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EFFICIENT MASS PROPAGATION OF *ORIGANUM ACUTIDENS* (HAND.-MAZZ.) IETSWAART UNDER *IN VITRO* CONDITIONS

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Abstract

This study describes germination of *Origanum acutidens* (Hand.-Mazz.) Ietswaart seeds followed by micropropagation from stem nodes of 58 days old seedlings that were cultured on MS basal medium containing 0.4, 0.8, 1.2, 1.6, and 2.0 mg/l BAP with or without 0.5 mg/l NAA cultured at 24ºC ± 1ºC under cool white fluorescent lights with 16 hrs light (42 µMol photons/m²/s) photoperiod. The results showed maximum number of 10.28 shoots per explant on MS medium containing 0.8 mg/l BAP. Comparing regeneration on MS medium containing various concentrations of BAP with and without 0.5 mg/l NAA; it was noted that shoot regeneration on MS medium containing BAP + NAA was partially inhibitory and induced comparatively reduced number of shoots per explant. Well developed shoots of *O. acutidens* on each concentration of BAP with or without NAA were rooted on MS medium supplemented with 0.75 mg/l NAA. The rooted plantlets were acclimatised in pots, followed by transfer to fields, where they bloomed and set seeds. The results suggest efficient practical production of the plant species for commercial purposes.

Introduction

Origanum is an important genus of the Lamiaceae and comprises about 900 species, widespread throughout the world, out of which 22 species and 32 taxa are found in Turkey (Davis 1982, Baser 2002). *Origanum* species are of great economic importance due to its use as spicy additive for food (Kokkini 1996, Kizil *et al*. 2008) herbal teas, culinary herb to flavour food products and alcoholic beverages (Kizil *et al.* 2009). *Origanum acutidens* (Hand.-Mazz.) Ietswaart is a locally endangered plant species and of dispersed distribution in South-Eastern and Eastern Anatolian Regions of Turkey (Bakis *et al.* 2011). It's essential oil contains carvacrol and *p*-cymene as major components that exhibit a significant antimicrobial activity against bacteria (Cosge *et al.* 2009).

At present, due to ever increasing abiotic and biotic stresses an increased pressure on natural resources is evident. Moreover, fast urbanization, increased industrial activities, increased fossil fuel consumption, unsustainable forestry and agricultural practices have also resulted in increased CO_2 pollution (IPCC 2010). Resultantly, *O. acutidens* populations are under great pressure and threat at their habitat.

No proper *O. acutidens* field propagation system is available. The plants belonging to *O. acutidens* are multiplied by seeds under natural conditions. The seedlings may suffer from uncertain biotic and abiotic stresses in nature during initial periods of growth. A possible alternative for conservation and commercial propagation to this could be *in vitro* multiplication through plant tissue culture techniques singly or interactively with conventional propagation methods. In addition, *in vitro* culture techniques can be an alternative method for the continuous provision of plantlet stocks for large scale field cultivation. Economic homogenous propagation of the plant, within each accession is desired characteristic for economic proliferation of the plant vegetative to shorten the juvenile period and induce precocity, a factor that has been observed in *Origanum* (Ravid and Putievsky 1987).

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There is a single report on *in vitro* multiplication of *O. acutidens* (Yildirim 2013). The study aimed to improve - determine if various concentrations of BAP + 0.5 mg/l NAA could improve regeneration ability on lateral stem nodes obtained from 58 days old *O. acutidens* seedlings under *in vitro* conditions.

Materials and Methods

The seeds (Fig. 1a) and plants of *O. acutidens* were collected at 1200 m altitude from district Maden of Elazig province, Turkey during the last week of July, 2009. The plants were identified in the Department of Biology, Dicle University, Diyarbakir, Turkey.

