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Effect of growth regulators on micropropagation of different olive cultivars (*Olea europaea* L.)

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Abstract
Olive (*Olea europaea* L.) is considered as the most extensively cultivated fruit crop due to its economic importance and nutritional values. Before the introduction of biotechnological techniques, it was propagated by laborious traditional methods which have limited growth efficiency. Most of the work has been carried out on plant regeneration using different explants and combinations of growth regulators. Keeping in view the importance of olive, attention should be paid to improve the technology to achieve 100% success of the micro propagation. Growth regulators and carbohydrate sources have a major role in micro propagation. Under particular conditions, olive cultivars display a high competence to propagate. Internal carbohydrates might interact with hormones and play a stimulatory effect on propagation. Various exogenously applied auxins primarily indole-butyric acid, naphthalene acetic acid and indole-acetic acid help promoting *in vitro* rooting of shoots. When used in combination with other hormones, they promote root initiation. L. For *in vitro* shoot initiation, cytokinins have been suggested; most common form is zeatin, which induces satisfactory growth. Gibberellins have been suggested to increase the fruit size and to control fruit drop. In this review, Olive micro propagation, with special focus on the effect of growth regulators on different olive cultivars has been reviewed.
Key words: Micropropagation; growth regulators; root initiation; proliferation; olive cultivars

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Introduction

Olive (*Olea eurovaea* L.) is the most cultivated and earliest fruit tree having an endless history; it covers a region of approximately 7.5 million hectares [1]. According to FAOSTAT, leading world producing countries of olive are Spain and Italy [2]. Exploration survey using morphological features of olive was conducted in Spain, which estimated 262 olive cultivars [3], and there are over 600 cultivars in Italy. Almost 25 olive cultivars are micro propagated and are under study for their performance. The most well documented olive cultivars include oueslati, cv zard, cv rowghani, moraiolo, dolce agogia, leccino, coratina, nocellara, and pendolino. Olive received maximum importance in the Mediterranean region and its cultivation in this region began 6,000 years ago. Olive cultivation is presently expanding from the Mediterranean region to various countries like Southern and Northern regions of America (Argentina, Chile, and United States) Australia, and Southern Africa [4].

Olive originated from the coastal areas of the Eastern Mediterranean Basin as well as Northern Iraq, and Northern Iran. Morphological and biological variations exist in different olive cultivars [5]. The olive fruit is usually oblong in shape, weighing 1 to 10 g or even more according to the cultivar. The skin of fruit is green when immature and dark blue, blue-violet or black when ripe [6]. Fresh and undressed olives are highly bitter due to a hydro-soluble glycoside called oleuropein. Some olives are naturally sweet due to low oleuropein. The glycoside is hydrolyzed with sodium hydroxide during processing and preserving. Oil can be extracted from fruit juice by hydrolyzing with water. Fresh fruits contain around 80% unsaturated fatty acids against 20% saturated ones, 20% oil and a very low quantity of cholesterol. The importance of olive oil was due to its use in cooking, salad dressing, food preparation, wool treatment, medicine, cosmetics and soap production [1]. Its wood is naturally long-lasting; in southern Italy olive is cultivated as a secondary source of profit [7].

Insect pests and diseases of olive

The olive tree and its products are susceptible to several diseases, pests and viruses. Latent viruses affect is round about 70% of olive trees. Among the various bacterial species, *Pseudomonas savastanoi*, is the most dangerous olive knot pathogen that damages the tree by producing tubercules forms or outgrowths called raised knots on the woody branches and stems, than herbaceous tissues of the plant [8]. Knot develops for many months and can expand from small smooth galls to irregular cluster of galls usually at nodes or at internodes [9]. Generally there are two strains of *Pseudomonas savastanoi,* olive strain and oleander strain. In New Zealand, it was recorded that *Pseudomonas savastanoi* (oleander strain) might also damage olive species [10]. In 1997, *Pseudomonas savastanoi* (olive strain) was identified in plants brought in through preservation [11]. The knot-forming pathogens after infection produce considerable quantities of phytohormones like auxins and

cytokinins that induce uncontrolled cell multiplication resulting gall formation [12].

