

Influence of Plant Growth Regulators on *In Vitro* Culture and Regeneration of *Dracocephalum kotschyi*

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Abstract – The study was conducted to develop a micro propagation system for *Dracocephalum Kotschyi*, an important-rare medicinal plant in Iran. MS media containing Zeatin or KIN (0, 1 and 2 mg.l⁻¹) in combination with IBA and NAA (0, 0.2 and 0.5 mg.l⁻¹) were investigated for shoot regeneration using nodal explants. The average length of microshoots was obtained on KIN (2 mg.l⁻¹) alone or combined with 0.5 mg. l⁻¹NAA or IBA. A maximum lateral shoots were produced on IBA at 0.2 mg.l⁻¹ or KIN 1 mg.l⁻¹. No sign of rooting was observed on treatments containing ZEA. Percentage of rooted microshoots reached to highest in some treatments such as KIN (1 mg.l⁻¹) + NAA (0.2mg.l⁻¹). Well-developed shoots with strong root were transplanted to the pots filled with a mixture of peat + cocopeat+ perlite (2:1:1) under controlled greenhouse condition. All plantlets were survived and showed normal growth.

Keywords – *Dracocephalum kotschyi*, IBA, KIN, ZEA, Node Culture.

Abbreviations – BAP, 6-Benzylaminopurine; IBA, Indole-3-Butyric Acid; KIN, Kinetin; MS, Murashige and Skoog Medium (1962); NAA, Naphthaleneacetic Acid; Pgrs, Plant Growth Regulators; ZEA, Zeatin.

I. INTRODUCTION

Lamiaceae is one of the largest and important flowering plants family containing about 440 species in 220 genera [1]. The eight species of the *Dracocephalum* genus have been found in Iran. *Dracocephalum Kotschyi* belongs to Lamiaceae family. This important- rare medicinal plant traditionally used for curing rheumatism, fever and relief the pain [2,3]. *D. Kotschyi* has ingredient compounds likes "Limonene" and "Alpha Terpinol"[3,4]. An anticancer drug (Spinalz) based on two medicinal plants; *Peganum harmala* L. and *D. Kotschyi* is successfully used in Iran [3, 4] for treatment of blood and digestion system cancers [2, 5] and is naturally propagated by seeds. The seeds have the poor viability and a hard coat which is almost impermeable to water and so need months to germinate. So, very few seedlings are produced from seeds. Because of excessive harvesting of this valuable medicinal plant,

the low traditionally reproduction rate could not be restored its habitats and therefore is at a great risk of extinction [6]. Plant regeneration by tissue culture technique would be a possible alternative for improving quality and producing *D. Kotschyi* plant. *In vitro* plant regeneration is the best available method for producing high-quality plants which are free of any disease and pest ensuring the maximum production potential of varieties that are genetically identical with the parent plant as well another [7]. To the best of our knowledge, a little information is available on clonal propagating *D. Kotschyi* such as Otroshy and Moradi [6]. So the objective of the present study was to develop an effective *in vitro* method for plant propagation of medicinally important *D. Kotschyi* by node culture technique.

II. MATERIALS AND METHODS

The experiment was carried out in the Tissue Culture Laboratory of the Agricultural Biotechnology Research Institute - Central region of Iran during 2013- 2014 to evaluate the effects of various plant growth regulators (PGRs) on plant regeneration of *D. Kotschyi* under *in vitro* condition.

Plant Materials and culture media

Seeds were taken from the Research Centre for Agriculture and Natural Resources, Isfahan, Iran 2013. Seeds were disinfected in 70 % ethanol for 1-minute, rinsed with sterilized water, disinfected with 1% sodium hypochloride + Tween 20 (two drops per 500 ml) for 15 minutes, and washed with sterilized water for five minutes under aseptic condition. Then, five disinfected seeds were cultured per bottle containing 25 ml of solid MS basal medium and incubated at growth chamber under 24°C and photoperiod 16/8 hd⁻¹ provided by fluorescent tube at a fluency rate of 2700 Lux at medium level for germination.

Four week- old seedlings were excised to 1-2 centimetrepieces including 1 lateral bud in aseptic condition and cultured on MS-based regeneration media supplemented with 0.3% activated charcoal and various concentrations of KIN or ZEA (0, 1 and 2 mg. l⁻¹) each

with combinations of IBA or NAA (0, 0.2 and 0.5 mg. l⁻¹). All cultures were kept in the same condition as mentioned above. After 30 days, parameters such as average length of stem and internode, average number of leaves, nodes and lateral shoots and percentages of rooted plantlets were recorded.

