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# MASS PRODUCTION OF *OLEA EUROPEA* L. (CV. ROWGHANI) THROUGH MICROPROPAGATION

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**Summary.** The effects of basal media (OM or DKW), carbohydrate source (mannitol or sucrose), different cytokinins (benzyl aminopurine (BAP), 2-isopentenyl adenine (2ip) solely or in combination) and different concentrations of $CoCl_2.6H_2O$ on micropropagation of an Iranian olive cultivar were investigated. The highest number of nods and new shoots per explant was produced in the DKW supplemented with 2ip (4 mg $l^{-1}$). Shoot proliferation rate was significantly decreased by using the combination of BAP and 2ip on DKW medium while it was increased on OM medium. Mannitol showed a positive effect on shoot proliferation. The number of nods and shoots were increased in all cobalt concentrations when mannitol was included in media while in the presence of sucrose these numbers decreased. *In vitro* shoots were rooted by indole-3- butyric acid IBA. The highest number of roots as well as length of the roots was obtained in those explants which grew in the medium supplemented with mannitol and $CoCl_2.6H_2O$ (0.037 mg $l^{-1}$). Viability of micropropagated shoots was higher by using subcultured media (OM or DKW) supplemented with mannitol.

***Keywords*:** micropropagation, cobalt, mannitol, sucrose, *Olea Europaea* L., ex vitro rooting.

***Abbreviations*:** OM – olive medium; DKW – Driver-Kuniyuki walnut medium; 2ip – 2-isopentenyl adenine; BAP – benzyl aminopurine, IBA – indole-3-butyric acid.

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## INTRODUCTION

The use of olive oil has been greatly increasing in recent years due to taste and nutritional economic importance. The plant is usually propagated by rooting leafy cutting under mist, but alternations in rooting ability through the seasons (Del Rio et al., 1991) and difficulties in finding suitable plant material with adequate health status for cutting, indicate that a more efficient method of mass propagation is needed (Chaari et al., 2002).

*In vitro* propagation allows producing high quality and rapidly growing plants. The first study on olive micropropagation was reported by Rugini (1984). The development of specific olive medium (OM), (Rugini, 1984) 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 trees (Garcia-Ferriz et al., 2002; Leva et al., 2004; 1991 Zacchini and De Agazio, 2004; Mendoza-de Gyves et al., 2008). Although it has been improved considerably for some olive cultivars, micropropagation of olive in general has not been very successful (Garcia-Ferriz et al., 2002; Lambardi and Rugini, 2003). Up to now the major difficulties encountered for *in vitro* micropropagation of mature tissues of olive cultivar was the establishment of axenic culture and subsequent initial growth of shoots (Lambardi and Rugini, 2003).

Cobalt is an essential element for the synthesis of vitamin $B_{12}$, which is required for human and animal nutrition (Young, 1983; Smith, 1991). The promotion effect of cobalt can be ascribed to its role in several physiological activities like growth, photosynthesis and respiration (El-Sheekh, 2003; Aziz Eman, 2007).The low level of cobalt ion promotes growth factors such as plant height, number of leaves per plant as well as fresh and dry weight of leaves and roots (Helmy and Gad 2002), and fruit per plant ( Aziz, Eman 2007 ). Cobalt increases the growth of the seedlings and alleviates the senescence of aged tissues as it inhibits the activities of ACC oxidase and reduces ethylene production (Lau and Yang, 1976; Li et al., 2005). Cobalt is an inorganic micronutrient used in most plant tissue culture media based on level established by Murashige and Skoog (1962) for tobacco tissue culture.

Root formation of in vitro explants is a crucial step of the micropropagation of woody species. 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, environment (light and temperature), and composition of the nutrient medium (Rugini, 1992; Rugini et al.,1993).

The aim of this study was to assess the effects of the carbohydrate source (mannitol or sucrose), hormones and cobalt salt concentrations on shoot proliferation and rooting of olive cv. Rowghani.