Conventional propagation techniques

Conventional methods of olive propagation are grafting and cutting [13]. Conventionally olive multiplication by seed is used to get rootstock material, for obtaining new cultivars with stronger root systems; it is believed that root systems obtained from seeds are deeper, consistent and stronger than any conventional method [14]. But seed multiplication usually gives a limited number of regenerated plants because of various reasons, including long juvenile non-bearing period (10–15 years), high heterozygosity of the species, existing self-incompatibility and genetic makeup of progeny [15]. So the genetic improvement by conventional methods has failed to give satisfactory results [16].

Modern propagation techniques

Tissue culture is a modern propagation technique of multiplying stock plant material of high quality, valuable genotypes, disease free within a short period of time [17-19]. It is applied to multiply such novel plants that have been genetically modified via traditional breeding methods, presenting numerous benefits not promising with conventional propagation techniques [20]. The micro propagation technique allows propagating those fruit species which are hard to propagate conventionally [17]. These include axillary bud stimulation, organogenesis, and somatic embryogenesis [21]. *In vitro* olive micro propagation was first reported in the mid-1970s, where suitable protocols for almost all cultivars were presented by optimizing the medium composition for all of the culture. Among different media, the olive medium [22], original Murashige and Skoog (MS) medium modified by Fiorino and Leva [23], and MS medium, are considered so far the most appropriate ones for *in vitro* olive culturing.

Growth regulators

Suitable *in vitro* protocols for olive culturing have been developed; studies indicated that the main factors for achieving elevated growth rates involved in tissue culture include medium formulation [24, 25, 26], growth hormones [27], rooting, genotype [17, 28] and acclimatization conditions. Specifically, among all factors the *in vitro* culture of *Olea europaea* species is widely dependent on the medium composition [4, 29]. Olive micro-propagation was first studied and documented by Rugini [22], who proposed the olive medium (OM), which has been proved to be efficient for the micropropagation of many olive cultivars [17, 22, 27, 30,]. Nevertheless, the success of the micropropagation of olive depends on the type of cultivar and its genetic background [31]. Some cultivars do not respond to *in vitro* conditions due to sensitivity, so their proliferation rate is prolonged [32], rooting of explants is also limited, and many plantlets die at the acclimatization stage [19]. The introduction of specific olive medium (OM), [22] for axillary bud stimulation and successive shoot multiplication has paved the way for further advancement of olive micro propagation. Moreover, the effect of different medium composition as well as culture conditions has also been evaluated on olive explants cultured *in vitro* [33].

Auxins

Auxins are the class of plant growth hormones that promotes root initiation by inducing both growth of pre-existing roots and adventitious root formation. The auxin treatment was one of the first factors to catch the consideration of researchers for *in vitro* rooting of shoots [7] as this phytohormone is involved in rooting for so many years and the positive role of various auxins on the initiation and development of rooting is well documented [34, 35]. Exogenous auxin is used as a growth regulator in micro propagation or *in vitro* culture at a concentration of 0.01-10.0 mg/L. In tissue culture systems, added auxin is generally related to the promotion of growth, induction of rooting, callus induction, cell elongation, tissue swelling, cell division, inhibition of adventitious and axillary shoot formation, and induction of embryogenesis. Exogenous auxins are widely used in cell culture, but their effect on the metabolism of endogenous auxins is not well known. The rooting capability of olive micro-cuttings varies strongly among various olive cultivars [36]. Internal concentrations of auxins and carbohydrates are really of great importance in rooting. It has been investigated that carbohydrates might affect auxin metabolism [36] and success rates was ranging from 25 to 85%, depending on the cultivar type tested and the time of the year when the experiments were conducted [22, 23, 37].

