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Full Length Research Paper

Micropropagation of medicinal plant *Dracocephalum kotschyi* Boiss. via nodal cutting technique

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An experiment was carried out to examine the effects of different combinations of plant growth regulators on the *in vitro* micropropagation of *Dracocephalum kotschyi* Boiss. *D. kotschyi*, of the family Lamiaceae, which is rich in aromatic essential oils and valuable for its pharmaceutical, aromatic and culinary properties. The present study showed the procedure for propagation of *D. kotschyi* using nodal segments from *in vitro* germinated plants. The highest efficiency and high frequency of root proliferation of shoot formation after 30 days occurred in the medium MS containing 2 mg/L 6-benzylaminopurine (BAP) plus 0.5 mg/L α-naphthalene acetic acid (NAA). After hardening, the rooted plants were transferred to the greenhouse condition where they normally grew, matured and flowered with a survival rate of 90 to 95%. We concluded that the present protocol can be efficiently used for mass propagation of *D. kotschyi*.

Key words: *Dracocephalum kotschyi,* Lamiaceae, explant, medicinal.

INTRODUCTION

Dracocephalum kotschyi Boiss, is a wild-growing flowering plant, belonging to the family Lamiaceae which is knowing for the essential oils, common to others members in the family (Golshani et al., 2004). Many biologically active essential oils have been isolated from various members of the family in southwestern Asia. *D. kotschyi* have been used as a medicinal herb for several years in Iran folk medicine for its antispasmodic and analgesic properties (Jahaniani et al., 2005). Anti-hyperlipidemic (Sajjadi et al., 1998) and immune-modulatory (Amirghofran et al., 2000) effects have been reported for *D. kotschyi*. The preferentially inhibit multiplication of tumor cells may offer means of developing drugs effective against cancer in human. One of the methods for finding such agents is division plant material, particularly those which have been reported to have anti-cancer, anti-inflammatory, anti-fungal or anti-bacterial effects (Cordell et al., 1991; Cox and Balick, 1994; Wu et al., 2002). Spinal-Z is a traditional Iranian anticancer remedy (Sobhani et al., 2002; Jahaniani et al., 2005). It was used by traditional medicine as a plant concoction for the treatment of many forms of cancer in humans.

These traditional medicines believed that the concoction was specifically effective against leukemia and GI tract malignancy. Spinal-Z is an extract which composed of two plants: *Peganum harmala* L. (Zygophyllaceae) seeds and *D. kotschyi* leaves (Sobhani et al., 2002; Jahaniani et al., 2005).

The natural propagation of *D. kotschyi* by seed distribution is restricted mainly because of the sever seed dormancy. This, besides incorrect usage of the plant per people and presence of suboptimal natural conditions makes it to have been considered as a rare plant. *In vitro* micropropagation is an effective mean for quick multiplication of species in which it is necessary to obtain high result uniformity. In recent years, the interests in using these techniques for rapid and large-scale propagation of medicinal and aromatic plants has been

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Abbreviations: BAP, 6-benzylaminopurine; **IBA,** indole-3-butyric acid; **KIN,** kinetin; **NAA,** α-naphthalene acetic acid; **AC,** activated charcoal.

Figure 1. Nodal segments of *Dracocephalum kotschyi* Boiss. taken from aseptic seedlings as explants on medium containing activated charcoal in combination with growth regulators.

significantly increased (Sahoo et al., 1997). There are many *in vitro* studies that have been conducted on Lamiaceae species using different explants, like nodal segments, leaf explants and axillary buds (Begum et al., 2002; Nirmal and Sehgal, 2010; Winthrop and Simon, 2000). The present study is the first report, it showed the procedure for the micropropagation of *D. kotschyi* using nodal cutting prepared from *in vitro* plants germinated.

MATERIALS AND METHODS

The experiment was carried out at the Tissue Culture Laboratory of the Agricultural Biotechnology Research Institute - Central region of Iran in 2010. Seeds of *D. kotschyi*, native cultivars were obtained from the Collection of Agricultural Research Center.

