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Research Report

An Efficient Plant Regeneration System via Direct Organogenesis with in Vitro Flavonoid Accumulation and Analysis of Genetic Fidelity among Regenerants of *Teucrium polium* L.

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Abstract. An efficient and rapid plant regeneration system via direct organogenesis was established for felty germander (*Teucrium polium* L.), an endangered and valuable medicinal plant. Induction of shoot organogenesis was obtained using nodal explants and preconditioned intact seedlings on the MS medium supplemented with various concentrations and combinations of plant growth regulators. The highest number of shoots (7.14/explants) was obtained from nodal explants cultured on MS containing 2.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} NAA. For preconditioning, Best response in terms of number of adventitious shoots per explant (6.85) and greatest shoot length (5.43 cm) was obtained with seedlings perpetrated in 0.5 mg·L^{-1} TDZ. The rooted plantlets were acclimatized successfully in the greenhouse with a survival rate of 100%. Significant differences ($p < 0.05$) were found between the greenhouse-grown tissue culture raised plants in total flavonoid contents. Methanolic extracts of plants raised on TDZ (0.5 mg·L^{-1}) containing medium showed the highest amount of flavonoid contents (1.67 mg Ru·g^{-1} DW) and a higher antioxidant activity (52.31%) in comparison with the field-grown mother plant. Randomly amplified polymorphic DNA (RAPD) analysis of plants raised pre-conditioning procedure revealed there were no somaclonal variations among them. Our results indicated that the cytokinin-preconditioning mediated micropropagation protocol could be used for the production of a large number of true-to-type plants of *T. polium*, which may contain higher quantities of valuable phytochemicals than those of wild plants.

***Additional key words*:** adventitious shoot formation, flavonoid content, in vitro propagation, seed pretreatment

Introduction

Felty germander (*Teucrium polium* L.), belongs to the Lamiaceae family, is one of the wild-growing flowering herb native to Iran and the Middle East (Abdollahi et al., 2003; Rechinger, 1982). It is found usually in rocky mountains and marginal areas, and is characterized as perennial dwarf, pubescent aromatic herb possessing oval leaves with enrolled margins 1-3 cm long, sessile, oblong, or linear; the stems end in a shortly paniculate or corymbose inflorescences that differ in color from white to pink (El-Oualidi et al., 1999). As a traditional remedy, felty germander is used for treatment of convulsion, headache, and digestive diseases in Iran (Baluchnejadmojarad et al., 2005). It is also well known for its diuretic, antipyretic, diaphoretic, antispasmodic, tonic, anti-inflammatory, antihypertensive, anorexic, analgesic, antibacterial, and antidiabetic effects (Nematollahi-Mahani et al., 2007). There are also some reports in the literature for antioxidant effects of crude extract of felty germander (Ljubuncic et al., 2006). Its antioxidant activity refers to the presence of flavonoids, iridioids, crisiols, volatile oils (Afifi et al., 2005) and diterpenes (Orhan and Aslan, 2009). An early study has shown that diterpenoids present in felty germander extract have in vivo antitumor activity in the P388 mouse model of leukemia (Nagao et al., 1982) and lately, the effect of its extract on human lung cancer has been reported (Haidara et al., 2011).

Biodiversity of *T. polium* has decreased dramatically in

recent years, and wild populations are currently at risk of rapid eradication and extinction because of irregular grazing as well as immoderate picking up by endemic peoples for folk medicine usage in Iran. Furthermore, very low rate of seed germination restrict growth and mass propagation of this plant (Nadjafi et al., 2006). Therefore, artificial propagation methods are necessary to conserve and maintain the germplasm. In addition, in vitro plant production via tissue culture technology has many potential applications to medicinal plant species such as further chemical analytical and pharmacological studies as well as enhancement of their secondary metabolite biosynthesis.

Published reports on in vitro propagation of *Teucrium* species are very limited and there are some in vitro studies with this genus such as an in vitro plant regeneration system for *T. stocksianum* Boiss (Bouhouche and Ksiksi, 2007) and *T. fruticans* (Frabetti et al., 2009). There is only one report on micropropagation of *T. polium* from Jordan origin via direct organogenesis using axillary buds isolated from wild plants (Al-Qudah et al., 2011). However, preparing the explants especially small size tissues from ex vitro plant materials and their establishment in in vitro conditions require extra care and using a complex sterilization procedure to avoid further contaminations. Use of seedling explants is advantageous for micropropagation and or transformation, due to easy planning of experiments, reduction in contamination during tissue culture, and reduction in labor and maintenance costs (Belide et al., 2010). In the present study, we examined the effect of cytokinin-pretreatment of intact seedlings on multiple shoots induction and plant regeneration in *T. polium*. The results of this procedure were compared with a propagation method using nodal stem explants. Total flavonoids accumulation, antioxidant activity and the genetic fidelity of in vitro-raised plants were also investigated.

