Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

In vitro organogenesis of *Scorzonera ahmet-duranii*

Received for publication, December, 20, 2014
Accepted, November, 2, 2016

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Abstract

The genus Scorzonera L. (Asteracea) is represented by fifty two species in Turkey. These species are being used in the treatment of several diseases due to their richness in secondary metabolites. In this study, endemic genus Scorzonera ahmet-duranii sp. nov. which is found in the South West Toros of Turkey was used as the experimental material. The species is only known in its type locality, and its population can be seen in a restricted area within the forest. For this reason, the continuity of these species is under threat. In addition, it is known that the species of the genus Scorzonera L. medicinal plants - due to their secondary metabolites - be used in the treatment of certain diseases among humans. So far, in vitro organogenesis of species of this genus has not been reported. In this study, in vitro organogenesis of S. ahmet-duranii is aimed for study. For this purpose, modified MS medium containing various concentrations of Kinetin and Naphthalene acetic acid (NAA) was used. In terms of percentage of callus formation, the most successful explant source is hypocotyl, documented as 90%. After the cultivation of apical meristems, cotyledon and hypocotyl explants in modified MS medium, shoot formation via organogenesis was observed.

Keywords: *Scorzonera ahmet-duranii*, organogenesis, plant regeneration, tissue culture.

1. Introduction

Even though the genus *Scorzonera* L. is widely spread in the arid zones of Eurasia and Northern Africa, its origin is Ancient Mediterranean and it involves about one hundred eighty species. (Lack [1]). According to Chamberlain [2], the genus *Scorzonera* was represented by forty nine taxa in Turkey, but then recent studies demonstrated fifty nine taxa (Coskuncelebi & al. [3], Dogan & al. [5], Dogan & al. [5]). *Scorzonera* species (Asteraceae) are mainly used as vegetables and folk-medicinal plants in Europe as well as in Turkey since 1800s. Plants of the genus *Scorzonera* were also employed as medicinal herbs in Turkish, and additionally European, Chinese and Mongolian folk medicines for different purposes such as to treat arteriosclerosis, kidney diseases, hypertension, diabetes mellitus, rheumatism as well as for pain relief and wound healing (Baytop [6], Jiang & al. [7], Tsevegsuren & al. [8], Turan & al. [9], Zidorn & al. [10], Zidorn & al. [11, Zidorn & al. [12]). *Scorzonera ahmet-duranii* sp. nov. is one of the endemic species of the Turkish flora (Makbul & al. [13]). Its type locality is known to be in C2 square according to grid square system given by Davis ([14]). The research area is located within the Sandras mountain throughout Southwest Toros. This species might be determined as “critically

endangered" due to its only known type locality and it spread around a very restricted range, which is less than 100 km^2 according to IUCN Red list Categories and Criteria (IUCN [15]).

Although there are no reports available on in vitro regeneration of the genus *Scorzonera*, one can find valid studies on the genus *Cichorium* and *Tragopogon*, which is close to the family of the genus *Scorzonera*. Explants of *Cichorium intybus* L. storage roots were grown in vitro on a modified Heller's medium lacking auxins and cytokinins, or were supplemented with auxins (either 2 4-Dichlorophenoxyacetic Acid (2,4 D) or NAA) alone or with a cytokinin (kinetin) or a combination of auxin and kinetin in different concentrations (Profuma & al. [16]). Germination of *Tragopogon heterospermus* was observed only in the case of immature seeds. Root formation was considerably depressed for *Tragopogon heterospermus* due to root wounding during transplantation after four months of cultivation leading to callus formation (Klavina & al. [17]).

In addition, this is the first comprehensive in vitro regeneration of taxa of the genus Scorzonera. The aim of this study was to detect in vitro organogenesis of *S. ahmet-duranii* sp. nov. on modified Murashige and Skoog (MS) medium containing various concentrations of Kinetin and Naphthalene acetic acid (NAA).

2. Materials and Methods

2.1. Plant material and sterilization

This study examined *Scorzonera ahmet-duranii* sp. nov. which was collected at an altitude of 1600-1700 m in a tight area in C2 square of the Sandras Mountain in Mugla, Turkey. It was identified by Makbul S. and Coskuncelebi K. ([13]). Mature achenes were cleaned and then saved at room temperature in our laboratory.

