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# MICROPROPAGATION OF SOME *Onobrychis* SPECIES THROUGH *in vitro* SHOOT REGENERATION

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**ABSTRACT**

*Onobrychis* species have an extensive spread in Turkey and adapted to various environmental conditions. In this study, it was aimed to propagate with tissue culture methods of endemic *Onobrychis fallax* Freyn & Sint. ex Freyn var. *longifolia* Aktoklu, *Onobrychis stenostachya* subsp. *sosnowskyi*, *Onobrychis elata* Boiss. & Balansa and worldwide commonly cultivated *Onobrychis viciifolia* Scop. species. For this purpose, cotyledon node explants were cultured in Murashige and Skoog (MS) media containing various concentration of 6-benzylaminopurine (BAP), thidiazuron (TDZ) or *meta*-Topolin (*m*T) alone or in combination with 0.5 mg $L^{-1}$ NAA. Significant variations in number of shoots per explant was observed depending on the species and growth regulators. The greatest number of shoots per explant was obtained from 1.2 mg $L^{-1}$ TDZ treatments in *O. stenostachya*, *O. fallax* and *O. elata* and from 0.3 mg $L^{-1}$ TDZ treatments in *O. viciifolia*, whereby 10.58, 9.50, 5.5, 6.42 shoots were recorded. Resultant shoots were rooted in half strength MS nutrient media containing 1 or 2 mg $L^{-1}$ indole-3-butyric acid (IBA). The rooting ratio was 86.11% in *O. viciifolia*, 50.00% in *O. stenostachya* and 36.11% in *O. fallax* and *O. elata*.

**Key words:** 6-benzylaminopurine, cotyledon node, *meta*-Topolin, *Onobrychis,* Thidiazuron

## INTRODUCTION

The genus *Onobrychis* is included in *Fabacaea* family and represented about 170 annual or perennial herbaceous or shrubby species and there are 55 species in Turkey (28 of them are endemic) [Aktoklu 2001, Avcı et al. 2016]. Most of the *Onobrychis* species with high protein contents constitute a great source for animal feed. Since *Onobrychis* species are classified in leguminous crops, they are able fixate atmospheric nitrogen into the soils, thus they play a significant role in improvement of pasture soils and nutrient contents [Erkovan et al. 2016]. Valuable characteristics such as palatability, drought tolerance, tannin and polyphenol composition which improves protein utilization, confer anthelmintic properties and prevents bloating have recently increased the interest in *Onobrychis* species as an animal feed source [Hayot Carbonero et al. 2011].

These gene sources have adapted to ecological conditions of the regions they grow in for centuries, thus with great gene diversity, they have a significant place in breeding programs [Avcı et al. 2014]. Today, biotic and abiotic stress factors highly restrict plant production activities and genetic bases of already cultured plants have narrowed. Therefore, plants in natural flora have become prominent genetic materials in breeding studies. Despite the widespread in natural flora, some *Onobrychis* species are not cultured and haven't been sufficiently used in breeding programs, too. Besides potential use of these plants as a feed source, knowledge on drought tolerance, salinity resistance and nutrient toxicity of these plants will provide great contributions to the science of agriculture and economy. Tissue culture techniques, started to be used

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widely, allow vegetative growth of plants in short time and also allows researchers to monitor plant response to different stressors and to select plants resistant to stress factors with the aid of somaclonal variation. However, genotype, nutrient media composition and cultural conditions play significant roles in plant regeneration with tissue culture. There isn't any global tissue culture regeneration methods for every plants and tissue culture regeneration methods should be optimized for each species, cultivars or varieties [Babaoğlu et al. 2001]. *In vitro* regeneration of sainfoin has been studied by using different explant and growth regulators [Özcan et al. 1996a, Özcan et al. 1996b, Özgen et al. 1998, Sancak 1999, Çeliktaş et al. 2006, Uzun 2012]. However, the studies about regeneration of endemic *Onobrychis* species with the tissue culture are quite limited. Therefore, the present study was designed for *in vitro* propagation of endemic *Onobrychis elata*, *Onobrychis fallax* var. *longifolia*, *Onobrychis stenostachya* subsp. *sosnowskyi* and commonly cultured *Onobrychis viciifolia* species. Three endemic species have poor germination due to hard seed coat [Avcı and Kaya 2013). While *Onobrychis elata* and *Onobrychis stenostachya* are classified as vulnerable according to IUCN criteria, *Onobrychis fallax* var. *longifolia* classified as endangered [Ambarlı et al. 2016, Karakuş 2016, Demirkuş et al. 2018]. Resultant *in vitro* regeneration method will allow vegetative propagation of the endemic species and to monitor the responds of *Onobrychis* species to different stress factors.

