In vitro regeneration in olive (Olea europaea L.) cv, ‘Frontio’ from nodal segments

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An efficient and reproducible protocol for plantlet regeneration from nodal segments of Olive cv ‘Frontio’ has been developed. Media and explants browning due to exudation of phenolics from the explants were controlled by fortification of the medium with 100 mg/L ascorbic acid. Best establishment of olive explants was observed on half-strength MS salts fortified with 2.0 mg/L 6-benzylaminopurine (BAP), which resulted in 56.2% of bud break and 93.7% survival whereas, a combination of full strength MS medium with 1.0 mg/L each of 3-indole-butyric-acid (IBA) and kinetin was found to be the best for shoot multiplication, in terms of number of shoots (3.6 shoots/explant) and shoot length (2.2 cm). The in vitro shoots were rooted on half-strength MS medium fortified with 0.2 mg/L IBA and 0.2 mg/L α-naphthalene acetic acid (NAA) with 1.5 g/L activated charcoal, which supported optimum rooting (60 %), with an average of 2-3 roots/shoot, about 2.4 cm length were produced on four weeks of culture.

Keywords: Micropropagation, Nodal segment, Olea europaea

Olive is one of the most important fruit trees cultivated in the Mediterranean basin. It tolerates moderate unfavourable environmental conditions such as high summer temperatures and drought, and has a good adaptation to different edaphic situations (ranging from heavy and clayey soils to light and sandy ones) and a long life. The use of olive oil has been greatly increasing in recent years due to its taste and nutritional value. The conventional method of olive tree propagation is based on vegetative multiplication using cuttings, grafting or suckers. The plant is usually propagated by rooting leafy cutting under mist, but alternations in rooting ability through the seasons¹ and difficulties in finding suitable plant material with adequate health status for cutting, indicate that a more efficient method of mass propagation is needed².

In vitro propagation allows production of high quality and rapidly growing plants. The establishment and in vitro regeneration of olive has been attempted by some unconventional techniques, such as protoplast technology and haploid culture, to obtain genetic improvements through somaclonal variation¹. In addition, cell and tissue culture techniques, i.e. somatic embryogenesis³, micropropagation and callus cultures⁴, have been applied to propagate selected clones or cultivars difficult to root with conventional techniques. The first study on olive micropropagation was reported by Rugini⁴. The development of specific olive medium for axillary bud stimulation and subsequent shoot multiplication marked an important step forward in the improvement of olive micropropagation. Over the past decade, many advances have been made for micropropagation from mature olive trees⁵. Although it has been improved considerably for some olive cultivars, micropropagation of olive in general has not been very successful⁶. Up to now the major difficulties encountered for in vitro micropropagation of mature tissues of olive cultivar were the establishment of axenic culture and subsequent initial growth of shoots⁶.

In fact, micropropagation techniques have been quite useful for producing olive tree cultivars of high quality and valuable genotypes. The present study has been carried out to investigate the effect of different anti-phenolics and sterilants on nodal explants and; effect of growth regulators on shoot establishment, multiplication and rooting of olive
plants, to overcome the inadequacies of the current conventional propagation methods.

**Materials and Methods**

*Plant material*—Seedlings of olive (*Olea europaea* L.) cultivar Frontio were established at Udheywalla campus of Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu, India to be used as the source of explants.

*Removal of phenolics*—For removal of phenolics different treatments such as frequent subculturing, (every alternate day for first week of culture and then at weekly interval for four weeks and thereafter, at monthly intervals) and fortification of media with antioxidants such as polyvinyl pyrrolidone (PVP-40) (molecular weight 40,000), ascorbic acid (50-300 mg/L), citric acid (50-300 mg/L), and charcoal (0.50-2.0 mg/L) were tried (Table 1). In addition, drying of explants before culturing was also tried for the control of browning. For this, after surface sterilization and washing the explants were placed on autoclaved filter paper discs for 10-30 min before culturing and the number of green explants after different durations of treatments as well as after different days of culture was recorded.

*Surface sterilization*—The nodal segments were dipped in water and brought to the laboratory; expanded leaves were removed, and nodal segments were washed thoroughly under running tap water. The explants were then kept in 2% bavistin solution for 30 min. Final sterilization was done using 0.1% HgCl₂ for 2 min and 70% ethanol for 2 min (Table 2) under aseptic conditions in Laminar Flow Systems (Yorco Sales Pvt. Ltd., New Delhi). The explants were then thoroughly rinsed 4-5 times with sterile distilled water. Both ends of the explants, which were exposed to sterilants, were trimmed and the explants were placed aseptically in 25×150 mm test tubes (Borosil Glassware India Ltd., Mumbai) containing 15 mL of the semi-solid medium. All the chemicals were of analytical grade (Hi-Media Laboratory Pvt., Ltd., Mumbai). The cultures were maintained at 25±2 °C and 60±5% RH under a 16 h photoperiod provided by cool white fluorescent tubes (Philips Electronic India Ltd., Gurgaon) with light intensity of 3000 lux.