## MATERIALS AND METHODS

The experiments were conducted in the National Institute of Genetic Engineering and Biotechnology of Iran during 2006-2007.

### Propagation of microshoots

Actively growing shoots of olive (cv. Rowghani) were collected from 4-year-old greenhouse grown plants. Leaves were removed and sterilized with bleach

(20%) for 5 min, then rinsed three times in sterile distilled water. Apical buds of sterile shoots were removed and shoots were cut into the single nod segments. The explants were cultured on olive medium (OM) supplemented with 2ip (4 mg $l^{-1}$), 3% (w/v) sucrose, and 6% (w/v) agar. OM medium was reported by Rugini (1984) (Table 1), who developed this specific medium composition by analyzing mineral elements from mature seeds. This medium has been used for commercial plant production in Italy for more than 50 olive cultivars (Rugini et al., 2006).

pH was adjusted to 5.8 before agar addition and autoclaving. *In vitro* shoots (4 -5 nods) raised from explants were used for further experiments.

**Effect of cytokinins**

Two nodal explants were subcultured in DKW (medim which was established by Driver and Kuniyuki (1984) for *in vitro* walnut propagation), (Table 1) or OM medium supplemented with BAP and or 2ip. The carbohydrate source was sucrose (30 g $l^{-1}$) (Table 2).

**Effect of cobalt salt**

Two nodal explants were subcultured in DKW medium supplemented with 2ip (4 mg $l^{-1}$). Four different concentrations of $CoCl_2.6H_2O$ (0; 0.025; 0.037; 0.05 mg $l^{-1}$) were used. The carbohydrate source was sucrose or mannitol (30 g $l^{-1}$).

All samples were kept in a growth chamber at a 16 h light/8 h dark photoperiod and 24±2°C. Forty five days after treatments, the number of nods and new microshoots (branches) from each explant were measured and then all samples were treated for rooting experiments.

Table 1. Macro/micro elements in olive medium (OM) and driver and Kuniyuki (DKW) medium.

| DKW [mg $l^{-1}$] | OM [mg $l^{-1}$] | Macro/Micro elements |
|---|---|---|
| 0.00 | 1100.0 | $KNO_3$ |
| 1416.0 | 412.0 | $NH_4NO_3$ |
| 1367.0 | 600.0 | $Ca(NO_3)_2.4H_2O$ |
| 112.5 | 440.0 | $CaCl_2.2H_2O$ |
| 0.00 | 500.0 | KCl |
| 1559.0 | 0.00 | $K_2SO_4$ |
| 361.49 | 1500.0 | $MgSO_4.7\ H_2O$ |
| 265.0 | 340.0 | $KH_2PO_4$ |
| 33.8 | 27.8 | $FeSO_4.7H_2O$ |
| 45.5 | 37.5 | $Na_2$-EDTA |
| 33.5 | 22.3 | $MnSO_4.4H_2O$ |
| 4.8 | 12.4 | $H_3B0_3$ |
| 17.0 | 14.3 | $ZnSO_4.7H_2O$ |
| 0.39 | 0.25 | $Na_2MoO_4.2H_2O$ |
| 0.25 | 0.25 | $CuSO_4.5H_2O$ |
| 0.00 | 0.025 | $CoCl_2.6H_2O$ |
| 0.00 | 0.83 | KI |
| 100.0 | 100.0 | MyoInositol |
| 2.0 | 0.5 | Thiamin-HCl |
| 2.0 | 2.0 | Glycine |
| 0.00 | 0.5 | Pyridoxine-HCl |
| 1.0 | 5.0 | Nicotinic acid |
| 0.00 | 0.05 | Biotin |
| 0.00 | 0.5 | Folic Acid |
| 0.00 | 2190.0 | Glutamine |

***Ex vitro* rooting and acclimatization**

*In vitro* shoots which were grown on different media, were treated by dipping the base of microshoots in the IBA (500 mg $l^{-1}$) for 15 min and then the explants were directly transferred to the jiffy pots. Shoots were kept in a transparent box (Fig. 1). Thirty days after treatments length and number of roots were measured.