## MATERIAL AND METHODS

Endemic *Onobrychis elata*, *Onobrychis fallax* var. *longifolia*, *Onobrychis stenostachya* subsp. *sosnowskyi* and commonly cultured *Onobrychis viciifolia* species were used in tissue culture studies. Three endemic species were collected and grown over the experimental fields of Ankara University Agricultural Faculty Field Crops Department in the years 2006–2008. Seeds of these growing seasons were used in this study (Tab. 1).

Seeds were deshelled (seed coats of 3 endemic species were also scratched with a bistoury since they have hard seed coat), immersed into 50% commercial bleach solution and mixed in a magnetic stirrer for 10 min to sterilize the seeds. Sterilized seeds then were rinsed 3 times through sterile distilled water. Sterilized seeds were germinated in petri dishes containing MS medium (Murashige and Skoog 1962, Duchefa Biochemie, M0222), 3% sucrose and 0.7% agar (Lab M). About 15–20 seeds were placed in each petri dish.

Explant isolation was performed from the plantlets about 10–15 days after taking the seeds for germination. Cotyledon and hypocotyl sections of the plantlets were cut and remaining cotyledon node explants were cultured in Magenta vessels (Sigma-Aldrich, 77 × 77 × 97) containing MS basal media with 3% sucrose, 0.7% agar and different concentration of TDZ (0.3–1.2 mg $L^{-1}$), BAP (0.5–2 mg $L^{-1}$) or *m*T (0.5–2 mg $L^{-1}$) alone or in combination with 0.5 mg/L NAA (Fig. 1a).

Developed shoots were rooted in half-MS nutrient medium containing 1 or 2 mg $L^{-1}$ IBA, 3% sucrose and 0.7% agar.

Sterilization of distilled water, nutrient media and the other tools and equipment used was performed in an autoclav (HMC brand HV-50 L model) at 1.5 atm pressure and 121°C for 20 min. The pH of all nutrient media was adjusted as between 5.6–5.8 by using 1N NaOH and 1 N HCI. Entire sterilized processes were performed in a sterile cabin (Nüve L120). All cultures were grown 16/8 light/dark photoperiod at 24 ±2°C temperature and 3000 lux light intensity.

Experiments were conducted in randomized plots design with 3 replications for *meta*-Topolin and rooting experiments and 4 replications for the other experiments. Each Magenta vessel had 6 explants. Percent values were subjected to "arcsine" transformation before the variance analyses. Statistical analyses were performed with "SPSS for Windows" software in accordance with randomized plots factorial experimental design. Treatment means were compared with Duncan's multiple range tests.

## RESULTS AND DISCUSSION

For *in vitro* regeneration of *O. stenostachya* subsp. *sosnowskyi*, *O. nobrychis fallax* var. *longifolia*, *O. nobrychis elata* and *O. viciifolia* species, cotyledon node explants were cultured in MS nutrient media containing BAP, BAP + NAA, TDZ, TDZ + NAA or

*m*T at different concentrations. About 8–9 weeks after the initiation of cultural practices, frequency of shoot regeneration and number of shoots per explant were determined (Fig. 1b).

**Regeneration in BAP and NAA Media.** In BAP-containing media, the greatest frequency of shoot regeneration (98.21%) was obtained from *O. stenostachya* and it was respectively followed by *O. fallax*, *O. viciifolia* and *O. elata* with 94.64%, 90.48% and 72.62%, respectively. Significant variations were observed in frequency of shoot regeneration values of the *Onobrychis* species ($P < 0.01$). The effects of BAP doses and NAA combinations on frequency of shoot regeneration values of the species were not found to be significant.