Materials and Methods

Plant Material and in Vitro Germination

Mature seeds of felty germander were collected in June 2011, from hillsides in the mountains of Urmia (37°33′10″ N, 45°04′33″E), West Azerbaijan province, Iran. These seeds were used for establishing seedlings in vitro. Seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 1 min and 2.5% (v/v) sodium hypochlorite (NaOCl), containing 5% of active chlorine, for 5 min; followed by a rinse with sterile distilled water three times. Due to very low germination of *T. polium* seeds, acid scarification of the seeds using H_2SO_4 (98%, 15 min) was also performed. Seeds were then cultivated onto petridishes containing agar-solidified half-strength (½) MS (Murashige and Skoog, 1962) medium.

Culture Media and Conditions

The MS (Murashige and Skoog, 1962) basal medium contained (MS) salts and B5 (Gamborg et al., 1968) vitamins with 3% (w/v) sucrose, and 0.7% (w/v) was used throughout the experiments. The pH of the media was adjusted to 5.8 with NaOH or HCl prior to the addition of 7 $g \cdot L^{-1}$ plant agar (Duchefa Biochemie, Haarlem, the Netherlands) and autoclaved at 121°C for 15 min. All cultures were incubated in a growth chamber at 24 ± 2°C, with a 16/8-h light/dark photoperiod and a photosynthetic photon flux density (PPFD) of approximately 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps.

Adventitious Shoot Induction

For adventitious shoot bud induction, in the first experiment, stem node explants from one month-old seedling were excited and cultured on shoot bud induction medium (SBIM) composed of the MS basal medium supplemented with different concentrations (0.0, 0.5, 1, 1.5, and 2.0 $mg \cdot L^{-1}$) of 6-benzylaminopurine (BAP) or kinetin (KIN) in combination with different concentrations (0.0, 0.25, and 0.5 $mg \cdot L^{-1}$) of α -naphthaleneacetic acid (NAA). Regenerated shoots were transferred onto shoot growth medium (SGM) composed of MS basal medium supplemented with 0.1 $mg \cdot L^{-1}$ of thidiazuron (TDZ) to promote further growth of shoots. Number of shoot bud and regenerated shoots per explant were recorded 4 weeks after culture initiation.

In the second experiment, surface-sterilized seeds were first cultured on preconditioning medium (PCM) composed of MS basal medium supplemented with different concentrations (0.0, 0.5, 1.0 $mg \cdot L^{-1}$) of BAP or TDZ. After seed germination, one-week-old seedlings from each treatment were transferred to the same PCM. Number of shoots per explant was recorded 6 weeks after initiation of culture.

Rooting and Acclimatization of Plantlets

For well development of root system, elongated shoots (> 20 mm) derived from both adventitious shoot induction procedures were transferred to half-strength (½) MS basal medium supplemented with different concentrations (0.0, 0.5, 1.0, and 1.5 $mg \cdot L^{-1}$) of NAA or indolebutyric acid (IBA). Data on number of roots per shoot and root length were recorded after 4 weeks of culture.

The plantlets with developed roots were transplanted into potting mixture of peat and perlite (4:1) and irrigated with water daily. The plantlets were maintained in the culture room (16/8-h light/dark, PPFD = 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 25 ± 2°C) and after two weeks of acclimatization, the plantlets were transferred to greenhouse conditions. The average survival percentage was calculated after five weeks of acclimatization.

Total Flavonoid Determination

The greenhouse-grown plants raised on media with plant growth regulators (PGRs), which induced the highest rate of regeneration (2.0 mg·L^{-1} KIN/BAP + 0.5 mg·L^{-1} NAA, 1.5 mg·L^{-1} BAP, 0.5 mg·L^{-1} TDZ) were selected for total flavonoid analysis. For extract preparations, leaf samples of selected plants and the field-grown mother plants (as the control) were lyophilized (48 hours) and 100 mg of dry weight (DW) was ground and then extracted with 25 mL of 75% methanol in a volumetric flask under sonication for 30 min. The mixture was filtered through Whatman filter paper. After filtration, extract was evaporated to dryness at 40°C in a rotary evaporator and then re-dissolved with 5 mL ethanol. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C until use.

Total flavonoid contents of extracts were measured according to the aluminum chloride colorimetric method (Djeridane et al., 2006), using rutin as a standard. One milliliter of extract was mixed with 1 mL of 2% $AlCl_3$ methanolic solution. After incubation at room temperature for 15 min, the absorbance was measured at 415 nm in a spectrophotometer. Concentration of flavonoids in extracts was expressed in terms of rutin equivalent (mg Ru·g^{-1} DW). All samples were analyzed in triplicate and the mean was calculated.

Antioxidant Activity

Antioxidant capacity (free radical scavenging activity) of extracts from tissue culture raised plants was assessed using 1,1-diphenyl-2-picryhydrazyl (DPPH) as a free radical (Hatano et al., 1988). The DPPH radical is a stable radical with a maximum absorption at 517 nm and it can readily undergo scavenging by antioxidant (Lu and Yeap Foo, 2001). One hundred µL of methanolic extracts with a concentration of 500 µg·mL^{-1} was taken in different test tubes with 4 mL of a 0.01% MeOH solution of DPPH. Ascorbic acid was used as the positive control. Absorbance at 517 nm was determined after 40 min of incubation at room temperature. Radical scavenging activity was calculated using the following formula:

Radical scavenging activity (%) = $[(A_{blank-} A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of blank at 40 min reaction (containing the sample + methanol instead of DPPH), and A_{sample} is the absorbance of the sample at 40 min. The experiment was carried out in triplicate and the mean value of radical scavenging activity was calculated.