Hardening and Acclimatization

Regenerated microshoots with the well-rhizogenesis system were carefully removed from culture bottle, washed under tap water to remove the remnants agar and then transplanted to the pots containing the mixture of peat + cocopeat + perlite (2:1:1) and covered with tight plastic covers to preserve the humidity. The plantlets were acclimatized in a controlled condition at 20-22°C under 16/8-hour (light-dark) photoperiod at phytotron and irrigated regularly. During the hardening stage, plastic covers were gradually punched and removed after 14-20 days. The surviving plants were transferred to pots containing garden soil and kept under greenhouse conditions for acclimatization.

III. RESULTS

The length of stem showed significant differences among treatments (df 24, MS 1.92, error 0.55, $p = 0.01$). Based on Table 1, a maximum length of *in vitro*

regenerated shoot was observed at (2 mg. l⁻¹) KIN alone or combined with NAA or IBA at a concentration of 0.5 mg. l⁻¹. Significant differences among treatments were observed on number of nodes (df24, MS 15.99, error 1.1, $p = 0.01$). Although, the plantlets grown on MS medium containing NAA (0.5 mg. l⁻¹) had the best value (5.66) of the mean number of nodes, but is not significantly different from NAA (1 mg. l⁻¹), KIN (2 mg. l⁻¹), KIN (2 mg. l⁻¹) + NAA (0.2 mg. l⁻¹) or IBA (0.2 or 0.5 mg. l⁻¹). Since the branches of lateral shoots and leaves originated from axillary buds, these two growth parameters were also significantly affected by applying treatments at $p=0.01$. As expected, the highest number of leaves and lateral shoots were obtained on NAA (0.5 mg. l⁻¹) and IBA (0.2 mg. l⁻¹) respectively. But, they were not significantly different from some treatments which listed by similar letters in the Table 1. The length of internodes showed significant differences among treatments (df24, MS 0.55, error 0.06, $p = 0.01$). As shown in the Table 1, the better results on the average length of internodes were generally getting on MS media fortified with various concentrations of ZEA than those supplemented by KIN. None of the seedlings grown in the medium containing ZEA did not produce roots. The percentages of rooted plantlets varied from 16.66 to 31.66 in all the treatments which contained no ZEA (Table 1).

Table 1: The effect of different concentration of some plant growth regulators on the average length of stem and internode, mean number of leaves, node, lateral shoots and percentage of rooting in *D.kotschy*