Mean number of shoots per explant varied significantly with the species, plant growth regulators (PGRs) and the interactions between species and PGRs ($P < 0.01$). The greatest number of shoots per explant was obtained from 4 mg $L^{-1}$ BAP medium of *O. stenostachya*, 4 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ NAA medium of *O. fallax* and *O. elata* and 2 mg $L^{-1}$ BAP medium of *O. viciifolia*. The medium yielding the greatest number of shoots per explant was different in all species, thus interactions were found to be significant (Tab. 2).

Considering the general averages of PGRs (BAP, BAP-NAA), the greatest number of shoots per explant was obtained only from 2–4 mg $L^{-1}$ BAP or their combination with 0.5 mg $L^{-1}$ NAA. Özgen et al. [1998] and Sancak [1999] also reported successful outcomes for embryonic axis, leaflet, petiole and stem explants of *O. viciifolia* with 2–4 mg $L^{-1}$ BAP and 0.05, 0.1 or 0.5 mg $L^{-1}$ NAA-containing media.

**Regeneration in TDZ and NAA Media.** While frequency of shoot regeneration were significantly affected by species ($P < 0.01$), mean number of shoots per explant was affected by species, the interactions between species and PGRs ($P < 0.01$). The greatest frequency of shoot regeneration (99.31%) was obtained from *O. fallax* and the lowest frequency of shoot regeneration (56.95%) was obtained from *O. elata*. The frequency of shoot regeneration was 93.05% in *O. stenostachya* and 90.97% in *O. viciifolia*. With regard to the mean number of shoots per explant of the species, the greatest value (9.01 shoots) was obtained from *O. stenostachya* and it was respectively followed by *O. fallax* (8.20 shoots), *O. viciifolia* (5.13 shoots) and *O. elata* (4.87 shoots). The greatest number of shoots per explant was obtained from 1.2 mg $L^{-1}$ TDZ of *O. stenostachya* and *O. fallax* and 0.3 mg $L^{-1}$ TDZ of *O. viciifolia*. The differences between the media were not found to be significant in *O. elata* (Tab. 3). TDZ has cytokinin-like effects and commonly used in

**Table 1.** Name of species and localities used in this study

| Name of species | Locations | Coordinates | | |
|---|---|---|---|---|
| | | latitude | longitude | altitude |
| *Onobrychis fallax* Freyn & Sint. ex Freyn var. *longifolia* Aktoklu | Malatya, Arguvan | 39°00'02" | 38°12'27" | 1410 m |
| *Onobrychis stenostachya* subsp. *sosnowskyi* | Erzurum, Tortum | 40°13'42" | 41°30'23" | 1987 m |
| *Onobrychis elata* Boiss.&Balans | Kayseri, between Hisarcık and Hacılar road | 38°38'17" | 35°29'36" | 1443 m |
| *Onobrychis viciifolia* | Local genotype grown around Sivas province | | | |