Table 2. Average number of nods and new shoots 45 days after subcultures in different media supplemented with BAP and/or 2ip. Different letters indicate significant differences ($p \leq 0.05$).

| No. of new shoots/explant | No. of nods / explant | Medium | 2iP [mg $l^{-1}$] | BAP [mg $l^{-1}$] |
|---|---|---|---|---|
| 0.65$^{c}$ | 3.91$^{c}$ | OM | 0 | 1 |
| 1.45$^{ab}$ | 5.22$^{b}$ | OM | 1 | 1 |
| 0.78$^{c}$ | 4.16$^{c}$ | OM | 0 | 2 |
| 1.36$^{b}$ | 5.16$^{b}$ | OM | 2 | 0 |
| 1.34$^{b}$ | 6.56$^{a}$ | OM | 4 | 0 |
| 0.68$^{c}$ | 3.41$^{c}$ | DKW | 0 | 1 |
| 0.95$^{c}$ | 3.29$^{c}$ | DKW | 1 | 1 |
| 0.78$^{c}$ | 4.06$^{c}$ | DKW | 0 | 2 |
| 1.34$^{b}$ | 6.04$^{ab}$ | DKW | 2 | 0 |
| 1.80$^{a}$ | 6.30$^{a}$ | DKW | 4 | 0 |

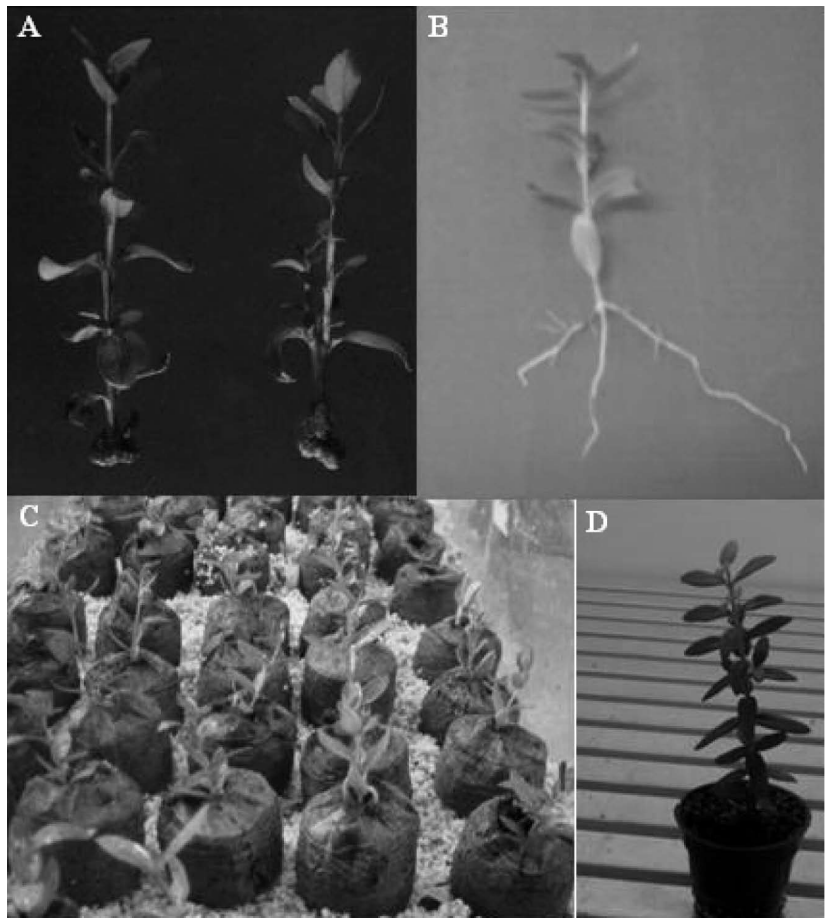


Fig. 1. Steps of micropropagation of olive cv. Rowghani. A) *In vitro* shoots; B) Rooted shoots after IBA treatment; C) plantlets in the jiffy pots: D) 4-month-old plant in the pot.

