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Effects of coconut water, plant growth regulators and light intensity on shoot bud proliferation and direct regeneration in olives (*Olea europaea* L.)

Sweta Mishra, Shailesh Kumar and Yuvraj Vala

Abstract
The present study determined the effect of combination of coconut water, cytokinin and light intensity for enhanced direct *in vitro* organogenesis of olives (*Olea europaea* L.). This optimized protocol showed that coconut water and light intensity are important factors for efficient shoot regeneration. These results show that the light intensity and the natural additive, coconut water olds the potential for efficient commercial *in vitro* propagation of olives. The meristematic explants were cultured on Murashige and Skoog (MS) medium supplemented with various combinations of coconut water, 2ip and kinetin under light intensity of 20, 30 and 40 µmol m^{-2} s^{-1}. The highest shoot induction (93.16 ± 0.09%) was obtained with 1.5 mg l^{-1} Kinetin + 2.0 mg l^{-1} 2ip under a light intensity of 30 µmol m^{-2} s^{-1}. The shoot proliferation and shoot length was highest (08.73±0.18 and 09.52±0.14, respectively) on media with 1.0 mg l^{-1} Kinetin + 2.0 mg l^{-1} 2ip and 10.0 % coconut water under a light intensity of 30 µmol m^{-2} s^{-1}. The highest root induction (92.28±0.12 %), rooting frequency (07.94±0.16 roots per shoot) and root length (07.73±0.17 cm) were obtained when adventitious shoots were inoculated on half-MS medium with 1.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA. The regenerated plantlets were acclimatized in coco peat, soil and sand mix for 15 days and were shifted to poly house for 2.5 months and thereafter they were transferred to field. The survival rate of the transplanted plantlets was about 85%.

Keywords: Olive, light intensity, direct organogenesis, coconut water, cytokinin

Introduction
Olive (*Olea europaea* L.) bears enormous impact on the economy, history and culture. In the last years, olive cultivation has expanded due to increased consumption of olive oil in many countries (Rugini *et al*., 2000) [19]. Improvement of olives is limited by various drawbacks such as long juvenile period, low level of fruit set and slow and asynchronous seed germination (Acebedo *et al*., 1997) [1]. However, application of biotechnological tools requires efficient *in vitro* regeneration methods, capable of giving rise to a whole plant from a single cell. In olive, somatic embryogenesis is the regeneration method most frequently used. However, it involves many uncontrolled genetic variation. This can be overcome by regeneration of plants through direct organogenesis method. Micropropagation methods of olive are available (Peixe *et al*., 2007) [18]; (Rugini, 1984) [20]; (Sghir *et al*., 2005) [22], and efforts are currently underway to cryopreserve shoot apices (Lynch *et al*., 2007) [14]. Capelo *et al*. (2010) [3] have been able to obtain somatic embryos from leaf and petioles of a mature wild olive tree. Micropropagation efficiency or success rate is affected by various factors, such as explant type, nutrients, plant growth regulators and other additives, temperature, and light intensity and duration.
The effect of light intensity on *in vitro* shoot proliferation of olives species has not been reported. Light is one of the important environmental factors that controls plant growth, development, morphogenesis, metabolism and chlorophyll content in plant cell, tissue and organ cultures (Dou *et al*. 2017) [8]. Light intensity and light source are important parameters that influence shoot regeneration, fresh weight, and secondary metabolite biosynthesis during micropropagation in a variety of crops (Farhadi *et al*. 2017) [9]; (Meziani *et al*. 2015) [15]; (Ouyang *et al*. 2003) [17]. Light can also influence the efficacy of plant growth regulators (PGRs). Light regulation of gibberellins and auxin metabolism has also been reported (García-Martinez and Gil 2001) [10]; (Halliday *et al*. 2009) [11]; (Stavang *et al*. 2007) [24]. Along this line, this study was aimed at evaluating the effect of coconut water, cytokinins and light intensity on shoot but proliferation and direct organogenesis of olives.

