Review Article

Techniques for Micropropagation of Olive (*Olea europaea* L.): A Systematic Review

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Abstract | Micropropagation is one of the most significant applications of biotechnology in the field of horticulture. This technique is used for proliferation of olive (*Olea europaea* L.) tree which is globally cultivated for centuries and its value added products are consumed worldwide. The traditional propagation techniques of olive are uneconomical with wastage of time while micropropagation of olive may expedite the development of important characteristics like biotic and abiotic resistance with high yield and premium quality olive oil. However, the commercial micropropagation of olive trees is currently not enough because of production cost and globally; it is mostly used for research and development on various aspects of olives. In this review, efforts are made to document the different techniques for successful initiation of olive micropropagation techniques because presently in Pakistan this technique is used at very limited research and development scale besides having basic infrastructure and necessary facilities. Therefore, authors planned to document different critical stages of olive micropropagation like; media preparation, culture establishment, shoot and root proliferation and acclimatization which would be applied for micropropagation of olives at Barani Agricultural Research Institute (BARI), Chakwal. Resultantly, olive expansion through micropropagation can be predicted in near future for sustainability of olive sector in Pakistan.

Received | September 08, 2020; **Accepted** | November 24, 2020; **Published** | March 04, 2021
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Citation | Haq, I.U., H. Umar, N. Akhtar, M.A. Iqbal and M. Ijaz. 2021. Techniques for micropropagation of olive (*Olea europaea* L.): A systematic review. *Pakistan Journal of Agricultural Research,* 34(1): 184-192.
DOI | http://dx.doi.org/10.17582/journal.pjar/2021/34.1.184.192
Keywords | Biotechnology, Critical stages of micropropagation, Olive Research and Development, Proliferation, BARI Chakwal

Introduction

Micropropagation is meant for the proliferation of different plant parts under controlled and intensive environment at mass scale along with production of virus free saplings (Boustany *et al.*, 2019). Generally, it has been used in different fields of agriculture like horticulture, forestry and plant breeding and genetics (Oseni *et al.*, 2018). This method had been used in olive non-conventional propagation and showed a greater advantage over the traditional propagation methods. This technique is used for the production of high quality plants of *olea europaea* L. (Bayraktar *et al.*, 2020).

During *in vitro* propagation; small pieces of mother plants/ tissues (explants) are used as a source for production of a huge amount of plants round the year. These explants have ability to develop millions of clone rapidly under less space and aseptic conditions as compared to conventional methods (Idowu *et al.*, 2009). This method also reduces storage space limitations for maintaining a large number of explants in an aseptic environment. Olive i*n-vitro propagation* technique is used for mass production of high quality plants during the shortest possible time. Gonzales *et al.*, 2010 stated that this technique ensures the production of virus free plants. Matmati et. al., (2020) confirmed that micropropagation ensures the

production of olive plants with high agronomic and phyto-sanitary qualities.

Olive (*Olea europaea* L.) is a member of *Oleaceae* family and among important fruit crops that originated in the Mediterranean region and whose products are used by people globally. The farming of olive trees has expanded from Mediterranean basin to various regions of North, South and Central Asia, South America and Australia (Afridi *et al*., 2015). Currently, olive is commercially growing in various parts of the world (Breton *et al*., 2008) and its commercial cultivation has also been started in Pakistan during past decade.

Currently, 10 million hectares of land is under olive farming worldwide. European Union is the largest producer of olives and its products having a share of 75% of world's olive production (FAO, 2020). While in Punjab; Pakistan, 4046 hectare area of Pothwar region is under olive cultivation. The main varieties grown in this region are BARI Zaitoon-1, BARI Zaitoon-2, Arbequina, Koroneiki, Gemlik and Pendolino etc. Resultantly, the demand for olive saplings is increasing during recent past. Approximately 70% olive nursery plants produced in Mediterranean countries (Cimato, 1999) and 100% in Pakistan are propagated through softwood, semi-hard and hardwood cuttings under mist unit of green house. Although, these techniques guarantees inherited characters; are unable to produce hard to root olive varieties because chances of success are fewer and the demand for disease free high quality plants always there (Martelli *et al*., 2001). These conventional methods are time consuming, laborious, have limited efficiency (Fabbri *et al*., 2004) and are affected by environmental factors, genetic diversity of cultivars, nutrition applied and hygienic status of the mother plants. Moreover, main drawback of these conventional methods exist, because of their weather dependency and large space requirements for extensive propagation of nursery plants.