**Data Analysis**

Experiments followed a randomized complete block design. Four explants per jar and eight replications per treatment were tested. Analysis of variance was performed using the General Linear Model procedure (SPSS ver. 14) and differences among treatments were evaluated by Duncan Test ($p \leq 0.05$).

## RESULTS

Micropropagation of olive cultivars ranged from easy to difficult, however, growth of microshoots (number of nods and new shoots per explant) of *Olea europaea L.* cv. Rowghani was high up to 10 nods and 4 shoots, depending on media, carbohydrate source and cobalt salt concentration of the medium.

**Effect of cytokinin type on proliferation rate**

Two nodal explants were subcultured in DKW or OM medium supplemented with BAP and/or 2ip. However, 2ip was more effective than BAP. In the DKW medium 2ip added at a concentration of 4 mg $l^{-1}$ was the best treatment because it resulted in a significant number of nodes (6.3) and new shoots (1.8). The highest number of nods were obtained at 2ip concentration of 4 mg $l^{-1}$ in OM medium with 6.56 nods per explant but with a little reduction in the new shoots number (1.34). BAP solely had a negative effect on shoot proliferation (number of nods and new shoots). Between the two carbohydrate sources at the same hormone conditions no significant differences of nods number were observed (Table 2). Based on these results, DKW medium supplemented with 2ip (4 mg $l^{-1}$) was chosen for further experiments.

**Effect of carbohydrate and cobalt on shoot proliferation**

Mannitol proved to be more effective than sucrose for shoot proliferation. The presence of different $CoCl_2.6H_2O$ concentrations together with mannitol improved the number of nods and shoots. Even though in the presence of mannitol no significant differences of nodes and shoots number among different cobalt salt concentrations were observed, the number of nods slightly increased with increasing $CoCl_2.6H_2O$ concentration up to 0.037 mg $l^{-1}$ (10.04) and the highest number of new shoots (2.00) was obtained in the presence of $CoCl_2.6H_2O$ (0.025 mg $l^{-1}$ ).

In the medium supplemented with sucrose, increasing $CoCl_2.6H_2O$ concentrations up to 0.025 mg $l^{-1}$ resulted in a considerable reduction of nods and shoots number. The minimum number of nods (6.65) and new shoots (1.53) were obtained in the medium containing sucrose and $CoCl_2.6H_2O$ at a concentration of 0.05 mg $l^{-1}$ (Table 3).

**Effect of carbohydrate sources and cobalt salt on rooting**

High percentage of rooting (100%) was observed in the explants which were taken from the media supplemented with mannitol and $CoCl_2.6H_2O$ (0.025 mg $l^{-1}$). The maximum root number (5.20) was obtained in the explants from the medium with mannitol and $CoCl_2.6H_2O$ (0.037 mg $l^{-1}$). In both carbohydrate treatments the $CoCl_2.6H_2O$ at a concentration of 0.05 mg $l^{-1}$ had a negative effect on the length and number of roots. The length and number of roots and rooting percentage showed a severe reduction in the explants taken from the medium supplemented with mannitol

Table 3. Average number of nods and new shoots per explant (45 days after subcultures at different cobalt concentration), percentage of rooted explants and number and length of roots from *in vitro* shoots grown in different media (30 days after treatment with IBA). Different letters indicate significant differences ($p \leq 0.05$).