Surface-sterilization of *Origanum acutidens* seeds was carried out for 15 min using 50% commercial bleach (containing 5% NaOCl) after addition of 0.5 ml of Tween 20 per 100 ml NaOCl followed by 3 × 3 min rinsing with sterile distilled water. Thereafter, they were germinated on MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar (Duchefa) and autoclaved at 121ºC, under pressure of 1.05 kg/cm^2 for 20 min. The pH of the medium was adjusted to 5.7 ± 0.1 with 0.1 N KOH or HCl before autoclaving.

The seeds were germinated after 13 days of culture that developed into full plantlets after 58 days of culture (Fig. 1b), that were used to obtain stem node explants (Fig. 1c) and cultured on MS medium supplemented with 0.4, 0.8, 1.2, 1.6 and 2.0 mg/l BAP with and without 0.5 mg/l NAA (two factors). Cultures were maintained at 24 ± 1ºC under 16 hrs white fluorescent light (42 µMol photons/m²/s) and 8 hrs dark photoperiod.

Thereafter, well developed shoots of *Origanum acutidens* were rooted on MS medium supplemented with 0.75 mg/l NAA at 24 ± 1ºC for 9 weeks under16 hrs light photoperiod. The rooted plants were acclimatised in peat moss contained in 2-litre plastic pots. Peat moss was prepared locally from leaves. It had pH of 6.2 and electrical conductivity (EC) of 0.12 dS/m with porosity of about (70 % v/w). These characteristics allowed high water absorption with low bulk density of 0.12 mg/m^3. Each *in vitro* regenerated plant was covered with polythene bag in greenhouse under 16 h light photoperiod and 24 ± 2ºC temperature for acclimatisation. Thereafter 2 weeks, when the plantlets began to show signs of growth, they were gradually exposed to reduced (40.0%) relative humidity by progressively removing the polythene bags over a period of three weeks.

The experiment made use of 60 explants for each treatment with 5 explants per replications (5 explants × 12 replications = 60 explants). The data were statistically processed by one way analysis of variance (One way ANOVA) using "IBM® - SPSS® Statistics Version 20 for Windows. Means were compared selecting DMRT with a significance level at $p < 0.05$.

Results and Discussion

The results (Table 1) of the experiment showed 80 - 100% shoot regeneration on MS medium containing various concentrations of BAP (Table 1). Shoot regeneration percentage showed statistically non significant differences of the effects of BAP concentrations, but had tendency of reduction with increasing concentration of BAP in numerical terms. The minimum shoot induction percentage was noted on MS medium containing 2.0 mg/l BAP.

Number of shoots per explant ranged 2.48 - 10.28. Maximum number of 10.28 shoots per explant was noted on MS medium containing 0.8 mg/l BAP (Table 1, Fig. 1d). Statistically similar number of shoots per explant was noted on MS medium containing 0.4 to 1.6 mg/l BAP followed by sharp decrease in number of shoots per explant on MS medium containing 2.0 mg/l BAP.

Mean shoot length ranged 1.83 to 3.25 cm. Maximum shoot length of 3.25 cm was recorded on MS medium containing 1.6 mg/l BAP (Table 1).

Table 1. Rate of shoot regeneration, number of shoots per explant, shoot length and percentage of acclimatised micropropagated *Origanum acutidens* plants on MS medium containing different concentrations of BAP.

BAP (mg/l)	Shoot regeneration (%)	Number of shoots per explant	Shoot length (cm)	Percentage of acclimatised plants
0.4	100 a	9.66 a	1.83 b	100.0 a
0.8	100 a	10.28 a	2.46 ab	81.8 ab
1.2	93.3 a	7.30 a	2.16 ab	100.0 a
1.6	93.3 a	8.40 a	3.25 a	88.2 a
2.0	80.0 b	2.48 c	2.26 ab	60.0 c

Mean values shown in a single column followed by different letters are significantly different at $p < 0.05$ using DMRT.