Carbohydrates, being the very important energy source in the rooting [13] are major factors of *in vitro* root initiation [38], and there is contentious relation between carbohydrate content and adventitious root formation in the rooting of cuttings. Rugini and Fedeli [4] reported that the major limitation in vegetative propagation, in different varieties, is the low ability of rejuvenation leading to the low percentage of rooting. Research in rooting has not been successful for some olive cultivars such as the Gordal cv. of Spain, and the Kalamata cv. of Greece as they are difficult to root

in vitro. Adventitious root formation of micro propagated shoots is a critical phase in plant rejuvenation and determines the effectiveness of any *in vitro* plant production systems. The *in vitro* rooting ability depends on many endogenous and exogenous factors such as genetic background, physiological influences, age and ontogenetic phase of the mother plant, and environment (light and temperature). Auxin involved in cell growth, is considered to be the controlling factor in the rooting process. Success in micropropagation depends on the production of good quality adventitious roots [39, 40]. Various auxins have been used for *in vitro* rooting of olive micro cuttings, mainly indole-butyric acid (IBA), naphthalene-acetic acid (NAA) and indole-acetic acid [41, 42]; however, the type and concentration requirement of each of these auxins varies considerably with different olive cultivars [25, 28, 43]. The IBA proved to be better rooting growth regulator for 'Moraiolo' olive cultivar in terms of its rooting percentage, root length and number of roots per rooted explants as compared to NAA which is less efficient for rooting of this cultivar of olive because of being more stable in nature in comparison with IBA. Therefore, studies have indicated that among various auxins, IBA is most frequently used plant hormone [44]. In most cases, the culture media are directly supplemented with IBA or NAA, at the rates increasing from 1 to 4 mg for highest rooted shoots, root number, root length and root quality. It was proved that IBA at 1.5 mg/L concentration is the best one for rooting of Moraiolo cultivar of olive, as it produces maximum rooting in 86.67% shoots [44]. Rooting ability vary significantly among different olive cultivars [45]. The roots generated in medium supplemented with IBA were longer with superior quality shoots whereas NAA produced poor growth [44]. Tetra nodal cutting is reported as the most appropriate one with highest rooting percentage (55.8%), number of roots (3.48), length of roots (1.76 cm) and survival percentage (45.33) [46]. The interaction of tetranodal micro-cuttings are much influenced with 1.25 mg/L IBA as it results in a promising outcome of 95.3% rooting, 5.61 roots per micro-cutting, and 3.40 cm root length with a survival percentage of 90.3%. IBA @ 1.25 mg/L was superior to other treatments with comparatively positive response towards rooting. An ascending trend was observed in rooting with increasing IBA concentration up to 1.25 mg/L and in the same way, increase in the micro-cutting size was positively associated with more favorable root development.

Medium darkening is considered as another important factor for efficient *in vitro* root development. Significant rise in the rooting rates was achieved by Rugini [47] by darkening the basal explants after painting in black the outside of the vessels and by placing black sterile polycarbonate granules on the surface of the solidified culture medium. The importance of medium darkening was first evaluated by Rugini [48] and later, modified by Mencuccini [49]. More economical alternative method was used by Mencuccini [49], who reported rooting rates between 86 and 100% with three Italian olive cultivars using the Brilliant Black commercial dye for medium darkening. Still on the subject of medium darkening, it is claimed that it is not importance for the induction stage of adventitious root formation, but seems essential for its further development [50]. Research conducted on uninodal cuttings of 'Koroneiki' olive cultivar indicated that when micro-cuttings were cultured on a modified Driver-Kuniyuki for Walnut medium lacking growth regulators for one month. After two months at the proliferation stage, the explants were again cultured for one week in the dark in 1 ml liquid Woody Plant Medium supplemented with IBA, NAA or IBA ± NAA. The explants were then shifted again to the solid medium deficient in growth regulators, with a small layer of perlite on the surface. The combination of the two auxins at 1+1 mg/L resulted in almost 76% rooting. The combination of olive knot extract at 50 mg/L with auxins increased the rooting percentage up to almost 87% [51].