Randomly Amplified Polymorphic DNA (RAPD) Analysis

RAPD technique was used to assess the genetic fidelity of greenhouse-grown 4-month-old in vitro-raised plants obtained via preconditioning procedure. Genomic DNA was extracted from leaf tissue of both mother plant and five randomly selected regenerants using CTAB protocol (Doyle and Doyle, 1990). A total 10 arbitrary primers (OPA 1-10; Operon Technologies Inc., Alameda, CA, USA) were used for RAPD analysis. The PCR was carried out via AccuPower PCR Premix (Biooner, Daejeon, Korea) in a total volume of 20 µL containing 50 ng of genomic DNA and 0.5 µM of each primer. Amplification conditions were an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 40 s, 36-45°C for 60 s and 72°C for 60 s. A final extension step of 7 min at 72°C was included after the last cycle. All PCRs were repeated twice to check their reproducibility. The gels were visualized and photographed using a Gel Documentation system (AlphaImager Mini System, Alpha Innotech Corp., San Leandro, CA, USA) and well-resolved fragments were scored.

Statistical Analysis

Thirty-two cultures (i.e. four replications with eight explants per replicate) and twelve cultures (i.e. four replications with three regenerated shoots per replicate) were used for the shoot induction, and rooting experiments, respectively and each experiment was repeated three times. A completely randomized design (CRD) was used in all experiments and data were subjected to analysis of variance (ANOVA) by the general linear model (GLM) procedure of SAS system (version 9.1; SAS Institute Inc., Cary, NC, USA). Comparisons of means were carried out using Fisher's least significant difference (FLSD) test at the 5% level of probability.

Results and Discussion

Adventitious Shoot Bud Induction Using Nodal Explants

Nodal segments are a preferred explant for micropropagation due to the presence of preexisting meristems, which can be developed easily into shoots while maintaining clonal fidelity (Ahuja, 1993). The axillary buds of the higher plants are dormant due to apical dominance and the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators specially auxin (Cline, 1996). Cutting the stem into segments and culturing them on medium supplemented with suitable Plant growth regulators (PGRs) can break the dormancy of the bud (Maleki Band et al., 2011; Punyarani and Sharma, 2010). Multiplication of plants through the sequential subculture of nodal segment explants has been achieved for a large number of plant species including *Olea europaea* (Benavente-García et al., 2000), and *Tectona grandis* (Mendoza de Gyves et al., 2007), *T. fruticans* (Frabetti et al., 2009) and wild *Medicago* spesies (Maleki Band et al., 2011). In this study, different BAP/KIN treatments were used to induce shoot proliferation via nodal segment explants of felty germander. Successful