Material and methods
Axillary and apical buds of olives were collected from 5-6 year old trees grown in the Centre for Crop Improvement, SDAU, S.K. Nagar, Gujarat, India.

This study was conducted in the Biotechnology section, Central Instrumentation Laboratory, Sardarkrushinagar Dantiwada Agricultural University, Gujarat. The explants collected were washed in running water and were rinsed with distilled water 2-3 times in the laboratory. The explants were excised in the laminar air flow hood and were treated for 4 minutes with 0.1% (w/v) mercuric chloride with 2 drops of tween-20 per 100 ml solution and rinsed 4-5 times with sterile distilled water.

Shoot Bud Induction and Proliferation

Murashige and Skoog (MS) [16] was used along with various concentrations of coconut water and cytokinins like 2ip and kinetin (KIN), individually or in combination for shoot bud induction (table 1) along with adenine sulphate (25 mg/l), glutamine (50 mg/l), ascorbic acid (100 mg/l), citric acid (50 mg/l), PVP (1.5 g /l), sucrose (30 g/l), agar (7.5 g/l). The chemicals used were of analytical grade (Duchefa Biochemie). All media were adjusted to pH 5.7 with 1 N NaOH or 1 N HCl before autoclaving at 121 °C for 20 min. The cultures were maintained at a temperature of 26 °C and a light intensity of 20, 30 and 40 µmol m^{-2} s^{-1} provided by cool white fluorescent light with a 16-h photoperiod. The shoot induction percentage was recorded after 6 weeks. Sub-culturing was dove every fortnightly. The proliferated shoots were excited and then again sub-cultured for further elongation and proliferation in the same media composition. Each experiment was repeated five times, each using 20 replicates (i.e., a total of 100 explants per treatment).

***In-vitro* rooting**

After 8 weeks of subculturing, the elongated shoots were excited individually and subcultured on ½ strength MS medium supplemented with NAA and 2,4-D (table 2) for rooting. The cultures for *in vitro* rooting were maintained at a temperature of 26 °C and a light intensity of 30 µmol m^{-2} s^{-1} provided by cool white fluorescent light with a 16-h photoperiod. Root induction was recorded after 4 weeks of culture.

Acclimatization and Hardening

In vitro raised plantlets with well-developed roots were removed from the culture medium and roots were washed thoroughly under tap water. Plantlets were transferred to plastic cups containing potting-mixture of sterilized sand, soil and coco peat (1:1:1). The plantlets were maintained inside the culture room covering them with polythene cover to maintain the relative humidity. The plantlets were watered with 1/2 strength MS basal salt solution devoid of sucrose and myo-inositol at 2 d interval for a period of 15 days. The regenerated plantlets were shifted to polyhouse, the polythene cover was removed and the plantlets were transferred to big polythene bags containing soil and kept under shade for another 2.5 months before transferring to the field.

Statistical Analysis

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. All experiments were performed drive times and were analyzed statistically by SAS 9.4 (Statistics Analysis System) software.

Results and discussions

Young twigs were collected from the 5-6 year old trees and were brought to the laboratory in ascorbic acid (50 mg/l) solution. Extensive sterilization of explants was carried out to establish aseptic cultures as the explants were collected directly from the field grown olive plantations. Olives have a tendency to produce a large amount of phenolic substances which are released from the cut ends of the explants, which restrict the culture response. Anti-phenolic agents like polyvinyl pyrrolidone (PVP), ascorbic acid and citric acid were used in this study to minimize browning of the explants and media. Incorporation of PVP (1.5 g/l), ascorbic acid (100 mg/l) and citric acid (50 mg/l) in the culture media effectively controlled the browning of the cultures and enhanced the culture establishment. The shoot induction was observed after 6 weeks of culture inoculation and another 8 weeks were required for shoot proliferation and elongation in the same media composition. Regenerated plantlets with normal green leaves were subjected to rooting which took 4-6 weeks to develop sturdy root system. The regenerated plantlets were acclimatized in coco peat, soil and sand mix for 15 days and were shifted to poly house for 2.5 months and thereafter they were transferred to field.