Plant growth regulators (mg. l ⁻¹)	Average stem length	Average number of leaves	Average number of nodes	Average number of branches	Percentage of rooting	Average length of internodes
Control	1.0633 ^d	2.1000 ^{f-i}	2.1000 ^{f-i}	1.1333 ^{c-e}	13.333 ^{b-d}	0.3500 ^{gh}
NAA (0.2)	1.8000 ^{b-d}	5.0000 ^{a-c}	5.0000 ^{a-c}	3.0000 ^{b-c}	16.667 ^{a-d}	0.3783 ^{f-h}
NAA (0.5)	1.6000 ^{b-d}	5.6667 ^a	5.6667 ^a	4.5000 ^{ab}	20.000 ^{a-c}	0.2833 ^h
Kin (1)	1.3833 ^{b-d}	3.3333 ^{d-g}	3.3333 ^{d-g}	1.8333 ^{c-e}	11.667 ^{cd}	0.3483 ^{gh}
Kin(1)+NAA (0.2)	1.7900 ^{b-d}	1.9333 ^{g-i}	1.9333 ^{g-i}	0.7333 ^{de}	31.667 ^a	0.3400 ^{gh}
Kin(1)+NAA (0.5)	1.5167 ^{b-d}	4.0000 ^{b-e}	4.0000 ^{b-e}	2.8333 ^{bc}	21.667 ^{a-c}	0.3183 ^h
Kin(2)	2.3000 ^{a-c}	4.5833 ^{a-d}	4.5833 ^{a-d}	2.1667 ^{cd}	18.333 ^{a-c}	0.5200 ^{e-h}
Kin(2)+NAA (0.2)	1.8667 ^{b-d}	4.5000 ^{a-d}	4.5000 ^{a-d}	2.5000 ^{b-d}	18.333 ^{a-c}	0.4083 ^{f-h}
Kin(2)+NAA (0.5)	3.0800 ^a	2.7000 ^{e-h}	2.7000 ^{e-h}	1.0000 ^{c-e}	23.333 ^{a-c}	0.7233 ^{b-f}
IBA(0.2)	1.0417 ^d	5.3333 ^{ab}	5.3333 ^{ab}	5.1667 ^a	30.000 ^{ab}	0.2017 ^h
IBA(0.5)	1.8333 ^{b-d}	4.5000 ^{a-d}	4.5000 ^{a-d}	2.8333 ^{bc}	18.333 ^{a-c}	0.3950 ^{f-h}
KIN(1)+IBA(0.2)	1.0500 ^d	3.5000 ^{c-g}	3.5000 ^{c-g}	2.3333 ^{cd}	25.000 ^{a-c}	0.3117 ^h
KIN(1)+IBA(0.5)	1.0833 ^d	3.6667 ^{c-f}	3.6667 ^{c-f}	1.3333 ^{c-e}	30.000 ^{ab}	0.2983 ^h
KIN(2)+IBA(0.2)	1.4333 ^{b-d}	4.6667 ^{a-d}	4.6667 ^{a-d}	1.8333 ^{c-e}	25.000 ^{a-c}	0.3467 ^{gh}
KIN(2)+IBA(0.5)	2.4167 ^{ab}	4.3333 ^{a-e}	4.3333 ^{a-e}	2.5000 ^{b-d}	16.667 ^{a-d}	0.5633 ^{d-h}
ZEA(1)	1.0833 ^d	1.6667 ^{hi}	1.6667 ^{hi}	0.0000 ^e	0.000 ^d	0.8333 ^{a-e}
ZEA(1)+NAA(0.2)	0.9167 ^d	1.3333 ^{hi}	1.3333 ^{hi}	0.0833 ^e	0.000 ^d	1.0833 ^{ab}
ZEA(1)+NAA(0.5)	0.8333 ^d	1.5000 ^{hi}	1.5000 ^{hi}	0.0000 ^e	0.000 ^d	0.8667 ^{a-e}
ZEA(2)	0.7500 ^d	1.1667 ^{hi}	1.1667 ^{hi}	0.0000 ^e	0.000 ^d	0.9167 ^{a-c}
ZEA(2)+NAA(0.2)	1.0833 ^d	1.1667 ^{hi}	1.1667 ^{hi}	0.0333 ^e	0.000 ^d	1.1167 ^a
ZEA(2)+NAA(0.5)	1.0833 ^d	1.1667 ^{hi}	1.1667 ^{hi}	0.0000 ^e	0.000 ^d	0.7000 ^{c-g}
ZEA(1)+IBA(0.2)	1.0000 ^d	0.8333 ⁱ	0.8333 ⁱ	0.0000 ^e	0.000 ^d	0.8733 ^{a-e}
ZEA(1)+IBA(0.5)	0.9167 ^d	0.5000 ⁱ	0.5000 ⁱ	0.0833 ^e	0.000 ^d	1.0000 ^{a-c}
ZEA(2)+IBA(0.2)	1.0833 ^d	1.5000 ^{hi}	1.5000 ^{hi}	0.0000 ^e	0.000 ^d	1.0833 ^{ab}
ZEA(2)+IBA(0.5)	1.2500 ^{cd}	1.5000 ^{hi}	1.5000 ^{hi}	0.0000 ^e	0.000 ^d	0.9500 ^{a-c}

Values followed by a similar letter are not statistically significantly different.

IV. DISCUSSION

Cytokinin promotes the growth of axillary buds by reducing the apical dominance of buds during the micropropagation phase [8] and low auxin concentrations are important for micropropagation [9]. Based on our findings; the length of the shoot was increased as the concentration of KIN (alone or in combination with NAA or IBA) rose from 1 mg. l⁻¹ to 2 mg. l⁻¹. Our results agree with previous study; reported a maximum shoot length at ½ MS + KIN 4 mg. l⁻¹ on organogenesis of *Coleus vettiveroides* (Lamiaceae) [10]. Rapid regeneration of *D. kotschy* from nodal explants was done by Otroshi and Moradi and reported the best number of shoot per explants and shoot length on MS medium containing BAP (2 mg. l⁻¹) + NAA (0.5 mg. l⁻¹) [6]. Sonanda kumar et al. (2003) reached the best results at the branching stage from the BA and KIN hormone treatment with a mean of 4.1 [11]. The best results from the bud samples in the BAP hormone treatment with a mean of 49.8 ± 1.71 and maximum height with a mean of (4.69 ± 0.08 cm) from the KIN hormone treatment were reported in tissue cultivating *Mentha*