Seed sterilization and cultivation

The seeds were washed with tap water for 5 to 10 min to remove surface contamination and then sterilized by immersing in 70% ethanol for 1 min with vigorous shaking followed by 20 min in 15% sodium hypochlorite containing one drop of Tween 20. The seeds were then rinsed three times with sterile distilled water in a laminar flow cabinet to remove minor amounts of disinfection liquid. For germinating, the seeds were cultured in a mug on 50 ml of standard (Murashige and Skoog, 1962) containing 3% (m/v) sucrose and 0.6% (m/v) agar. Cultures were incubated in a growth chamber at temperature of 24°C, a 16 h photoperiod provided and with a light intensity of 2000 lux provided by white fluorescent lamps. After four weeks, the germinated seeds had produced young seedlings with 5 to 9 leaves. Nodal segments (with one node) derived from these aseptic seedlings were used as explants.

Nodal cutting and culture

Nodal segments (0.5 to 1 cm length) were cultured on MS medium supplemented with (6-benzylaminopurine) or kinetin (KIN) with three concentrations (0, 1.0, 2.0 mg/L) combined with Indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA). Three concentrations (0, 0.2, 0.5 mg/L) of IBA or NAA were applied to enhance shoot formation. Sterilization was performed by autoclaving at 121°C for 20 min. pH was adjusted at 5.8 before adding 0.6% (w/v) agar and 0.4% (w/v) activated charcoal. Five explants were aseptically cultured in a mug containing 50 ml of the induction medium. The mugs were covered and sealed with household plastic foil for a period of 5 weeks and then transferred to the same conditions as mentioned previously.

Hardening and transfer

After 5 weeks, the rooted plantlets were acclimatized and transferred to pots containing sterilized peat mass and vermiculite (3:1 ratio). The pots were covered with a clear beaker with a few holes and were frequently watered to maintain a high humidity and kept in a phytotron for 15 days for hardening. Hardened plantlets were transferred to a greenhouse set at a day temperature of 21°C, a night temperature of 17°C, a relative humidity of 85% and a day length of 12 h. Immediately after planting, the plantlets were irrigated and adequate soil moisture was maintained through daily watering.

Data collection

Five weeks after transplanting, growth parameters including the number of branches, the number of leaves, the number of roots, the stem length, the average length of internodes, the average root length, and the percentage of rooting were recorded and the effect of different shoot induction media was evaluated.

Experimental design and statistical analysis

The experiment was carried out in a completely randomized factorial design. Data were statistically analyzed using the SAS software (version 8). When the ANOVA indicated significant treatment effects (5 or 1%) based on the F-test, the Duncan's Multiple Range Test ($P \leq 0.05$) was used as a method to determine which treatments were statistically different from other treatments.

RESULTS

In a preliminary experiment, nodal explants from grown plants of *D. kotschyi* were inoculated on MS supplemented with growth regulations BAP and Kin alone or in combination at different concentrations (0, 1.0, 2.0 mg/L) plus three concentrations (0, 0.2, 0.5 mg/L) of IBA or NAA, for production of multiple shoots (Figure 1). The multiplication efficiency of nodal segments from plants was significant, when estimated four to five weeks. Different concentrations and combinations of growth regulators showed significantly different responses in terms of number of shoots per explant, roots per explant, shoots length per explants (cm) the length of the stem, internodes and roots, and the percentage of rooting (Tables 1 and 2). After 4 weeks of culture, as a supplement 2.0 mg/L BAP plus 0.5 mg/L NAA resulted in maximum proliferation was noted in 90% cultured explants (Figure 2 and Table 1). Media containing KIN

Table 1. Effect of growth regulators BAP, IBA and NAA on the number of shoots per explants shoots length (cm) per explants and percent of response on shoots induction from nodal and explants of *Dracocephalum kotschyi* Boiss. (Lamiaceae).

