAA 2-4 mg/l	Hegazi et al., 2021
ZDH4 Lucques Haouzia Dahbia Amellau Salonenque Picholine marocaine Picholine du Landuedoc	52 30 57 54 70 20 65 40	OM	NAA 5,37μM or IBA 24,6 μM	Sghir et al., 2005

*AC-activated charcoal, m-modified medium, media: DKW (Driver and Kuniyuki, 1984), MS (Murashige and Skoog, 1962), OM (Rugini, 1984), WPM (McCown and Lloyd, 1981), NR-not reported

The most relevant results based on the rooting of olive cultivars data (Table 2) proved the effect of the genotype. Moreover, the rooting efficiency of eight Moroccan olive genotypes grown on the same medium varied between 70% and 20% (Sghir et al., 2005), confirming the complexity of the interactions between the genotype, growth regulators, and rhyzogenesis. In most studies, the rooting stage is critically important for the successful intensive propagation of olives because it is directly correlated with the stage of acclimatization to the *ex vitro* environment and the rate of survival plants.

ACCLIMATIZATION

Acclimatization is a crucial phase in micropropagation due to the changes in plant physiology and environmental conditions - transition to autotrophic nutrition, humidity, light intensity, and non-sterile conditions. Ex vitro adaptation is carried out in containers with various sterile substrates. After transplanting, a gradual decrease in humidity from 95% to 70% and a temperature of 22 - 25 ^{o}C, depending on the season, is necessary. Treatment with fungicides is recommended against developing fungal diseases that can cause additional losses.

Furthermore, the application of mycorrhiza (*Glomus mosseae*) significantly improved plant survival and subsequent plant development and growth (Binet et al., 2007).

The acclimation stage in high relative humidity with constant light is essential to obtain olive plants with lateral branches ready for transplanting. After that, one year of further cultivation and hardening in a nursery is recommended before establishing in industrial plantations (Mencuccini, 1998). Subsequent studies for the field performance have shown that micropropagated plants start flowering at the same time as plants obtained from cuttings (Rugini et al., 1986), but in some cultivars, micropropagated plants start fruiting earlier (Briccoli Bati et al., 1999).

GENOTYPE

The micropropagation technique is relatively tricky in propagating economically significant olive varieties. The mentioned above studies (Table 1 & 2) have illustrated the lack of a universal protocol for micropropagation due to the marked heterogeneity of the studied genotypes. The in vitro propagation efficiency of the olives depends mainly on the genotype, and it further determines the choice of the type and concentration of growth regulators, the carbon source used, and cultivation conditions, especially (Sghir et al., 2005; Chaari Rkhis et al., 2011). As an example, the study done by Sghir et al. (2005) with the olive cultivars (ZDH4, Lucques, Haouzia, Dahbia, Amellau, Salonenque, Picholine marocaine, and Picholine du Landuedoc) recorded a multiplication rate between 0.4 and 3, concluding that Haouzia was most susceptible to micropropagation in the presence of zeatin riboside (ZR). The same cultivars demonstrated different rooting abilities, the highest in Salonenque (70%) and the lowest in Amellau (20%), while in the best proliferative variety Haouzia, the rooting was 57%. Comparing the regeneration ability of the olive cultivars, Ozakaya et al. (2003) pointed out that the variety Kalamon lends itself to multiplication in vitro, which is much more complicated than Koroneiki. All of the abovementioned for olive rooting proved that such a complex process demands an integrated approach, and only this way could be achieved to root any olive cultivar (Porfírio et al., 2016).

ALTERNATIVES FOR MICROPROPAGATION EFFICIENCY IMPROVEMENT

Micropropagated plants are extremely labor intensive; therefore, it is necessary to focus research on the automation of the process to reduce the costs. *In vitro* cultivation requires enough monetary inputs to be rationalized, and to achieve economic benefits, this technique must be used more efficiently. Nowadays, researchers have developed a Temporary Immersion System (TIS) for olive intense proliferation, which is available (Benelli & Carlo, 2018). Similarly, Lambardi et al., 2006 used the RITA® system for shoot proliferation of olive and achieved promising results compared to the traditional use of zeatin-containing solid media (Haq et al., 2021).

Plant biotechnology has been widely used in fundamental and applied research on various biological species, and the scientific interest in transferring that technology on an industrial scale has been rapidly growing. During the last decades, the improvement of the efficiency of plant tissue methods was directed to the commercialization of micropropagation by robotization or automation of the process and application of different bioreactor systems (Yancheva & Kondakova, 2018).

CONCLUSION

The analysis of the achievements of the studies for olive in vitro propagation allows the following general conclusions:

- Genotype is the crucial factor for the propagation efficiency.

- Appropriate initial explants are the nodal cuttings taken 5-10 cm below the shoot apex, isolated from greenhouse-grown plants.
- Sterilization of olive explants is critical, where using antioxidants and adding silver nano-particles to the culture medium has a beneficial effect.
- The most suitable basal nutrient medium for olive micropropagation is the original or modified OM (Rugini et al., 1984).
- •The replacement of sucrose with mannitol positively affects reproduction in some genotypes.
- Zeatin remains the most suitable cytokine for olive micropropagation despite its high cost.
- Dikegulac and substances of natural origin, such as coconut milk and neem oil, could replace expensive cytokinins, lowering micropropagation costs.
- Rooting efficiency could be enhanced by auxin applied alone or combined, adding putrescine, and culturing the explants in the dark.

The present review underscores the critical importance of a well-informed approach to olive micropropagation system development. Insightful decision-making and an understanding of tissue culture techniques are paramount. The guidance offered enables the development of propagation systems that are not only functional but also highly efficient. By carefully discussing the nuances of some critical stages, olive physiological abnormalities, and various plant cultivation protocols, this study contributes to the knowledge necessary to create an entire micropropagation system, providing a sustainable approach for efficient olive propagation.

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