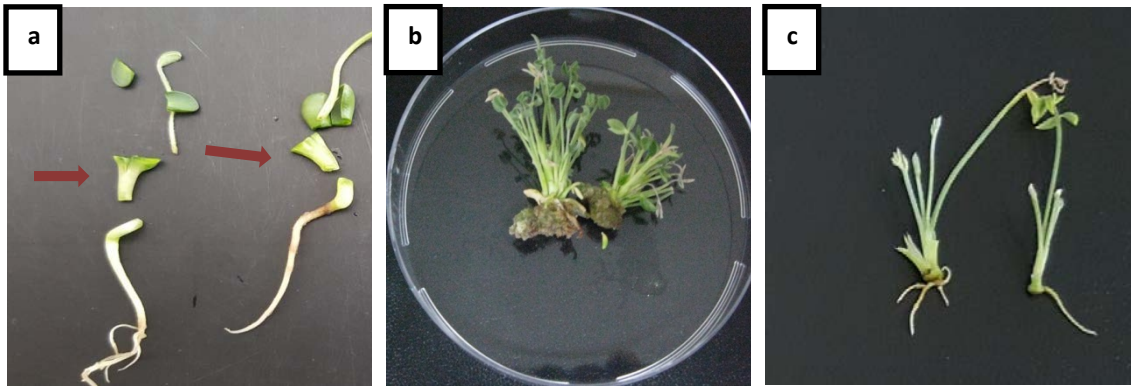


**Fig. 1.** Shoot regeneration and *in vitro* rooting in *Onobrychis*; a) preparation of cotyledon node explants from 10–15 days old plantlets; b) shoot regeneration on MS medium containing 2 mg $L^{-1}$ *m*T; c) rooting in half strength MS nutrient medium containing 1 mg $L^{-1}$ IBA

**Table 2.** Effects of different BAP-containing media on number of shoots per explant of *Onobrychis* species

| PGR (mg $L^{-1}$) | | Species | | | | Means |
|---|---|---|---|---|---|---|
| BAP | NAA | *O. stenostachya* | *O. fallax* | *O. elata* | *O. viciifolia* | |
| – | – | 1.79 d* | 1.22 c* | 1.05 b* | 2.01 c* | 1.52 d* |
| 1 | – | 3.13 bc | 2.91 b | 1.66 b | 4.17 b | 2.97 c |
| 2 | – | 3.75 abc | 3.38 ab | 2.82 a | 6.25 a | 4.05 a b |
| 4 | – | 4.64 a | 3.42 ab | 3.32 a | 5.62 a | 4.25 a |
| 1 | 0.5 | 1.64 d | 2.81 b | 2.35 a | 3.76 b | 2.64 c |
| 2 | 0.5 | 2.92 c | 3.04 ab | 2.90 a | 5.59 a | 3.61 b |
| 4 | 0.5 | 3.88 ab | 3.92 a | 3.65 a | 4.53 b | 3.99 ab |
| Means | | 3.12 b* | 2.96 b | 2.54 c | 4.56 a | |

* Values within the same column or row followed by different letters are significantly different at P < 0.05 of Duncan's multiple range test

**Table 3.** Effects of different TDZ-containing media on number of shoots per explant of *Onobrychis* species

| PGR (mg $L^{-1}$) | | Species | | | | Means |
|---|---|---|---|---|---|---|
| TDZ | NAA | *O. stenostachya* | *O. fallax* | *O. elata* | *O. viciifolia* | |
| 0.3 | | 8.53 bcd* | 7.70 bc* | 5.10 a* | 6.42 a* | 6.93 ab* |
| 0.6 | | 9.45 abc | 8.65 ab | 4.77 a | 5.54 ab | 7.10 ab |
| 1.2 | | 10.58 a | 9.50 a | 5.50 a | 4.61 b | 7.55 a |
| 0.3 | 0.5 | 7.88 d | 7.85 bc | 4.57 a | 5.25 ab | 6.39 b |
| 0.6 | 0.5 | 9.53 ab | 6.55 c | 5.13 a | 4.66 b | 6.47 b |
| 1.2 | 0.5 | 8.08 cd | 8.95 ab | 4.18 a | 4.29 b | 6.38 b |
| Means | | 9.01 a* | 8.20 b | 4.87 c | 5.13 c | |

* Values within the same column or row followed by different letters are significantly different at P < 0.05 of Duncan's multiple range test

shoot regeneration studies in recent years [Guo et al. 2011]. Previous researchers also indicated that TDZ promoted *in vitro* shoot regeneration [Erişen et al. 2011, Yorgancılar and Erişen 2011]. TDZ-containing media of the present study also had higher number of shoots per explants. Optimum TDZ concentration varied with the species. While *O. stenostachya* and *O. fallax* had greater number of shoots per explant at high TDZ concentrations, *O. viciifolia* had greater number of shoots at low TDZ concentrations. There were not any significant differences between the growth media in *O. elata*. Similarly, Magyar-Tabori et al. [2010] indicated that optimal TDZ concentration varied largely based genotypes in apples.