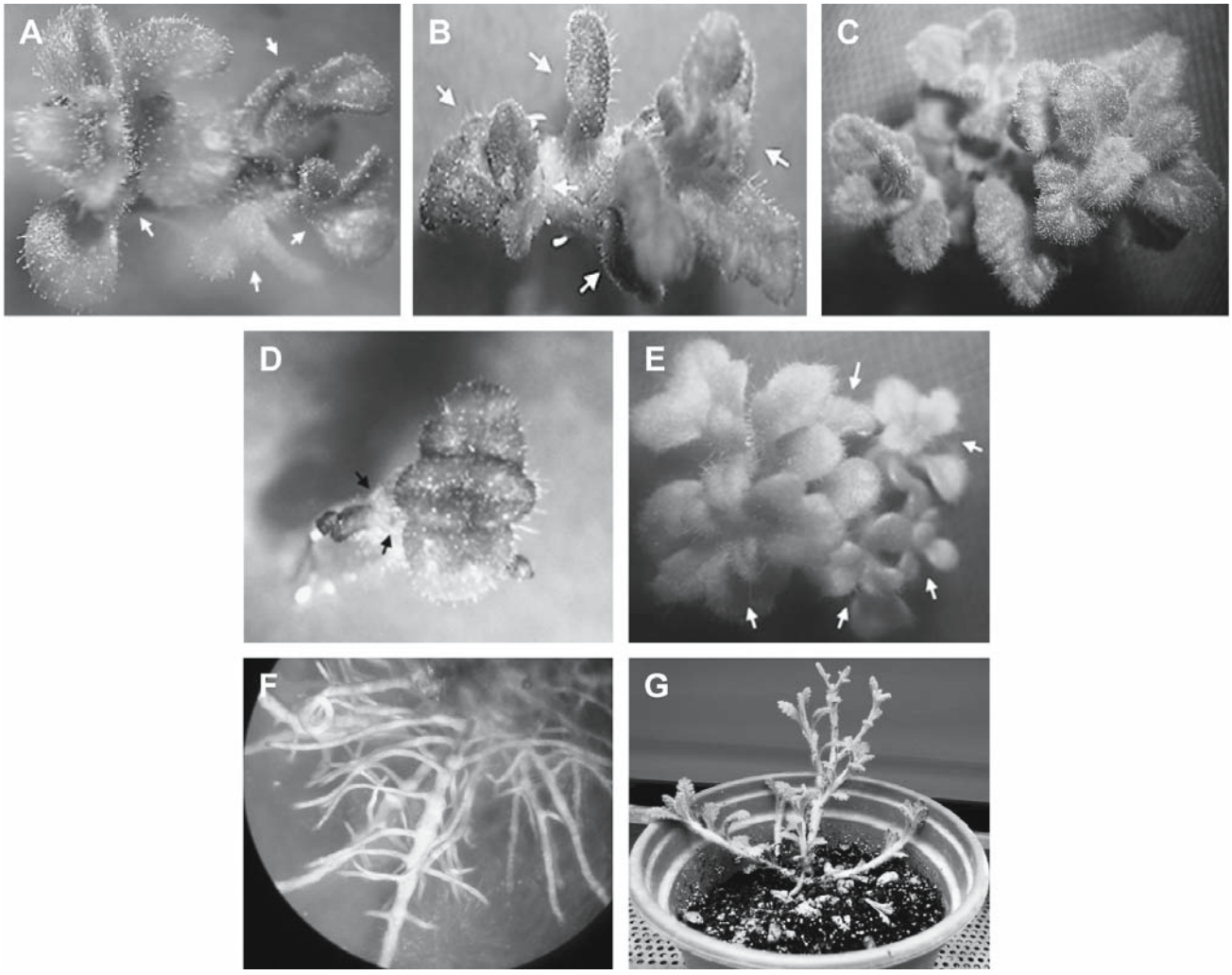

Fig. 1. In vitro propagation of *Teucrium polium* via direct organogenesis pathways: A-C, shoot induction and regeneration from nodal explant (arrows); D-E, multiple shoot initiation (arrows) from pre-conditioned seedling; F, Rooting of regenerated shoot; and G, acclimatized plants in pot.

adventitious shoot buds (SBs) were induced at the periphery of the petiole base on nodal segment explants cultured on SBIM media supplemented with various PGRs but the explants on MS basal medium devoid of PGRs did not initiate adventitious buds and only differentiated into single shoot per an explant. Fig. 1A shows formation of multiple shoots on the nodal explants. Regeneration of adventitious shoots was observed without an intervening callus phase and well-defined shoots were visible within 3 weeks of culture. Mean number of SBs and regenerated shoots were significantly affected ($p < 0.05$) by the types of cytokinin and concentrations (Fig. 2A). The SBs induction and regeneration capacity were significantly improved by increasing of cytokinins (BAP or KIN) and auxin (NAA) concentrations. However, in comparison, the mean number of shoots formation was significantly higher in medium containing BAP relative to medium with KIN at all of the concentrations. The highest number of adventitious shoot formation was obtained on SBIM supplemented with 2.0 $mg \cdot L^{-1}$ BAP or KIN in combination with 0.5 $mg \cdot L^{-1}$ NAA, with mean values of 7.14 and 5.46 shoots per explant, respectively (Figs. 2A and 2B). Induced shoots elongated well up to 4 cm in SEM (Fig. 1C), while their growth was slowly.

Suitability of BAP at higher concentrations with combination with lower level of an auxin for shoot regeneration from nodal segments have been reported in *T. fruticans* (Frabetti et al., 2009), *Portulaca grandiflora* (Safdari and Kazemitabar, 2010) and wild medics (Maleki Band et al., 2011). However, KIN in combination of an auxin has been also reported for adventitious shoot induction in several plants, e.g. *Rotula aquatica* (Martin, 2003), *Saussurea obvallata* (Joshi and Dhar, 2003) and *T. stocksianum* (Bouhouche and Ksiksi, 2007).