Effects of coconut water, cytokinins and light intensity on shoot bud induction

18 media combinations were evaluated under 3 light intensities for shoot induction and proliferation. The shoot induction was found to be dependent on the concentrations of the different cytokinins, coconut water and light conditions. Out of the 18 combinations of media tested, the explants showed the highest shoot induction frequency of 93.16±0.09 per cent observed on MS medium supplemented with 1.5 mg/l Kinetin + 2.0 mg/l 2ip with 5% coconut water under 30µmol $m^{-2}s^{-1}$ light intensity. The lowest shoot induction of 18.83±0.14 was recorded on MS medium supplemented with 0.5 mg/l Kinetin + 1.0 mg/l 2ip with 5% coconut water under 40µmol $m^{-2}s^{-1}$ light intensity. Light intensity had a remarkable effect on shoot induction. Under 20µmol $m^{-2}s^{-1}$ light intensity the shoot induction per cent varied from 28.45±0.15 to 89.26±0.07 per cent. The media composition supplemented with 1.0 mg/l kinetin + 2.0 mg/l 2ip and 10 % coconut water was showed efficient shoot induction under 20µmol $m^{-2}s^{-1}$ light intensity. Under 30µmol $m^{-2}s^{-1}$ light intensity the shoot induction per cent varied from 27.03±0.06 to 93.16±0.09 per cent. The media composition supplemented with 1.5 mg/l kinetin + 2.0 mg/l 2ip and 5 % coconut water was showed efficient shoot induction under 30µmol $m^{-2}s^{-1}$ light intensity. However, under 40µmol $m^{-2}s^{-1}$ light intensity the shoot induction per cent varied from 18.83±0.14 to 78.16±0.12 per cent. The media composition supplemented with 1.5 mg/l kinetin + 2.0 mg/l 2ip and 5 % coconut water was showed efficient shoot induction under 40µmol $m^{-2}s^{-1}$ light intensity. The results showed that a light intensity of 30 µmol m^{-2} s^{-1} was best for attaining high frequency of shoot induction. The most cost effective media combination with a high rate of shoot induction was 1.5 mg/l kinetin + 2.0 mg/l 2ip and 5 % coconut water. (Table 1; Fig. 1).

Effects of coconut water, cytokinins and light intensity on shoot proliferation

The shoot proliferation rates varied with the concentrations of the different cytokinins, coconut water and light conditions. Out of the 18 combinations of media tested, the induced shoots produced the maximum number of shoots (08.73±0.18) per explant on MS medium supplemented with 1.0 mg/l Kinetin + 2.0 mg/l 2ip with 10% coconut water under 30µmol $m^{-2}s^{-1}$ light intensity. The minimum number of shoots per

explant (01.41±0.21) was recorded on MS medium supplemented with 1.0 mg/l Kinetin + 2.0 mg/l 2ip with 15% coconut water under 40µmol $m^{-2}s^{-1}$ light intensity. The shoot length was also affected by the light intensity. Maximum shoot length (09.52±0.14) was recorded on MS medium supplemented with 1.0 mg/l Kinetin + 2.0 mg/l 2ip with 10% coconut water under 30µmol $m^{-2}s^{-1}$ light intensity. The minimum shoot length (03.11±0.13) was recorded on MS medium supplemented with 0.5 mg/l Kinetin + 2.0 mg/l 2ip with 15% coconut water under 20µmol $m^{-2}s^{-1}$ light intensity.