| $CoCl_2.6H_2O$ [mg $l^{-1}$] | Carbohydrate [30 g $l^{-1}$] | No. of Nods | No. of new shoots | No. of roots | Root length [cm] | Rootong% |
|---|---|---|---|---|---|---|
| 0.000 | Sucrose | $8.19^{cd}$ | $1.74^{bc}$ | $4.69^{a}$ | $4.90^{ab}$ | $97.2^{ab}$ |
| | Mannitol | $9.00^{abc}$ | $1.92^{ab}$ | $4.70^{a}$ | $6.00^{a}$ | $97.3^{ab}$ |
| 0.025 | Sucrose | $8.57^{bc}$ | $1.93^{ab}$ | $4.43^{ab}$ | $4.5^{ab}$ | $90.6^{abc}$ |
| | Mannitol | $9.75^{a}$ | $2.00^{a}$ | $4.75^{a}$ | $5.41^{ab}$ | $100^{a}$ |
| 0.037 | Sucrose | $8.03^{cd}$ | $1.78^{bc}$ | $4.36^{ab}$ | $6.76^{a}$ | $91.6^{abc}$ |
| | Mannitol | $10.04^{a}$ | $1.88^{ab}$ | $5.20^{a}$ | $5.61^{ab}$ | $80.0^{cd}$ |
| 0.050 | Sucrose | $6.65^{d}$ | $1.53^{c}$ | $3.68^{ab}$ | $4.18^{b}$ | $82.76^{abc}$ |
| | mannitol | $9.60^{ab}$ | $1.93^{ab}$ | $3.00^{b}$ | $2.06^{c}$ | $72.2^{d}$ |

and $CoCl_2.6H_2O$ at a concentration of 0.05 mg $l^{-1}$ (Table 3). The survival rate of rooted plants in the jiffy pots was 100%.

## DISCUSSION

The present study proved that DKW depended on the hormone composition as well as on OM for this cultivar. Different culture media have been used for different olive cultivars, indicating differences in adaptability and requirements of *in vitro* cultures (Chaari et al., 2002; Rugini et al., 2006).

Although reports on olive cultivars Kalamon (Dimassi-Theriou, 1994) have shown that BA alone is more effective than 2ip, we found that 2ip raised the shoot proliferation rate. Since shoot proliferation in the DKW supplemented with 2ip at 4 mg $l^{-1}$ showed a significant increase, this medium will be used for future studies.

Our results indicated that mannitol as the carbon source in the medium improved the growth rate. As regards the carbon source, it has been shown that polyalcohol promoted significantly growth compared with sucrose by increasing shoot length, pairs of leaves formed, breaking apical dominance, improving the general quality and uniformity of shoot cultures and reducing basal callus formation in some olive cultivars (Garcia et al., 2002; Leva et al., 1994). In some plant species, polyols (or sugar alcohol) are direct products of photosynthesis in mature leaves in parallel with sucrose (Noiraud et al., 2001; Conde et al., 2007).

Cobalt, given as chloride, increased the elongation of *in vitro* olive shoots and the quality of shoots was improved. Cobalt strongly promotes the growth of certain isolated plants parts. This remarkable fact was reported earlier by Miller (1951). Cobalt salts promote many growth process, including stem and coleoptiles elongation, opening of hypocotyls hooks, and bud development (Grover and Purves, 1976; Aziz, Eman, 2007). It was suggested that cobalt affected the elongation of stems through its effect on ethylene level. A number of possible mechanisms of $Co^{2+}$ action such as blockage of IAA oxidation have been proposed. A more recent hypothesis is that $Co^{2+}$ interferes with both the biosynthesis and the action of ethylene (Tamimi and Timko, 2003; Li et al., 2005).

In the past decades great advances have been made in rooting of micropropagated shoots. *In vitro* propagated shoots can be rooted in vitro (Briccoli et al., 2002; Rugini et al., 1993; Mendoza-de Gyves et al., 2008) by using different auxin treatments. Alternatively to traditional use of rooting media, a "dip method" was proposed (Bartolini and Leva 1990), which consisted in dipping the base of olive microcutting for 30 min in potassium-salt IBA solution, before culturing in a hormone-free medium. Using of *ex vitro* rooting of microshoots could shorten the period of micropropagation.

In conclusion, the optimal result on number of shoots and nods was obtained with cv. Rowghani at 4 mg $l^{-1}$ 2iP in DKW supplemented with 0.025 mg $l^{-1}$ $CoCl_2.6H_2O$ when mannitol was included in the medium.

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