The achenes were cleared of their pappus bristles and their coat was removed; they were treated with sodium hypochlorite solution (10%) for 10 min (commercial sodium hypochlorite was used in sterilization process), followed by three rinsing stages with sterile distilled water.

2.2. Medium and culture conditions

The seeds were surface-sterilised and aseptically germinated at half-strength MS medium (Murashige & al. [18]). The nutrient medium consisted of MS salts and vitamins gelled with 0.7% (w/v) agar (Sigma). This basal medium was supplemented with various growth regulators (NAA, Kinetin) at various concentrations combined together (Table 1). The pH of all media was 5.8. Media were sterilized by autoclaving for 15 min at 121°C and 108 kPa. Cultures were grown at 24±2°C on a 16/8 photoperiod (42 μmol m^{-2} s^{-1}, cool white fluorescent light).

Table 1. Composition of modified MS medium for organogenesis

Media	**Components**
S1	MS Salts, vitamins and casein hydrolysate 0.5 (g L^{-1}) + kinetin 2 (mg L^{-1}) + NAA 0.05 (mg L^{-1}) + Agar (0.7%) + Sucrose (2%)
S2	MS Salts, vitamins and casein hydrolysate 0.5 (g L^{-1}) + kinetin 1.5 (mg L^{-1}) + NAA 0.1 (mg L^{-1}) + Agar (0.7%) + Sucrose (2%)
S3	MS Salts, vitamins and casein hydrolysate 0.5 (g L^{-1}) + kinetin 1 (mg L^{-1}) + NAA 0.5 (mg L^{-1}) + Agar (0.7%) + Sucrose (2%)
S4	MS Salts, vitamins and casein hydrolysate 0.5 (g L^{-1}) + kinetin 0.5 (mg L^{-1}) + NAA 1 (mg L^{-1}) + Agar (0.7%) + Sucrose (2%)

Seeds of *Scorzonera ahmet-duranii* were germinated in Petri dishes (90×15 mm) containing hormone-free MS basal medium.

Explants (Apical meristem: 0.2-0.3 cm, hypocotyls, cotyledon, young leaves and the roots (0.8-1.0 cm) of 25 days old aseptic seedlings (10 to 12 cm in height) germinated from achene in vitro medium were taken up by modified MS medium with various growth regulators for direct and indirect organogenesis. Shoot tips and calli were subcultured every 4-6 weeks. Experiments were completed by taking the obtained clone seedlings in the soil.

2.3. Medium and culture conditions

Modified MS with different concentrations of NAA, growth regulator, was used for rooting (Table 2). The regenerated shoots were rooted using rooting media (pH=5.8). Media were sterilized by autoclaving for 15 min at 121°C and 108 kPa. Cultures were grown at 24±2°C on a 16/8 photoperiod (42 μmol $m^{-2}s^{-1}$, cool white fluorescent light). The obtained aseptic seedlings via organogenesis were placed perpendicularly and then the growth of the roots was followed.

Table 2. Rooting media of explants with indirect and direct organogenesis.

Media	**Components**
RS1	MS Salt, Vitamins and Casein Hydrolysate (0.5 g L^{-1}) + NAA (0.5 mg L^{-1}) + Agar (0.7%) + Sucrose (2%)
RS2	MS Salt, Vitamins and Casein Hydrolysate (0.5 g L^{-1}) + NAA (0.75 mg L^{-1}) + Agar (0.7%) + Sucrose (2%)
RS3	MS Salt, Vitamins and Casein Hydrolysate (0.5 g L^{-1}) + NAA (1 mg L^{-1}) + Agar (0.7%) + Sucrose (2%)

2.4. Acclimatization

Shoots, which rooted in modified MS medium, became a complete clone seedling. Ex vitro rooting was attempted in order to prevent the waste of time spent for going to the field. The seedlings were taken out from the culture jars, washed to remove media and transplanted into the small plastic pots pre-filled with autoclaved sterile garden soil. The seedlings were subsequently transferred to the larger pots and gradually acclimatized to outdoor condition and eventually became suitable for transplantation in the field. The ratio of humidity in the laboratory was gradually decreased from 90% to 55% in 15 days. The seedlings that developed fully in the laboratory exhibited morphological characters (stem colour, leaf morphology, growth pattern, flower colour, etc.) that are identical to those of the mother plant (Fig 4). The seedlings, that lived healthy for two months, had brown marks. They later lost their vividness.