Browning and vitrification in cultures on medium with

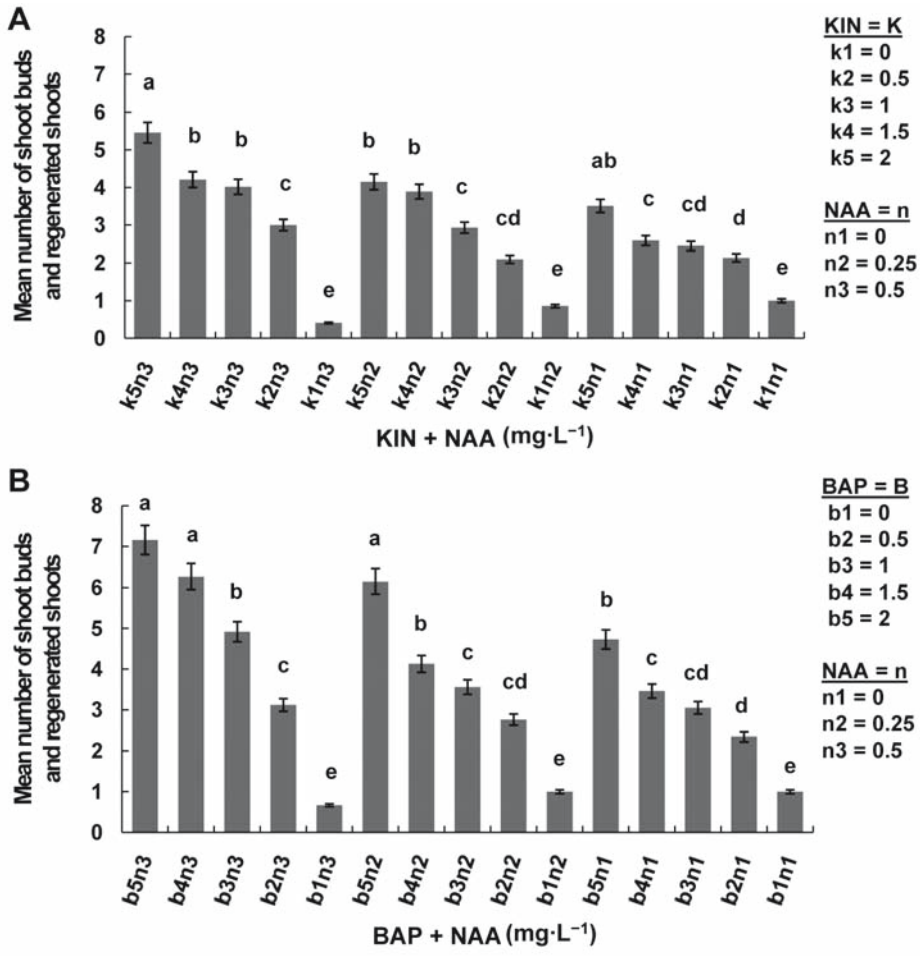

Fig. 2. The effects of various concentrations of KIN (A) or BAP (B) in combination with different concentrations of NAA on adventitious shoot regeneration from leaf and nodal explants of *Teucrium polium*. Error bars represent the standard error of the mean of three repeated experiments. Means with the same letter are not significantly different ($p < 0.05$).

higher level of cytokinins have been previously reported (Ivanova and Van Staden, 2008), but in the present study, these problems were minimal as the percentage of vitrified regenerated shoots under influence of both the cytokinins was estimated very low (3.7%).

Adventitious Shoot Formation by TDZ-preconditioned Seedlings

The effect of different TDZ/BAP concentrations used in the germination medium on subsequent direct shoot development from seedlings of felty germander was examined. Our previous study on seed dormancy of felty germander showed plentiful germination of the seeds and well growing of seedlings under cytokinin-pretreatments, however, TDZ, in comparison, improved significantly the germination factors of *T. polium* than BAP (unpublished data). The prominent effect of TDZ on seed germination has also been reported in other plants such as *Lotus corniculatus* (Nikolic et al., 2007) and *Nymphaea alba* (Sumlu et al., 2010). In the present study, seeds subjected to cytokinin-pretreatment for the possible shoot induction on seedlings. Preconditioned seedlings (as explants) when cultured in PCM medium resulted in multiple shoot formation the base of the seedlings within two weeks of culture (Fig. 1B). There was significant difference in shoot induction potential between cytokinins and mean number of shoots formed per explant varied considerably and showed significant differences at different concentration (Fig. 3). However, seedlings arose from untreated (i.e., germinated and further grown on medium containing no PGRs) produced no shoots at all.

Shoot induction rate improved with increasing BAP concentration but this was not the case with TDZ. In other words, shoot formation intensified with decreasing concentration of TDZ (Figs. 3A and 3B). A similar result was found for growth of shoots. The maximum number of induced shoot was observed on MS basal medium supplemented with 0.5 $mg \cdot L^{-1}$

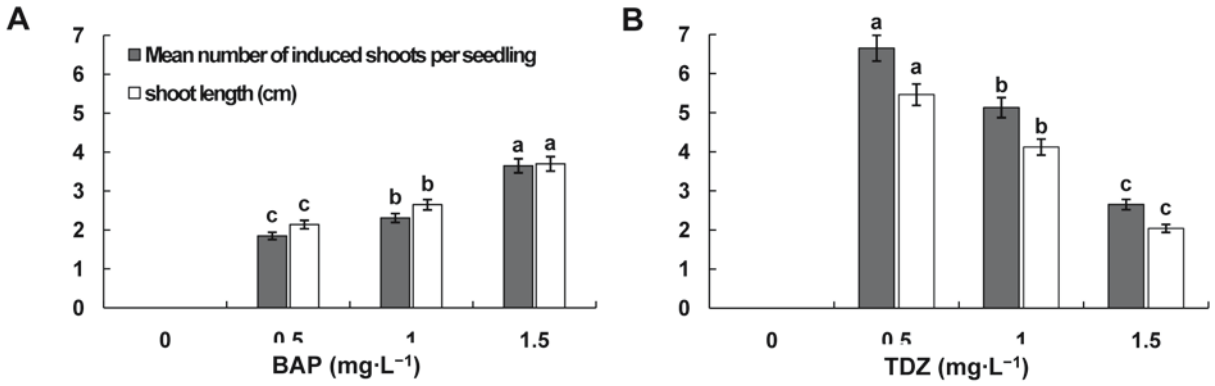

Fig. 3. The effects of various concentrations of BAP (A) and TDZ (B) on multiple shoot induction from pre-conditioned seedlings of *Teucrium polium*. Error bars represent the standard error of the mean of three repeated experiments. Means with the same letter are not significantly different ($p < 0.05$).