Well developed single shoots on each regeneration medium were rooted on 0.75 mg/l NAA. These single shoots induced multiple roots and 7 - 9 shoots per explant as well (Fig. 1e). Development of multiple shoots on rooting explants was very helpful in easy acclimatisation of *in vitro* regenerated plants in peat moss (last column Table 1). Percentage of acclimatised plants ranged 60 to 100. The minimum percentage of acclimatisation was noted on the plants regenerated on MS medium containing 2 mg/l BAP. Hundred percent (100%) acclimatisation was noted on plantlets regenerated on MS medium containing 0.4 and 1.2 mg/l BAP (Table 1, Fig. 1f). These plants were transferred to pots followed by transfer to open fields for blooming and seed set.

The result showed 80 to 100% shoot regeneration on stem node explants on MS medium containing 0.4 to 2 mg/l BAP + 0.5 mg/l NAA (5 combinations) and showed variations among them in terms of statistics (Table 2). One hundred per cent or statistically similar shoot regeneration was noted on MS medium containing 0.4, 0.8, 1.2 and 1.6 mg/l BAP - 0.5 mg/l NAA followed by sharp fall in regeneration percentage (80) on MS medium containing 2 mg/l BAP + 0.5 mg/l NAA.

Maximum number of 9.06 shoots per explant was inducted on MS medium containing 0.4 mg/l BAP + 0.5 mg/l NAA followed closely by 6.25 shoots per explant on MS medium containing 0.8 mg/l BAP + 0.5 mg/l NAA. All other BAP + NAA combinations were inhibitory variably (Table 2).

Mean shoot length ranged 0.82 - 2.07 cm. Minimum and maximum shoot length was recorded on MS medium containing 1.2 mg/l BAP + 0.5 mg/l NAA and 0.4 mg/l BAP + 0.5 mg/l NAA respectively. However, no statistical difference among shoot length was recorded on any concentration of BAP + 0.5 mg/l NAA (5 combinations) (Table 2).

Well developed single shoots that regenerated on each treatment containing MS medium with different concentrations of BAP + 0.5 mg/l NAA were rooted on (MS medium containing) 0.75 mg/l NAA. These shoots also developed multiple roots and shoots (Fig. 2a). No problem was recorded in rooting of these shoots irrespective of their origin.

All plants (100%) acclimatised (Table 2, Fig. 2b) under greenhouse conditions. They bloomed and set seeds under open field conditions.