2.5. Statistics

The data were analysed by one-way analysis of variance (ANOVA) and the means was compared by Duncan's multiple-range test (Duncan [19]). Each experiment was replicated three times and arranged in completely randomized design. The data given in percentages were subjected to arcsine transformation (Snedecor & al. [20]) before statistical analysis.

3. Results

3.1. Achenes germination of Scorzonera ahmet-duranii

Sterilized mature achenes were placed on hormone-free MS medium (5 seeds per jar). The first germination started on the fourth day. Next, apical meristem, hypocotyls, cotyledon, young leaves and the root explants of aseptic seedlings (25-30 days) germinated in the in

vitro medium were placed into modified MS medium with various concentrations (Kinetin and NAA) for organogenesis.

3.2. Achenes germination of Scorzonera ahmet-duranii

Calli were initiated on the MS basal medium supplemented with different concentrations of NAA in combination with Kinetin, and on the fifth day of the experiment, an explant was derived from the apical meristem (Fig 1). The initial calli were small, globular shape and pale yellow; the greenish colour developed on the surface of the explant.

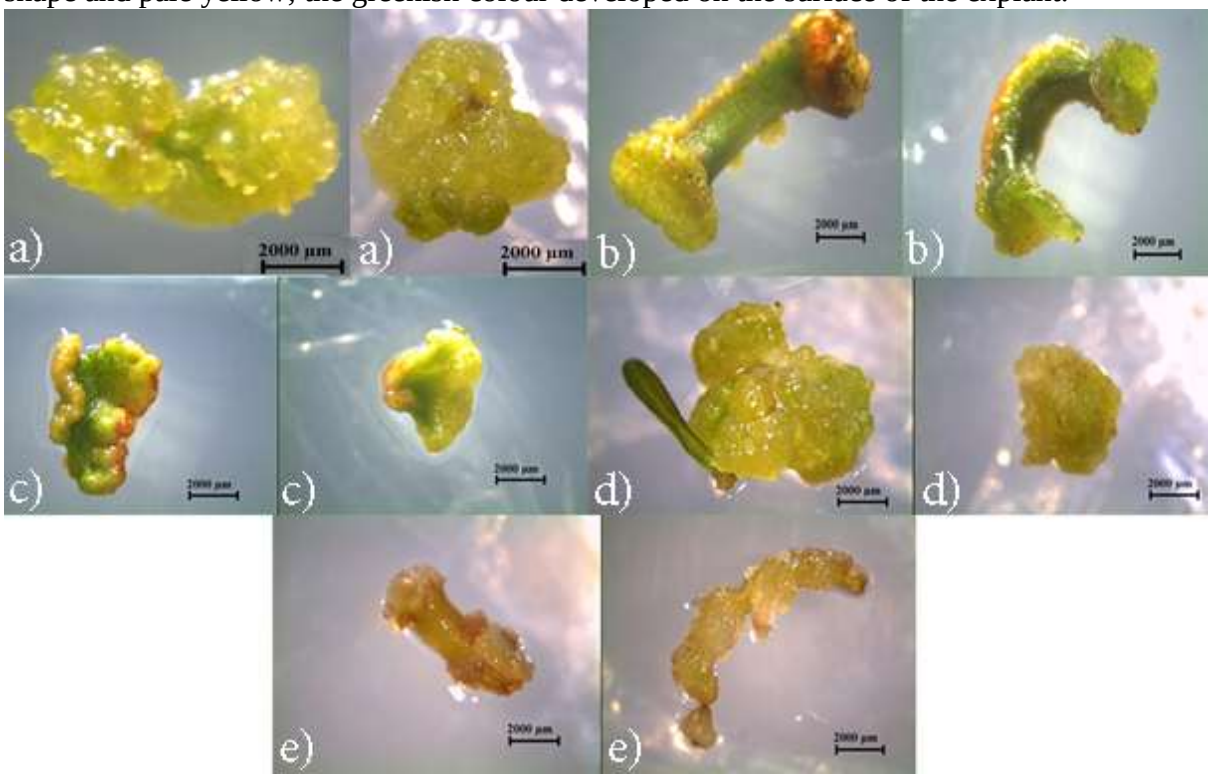

Figure 1. Calli formation from explants of *S. ahmet-duranii* with indirect and direct organogenesis. a) apical meristem explants of seedling b) cotyledon explants of seedling c) young leaves explants of seedling d) hypocotyl explants of seedling e) root explants of seedling