These bottlenecks allow olive *in-vitro* propagation for asexual proliferation that may assume a good alternative and possible solution for propagation of olive cultivars, by minimizing and even restraining some of these constraints (Rugini and Caricato, 1995). Leva, 2011 explained that *olive* plants produced by *in vitro* method do not require "quarantine" procedure during introduction of exotic plant material. Plants produced by intensive propagation i.e. *in vitro* propagation have superior traits as compared with plants produced by extensive propagation i.e. conventional methods through cuttings and air layering etc. (Thorpe, 2007).

Olive *in vitro* propagation has many different stages namely preparation of nutrient media, selection of explants and its sterilization, inoculation, root induction and acclimatization etc. These stages are applied generally across the world and are briefly described below;

Nutrient medium

Nutrient medium used for micropropagation has all the essential nutrient elements which are inevitable for optimum growth and development of saplings. It comprises of major elements, minor elements, vitamins, organic compounds, plant growth regulators, carbon source and other gelling agents. Murashige and Skoog medium (MS medium) is most commonly used for micropropagation of many plant species. The pH of the medium is very critical which affects both the growing saplings as well as plant growth regulators. It is adjusted between 5.4 to 5.8. The optimum growth and development of plant cells and tissues in culture medium is directly affected by the type of plant growth regulators. The most commonly used plant growth regulators are; auxins, cytokinins and gibberellins. The higher concentration of auxins in culture medium promotes root formation while cytokinins higher concentration promotes shoot proliferation (Oseni *et al*., 2018).

Explant material

Selection of healthy explants is the foremost step of *in vitro* propagation. Before collection of explants, a fungicide spray is applied to the mother plants. The smaller explants such as shoot tip culture lead to the establishment stage of micropropagation which are free from viruses among most plant species (Hassan and Zayed, 2018). These explant received from auxiliary buds of uninodal explants taken from selected mother plants (Michelia and Da Silva, 2020).

Explant sterilization

Surface sterilization of explants is carried out through; washing under running tap water for an hour and rinsed with distilled water 2-3 times; immersion in fungicide and 70% ethanol for some time followed by multiple washing in double distilled water and 0.1% (w/v) mercuric chloride solution with 03 drops of tween twenty on 0.5 liter distilled water for a few

minutes followed by multiple washing in sterilized distilled water. Both ends of explants are cut off and transferred in culture vessels containing semi-solid medium.

Inoculation

After surface sterilization, the explants are inoculated in culture vessels/ containers and this activity is performed under asepsis environment. At this stage micro-cuttings are shifted on to sterilized MS media for shoot proliferation. Increased shoot proliferation of olive obtained with increasing BAP concentration up to 2 mg L^{-1} in the culture medium (Hassan and Zayed, 2018).

Establishment of micro-cuttings in growth chamber

After inoculation, the culture vessels are shifted to growth chamber for initiation of proliferation stage under photoperiod of 16 hour having optimum temperature (25±3°C), relative humidity (60 to 80%) and controlled light (1500-2000 lux) respectively. The microcuttings are kept under observation on daily basis for data recording including symptoms of any infection or contamination. The healthy microcuttings are sub-cultured on to fresh media after one month.

Root induction

Generally, rooting is induced on MS or Olive media by minimizing the concentration of major nutrient elements to half strength. For olive root induction, IBA and NAA are well known auxins. IBA is able to induce earlier rhizogenesis (Briccoli *et al*., 1999). Bartolini *et al*., 1990 developed a method for root initiation that comprises of dipping the microshoot's base into potassium salt of IBA. MS medium containing 2 mg L^{-1} IBA is essential for obtaining good rooting during micropropagation. Moreover, the culture media are provided with darkening agents like charcoal or black dye @ 100 mg l^{-1} to promote *in vitro* rooting of explants.