Growth regulators (mg/L)			No. of shoots/ explant	Shoots length/ explants (cm)	No. of roots/ explant	Rooting (%)
BAP	IBA	NAA				
0	-	-	0.27^{e}	3.00^{e}	1.00^{e}	18.43^{e}
1	-	-	3.50^{d}	4.08^{d}	2.45^{d}	38.2^{d}
2	-	-	4.53^{c}	4.66^{d}	2.18^{d}	39.00^{d}
0	0	-	0.80^{e}	3.90^{e}	1.32^{e}	20.23^{e}
1	0.2	-	3.73^{d}	5.00^{c}	2.81^{d}	48.2^{c}
2	0.5	-	5.18^{b}	5.60^{c}	3.00^{c}	50.08^{c}
0	-	0	0.82^{e}	3.20^{e}	2.40^{d}	18.23^{e}
1	-	0.2	5.10^{b}	6.00^{b}	4.01^{b}	68.4^{b}
2		0.5	7.11^{a}	8.5^{a}	8.00^{a}	90.08^{a}

Table 2. Effect of growth regulators KIN, IBA and NAA on the number of shoots per explants, shoots length (cm) per explants and percent of response on shoots induction from nodal and explants of *Dracocephalum kotschyi* Boiss.

Growth regulators (mg/L)			No. of shoots/ explant	Shoots length/ explants (cm)	No. of roots/ explant	Rooting (%)
KIN	IBA	NAA				
0	-	-	0.27^{d}	2.00^{e}	0.20^{d}	8.43^{f}
1	-	-	0.50^{d}	3.08^{d}	0.45^{d}	10.2^{e}
2	-	-	1.53^{c}	4.66^{c}	1.86^{c}	16.00^{c}
0	0	-	0.80^{d}	3.5^{d}	0.46^{d}	10.20^{e}
1	0.2	-	2.00^{b}	4.2^{c}	1.76^{c}	15.23^{d}
2	0.5	-	2.13^{b}	5.0^{b}	2.00^{b}	18.12^{b}
0	-	0	0.82^{d}	3.5^{d}	0.40^{d}	15.2^{d}
1	-	0.2	2.84^{a}	4.2^{c}	1.21^{c}	18.23^{b}
2		0.5	2.93^{a}	5.5^{a}	2.60^{a}	25.12^{a}

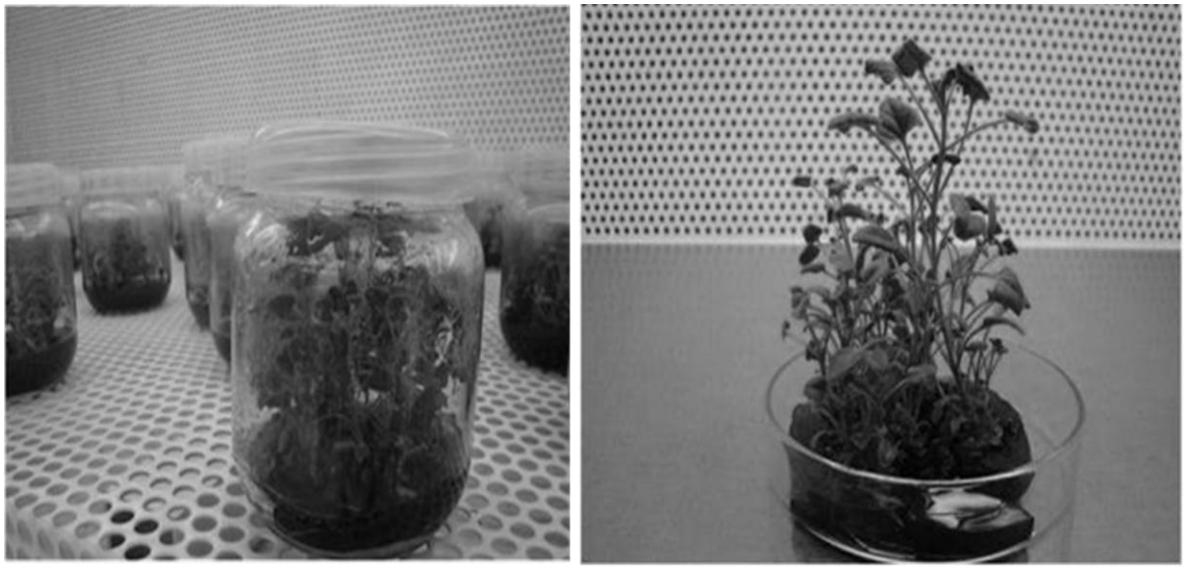

Figure 2. Seedling of *Dracocephalum kotschyi* Boiss. with expanded buds and extended roots after 4 weeks of regenerating in medium containing activated charcoal plus plant growth regulators.