*Effect of different growth regulators on explants establishment and shoot multiplication*—To identify the most suitable medium for explants establishment, sterilized explants were inoculated into different types of media with or without modifications and supplemented with different concentrations of 6-benzylaminopurine (BAP) (Table 3). After 15 days of incubation percentage of survival and bud proliferation was recorded. *In vitro* established shoots were multiplied on MS medium with 1.0 mg/L each of 3-indole-butyric-acid (IBA) and kinetin.

*Effect of different growth regulators on rooting*—*In vitro* multiplied shoots of about 1.5-2.5 cm were harvested for rooting experiment and cultured on MS³ (half or full strength) medium supplemented with 3-indole-butyric-acid (IBA) or α-naphthalene acetic acid (NAA) alone or in combinations. Data on percentage rooting, root number, and root length were recorded after 4 weeks of culture in rooting medium. Well rooted shoots were removed from culture tubes after four weeks in rooting medium. Rooted shoots were washed thoroughly with tap water to remove adhering medium and transferred to thermocol pots (5 cm diameter) containing cocopeat + sand + soil (1:1:1). Transparent polythene bag was inverted on each plantlet to maintain high humidity and watered on alternate day. Plantlets were monitored under the same environmental conditions as *in vitro* cultures for 3-4 weeks, and thereafter transferred to polyhouse for further growth. The transparent polythene bag was removed permanently after new leaf emergence.

*Statistical analysis*—All the experiments were conducted with a minimum of 16 replicates per treatment and repeated three times. The data were analyzed statistically using one-way analysis of variance (ANOVA). The significances of differences among means were carried out using Duncan’s multiple range test (DMRT) at *P* < 0.05.

*Results*  
*Medium and explant browning*—Out of the different antioxidant treatments tried wherein the establishment medium was supplemented with the antioxidants, the fortification with 100 mg/L ascorbic acid proved to be the best for control of browning. This treatment resulted in 87.5% survival of explants (green explants) after eight days of culture, and was followed by 100 mg/L citric acid and 2 g/L charcoal, which resulted in 75.0 and 62.5% green cultures respectively (Table 1). These cultures remained green thereafter.

An additional precaution was also followed in all experiments that the explants were kept in running tap water for half an hour and then kept in 0.2% solution of ascorbic acid for 1-2 h. Once the browning was controlled, the explants showed proliferation of buds in the establishment medium.
Effect of different sterilants on survival of explants—Out of the different sterilization treatments tried during establishment of culture, a treatment of 2% (w/w) bavistin followed by 0.1% HgCl₂ and 70% ethanol, each for 2 min, proved to be the best for control of contamination as it resulted in 75% survival of explants after 21 days of culture. This was followed by 2% bavistin, 0.075% HgCl₂, 70% ethanol each for 3 min and 2% bavistin, 2% NaHPO₄, 70% ethanol, each for 4 min. These treatments resulted in 62.5% and 50% culture establishment after twenty one days of culture (Table 2).

Table 1—Effect of different antioxidants on explant browning of Olive (Half strength MS + 2.0 mg/L BAP)

<table>
<thead>
<tr>
<th>Type of antioxidant</th>
<th>Survival of explants (%) (after 2 - 8 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP (100 mg/L)</td>
<td>100 - 18.75d</td>
</tr>
<tr>
<td>Charcoal (2 g/L)</td>
<td>100 - 62.50c</td>
</tr>
<tr>
<td>Citric acid (100 mg/L)</td>
<td>100 - 75.00b</td>
</tr>
<tr>
<td>Ascorbic acid (100 mg/L)</td>
<td>100 - 87.50a</td>
</tr>
</tbody>
</table>

LSD (P<0.05) 1.0

ANOVA \( F_{3, 8} = 8163.0 \)

Means values within the column sharing the same superscript are not significantly different at \( P < 0.05 \).

Shoot establishment and multiplication—For culture establishment of olive, different types of media such as full and half strength MS medium, Knop’s macro salts+Heller’s micro salts, Lloyd and McCown (Woody Plant Media), Rugni olive media with or without certain modification, were tried (Table 3). Best establishment of olive explants was observed on half strength MS salts with 2.0 mg/L BAP, which resulted in 56.2% of bud break and 93.7% survival (Fig. 1A and B). Shoot multiplication in olive was studied using IBA/kinetin (0.25-2.0 mg/L) as well as BAP (2.0 mg/L) (detailed data not shown). The medium supplemented with IBA and kinetin both at 1.0 mg/L gave good result in olive.

Regarding shoot multiplication, full strength MS medium with 1.0 mg/L each of IBA and kinetin produced on an average 3.4 shoots/explant with an average shoot length of 2.2 cm in olive (Fig. 1C). The number of shoots formed per explant increased significantly upon subculture of the responding explant after three weeks on the same medium (MS+1.0 mg/L IBA+1.0 mg/L kinetin).

Rooting—MS medium (half strength) supplemented with IBA and NAA both at 0.2 mg/L concentration with 1.5 g/L activated charcoal supported best rooting (60%), in which on an average 2-3 roots/shoot, each of about 2.4 cm were produced within four weeks of culture (Fig. 1D). This treatment was followed by 55% rooting in half strength MS medium supplemented with IBA and NAA both at 0.2 mg/L concentration but without activated charcoal. Rooted plantlets were hardened and acclimatized according to the protocol described in materials and methods with final success rate of 78%.