**Regeneration in *meta*-Topolin Media.** In mT-containing media, frequency of shoot regeneration was observed as 96.3% in *O. viciifolia*, 94.44% in *O. stenostachya*, 92.59% in *O. fallax* and 77.78% in *O. elata*. The differences in frequency of shoot regeneration values of the species were not found to be significant. Mean number of shoots per explant was affected by species, PGRs and interactions (species and PGRs, $P < 0.01$; interaction $P < 0.05$). Number of shoots per explant varied between 2.65–5.86 in *O. stenostachya*, between 2.91–5.02 in *O. falla*x, between 1.97–4.00 in *O. elata* and between 2.39–3.60 in *O. viciifolia*. The greatest number of shoots per explant was obtained from 2 mg $L^{-1}$ *m*T of all species and the lowest number of shoots per explant was obtained from 0.5 mg $L^{-1}$ *m*T of all species. However, significant differences were not observed between 2 mg $L^{-1}$ *m*T and 1 mg $L^{-1}$ *m*T treatments of *O. fallax* and *O. viciifolia* (Tab. 4). In recent tissue culture studies (during the last 15–20 years), topolins existing in *Populus* species were investigated as a natural aromatic source of cytokinin [Gentile et al. 2014]. *m*T is a natural growth substance and it is metabolized in different fashion from the BA [Strnad et al. 1997, Magyar-Tabori et al. 2010]. *m*T is used for micro-propagation and shoot regeneration of several species such as safflower and *Prunus* spp. [Gentile et al. 2014, Vijayakumar et al. 2017]. In *O. stenostachya, O. fallax, O. elata* and *O. viciifolia* species, respectively 5.86, 5.02, 4.00 and 3.60 shoots were obtained in 2 mg $L^{-1}$ *m*T-containing media. Number of shoots per explant increased with increasing *m*T doses. Similarly, Dimitrova et al. [2016] indicated that increasing *m*T doses resulted larger number of shoot in *Pyrus communis*. However, unfavorable response was obtained in *Prunus* by Gentile et al. [2014].

Considering the frequency of shoot regeneration and number of shoots per explant, it was observed that species had different values from each other and the lowest regeneration capacity was observed in *O. elata*. Honarmand et al. [2016] carried out a study with Shahrkord and Khansar sainfoin cultivars and reported greater callus induction percentage, shoot regeneration frequency and number of shoots per explants for 'Khansar' cultivar than for 'Shahrkord' cultivar. Similarly in studies carried out with common vetch and alfalfa significant effects of plant genotype on *in vitro* shoot regeneration were reported [Çöçü et al. 2003, Erişen 2005].

Significant differences were observed in number of shoots per explant of the species with growth regulator doses and combinations. Optimum cytokinin concentrations significantly varied with the species and TDZ-containing media had higher number of shoots per explant. Success in regeneration of a plant is influenced by the type and concentration of the cytokinin applied since cytokinin uptake, transport and

**Table 4.** Effects of different *meta*-Topolin doses on number of shoots per explant of *Onobrychis* species

| *m*T (mg $L^{-1}$) | Species | | | | Means |
|---|---|---|---|---|---|
| | *O. stenostachya* | *O. fallax* | *O. elata* | *O. viciifolia* | |
| 0.5 | 2.65 b* | 2.91 b* | 1.97 b* | 2.39 b* | 2.48 c* |
| 1 | 2.97 b | 4.70 a | 2.85 b | 3.30 ab | 3.46 b |
| 2 | 5.86 a | 5.02 a | 4.00 a | 3.60 a | 4.62 a |
| Means | 3.83 a* | 4.21 a | 2.94 b | 3.10 b | |

* Values within the same column or row followed by different letters are significantly different at $P < 0.05$ of Duncan's multiple range test

metabolism vary with the species and cytokinin can react with the endogenous cytokinin of the explants [Magyar-Tabori et al. 2010]. Catterou et al. [2002] and Hill and Schaller [2013] indicated that natural differences in cytokinin synthesis and respond capability of the species played significant roles in *in vitro* regeneration capacity of the plants and the plants with greater bioactive cytokinin accumulations were quite open for the methods used to induce de novo shoot organogenesis.