Acclimatization

Acclimatization is the final stage of olive *in vitro* propagation; in which abrupt change in environmental conditions occur from intensive cultivation to extensive cultivation of newly developed saplings. It could be accomplished by shifting of culture plants from *in vitro* to *ex vitro* under conventional propagation structures having mist system as developed by Roussos and Pontikis, 2002. In which plants removed from the sterile medium are washed and shifted into agriculture media having equal parts of sand: peatmoss (1:1). These transplanted saplings then maintained under intermittent mist.

Keeping in view all the aforementioned factors, this review was formulated to gather different standard protocols for successful *in vitro* propagation of olive under autotrophic conditions.

Review of literature

In vitro propagation provides an opportunity for those horticultural fruit crops which have poor proliferation rate by conventional techniques together-with proliferation of disease free high quality clonal plants (Cancado *et al*.,2013). *In vitro* propagation methods of olive resulted in the production of genetically identical clones with superior and improved characteristics (Saghir, *et al*., 2005). If explants would be collected from large bearing olive plants then many constraints should be faced during intensive propagation of olive like; significant oxidation of explants cells, difficulties during surface sterilization of explants and the arduousness during initial proliferation stage with few hardy olive varieties.

Establishment of aseptic culture

Rostami and Shahsavar, 2012 reported that aseptic cultures of olive explants can be established by placing them in dark conditions. The additional treatment of antioxidants and activated charcoal remained most effective in controlling phenols and browning as well as submerging the explants in water for periods of time achieved the desirable results. Submersing the explants in water prevents phenolic compounds accumulation due to delusion of phenolic compound and or their precursors in the explants (Rugini, 1984). Roussos and Pontikis (2001) observed that antioxidants work as anti-phenolic and anti-browning agent. During *in vitro* propagation, establishment of aseptic culture is the most critical factor especially in evergreen perennial plants. This is time consuming and expensive. Rostami and Shahsavar, 2009 conducted a study in which approximately 95% of the node explants remained aseptic by adding 4 mg L^{-1} nano silver particles to proliferation medium. These particles discharge silver ions, which can break down the membrane structures of microorganisms (Dibrov *et al*., 2002). Rostami and Shahsavar, 2012 studied that submersion of micro-cuttings in ascorbic and citric acid for 30 minutes as antioxidants controls the phenolic compounds. After injury, many woody

plants exude anti growth agents which impede the establishment of *in vitro* cultures. Roussos and Pontikis, 2001 stated that these anti-growth agents are lethal for olive clones. Hassan and Zayed, 2018 reported that surface sterilization of explants obtained by use of Clorox and tween 20 solution. Citric acid and ascorbic acid at 150 mg per lit. and 100 mg per lit. are best treatment for controlling phenol exudation. The similar findings were reported by Mangal *et al.*, 2014. Ahmed *et al.*, 2016 stated that mancozeb prevented fungal contamination up to 90% and 20% chlorox maximize sterilization. These findings are in line with Afridi *et al.*, 2015. Zacchini and Agazio, 2004 used Mercuric chloride and sodium hypochloride to overcome this problem (Table 1).

Table 1:

Chemicals/ compounds to establish aseptic culture	Action
Anti-oxidants	Submersion of micro-cuttings in ascorbic (100 mg l^{-1}) and citric acid (150 mg l^{-1}) for 30 minutes controls the phenolic compounds
Activated charcoal	Prevents the browning by creating dark environment
Nano-silver particles	95% of the nodal explants remained aseptic by adding 4 mg L^{-1} nano silver particles to proliferation medium
Chlorox and tween 20	Surface sterilization of explants obtained by use of Clorox and 03 drops of tween 20 solution
Mancozeb	Prevents 90% fungal contamination
Mercuric chloride	0.1% (w/v) mercuric chloride solution with tween twenty on 0.5 liter distilled water for few minutes followed by multiple wash in sterilize distilled water