Discussion

Olive tree is a valued, long-lived evergreen tree that has been grown in the Mediterranean region from time immemorial. The conventional method of olive tree propagation is based on vegetative multiplication using cuttings, grafting, or suckers. Although occasionally yielding satisfactory results, these methods have been frequently criticized as being very slow and inefficient for some highly valued cultivars. Micropropagation is a reliable technology applied commercially worldwide for large-scale plant multiplication, germplasm conservation, pathogen elimination, genetic manipulations and supply of
Table 3—Effect of different culture media on the culture establishment of olive

<table>
<thead>
<tr>
<th>Media used</th>
<th>Status of explants after 6 days in culture</th>
<th>Status of explants after 10 days in culture</th>
<th>Survival (%)</th>
<th>% of bud proliferation after 10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knop’s macro salts Heller’s microsalts + 3 % sucrose + 2.0 mg/l BAP</td>
<td>Swelling of 2 bud, 10 green</td>
<td>2 bud swelling, 8 green</td>
<td>31.25</td>
<td>0.00c</td>
</tr>
<tr>
<td>WPM+3 % sucrose + 2.0 mg/l BAP</td>
<td>Swelling of 2 bud, 18 green</td>
<td>8 bud swelling but no proliferation, 4 green</td>
<td>37.5d</td>
<td>0.00c</td>
</tr>
<tr>
<td>MS half strength + 3 % sucrose + 2.0 mg/l BAP</td>
<td>18 buds show swelling, 12 green</td>
<td>18 proliferated, 12 swelling</td>
<td>93.7a</td>
<td>56.25a</td>
</tr>
<tr>
<td>Rugini salts + 3 % sucrose + 2.0 mg/l BAP</td>
<td>8 swelling, 16 green, 2 dead</td>
<td>4 proliferated 6 swelling, 12 green</td>
<td>68.7b</td>
<td>12.5b</td>
</tr>
<tr>
<td>LSD (P&lt;0.05)</td>
<td></td>
<td></td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td></td>
<td>$F_{3, 8} = 5013.5$</td>
<td>$F_{3, 8} = 6011.3$</td>
</tr>
</tbody>
</table>

Means values within a column sharing the same superscript are not significantly different at $P < 0.05$

Total number of explants inoculated during each medium combination = 32

Fig. 1—Different stages of micropropagation in Olive. (A) Initiation of bud proliferation; (B) Initiation of shoot multiplication; (C) Established shoots; (D) A rooted shoot.

selected plants. Micropropagation techniques have been quite useful for producing olive tree cultivars of high quality and valuable genotypes.9-11

The leaching of phenolic compounds from explant in media is a serious problem for establishment of cultures of woody crops which results in browning of the medium as well as the explant, rendering it unsuitable for proliferation.12 In *Psidium guajava*, fortification of the medium with 500 mg/L citric acid and initial incubation of cultures in complete dark for 24 h reduced browning considerably.13 During our earlier investigations on *Psidium guajava*, the effect of different antioxidants on explants browning was investigated in detail and therefore keeping in view the result of that investigations as well as considering the fact that explants browning was more severe in guava as compared to olive, the doses of different antioxidants for the control of explants browning in olive were decided. During the present investigations, a treatment of 100 mg/L ascorbic acid proved to be better than PVP or citric acid for control of browning in olive.

The composition of the micropropagation medium represents an important factor for the achievement of high multiplication rates.15-16 Several studies have been conducted to evaluate the effect of alternative medium compositions or culture conditions on the propagation of explants taken from adult plants or vegetative explants cultured in vitro. Olive medium (OM), proposed by Rugini, has been reported to be efficient for the micropropagation of a wide array of olive cultivars.8,10,17 Mannitol (as a carbon source) and zeatin (as a growth regulator) in culture media has been suggested to be essential to achieve satisfactory results in shoot proliferation.4,18 However, Revilla et al.15 achieved micropropagation from mature olive trees using a Driver and Kuniyuki walnut (DKW) medium containing sucrose as well as BAP and IBA as phytohormones. Peixe et al.20 proposed coconut water and BAP to replace zeatin in Olive micropropagation.

During the present investigations MS medium fortified with IBA (1.0 mg/L) and Kn (1.0 mg/L) showed best shoot multiplication. Appropriate ratio of auxins and cytokinins is required for efficient regeneration of shoots as has also been reported
in *Bacopa monnieri*\(^{21}\), *Psidium guajava*\(^{14}\) and *Momordica cymbalaria*\(^{22}\). The number of shoots formed per explant increased upon subculture of the responding explant after three weeks on the same medium. This is in accordance with earlier observations reported in *Simmondsia chinensis*\(^{23}\) and *Dendrocalamus giganteus*\(^{24}\).

A combination of IBA and NAA resulted in well developed roots. Several previous studies reported that the *in vitro* rooting of Olive varied from 25 to 100%, depending on the cultivar, auxin type and concentration. Addition of the external auxin in the medium for the rooting was also reported in many species\(^{24,25}\).

**Reference**