Previous studies about *in vitro* regeneration of sainfoin mostly focused on *Onobrychis viciifolia*. Researchers used different plant explants and applied plant growth regulators at different concentrations and combinations to have an efficient shoot regeneration in sainfoin plants. Özcan et al. [1996a] had high shoot regeneration frequencies from the hypocotyl samples cultured in MS medium containing 0.5 mg $L^{-1}$ BAP and 0.2 mg $L^{-1}$ NAA. Özcan et al. [1996b] obtained similar high values from immature embryos cultured in MS medium containing 0.5 mg $L^{-1}$ BAP and 2 mg $L^{-1}$ NAA. Özgen et al. [1998] from the stem explants of plants cultivated under field conditions cultured in MS medium containing 20 µM BA and 0.5 µM NAA. Sancak [1999] from embryo in MS medium with 2 mg $L^{-1}$ BAP and 0.05 or 0.1 mg $L^{-1}$ IBA. Çeliktaş et al. [2006] from apical meristem in B5 medium with 10.7 µM NAA and 2.3µm kinetin. It was observed in those studies carried out with *Onobrychis viciifolia* that shoot regeneration generally varied with type of explant, type and concentration of plant growth regulators. Current findings were similar with the results of these studies. In cotyledon node explant, significant differences were observed in number of shoots per explant with growth regulator doses and combinations.

**Rooting of Shoots:** Regenerated shoots were cut and rooted in half MS nutrient media containing 1 or 2 mg $L^{-1}$ IBA. About 7–8 weeks after the initiation of culture, rooting ratios and number of roots per shoot were determined (Fig. 1c). There were highly significant differences in rooting ratios of the species. The rooting ratio was 86.11% in *O. viciifolia*, 50.00% in *O. stenostachya* and 36.11% in *O. fallax* and *O. elata*. Effects of nutrient medium on rooting ratios were not found to be significant. Effects of IBA doses on number of roots per shoot varied with the species. The greatest number of roots per shoot was obtained from 2 mg $L^{-1}$ IBA treatment of *O. stenostachya* and *O. elata*. The differences between the growth media were not found to be significant in *O. fallax* and *O. viciifolia* (Tab. 5). The greatest rooting ratio was observed in *O. viciifolia* and the lowest rooting ratio was observed in *O. elata* and *O. fallax*. Effects of genotypes on rooting were also reported by Gomes et al. [2010] and Cézar et al. [2015] for *Arbutus unedo* and *Pinus taeda*. Previous researchers reported rooting ratios of *O. viciifolia* in 1 mg $L^{-1}$ IBA-containing MS medium as between 50–60% [Özcan et al. 1996a, Özcan et al. 1996b, Sancak 1999].

**Table 5.** Effects of different IBA doses on number of roots per shoot in *Onobrychis* species

| IBA (mg $L^{-1}$) | Species | | | | Means |
|---|---|---|---|---|---|
| | *O. stenostachya* | *O. fallax* | *O. elata* | *O. viciifolia* | |
| 1 | 2.83 b* | 2.89 a* | 1.22 b* | 3.39 b* | 2.59 b* |
| 2 | 3.56 a | 2.50 a | 4.39 a | 3.62 a | 3.52 a |
| Means | 3.20 | 2.70 | 2.81 | 3.51 | |

* Values within the same column or row followed by different letters are significantly different at $P < 0.05$ of Duncan's multiple range test

## CONCLUSIONS

In general, the greatest number of shoot per explant in BAP and NAA-containing media was observed from only 2–4 mg $L^{-1}$ BAP or their combinations with 0.5 mg $L^{-1}$ NAA. TDZ-containing media also had higher number of shoots per explant, but optimum TDZ concentrations varied with the species. Number of shoots per explant increased with increasing *m*T doses.

Present outcomes can be used in clonal propagation of investigated *Onobrychis* species. Present findings can also be used to determine tolerance of these *Onobrychis* species to biotic and abiotic stress factors and gene transfer studies.

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