Culture medium

The culture media is a critical factor in the success of *in-vitro* proliferation (Sertkaya and Cinar, 1999). Many research findings are documented to standardize the composition of different culture media (Lambardi *et al.*, 2006). Peyvandi *et al.*, 2009 conducted a study on two different media i.e. olive (OM) and driver-kuniyuki walnut (DKW) for *in vitro* propagation of an Iranian olive variety and found that maximum shoots and nodes were obtained in DKW media. Rkhis *et al.*, 2010 investigated on culture media and plant growth regulators. The scientists concluded that Rugini olive media produced maximum shoots in the presence of Zeatin. Moradnezhad *et al.*, 2017 did comparison among woody plant medium, Olive medium and Murashig and Skoog. Their findings support the olive medium for obtaining high quality olive clones during *in vitro* studies. Many scientists across the globe acknowledged the Rugini olive media having essential macro and micro-elements inevitable for *in vitro* proliferation (Zuccherelli and Zuccherelli, 2002; Rkhis *et al.*, 2011) (Table 2).

Table 2:

Culture medium	
Rugini olive media	Maximum shoots produced in the presence of Zeatin
Murashig and skoog	Most commonly used for micropropagation of many plant species
Olive Media	Obtained high quality olive clones during *in vitro* studies
Driver-kuniyuki walnut (DKW)	Maximum shoots and nodes obtained in Iranian olive varieties

Shoot proliferation

During olive *in vitro* propagation, shoot proliferation is a major difficulty encountered during culture establishment. Shoot multiplication under *in vitro* condition is largely depends on plant genotype and cytokinin concentration (Radmann *et al.*, 2011) so cytokinin concentration in medium must be optimized in order to get effective shoot proliferation (Micheli *et al.*, 2010). Rugini, 1984 documented that among zeatin; cytokinin is the most desirable plant growth regulator for rapid proliferation of olive explants because it does not easily degrade or breakdown hence remain in a nutrient media. Saghir *et al.*, 2005 supported the earlier research findings on zeatin use for intensive proliferation of olive but also pointed out that its high cost could be an obstacle for commercial employment of this useful tool. Due to this fact, olive tissue culture practices are not applied in commercial nursery sector. This failure is correlated with hard to shoot and root ability of olive mature plants (Lambardi and Rugini, 2003; Rostami and Shahsavar, 2012). To resolve this important issue; many scientists in the world developed different technologies during past two decades (Gyves *et al.*, 2008; Ali *et al.*, 2009).

Possible replacement of zeatin for shoot proliferation

Peixe *et al.*, 2007 conducted a study in which they proved the successful replacement of zeatin with natural source of growth regulator i.e. coconut water. They concluded that 50 ml L^{-1} coconut water resulted

in maximum shooting percentage on monthly basis. Micheli *et al*., 2018 proved with experimentation that neem oil could also be used for successful intensive proliferation. Moreover, it is a naturally occurring compound and very economical. Antonopoulou *et al*., 2018 studied the use of Dikegulac on olive intensive proliferation and found that it is not phytotoxic and much cheaper than zeatin. Sweeta *et al*., 2019 determined the effect of combination of coconut water, cytokinin and light intensity for enhanced direct *in vitro* organogenesis of olives (Table 3).

Table 3:

Compounds	Mode of action
Coconut oil	50 ml L^{-1} coconut water and 2.22 μM BAP in olive medium resulted in maximum shooting percentage
Neem oil	Addition of 0.1 ml L^{-1} of neem oil to the olive medium resulted in shoot regeneration improvement
Dikegulac	Rugini olive medium supplemented with dikegulac at 100.5 μM produced desireabl shooting

Alternate methods of shoot proliferation

In vitro culturing requires enough monetary inputs that should be rationalized and it is essentially needed to use this technique more efficiently to achieve economic benefits. The researchers developed a Temporary Immersion System (TIS) for olive intense proliferation which is now available (Benelli and Carlo, 2018). Similarly, Lambardi *et al*., 2006 used the RITA® system for shoot proliferation of olive and achieved good results in comparison with use of zeatin. Leva, 2011 developed an *ex vitro* rooting method for olive micro cuttings and found that it is a viable solution for lowering the production cost and time required for propagation of intensively grown olive plants (Table 4).