Table 3. Effect of different concentrations of IBA and NAA on number of roots per explants, root length (cm) and rooting percent shoots induction from nodal and explants of *Dracocephalum kotschyi* Boiss.

Growth regulators (mg/L)		No. of roots/ explant	Rooting (%)	Root length (cm)
IBA	NAA			
0	-	1.32^{e}	20.23^{e}	3.24^{d}
0.2	-	2.81^{d}	48.2^{c}	6.51^{c}
0.5	-	3.00^{c}	50.08^{c}	6.54^{c}
-	0	2.40^{d}	18.23^{e}	2.38^{e}
-	0.2	4.21^{b}	68.4^{b}	8.76^{b}
-	0.5	5.00^{a}	90.08^{a}	12.49^{a}

Figure 3. Hardened plantlet of *Dracocephalum kotschyi* Boiss. in a phytotron after transferring from *in vitro* conditions.

alone or in combinations with other growth regulators auxin had the least effectiveness in inducing regeneration (Table 2). The maximum number of roots per explant was obtained in MS combined with BAP (2 mg/L) plus 0.5 mg/L NAA (Table 1). The percentage of explants that had no rooting on regeneration medium after transportation to the rooting medium included 0.2 mg/L NAA, which was safely used for the treatment of almost all the explants that were properly rooted (Table 3). NAA had a higher potential with respect to the induction of roots in this cultivar than IBA (Table 3). Shoots was rooted properly after 4 weeks of subculturing. Regenerated plantlets were used repeatedly as new materials for the next cycles of regeneration (Table 3).

The proliferated plants showed 90 to 95% survival during hardening and acclimatization (Figure 3). There were no observable variations between the parent plants and *in vitro* propagated plants. The transplanted plantlets established well in a glasshouse (Figure 4).

DISCUSSION

The literature on regeneration of *D. kotschyi* and the role of plant growth regulators are not reported yet, but there is different reported regeneration of the family

Figure 4. *Dracocephalum kotschyi* Boiss. whole plants planted in a greenhouse.

Lamiaceae. These studies used different species, varieties and explants and a vast range of *in vitro* regeneration media and/or strategies. The regeneration of that family from nodal segments has been considered as a relative simple method, which could be potentially applied for mass propagation of the species (Dode et al., 2003; Irina et al., 2004; Naghibi et al., 2005; Lucia, SA et al., 1994; Luis, JG et al., 1992; Mederos, 1991; Mederos, et al., 1997).

The importance of BAP for regeneration of different plants of the Lamiaceae family, for example *Ocimum* spp., has been emphasized by Dode et al. (2003), Nirmal and Sehgal (1999) and David and Arockiasamy (2008), whom showed that MS medium supplemented with 5 mg/L BAP plus 0.5 mg/L NAA was effective for shoot multiplication in nodal explants of *Ocimum basilicum* L. Kiran et al. (2004) showed that MS medium supplemented with 1 mg/L BAP was effective for shoot multiplication in nodal segment of *Mentha piperita* L. In our study, the concentration of BAP lesser than 2 mg/L was less effective for regeneration of *D. kotschyi*. Another cytokinin, KIN, alone or in combination with auxins was not as effective as BAP was for the regeneration of *D. kotschyi*. The minimum number of shoots was regenerated in media containing KIN in combination with IBA or NAA. This was in accordance with previously report (David and Arockiasamy, 2008).

In our experiment NAA was more effective than IBA with respect to rooting of the regenerated shoots. The shoot buds proliferating from axillary shoot nodal explants rooted easily in medium supplemented with NAA. The axillary shoots further produced multiple shoot buds when cultured in bud induction medium. The *in vitro* established plantlets were hardened in a phytotron with a survival rate of 90 to 95% and were then transplanted in glasshouse (David and Arockiasamy, 2008).

Overall findings of the present study are significant in obtaining the maximum regeneration with minimum concentrations of growth regulator. In conclusion, we have developed a promising method for an efficient regeneration from nodal explants of *D. kotschyi* Boiss. using BAP and NAA. The protocol could be useful for

large scale production of single genotypes and provides a possible system towards genetic improvement of the crop.

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