Hypocotyl explants in terms of percentage of callus formation were the most successful in the experiments. Callus formation of hypocotyl explants was 90% in 4 different MS media (S1, S2, S3, and S4). The percentage of the lowest callus formation was 50.77 for the root explants in S3 medium (Table 3).

Table 3. Percentage of calli formation from explants of *S. ahmet-duranii* on modified MS (%) (± Standard error).

Media	**Apical meristem**	**Cotyledon**	**Hypocotyls**	**Young leaves**	**Root**
S1	71.14 ± 9.47a	69.38 ± 10.61a	90 ± 0.0	90 ± 0.0a	76.92 ± 13.07b
S2	90 ± 0.0a	76.92 ± 13.07a	90 ± 0.0	90 ± 0.0a	90 ± 0.0b
S3	90 ± 0.0a	81.14 ± 8.85a	90 ± 0.0	90 ± 0.0a	50.77 ± 0.0a
S4	81.14 ± 8.85a	81.14 ± 8.85a	90 ± 0.0	81.14 ± 8.85a	90 ± 0.0b

Means with the same lowercase letter in the column, for each species, are not significantly different by Duncan's test ($p \leq 0.05$).

3.3. Induction of Shoot

The shoot formation that was obtained from the explants of apical meristem, cotyledon and hypocotyls was observed via indirect organogenesis on modified MS medium.

Shoot formation from the explants of the first leaf and the root was not detected. However, it was observed on the explants of cotyledon via direct organogenesis (Fig 2).

In the apical meristems saved in modified MS medium, the highest shoot formation was observed in S2 and S3 media (90%), and the lowest shoot formation was seen in S4 medium (34.63%). In cotyledon explants, the highest shoot formation was found in S1 and S2 media (30.78%), while the lowest shoot formation was noticed in S4 medium (17.70%). In hypocotyls, the highest shoot formation was detected in S2 medium (43.07%), while the lowest shoot formation was seen in S4 medium (17.70%) (Fig.2).

