

Full Length Research Paper

Micropropagation of *Origanum sipyleum* L., an endemic medicinal herb of Turkey

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Accepted 9 October, 2009

Origanum sipyleum L. (Lamiaceae) is an endemic species of the Western Anatolia, Turkey. Essential oils of oreganos are utilized in pharmaceutical and cosmetic industries. A micropropagation protocol was developed using seedlings-derived explants. Shoot apices of 17d-old seedling were cultured and shoots multiplied on Murashige-Skoog modified (MSM) containing 550 mg/l of CaCl₂ for sustained growth. Multiple shoots (3.7 ± 0.3 shoot/explant) were produced on medium containing 1 mg/L benzylaminopurine (BAP). On subculturing rate of shoot multiplication increased to 7.8 ± 0.4. 96% of the shoots rooted in a culture medium with 0.5 mg/L indolebutyric acid (IBA) after 3 weeks. The plantlets were acclimatized into outdoor conditions. 76% of these survived in the greenhouse. These *in vitro* derived microplants are already under the evaluation for their essential oil composition.

Key words: *Origanum sipyleum*, micropropagation, benzylaminopurine, indolebutyric acid.

INTRODUCTION

Oregano (*Origanum* sp.) is a perennial herb of the family Lamiaceae. The genus *Origanum* comprises about 38 species, most of which are indigenous to the Mediterranean region. The essential oils of oregano have antibacterial (Vokou et al., 1993) and antifungal (Paster et al., 1993) actions. Many of them are commercially exploited and are in high demand (Schiuma, 1993). Turkey is one of the leading exporter of Mediterranean type of oregano (Başer, 2002).

Several species of genus *Origanum*, such as *Origanum majorana*, *Origanum syriacum*, *Origanum vulgare*, naturally grow in Turkey, while some of them is either cultivated (e.g. *Origanum onites*). *Origanum sipyleum*, is an endemic to Western Anatolia and grows on calcareous rocks and hillsides and in *Pinus* grove and oacen maquis during April - May to October. The life span of the plant is about 3 - 4 years under favorable climatic conditions. Traditionally vegetative parts and biochemical extracts of the plant are used as medicinal tea or food additives (Özçelik, 2000; Ozkan et al., 2007). Collection of plants

from the nature has endangered the species. There is need for application of tools and techniques for multiplication and conservation of this species. Plant tissue culture offers several advantages (Nadeem et al., 2000; Nalawade et al., 2003). There is no report on application of tissue cultures for propagation and multiplication of *Origanum sipyleum*. There are reports on micropropagation of *Origanum bastetanum* (Scorro et al., 1998) and *Origanum vulgare* (Goleniowski et al., 2003). We report here on development of tissue culture protocol for propagation using apical tips of this species.

MATERIALS AND METHODS

Plant material

O. sipyleum seeds were collected from Spil Mountain in Turkey during September. These were surface sterilized by immersing in a 70% (v/v) ethanol for 30 s and then in a 20% (v/v) commercial bleach (5% NaOCl) for 15 min. The surface-sterilized seeds were rinsed three times with autoclaved water. 10 seeds were placed on 0.8% (w/v) water-agar in per 100 ml erlenmeyer flasks incubated in a growth chamber at 18 ± 2°C in the dark. After 4 days these were transferred under 16/8 photoperiod. Seedlings were transferred to modified Murashige Skoog (MS) (1962) with CaCl₂ 550 mg/l. This medium is referred to as MSM.

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Table 1. Effect of BA and subcultivation on shoot multiplication and effect of transplantation substrates on plantlet acclimatization of *Origanum sypyleum* L.

Medium	Shoot no ^a per explant	Shoot length ^a (cm)	Transplantation substrate	% of acclimatized plantlets
MSM + 1 mg/l BAP	3.7 ± 0.3*	1.8 ± 0.4*	Send : Peat : Perlite (1:1:1; v/v)	98
MSM + 1 mg/l BAP	7.8 ± 0.4**	1.2 ± 0.1**	Peat : Perlite (1:3; v/v)	48

*At the end of the first cultivation (5 wks).

**At the end of the subcultivation (with 3 wks of interval).

^aValues are mean ± SE of the 60 explants (assay repeated twice).

Micropropagation, rooting and acclimatization

Apical shoot tips (5 mm) of 17 days old seedlings were excised and cultured on MSM medium containing 1 mg/l benzylaminopurine (BAP). The pH of the media was adjusted to 5.8 with 1 N KOH before autoclaving at 121 °C, 1.4 kg/cm² for 20 min. The cultures were kept at 20 ± 2 °C under cool white fluorescent light (4000 lux) with a 16 h photoperiod. After 5 weeks, the number and length of the shoots per explant were recorded. Produced shoots were subcultured with 3 weeks of intervals.