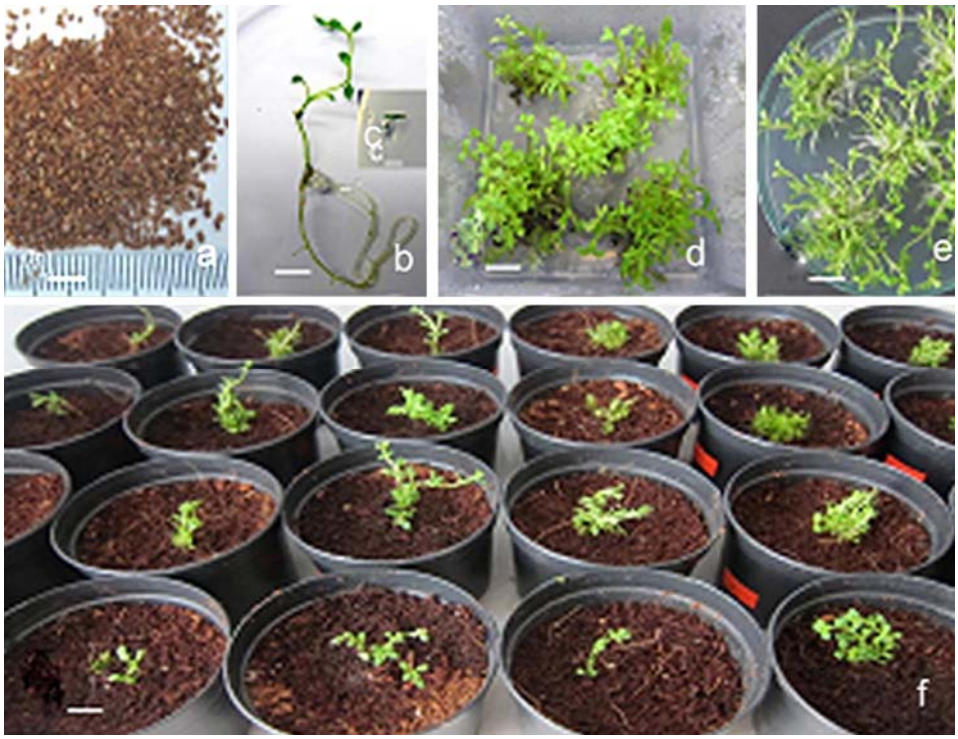

Fig. 1. Plant regeneration from *Origanum acutidens* on MS medium containing different concentrations of BAP (a) seeds of the plant (b) 58 days old seedlings, (c) stem explant, (d) shoot regeneration on MS medium containing 0.8 mg/l BAP stem node explants sown in the MS medium contain BAP, (e) multiple roots and shoots developed from single shoot and (f) acclimatisation of plantlets regenerated on MS medium containing 1.2 mg/l BAP.

Table 2. Rate of shoot regeneration, number of shoots per explant, shoot length and percentage of acclimatised micropropagated *Origanum acutidens* plants containing different concentrations of BAP-NAA.

BAP (mg/l)	NAA (mg/l)	Shoot regeneration (%)	Number of shoots per explant	Shoot length (cm)	Percentage of acclimatised plants
0.4	0.5	100 a	9.96 a	2.07	100
0.8	0.5	100 a	6.25 b	1.92	100
1.2	0.5	100 a	3.53 c	0.82	100
1.6	0.5	93.3 a	4.70 c	1.35	100
2.0	0.5	80.0 b	4.10 c	1.42	100

Mean values shown in a single column followed by different letters are significantly different at $p < 0.05$ using DMRT.

The results of the study showed that *O. acutidens* seeds germinated readily in 13 days time and as such had no dormancy problem. The results contradict findings of Putievsky (1983), Thanos *et al*. (1995) and Ellis *et al.* (1995) who observed partial seed dormancy and poor germination ability in fresh seeds of *O. majorana* and *O. vulgare*.

Development of *in vitro* micropropagation protocols for medicinally important and neglected plant species is highly desirable. The study compared effects of MS medium containing 0.4, 0.8, 1.2, 1.6, 2.0 mg/l BAP with and without 0.5 mg/l NAA to achieve pragmatic clonal propagation of *Origanum acutidens* on stem node explants obtained from 58 days old seedlings. The basic idea was to know if this variation could induce any effect on shooting and rooting potential of the plant with aim to improve previously reported results (Yildirim 2013).

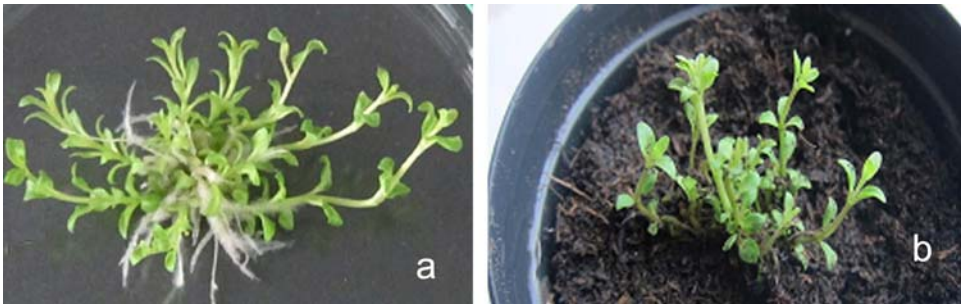

Fig. 2. Plant regeneration from *Origanum acutidens* on MS medium containing different concentrations of BAP + NAA. (a) Multiple roots and shoots induction on BAP+NAA regenerated single shoots. (b) Acclimatisation of plantlets in pots.