Table 4:

Methods	Shoot proliferation
Temporary immersion system (TIS)	Olive shoots propagated through immersion frequency of 8 minutes after every 16 hour and additional ventilation showed good adaptability and better growth.
RITA®	30% shoot proliferation achieved for olive shoots with immersion frequency under this system

Root proliferation

Lambardi and Rugini, 2003 reported that auxin application for root induction in woody plants is being used since long and its influence on root primordia induction is well understood (Frett *et al*., 2001). Among auxins, IBA and NAA are most commonly used for root induction and proliferation. However, due to many positive attributes of IBA it is preferable auxin than others like its non-toxic behavior and high stability factor (Hartmann *et al*., 2007). Saghir *et al*., 2005 reported that IBA produced maximum roots in dark culture conditions (Table 5).

Table 5:

Rooting hormone	Concentration
IBA in olive medium	3 g L^{-1} IBA resulted upto85% rooting
IBA in MS medium	2 mg L^{-1} IBA essential for rooting
IBA in modified olive medium	1.25 mg L^{-1} showed fairly alluring response towards rooting

Alternate methods to enhance in vitro rooting

Druart, 1997 reported that higher dose of auxins in culture media could be a limiting factor for poor *in vitro* rooting. Olive is a hard to root plant and its rooting ability could be altered by using different types of rooting hormones. Mencuccini, 1995 documented that intensive root proliferation could be achieved by keeping the cultures in dark environment or by incorporation of chemicals into media which have darkening effects. Significant results were achieved by Rugini *et al*., 1993 when they painted vessel base and used polycarbonate granules on media. Mencuccini, 2003 and Haq *et al*., 2009 achieved maximum rooting by using darkening agent during root proliferation stage. Rhizogenesis is the most important step for successful intensive propagation of olives because it is directly correlated with acclimatization of the young saplings in *ex vitro* environment (Rocha *et al*., 2008).

Acclimatization

During acclimatization abrupt change observed in growth conditions of intensively grown clonal plants due to shifting from intensive to extensive growth environment. Resultantly, these clones faced biotic and abiotic stress and to protect them from possible stress their appropriate transplanting must be ensured. For their transplanting different agriculture media like sand, peat moss and perlite etc. are used (Hassan and Zayed, 2018) and these intensively grown saplings must be kept under glass house infrastructure for a period of one year hardening to ensure high humidity and low temperature before

shifting into *in vivo* environmental conditions. Mencuccini, 1995 reported that a mixture of potting medium including vermiculite, sand and peat-moss in equal proportions produced best results for hardening of olive saplings (Table 6).

Table 6:

Conditions for acclimatization	*In vitro* grown saplings kept under glass house conditions to ensure high humidity (80-90%) and low temperature (±23°C) before open field transplantation

Conclusions and Recommendations

The aim of this review was to combine the study of olive *in vitro* propagation performed by different scientists worldwide. Although many protocols are documented for the intensive propagation of olive; out of these, most competitive and economical methods would be applied for initiation of *in vitro* research studies on olives at Barani Agricultural Research Institute, Chakwal. Therefore, the findings of this publication would be helpful in future for developing certain protocols for available olive germplasm in Pakistan which could be used in public sector for commercial propagation of disease free olive plants. Consequently, the expansion of olive *in vitro* propagation can be predicted in near future in Pakistan.

Novelty Statement

Olive is a hard to root woody plant specie which is conventionally propagated vegetatively through cuttings and air layering but the overall success rate of nursery plants varies from 25-30 percent only and most of the plants remained contaminated with different pathogens. However, the olive plants produce through micropropagation are disease / virus free and can be propagated round the year under controlled conditions with maximum survival rate. Therefore, micropropagation should be explored for sustainability of Pakistani olive sector.

Author's Contribution

Inam Ul Haq: Conceived the idea, wrote abstract, introduction and methodology.
Humara Umar: Technical input at every step, wrote results, discussion and conclusion.
Naeem Akhtar: Data collection, analysis and review of literature.
Dr. Muhammad Azhar Iqbal: Data collection and references.
Dr. Muhammad Ijaz: Overall management of the article.

Conflict of interest

The authors have declared no conflict of interest.

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