TDZ or 1.5 mg·L^{-1} BAP forming 6.85 and 3.74 shoots per explant, respectively. Longer shoots (5.43 cm) was developed on seedlings preconditioned with 0.5 mg·L^{-1} TDZ (Fig. 3B). Studies on the shoot induction and regeneration of *Beta vulgaris* (Gurel et al., 2003), *Hyoscyamus niger* (Uranbey, 2005), *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu, 2007), *Paulownia tomentosa* (Corredoira et al., 2008) *Medicago sativa* (Li et al., 2009), wild *Medicago* species (Maleki Band et al., 2011) also proved the effectiveness of TDZ at lower concentrations. It has also been demonstrated that cytokinins at high concentrations retarded the plant growth. TDZ is known to inhibit shoot elongation. Retarded elongation in TDZ-induced shoots has been reported in *Tamarindus indica* (Mehta et al., 2004) and *Zingiber officinale* (Lincy and Sasikumar, 2010). This may be due to the high cytokinin activity of TDZ and its extreme stability in plant tissues (Jahan et al., 2011; Mok et al., 2000). However, in the present study, growth inhibition was not substantial with using both cytokinins.

The technique of pretreating the explants with different plant growth regulators was applied with a view to getting maximum results, as it helped in better uptake of nutrition where the explants itself is submerged in a sea of nutrients and was in direct touch with hormone (Jahan et al., 2011; Shan et al., 2005). It was reported that pretreatment of explants in TDZ was more effective than any other growth hormone for preconditioning. It was suggested that the capacity for shoot regeneration was predetermined at an early stage of leaf differentiation and, therefore, TDZ was required for maintaining the regenerative capacity (Gurel et al., 2003). The effects of preconditioning seedlings on the subsequent shoot regeneration were previously examined in several plant species, e.g. *Beta vulgaris* (Gurel et al., 2003, 2011; Krens et al., 1996), *Pongamia pinnata* (Belide et al., 2010), *Nyctanthes arbor-tristis* (Jahan et al., 2011). In the present study, the effect of adding cytokinins during seed germination (preconditioning) proved to be beneficial for early multiple shoot induction from seedlings of felty germander. In addition, our results are in accordance with the previous reports, as mentioned above; that TDZ is more effective than other cytokinin (BAP) for preconditioning.

Rooting of Shoots and ex Vitro Acclimatization of Plantlets

For the development of healthy root system, 4-8 mm long shoots were transferred to rooting medium supplemented with different concentrations of NAA or IBA. After three weeks of culture, adventitious roots were observed from the basal end of the shoots (Fig. 1C). Rooting capacity was significantly influenced ($p < 0.05$) by type and concentration of auxin tested. Number of adventitious roots increased with increasing auxin concentration and a greatly reduced root length (Figs. 4A and 4B). A higher number of roots and higher rate of root growth occurred at 1.5 mg·L^{-1} and 0.5 mg·L^{-1}, of both the auxins, respectively. However, IBA was the most effective auxin in terms of mean number of roots per shoot (13.34) and root length (7.4 cm) (Fig. 4A). High concentrations of auxin inhibited root elongation and instead enhanced adventitious root formation (Krieken et al., 1993). We selected the middle concentration (1.0 mg·L^{-1}) of IBA as an optimal concentration for a well-developed root system due to the induction of an acceptable root number and longer roots (Fig. 4A) that resulted in better acclimatization and survival of plantlets. Favored effects of IBA, in comparison with NAA, on rooting in several medicinal plant species have also been reported (Goel et al., 2009; Leonardi et al., 2001; Sujana and Naidu, 2011).