The *in vitro* produced shoots were cultured on MSM medium for root induction. Indolebutyric acid (IBA), as 0.5 or 1 mg/l, was used for rooting agent. The ratio of rooting of the shoots and the length of the roots per shoot were evaluated after 3 weeks. Shoot number and shoot length were also evaluated upon observation of shooting increased by treatment of IBA. A minimum 20 erlenmeyers (replicates) with three shoots or explants were used in each experiment and each experiment repeated twice.

Rooted shoots were transferred onto a peat : sand : perlite (1:1:1; v/v) or peat : perlite (1:3; v/v) containing culture vessels and covered with parafilm. The lids of the cultures were loosened after 10 days and they remained in the 90% humidity environment for 20 days more. Then, plantlets were transferred into multivials with garden soil (soil : peat : perlite; 1:1:1; v/v) and moved to greenhouse conditions. The final tally of acclimatized plants were recorded after 5 weeks.

RESULTS AND DISCUSSION

Germination and growth of seedlings

A few seeds of *O. sypyleum* germinated within one week on water-agar. Darkness at the beginning of cultivation of the seeds seemed necessary. Earlier, Akçam and Yürekli (1993) found that for better growth of juvenile plants MSM containing higher amount of CaCl₂ (550 mg/l instead of 440 mg/l as is in MS basal medium) is suitable. Therefore for sustained growth and development this medium was used. The culture grew at relatively low temperature (app. 20 °C) for growth of all the cultures.

Micropropagation, rooting and acclimatization

Apical tips (0.5 cm) of 17 days old seedlings were established on MSM medium with 1 mg/l BAP (Figure 1A). Ueno and Shetty (1998), Socorro et al., (1998) and Moreno-Fortunato and Avato (2008) reported that 1 mg/l

of BAP was suitable for tissue cultures and multiple shoot production in *Origanum* species. In *O. sypyleum*, each apical tips produced 3.7 ± 0.3 shoots (length of each shoot was 1.8 ± 0.4 cm) at the end of first cultivation period (5 weeks) and the (Table 1). Further subculture of proliferating explant resulted in production of 7.8 ± 0.4 (Figure 1B and Table 1). But the shoots were relatively shorter (1.2 ± 0.1 cm) and some hyperhydricity occurred. Increase in shoot production during ongoing subcultures is explained by some physiological changes as termed "rejuvenation" (Webster and Jones, 1989). In the subsequent culture cycles the rate of shoot multiplication was 8 shoots per explant every three weeks. The micro-propagated shoots were cultured on MS medium with 0.5 to 1 mg/l IBA or NAA. These auxins are reported to be root-inducing in several plant species (Thomas and Philip, 2005). Goleniowski et al. (2003) reported spontaneous rooting in shoot multiplication medium supplemented with BA (0.28 µM) + NAA (0.53 µM) for *O. vulgare*, whereas Socorro et al. (1998) reported on rooting of micropropagated plantlets of *O. bastetanum*, on peat substrate. During the present investigation we obtained rooting in 96% of shoots (an average root length of 5.52 ± 0.2) on medium containing in 0.5 mg/l IBA (Figure 1C). While on 1.0 mg/l of IBA only 12% of the shoots was rooted within 3 weeks (Table 2). Also on this medium the multiplication of shoots occurred simultaneously reaching to rate of shoot multiplication to 23.7 ± 0.3 shoot per explant. Such effects of IBA on shoot propagation have been described in several works (Zhu et al., 2005; Azad et al., 2006). IBA (Zhu et al., 2005) and BA (Kadota and Niimi, 2003) may affect shoot development negatively by giving rise to over hyperhydricity when was used continuously in following subcultures. Henceforth, the shoots with roots subcultured once in the medium devoids plant growth regulators (PGRs) prior to acclimatization (Figure 1E).

A peat : sand : perlite (1:1:1; v/v) mixture was much more effective (98%), in comparison to peat : perlite (1:3; v/v) (48%) for acclimatization of the plantlets under 90% humidity (Table 1). The plantlets, derived through *in vitro* propagation, survived under greenhouse conditions (76%) (Figure 1F) and mimicked the growth and morphological characteristics of the donor plants. In conclusion, we described for the first time an efficient *in vitro*