Cytokinins

Cytokinins are phytohormones which stimulate shoot proliferation, control differentiation, and activate the transcription by regulating enzyme activity. Today, more than 200 types of natural and synthetic cytokinins exist. Suitable concentrations of cytokinins in culture medium may enhance growth by stimulating morphogenesis (shoot initiation/bud formation). Cytokinin concentrations vary in different parts of plant; its concentration is highest in meristematic section and continually growing areas like roots, infantile leaves, mounting fruits, as well as seeds [52]. Different cytokinins have different response on particular plant species [53]. Single node explants of 'Koroneiki' olive trees were cultured for one month on a modified Driver-Kuniyuki for walnut medium deficient in growth regulators. The explants were sub-cultured once a month on a medium blended with zeatin riboside, 6-dimethylallylamino purine, 6-benzyladenine or thidiazuron. Zeatin riboside has proved to be better cytokinin in inducing shooting of olive [51].

The effect of olive medium (OM) [54], woody plant medium (WPM) and growth regulators (Zeatin and BAP) on the shoot proliferation of 'Moraiolo' cultivar of olive has been studied to find the best one for micro-propagation. Effect of woody plant medium (WPM) and olive medium (OM)

supplemented with different concentrations of cytokinins particularly Zeatin and BAP was investigated [44]. Among these two, olive medium (OM) proved to be suitable one, for inducing shoot proliferation. Zeatin (3.0 mg/L) blended with 0.5 mg/L BAP produced the maximum number of micro shoots per explant (0.84), with the shoot length of 2.25 cm and 1.88 numbers of nodes on olive medium, only zeatin is not enough to induce shooting. The best results of both media with cytokinins were obtained when zeatin in a concentration range of 3.0 mg/L was used with 0.5 mg/L BAP [44]. Many researchers have put an effort to replace zeatin from the protocols of tissue culture [55, 56] as it is not economically adequate for research purposes. Garcia-Ferriz et al [56] recently reported the use of BAP and thidiazuron as zeatin substitutes. However, being costly chemical, it is not economical to use thidiazuron as zeatin substitute. So zeatin has remained in use in recent *in vitro* research on olive culture [18]. Portuguese olive cultivar 'Galega vulgar' is known to be hard to proliferate *in vitro*. Hence, experiments were carried out on the Galega vulgar during 24 months. Studies have indicated that zeatin was successfully substituted by coconut water in combination with BAP or kinetin [57]. The best results were obtained at a concentration of 50 ml/L for coconut water and 2.22 mM for BAP. For *in vitro* multiplication, concentration of BAP was increased to 8.87 mM and highest proliferation rates were obtained.

Gibberellic acid

Gibberellins (GAs) generally regulate growth and encourage stem elongation, flowering, and break dormancy of seeds, buds, and bulbs, influencing various developmental processes. More than 90 types of gibberellins exist, but GA3 is the most frequently used in tissue culture. Gibberellins potentially increase fruit size and promote parthenocarpic (seedless) fruit development. The use of GA3 as a growth regulator to promote size and to control fruit drop was reported by Swietlik [58]. Recently, studies about seed multiplication usually give a limited number of regenerated plants because of long juvenile non-bearing period (10–15 years), high heterozygosity of the species, existing self-incompatibility of the species and genetic makeup of progeny frequently do not even look like the original plant [15]. Research has been conducted to analyze the effect of gibberellic acid with zinc sulphate applied during fruit growth for the improvement of yield and fruit characteristics in 'Shengeh' olive cultivar [59]. Results showed that fruit weight was considerably increased due to an increase in fruit size. GA3 spray @ 30 ppm concentration was more effective in improving yield. Thus, fruit growth and total yield of olive was enhanced, allowing for an increase in its economic value. Use of 0.5% $ZnSO_4$ + 30 ppm GA3 treatments was found optimum for the improvement of olive fruit yield [59]. Very little is known about the effect of gibberellic acid on olive fruiting.

Conclusions

In conclusion, the results obtained from the application of tissue culture technique are encouraging which opened new potential pathways for the micro propagation of olive plants. This technique presents numerous benefits not promising with conventional propagation techniques. But further research on acclimatization is needed to make the steps of tissue culture technique easier and faster. The biotechnological methods for micro propagation of olive could be used for commercial mass propagation of different olive cultivars, in the period of 90 to 120 days. But it is recommended that the applicability of this method should be evaluated for different cultivars. More sophisticated techniques could bring improvement in the regeneration protocol, which is currently being used for the genetic transformation of olive.

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