Almost, similar shoot regeneration frequency was noted on MS medium containing different concentrations of BAP with and without 0.5 mg/l NAA. However, a comparison in number of shoots per explant showed that addition of 0.5 mg/l NAA to the culture medium induced partial inhibition on shoot induction percentage and number of shoots per explant. The results are not in agreement with Aasim *et al*. (2009), who noted that MS medium containing various concentrations of BAP + 0.1 mg/l NAA increased callusing number of shoots per plumular apice explant and shoot length in cowpea cv. Akkiz. However, Aasim *et al*. (2013) found that immature cotyledons cultured on MS medium containing 0.25 - 0.75 mg/l BAP with 0.25 mg/l showed partial inhibition in shoot regeneration on immature embryos of cowpea cv. Akkiz compared to BAP used singly. Similarly, Kendir *et al*. (2009) found partial inhibition of BAP + 0.5 mg/l NAA on shoot regeneration of grass pea. Percentage of acclimatised plants was slightly better when the culture media contained different concentrations of BAP + 0.5 mg/l NAA. This study reports 80 - 100% shoot induction on different concentrations of BAP with and without 0.5 mg/l NAA. This study further reports induction of maximum number (10.28) of shoots per explants on 0.8 mg/l BAP and 9.96 shoots per explants on 0.4 mg/l BAP + 0.5 mg/l NAA. The results of this study suggest that shoot regeneration from stem nodes varies depending on the concentration of BAP and presence or absence of NAA in MS culture medium in agreement with Sahin-Demirbag *et al*. (2008).

Increased concentration of BAP was inhibitory both in terms of shoot regeneration, number of shoots per explants. The increased BAP concentrations also had negative impact on percentage of greenhouse acclimatised plants in agreement with Aasim *et al*. (2009). They reported that addition of any concentration of NAA resulted in significant decrease in the frequency (%) of shoot regeneration and mean number of shoots per explant on cowpea shoot meristems. The results of this study show improvement over the results of Yildirim (2013), who reported induction of 9.31 shoots per explant with shoot length of 1.81 cm using 2.4 mg/l BAP + 0.2 mg/l NAA on stem node explants. The results show compatibility with Socorro *et al*. (1998), Goleniowski *et al*.

(2003) and Moreno-Fortunato and Avato (2008); who used MS medium containing BAP with and without NAA for micropropagation in other *Origanum* species. Akcam and Yurekli (1993) reported callus cultures studies in *Origanum sipyleum* and Oluk and Cakir (2009) reported micropropagation of *O. bastetanum* using seedlings derived explants. The researchers used shoots obtained from 17 day-old seedlings that were multiplied on modified MS medium containing 550 mg/l of $CaCl_2$ for sustained growth. They were successful to induce multiple shoots (3.7 ± 0.3 shoot/explant) on medium containing 1 mg/l BAP. Contrarily, this study reports higher number of shoots per explant obtained from 54 day-old plants and did not need any concentration of $CaCl_2$ to induce shoots.

This study reports 100% rooting success on of 0.75 mg/l NAA in MS medium compared to Yildirim (2013), who has reported 96% rooting of *Origanum acutidens* using 0.5 mg/l NAA. Barpete *et al*. (2014) also emphasize role of NAA in difficult to root plants. Contrarily, Goleniowski *et al*. (2003) reported spontaneous rooting in shoot multiplication medium containing BA with NAA for *Origanum vulgare*. Whereas, Socorro *et al*. (1998) rooted micropropagated shoots of *Origanum bastetanum*, on peat substrate. Differential rooting protocols used in these studies show that rooting of different *Origanum* is species dependent and may vary from situation to situation.

The results of this study further report 60 - 100% and 100% cent acclimatisation on all plantlets induced on cultures containing BAP and BAP+NAA, respectively. These results show improvement over previous results by Yildirim (2013), who did not report acclimatization of micropropagated plants. All acclimatised plants grew to flower and seed set.

The establishment of a successful micropropagation protocol of *Origanum acutidens* using stem node explants provides an opportunity for application of biotechnological tools providing information for commercial propagation. Multiplication of economically important medicinal plant *O. acutidens* through *in vitro* microproliferation techniques may also have impacts on conservation and maintenance of this important plant species.

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