piperrita [12,13]. The best condition for shoot growth was also reported with Kinetin 1.0 (mg. l⁻¹) and IAA 0.5 (mg. l⁻¹) in MS medium for *in vitro* micro propagating *Ocimum americanum* (Lamiaceae) [14]. An efficient and rapid *in vitro* propagation system by node culture technique of *Thymus hyemalis* (Lamiaceae) was adopted. Compared with white medium, B5 or ½ MS media, MS basal salt medium was found to be best for *in vitro* establishment. After 4 weeks, the maximum proliferation was observed (corresponding to 6.58 ± 0.22 number of shoots) for nodal explants cultured on MS medium containing 3% (w/v) of sucrose and supplemented with 1.8 μM of Kinetin [15]. *In vitro* propagation of *Ziziphora tenuior* L. (Lamiaceae) was done. The number and length of shoots were 4.21 and 6.17 cm on MS medium supplemented with 1 (mg. l⁻¹) KIN + 0.1 (mg. l⁻¹) NAA, respectively [16]. The study of several morpho-physiological indices of *in vitro* regenerants of *Rhodiola rosea* (Crassulaceae) and *Stachys sieboldii* (Lamiaceae) were made and the culture medium variant enriched with NAA, recommended for a high number of nodes per stem [17].

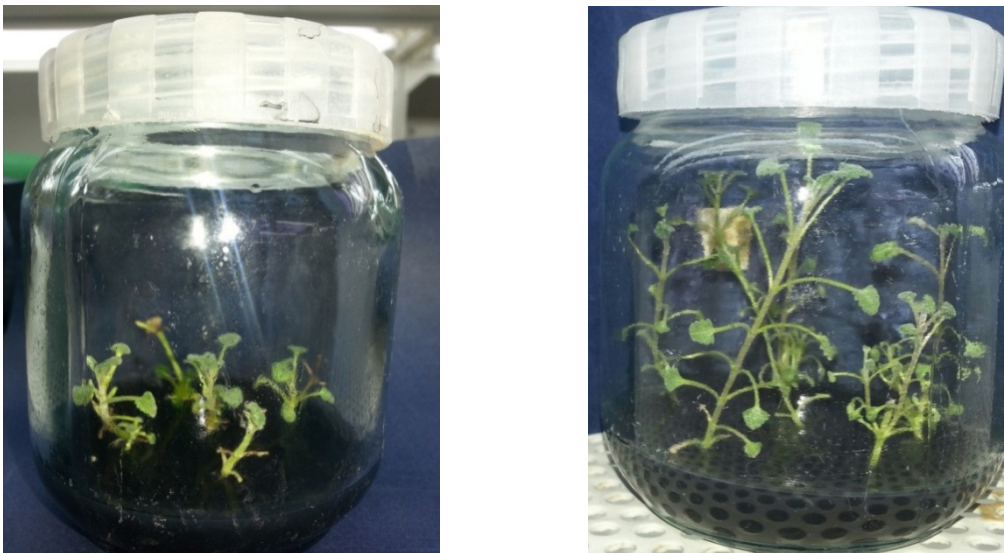


Fig.1. The effect of MS medium containing KIN (Right) compared with ZEA (Left)



Fig.2. Plantlets of *D. kotschy* with normal growth after hardening of *in vitro* gathered micro shoots.

Rapid axillary bud proliferation in *Mentha piperita* (Lamiaceae) was reported and revealed that MS medium fortified with 4.44 μM BA and 2.32 μM KIN was best for proliferating shoots and induced a mean of 4.1 shoots per node explants [11]. The best results at the rooting stage of the NAA hormone treatment with a mean of 11.1 was reported for *Mentha piperita* [11]. Sujana et al (2011) achieved also their best results at the rooting stage of *in vitro*-derived micro shoots of *Mentha piperita* from the IBA hormone treatment with a mean of (48.5 \pm 1.45) [18].

V. CONCLUSION

In conclusion, cytokinin (KIN) and auxin (NAA, IBA) had an effective role on proliferation, shoots length and node formation of *in vitro* micro propagation of *Dracocephalum Kotschyi*. This simple protocol would be expected to contribute to the future efforts for a large-scale production of cardenolides, germplasm conservation, and genetic transformation studies in this medicinally important species.

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