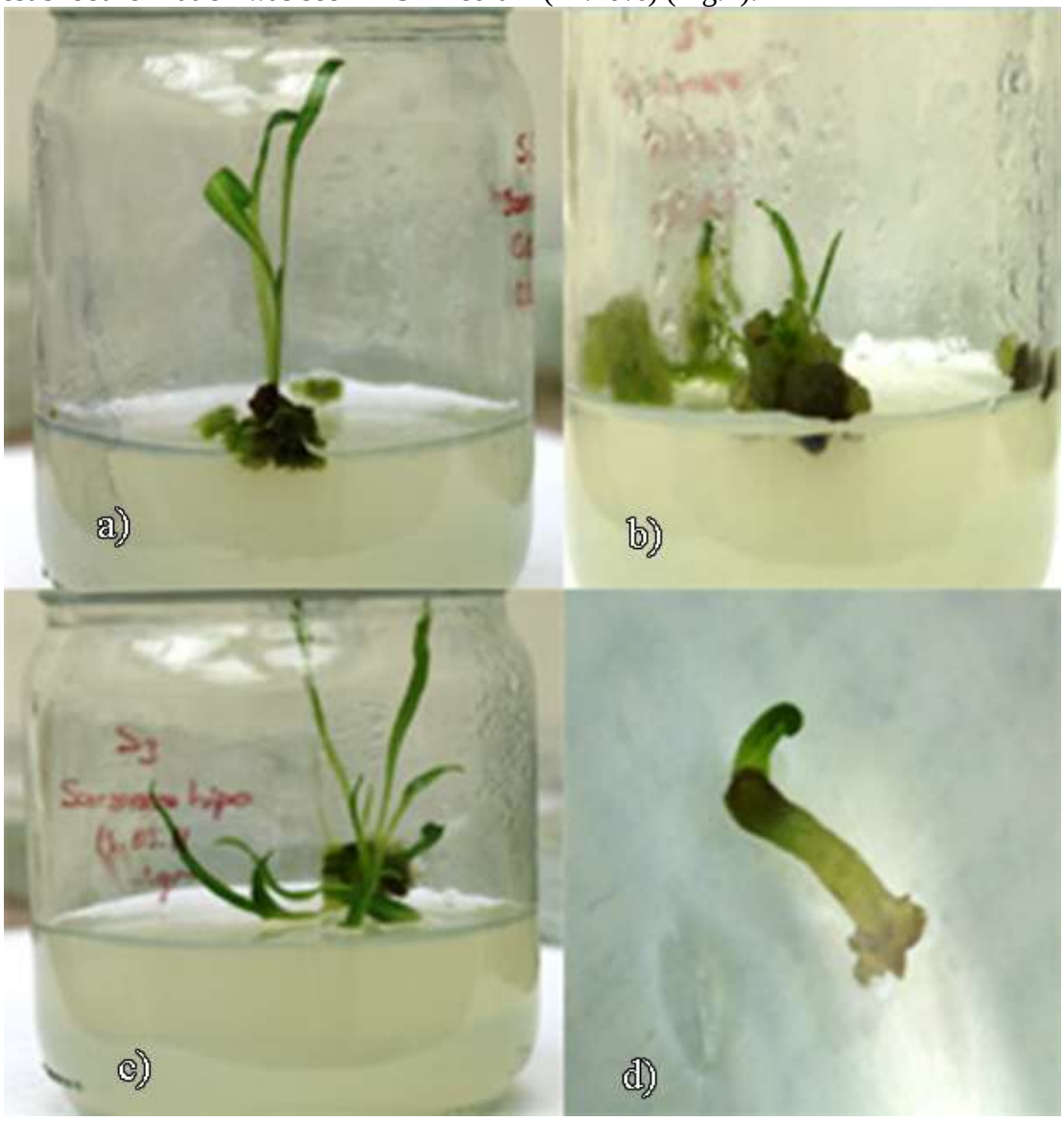

Figure 2. a) apical meristem in vitro organogenesis indirectly b) cotyledon in vitro organogenesis indirectly c) hypocotyl in vitro organogenesis indirectly d) cotyledon in vitro organogenesis directly

Shoot formation from apical meristem was the highest in S2 and S3 media (90%) (Fig 2, Table 4), and the lowest shoot formation was in S4 medium (34.63%). When the aseptic shoots became at least 10 cm in height, they were subcultured in modified MS medium. With an increased number of leaves, young seedlings were taken into the rooting medium.

Table 4. Percentage of shoot formation from explants of *S. ahmet-duranii* on modified MS (%) (± Standard error).

Medium	Apical Meristem	Cotyledon	Hypocotyl
S1	46.92 ± 3.84b	30.78 ± 4.22a	26.56 ± 0.0a
S2	81.14 ± 8.85a	30.78 ± 4.22a	43.07 ± 10.81a
S3	35 ± 4.22b	26.56 ± 0.0a	21.93 ± 11.55a
S4	34.63 ± 8.07b	17.70 ± 8.85a	17.70 ± 8.85a

Means with the same lowercase letter in the column, for each species, are not significantly different by Duncan's test ($p \leq 0.05$).

3.4. Subculture

Three weeks later, the explants started to blacken, which put the continuity of organogenesis under risk. Therefore, all the cultures required subculture every three weeks in the same medium concentration.

3.5. Induction of root

Shoots of body organogenesis completed in modified MS medium were taken in rooting medium containing different concentrations of NAA (RS1, RS2 and RS3). In the rooting experiments, 100% of the rooting occurred by putting the shoots obtained from apical meristem into RS2 medium (Fig 3, Table 4). The first rooting was observed right after the first week. The obtained roots continued to grow gravitropismic into agar medium.