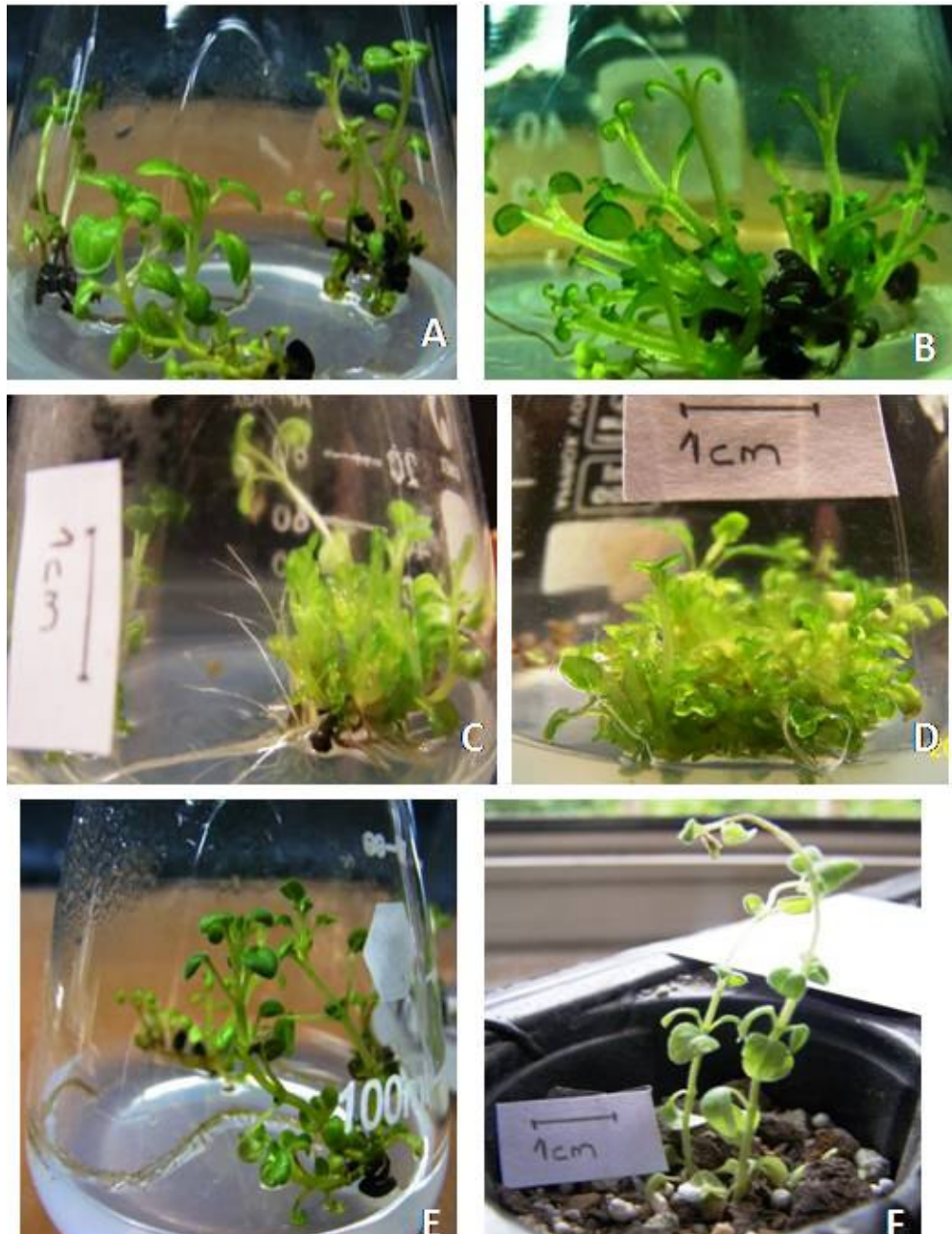


Figure 1. A. Shoot multiplication in 1 mg/l BAP, 5 weeks after culture beginning, B. Increase in shoot propagation upon subculturing, C. Rooting and shoot propagation and D. Shoot propagation in 0.5 mg/l IBA, E. Improvement of the shoots and the roots upon transferring to medium lacking PGRs, F. Five weeks old plants in garden soil.

Table 2. Effects of IBA on rooting of micropropagated plantlets and shoot multiplication in *Origanum sipyleum* L., after 3 wks.

Medium (MSM)	% of rooted shoots	Root length ^a (cm)	Shoot no. per explant ^b	Shoot length ^b (cm)
0.5 mg/l IBA	96	5.5 ± 0.2	23.7 ± 0.3	0.8 ± 0.3
1 mg/l IBA	12	1.6 ± 0.1	-	-

^aValues are mean ± SE of the 60 shoots (assay repeated twice)

^bValues are mean ± SE of the 60 explants (assay repeated twice).

multiplication system of *O. sipyleum* in the present study. This system provides recovery of whole, uniform plants from apical tips after approximately 5 months. The procedure described here may help to reduction in genetic erosion and extinction in the wild of this species. Further studies will be carried out on the analysis of essential oil constituents of these micropropagated plantlets.

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