Light intensity also had a remarkable effect on shoot proliferation. Under 20µmol $m^{-2}s^{-1}$ light intensity the average number of shoots per explant varied from 02.52±0.12 to 07.53±0.15 and the shoot length varied from 03.11±0.13 to 07.54±0.16 cm. The media composition supplemented with 1.0 mg/l kinetin + 2.0 mg/l 2ip and 10 % coconut water produced maximum number of shoots per explant, while media supplemented with 1.5 mg/l kinetin + 2.0 mg/l 2ip and 5 % coconut water produced longest shoots under 20µmol $m^{-2}s^{-1}$ light intensity. Under 30µmol $m^{-2}s^{-1}$ light intensity the average number of shoots per explant varied from 02.87±0.21 to 08.73±0.18 and the shoot length varied from 04.11±0.21 to 09.52±0.14 cm. The media composition supplemented with 1.0 mg/l kinetin + 2.0 mg/l 2ip and 10 % coconut water produced maximum number of shoots per explant and longest shoots under 30µmol $m^{-2}s^{-1}$ light intensity. Under 40µmol $m^{-2}s^{-1}$ light intensity the average number of shoots per explant varied from 01.41±0.21 to 05.32±0.08 and the shoot length varied from 03.24±0.14 to 07.42±0.18 cm. The media composition supplemented with 1.5 mg/l kinetin + 2.0 mg/l 2ip and 5 % coconut water produced maximum number of shoots per explant and longest shoots under 40µmol $m^{-2}s^{-1}$ light intensity. The results showed that a light intensity of 30 µmol m^{-2} s^{-1} was best for attaining maximum number of shoots per explant and the longest shoots (Table 1).

In plants, growth regulators play essential roles in controlling the growth and development. Supplementation of tissue culture media with some plant growth regulators is essential. Cytokinins are required for cell division in a wide variety of plant tissue cultures (D'Agostino and Kieber 1999) [5]; (Dewitte *et al*. 1999) [6]. Cytokinins can promote stem elongation in plants grown under light conditions (Lin and Cheng 1997) [13]; (Smets *et al*. 2005) [23]. An efficient adventitious shoot regeneration system from direct organogenesis is an essential method for producing a large number of elite genotypes and avoiding somaclones. Multiple adventitious shoots were proliferated under a higher light intensity. Light intensity or quality-dependent changes in plant physiology and morphogenesis are regulated by plant hormones (Afshari *et al*. 2011) [2]; (Dietz 2015) [7]; (Kissoudis *et al*. 2017) [12]. This result may be due to the interaction between light intensity, endogenous auxin and endogenous cytokinins, which directly or indirectly affect shoot proliferation.

Effect of auxins on *in vitro* rooting

The multiple shoots regenerated were excised individually and were transferred to half strength MS medium supplemented with 2,4-D (0.0-2.0 mg/L) and NAA (0.0-2.0 mg/L). The shoots that were transferred to half strength-MS medium with 2,4-D (1.0 mg/l) + NAA (1.0 mg/l) showed the highest rooting response (92.28±0.12 %) with the highest number of roots per regenerated shoot (07.94±0.16) and root length (07.73±0.17 cm) (Table 2; Fig. 1). In general, root induction can be initiated by adding auxins such as NAA, IBA, and IAA (Sauer *et al*. 2013) [21]. Previous studies have reported the auxin requirements for olive species for inducing adventitious root formation (Chaari-Rkhis, *et al*., 2000) [4]; (Rugini *et al*., 2000) [19].

Acclimatization and hardening

The sturdy regenerated plantlets with the long and strong roots were transferred to plastic pots containing mixture of sterilized sand: soil: coco peat (1:1:1) and covered with transparent polythene cover punctured with holes to maintain the relative humidity. These plantlets were initially kept in the culture room for 15 days and were later transferred to the poly-house for acclimatization for 2.5 months before transplanting to the field (Fig. 1). The survival percentage of the acclimatized plants in the field was 85.0%.