The rooted plantlets were subjected to acclimatization. Plants were successfully adapted to *ex vitro* conditions with

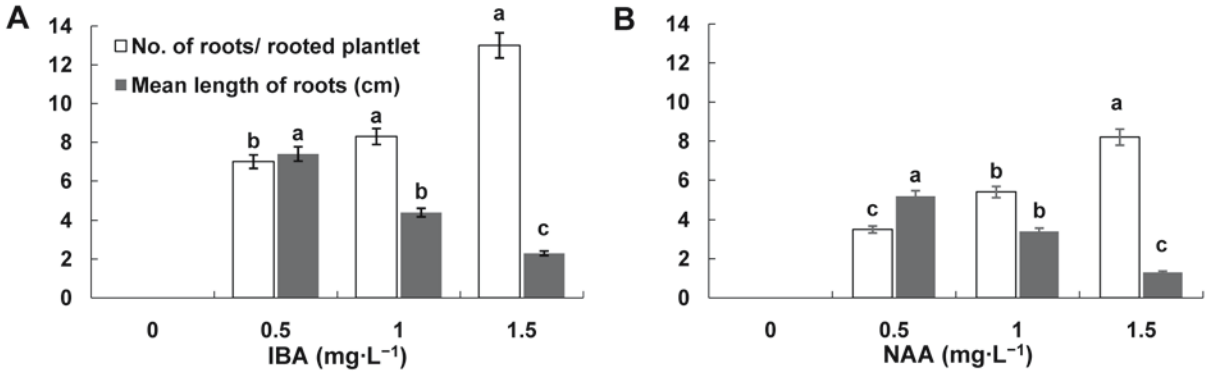

Fig. 4. In vitro rooting of *Teucrium polium* shoots in ½ MS medium supplemented with various concentrations of IBA (A) or NAA (B). Error bars represent the standard error of the mean of three repeated experiments. Means with the same letter are not significantly different ($p < 0.05$).

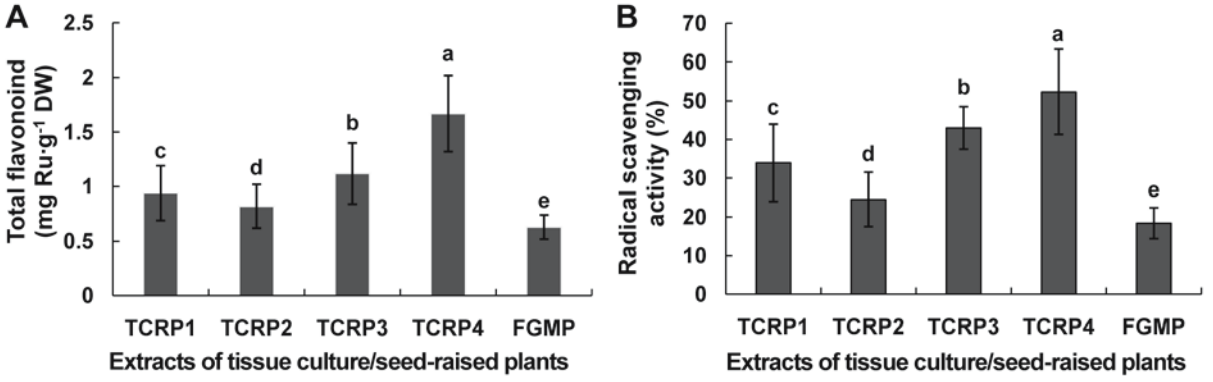

Fig. 5. Total flavonoid contents (A) and radical scavenging activity (B) in extracts of micropropagated *T. polium* plants raised on media containing different plant growth regulators. TCRP1-TCRP4, tissue culture raised plants raised on MS medium containing 2.0 mg·L^{-1} KIN + 0.5 mg·L^{-1} NAA, 2.0 mg·L^{1} BAP + 0.5 mg·L^{-1} NAA, 1.5 mg·L^{-1} BAP and 0.5 mg·L^{-1} TDZ, respectively. FGMP, field-grown mother plants as the control. The total flavonoid content was expressed in trems of rutin equivalent (mg Ru·g^{-1} DW). Error bars represent the standard error (SE) of the mean of three repeated experiments. Means with the same letter are not significantly different ($p < 0.05$) according to FLSD.

100% survival rate (Fig. 1D). All the acclimatized plants appeared morphologically uniform with normal growth pattern.

Accumulation of Flavonoids in Tissue Culture Raised Plants

Phenolic compounds such as flavonoids are a class of antioxidant agents, which possess diverse biological activities including anti-inflammatory, anti-carcinogenic, and anti-atherosclerotic activities. These activities might be related to their antioxidant activity (Chung et al., 1998). Therefore, dietary intake of flavonoid-containing foods was suggested to be of benefit as free radical preservatives. By regarding the potentially antioxidant activity and rich content of flavonoids of *T. polium* (Sharififar et al., 2009), this plant have great potential as a source of natural antioxidants in place of synthetic antioxidants for health-promoting effects and attenuating oxidative damages of degenerative diseases.