Table 1: Effect of cytokinins, coconut water and light intensity on shoot proliferation and direct regeneration

KIN (mg/l)	2ip (mg/l)	CW (%)	Light Intensity								
			20µmol $m^{-2}s^{-1}$			30µmol $m^{-2}s^{-1}$			40µmol $m^{-2}s^{-1}$		
			Shoot induction %	Average Number of shoots	Shoot length (cm)	Shoot induction %	Average Number of shoots	Shoot length (cm)	Shoot induction %	Average Number of shoots	Shoot length (cm)
0.50	1.00	5.00	51.82±0.15	04.41±0.21	04.11±0.13	48.82±0.14	04.41±0.07	05.11±0.13	18.83±0.14	03.57±0.23	03.37±0.17
0.50	1.00	10.00	59.75±0.24	04.79±0.23	04.64±0.28	27.03±0.06	04.79±0.33	04.64±0.22	29.64±0.24	02.83±0.17	05.48±0.26
0.50	1.00	15.00	64.07±0.19	05.68±0.26	04.82±0.24	74.60±0.27	06.53±0.14	07.66±0.12	69.38±0.27	04.69±0.17	05.62±0.21
0.50	2.00	5.00	37.53±0.17	02.52±0.12	03.52±0.22	31.67±0.14	03.52±0.22	05.52±0.18	64.52±0.17	03.74±0.24	05.72±0.24
0.50	2.00	10.00	45.86±0.24	02.54±0.26	03.51±0.13	39.71±0.09	04.54±0.18	06.51±0.11	64.03±0.19	02.43±0.13	04.38±0.16
0.50	2.00	15.00	48.44±0.18	04.87±0.13	03.11±0.13	60.42±0.15	02.87±0.21	04.11±0.21	33.41±0.25	04.87±0.27	03.91±0.11
1.00	1.00	5.00	38.15±0.21	03.82±0.26	05.28±0.14	47.18±0.21	05.82±0.18	05.28±0.16	48.17±0.23	02.62±0.22	03.26±0.14
1.00	1.00	10.00	58.32±0.16	04.59±0.11	04.03±0.17	33.95±0.26	05.59±0.21	06.03±0.18	32.35±0.15	03.71±0.03	03.73±0.19
1.00	1.00	15.00	32.11±0.12	04.88±0.16	04.89±0.21	68.86±0.07	03.88±0.24	06.89±0.13	56.94±0.12	02.58±0.12	04.56±0.21
1.00	2.00	5.00	62.18±0.23	05.22±0.20	06.04±0.18	73.14±0.16	05.22±0.12	04.34±0.22	65.68±0.12	04.93±0.13	03.24±0.14
1.00	2.00	10.00	89.26±0.07	07.53±0.15	06.24±0.14	82.71±0.23	08.73±0.18	09.52±0.14	62.85±0.23	03.07±0.24	05.36±0.22
1.00	2.00	15.00	74.52±0.16	03.44±0.12	03.35±0.20	80.22±0.21	03.24±0.16	04.35±0.15	54.82±0.14	01.41±0.21	03.68±0.12
1.50	1.00	5.00	42.06±0.21	06.82±0.16	04.28±0.12	52.32±0.18	06.82±0.12	07.28±0.12	32.61±0.27	03.52±0.16	05.44±0.12
1.50	1.00	10.00	28.45±0.15	05.59±0.21	05.03±0.14	49.82±0.15	05.59±0.31	07.03±0.17	55.24±0.15	01.75±0.15	06.53±0.31
1.50	1.00	15.00	55.37±0.13	05.88±0.18	04.89±0.21	46.17±0.13	05.88±0.14	04.89±0.23	65.87±0.21	02.46±0.14	04.32±0.22
1.50	2.00	5.00	85.23±0.13	06.35±0.14	07.54±0.16	93.16±0.09	07.74±0.13	08.41±0.07	78.16±0.12	05.32±0.08	07.42±0.18
1.50	2.00	10.00	82.64±0.12	06.53±0.23	05.36±0.14	88.24±0.14	05.53±0.17	07.36±0.12	72.43±0.11	05.13±0.31	04.16±0.12
1.50	2.00	15.00	77.12±0.23	03.44±0.22	05.35±0.15	81.35±0.06	03.44±0.18	05.35±0.15	63.62±0.14	02.34±0.18	03.73±0.16