In the present study, significantly different ($p < 0.05$) amounts of total flavonoids were found among extracts of tissue culture raised plants of *T. polium* and the flavonoid content was higher in all extracts as compared with the filed-grown mother plant as the control (Fig. 5A). The total flavonoid content in tissue culture raised plant extracts depends on the type of PGRs used in regeneration process of the plants. Plants raised on TDZ (0.5 mg·L^{-1})-supplemented medium showed the maximum amount of flavonoid content (1.67 mg Ru·g^{-1} DW), which was 2.65-fold higher than the control plant. Levels of flavonoids were lower in extracts of tissue-cultured plants raised on medium supplemented with higher concentrations of tested cytokinins (2.0 mg·L^{-1} KIN or BAP). It has been suggested that plants "remember" the treatments to which they were exposed during the initial phases of their growth, thus, conditioning their future development (Moncaleán et al., 2001). The PGR composition of the culture media is known to be one of the main causes

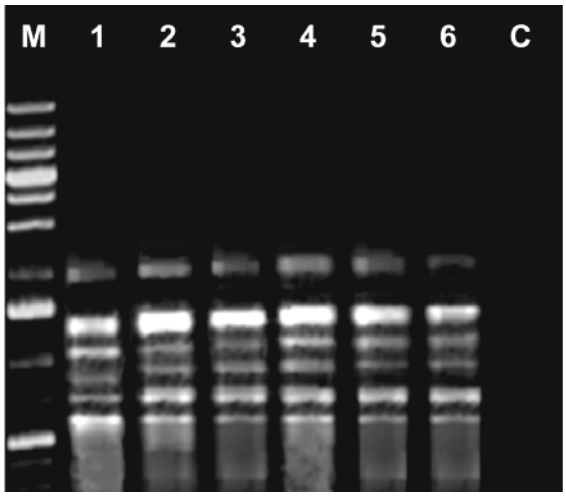

Fig. 6. RAPD banding profile of both mother plant and greenhouse grown in vitro-raised plants of *T. polium* using OPA-8 primer: M, 1 Kb plus DNA ladder (Fermantas); 1, mother plant; 2-6, greenhouse grown 4-month-old in vitro-raised plants; and C, negative control.

of morphological and physiological modification in regenerated plantlets; in turn, it could alter the plant biomass capacity to produce secondary metabolites (Patnaik et al., 1999; Van Staden et al., 2006).

In the present study, total flavonoid content was measured during advanced plant development stage; however, it was notable to investigate the patterns of flavonoid production in different developmental stages during in vitro culture process of this plant. Several in vitro studies in various plants have demonstrated that phenolic compounds accumulate in whole plants at a higher level than undifferentiated tissues and regenerated shoots (Danova et al., 2010; Karalija and Parić, 2011; Palacioa et al., 2012).

The DPPH Scavenging activity has been widely used to evaluate the antioxidant activity of natural products from plant sources. In the present study, the flavonoids of *T. polium* tissue culture raised plants showed an increased antioxidant activity by scavenging DPPH (Fig. 5B). A significant correlation was obtained between antioxidant activity and flavonoid content, indicating that phenolic compounds contribute significantly to the antioxidant activity of the investigated different extracts. The result of the present study showed that the extracts of tissue culture raised plants, which contained the highest amount of flavonoid content, exhibited the greatest antioxidant activity compared to field-grown wild plant and extracts of plants raised from TDZ (0.5 $mg \cdot L^{-1}$)-supplemented medium exhibited highest antioxidant activity of 52.31%. The radical scavenging activity of plant extracts depended on the amount of polyphenolic compounds in the extracts (Benavente-García et al., 2000; Zayova et al., 2013).

Genetic Fidelity of in Vitro-raised Plants

Total 10 primers were used for PCR amplification out of which 2 primers did not give any amplification. About 52 amplified reproducible bands were produced by RAPD analysis of 5 in vitro raised plants. The number of bands for each selected primer varies from 3 to 13. The OPA-8 and OPA-3 primers showed the highest number of bands, 6 and 9 bands, respectively. Fig. 6 showed the representative of the RAPD profile generated by with one of the best primer (OPA-8 primer). All bands generated by the RAPD techniques were monomorphic in nature, no polymorphic bands were observed. RAPD analysis revealed no evidence of genetic variation either within or between the micropropagated plants and the mother plant. Therefore, all the tissue-raised plants were found to be genetically uniform and true-to-type with their parent. The results obtained suggested that direct organogenesis from preconditioned seedlings of *T. polium* induced by TDZ carry no risk of generating somaclonal variants.

It is demonstrated that genetic fidelity of in vitro micropropagated plants should be ascertained before transferring hardened plants to field (Goel et al., 2009; Rani and Raina, 2000). The somaclonal variations are common problem among in vitro raised plants, which can be detected by various PCR-based techniques such as RAPD. However, some of techniques such as RFLP are more complex and have some the disadvantages for its routine application in micropropagation system. RAPD can bypass some of the problems associated with complicated molecular markers. This method was used for clonal fidelity analysis of many tissue-cultured plants (Goel et al., 2009; Mishra et al., 2011; Rani and Raina, 2000).

In conclusion, this study presents the first report of plant regeneration via direct organogenesis from nodal stem as well as pre-condtioned seedlings of *T. polium*. t was evident that both types of explants were able to produce adventitious shoots. The results revealed that pretreated explants were more vigorous for multiple shoot formation. The results RAPD analysis revealed that the micropropagation protocol does not induce changes in genetic structure of regenerants, and guarantees genetic stability of in vitro-raised *T. polium* plants. This simple and efficient method could be used for mass propagation and genetic transformation studies of this medicinally important plant.

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