Table 2: Rooting of *in vitro* regenerated shoots of Olive

2,4-D	NAA	Rooting %	Average Number of roots per shoot	Root length (cm)
0.00	0.00	12.03±0.13	02.53±0.12	04.68±0.12
0.00	1.00	32.12±0.14	03.86±0.13	03.54±0.16
0.00	2.00	41.37±0.16	02.74±0.16	03.71±0.13
0.50	0.00	18.42±0.26	02.49±0.13	02.24±0.12
0.50	1.00	72.26±0.12	06.91±0.19	05.93±0.16
0.50	2.00	88.65±0.20	07.84±0.14	05.46±0.14
1.00	0.00	22.53±0.17	02.28±0.20	04.51±0.11
1.00	1.00	92.28±0.12	07.94±0.16	07.73±0.17
1.00	2.00	79.53±0.19	06.57±0.16	05.28±0.12
1.50	0.00	25.36±0.24	03.27±0.22	04.37±0.23
1.50	1.00	58.62±0.12	05.46±0.12	03.29±0.17
1.50	2.00	84.14±0.16	06.23±0.17	05.79±0.17
2.00	0.00	38.26±0.12	02.42±0.12	03.57±0.21
2.00	1.00	62.18±0.16	04.38±0.16	03.29±0.13
2.00	2.00	41.94±0.12	03.15±0.25	04.77±0.22

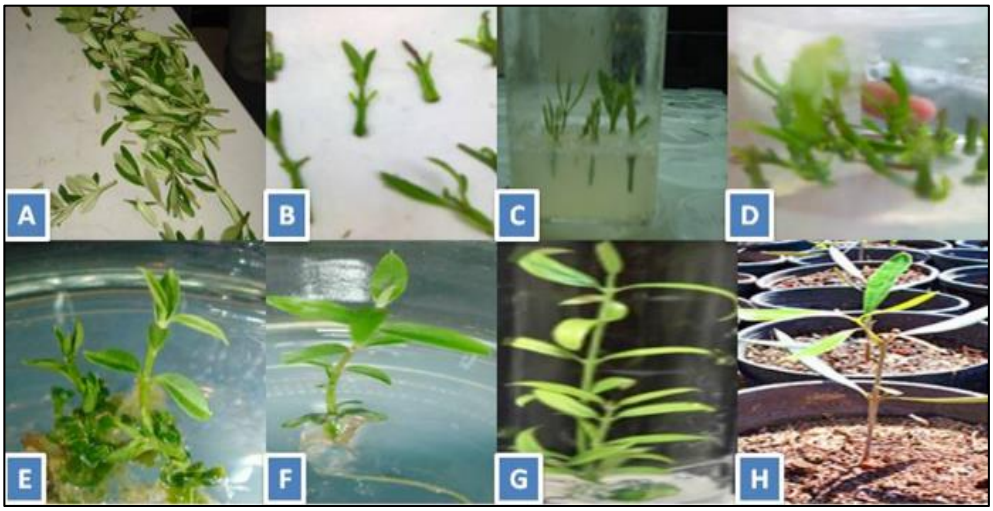

Fig 1: A-olive explants; B-axillary and apical buds prepared for inoculation; C- shoot induction; D- initiation of multiple shooting; E-shoot elongation; F & G- regenerated plantlets; G- Acclimatization

Conclusions

We report for the first time the effect of light intensity and coconut water on regeneration of olive explants derived from physiologically mature trees. The best and the most economical combination for olive regeneration through direct organogenesis is 30µmol m-2s-1 light intensity + 1.0 mg/l kinetin + 2.0 mg/l 2ip and 10 % coconut water. The survival percentage of the plants in the field after acclimatization was 85.0%. These results could promote research on the physiology, genetic engineering, and molecular biology of the olives.

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