Figure 3. The shoot rooting obtained from apical meristem explants

Figure 4. Seedling in a small cup (30 days after transfer)

4. Discussion

In Turkish folk medicine, *Scorzonera* species are used to treat arteriosclerosis, kidney diseases, hypertension, diabetes mellitus, rheumatism, wound healing and for pain relief. (Jiang & al. [7], Turan & al. [9], Zidorn & al. [11], Zidorn & al. [12]). Seskiterpenoid and its derivatives were obtained from methanol extraction of the roots of *S. hispanica*. (Zidorn & al. [10]). The cytotoxicity of lignan and bisabolane derivatives from *S. hispanica* against several cancer cell lines were researched by Granica & al. 2015. Syringaresinol was the only active compound against myeloma and colon cancer cell lines. However, this lignan was seen to be cytotoxic for normal peripheral blood mononuclear cells (PBMCs) (Granica & al. [21]). Five flavonoid glycosides and two derivatives were isolated from *S. austriaca* by silica gel column chromatography and preparative HPLC (Xie & al. [22]). Profuma & al. [16], callus occurred from roots explants cultured of *Cichorium intybus* L. on a modified Heller's medium supplemented with different concentration of auxins and cytokinins (2,4-D (0.5 mg L^{-1} 0.1 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1}), NAA (0.05 mg L^{-1}, 0.1 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1}), Kinetin (0.05 mg L^{-1}, 0.1 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1})). The highest callus formation was medium supplemented with 2,4-D. Only using kinetin was effective for shoot formation. As for NAA, it was effective in differentiating between shoot and root formation. Alternatively, in this study, MS medium supplemented with kinetin and NAA was used for callus formation. In both studies, the first callus formation were detected on the 4th and 5th day and were found to be pale yellow and globular. The percentage of callus formation found by Profuma & al. ([16]) was less than the percentage of callus formation found in this study.

According to our knowledge, this paper is the first report on shoot regeneration via organogenesis from the explants of *Scorzonera ahmet-duranii*. It is important to provide guidance for future studies of genus *Scorzonera*. In conclusion, this study indicated that a high frequency shoot differentiation from callus culture of *Scorzonera ahmet-duranii* was dependent on the critical balance of various growth regulators and culture conditions.

As a result, depending on the fact that no issue culture study has been carried on *Scorzonera* genus so far as well as the rich chemical contents of these plants enhances the

value of these plants for our country. Under these circumstances, the in vitro culture of the plant material we have used in our study is important in the sense that it will not only help protect the species from extinction, but also it will help us to detect and produce secondary metabolites that the plant might have in various numbers. We expect our study to inspire other studies in future.

5.Acknowledgements

The authors gratefully thank Ayse Ceylan Hamamcioglu (Faculty of Pharmacy, Bulent Ecevit University) for proof reading.

References

1. H.W. LACK. Tribe Cichorieae Lam. and DC. – In: Kubitzki (ed.): The Families and Genera of Vascular Plants, Vol. VIII, Flowering Plants. Eudicots, Asterales: Kadereit and Jeffrey (eds.): 180-199. Heidelberg, Berlin: Springer-Verlag, (2007).
2. D.F. CHAMBERLAIN. *Scorzonera* L. – In: Davis PH (Ed.). Flora of Turkey and the East Aegean Islands, volume 5: 632-657. Edinburgh: Edinburgh University Press (1975).
3. K. COSKUNCELEBI, S. MAKBUL, M. GULTEPE, S. OKUR, M.E. GUZEL. A conspectus of *Scorzonera* s.l. in Turkey, *Turk. J. Bot*, 39: 76-87 (2015).
4. B. DOGAN, A. DURAN. A new record for the flora of Turkey: *Scorzonera renzii* Rech. f. (Asteraceae). *Biological Diversity and Conservation*, 3/3: 133-136, (2010).
5. B. DOGAN, A. DURAN, S. MAKBUL. *Scorzonera tuzgoluensis* sp. nov. (Asteraceae), a new halophytic species from central Anatolia, Turkey. *Nordic Journal of Botany*, 29: 20-25 (2011).
6. A. BAYTOP. Farmasotik Botanik. Istanbul Universitesi Basimevi. pp. 310-316 (1983).
7. T.F. JIANG, Y.H. WANG, Z.H. LV, M.E. YUE. Determination of kava lactones and flavonoid glycoside in *Scorzonera austriaca* by capillary zone electrophoresis. *J. Pharm. Biomed. Anal*, 43: 854-858 (2007).
8. N. TSEVEGSUREN, R.A. EDRADA, W. LIN, R. EBEL, C. TORRE, S. ORTLEPP, V. WRAY, P. PROKSCH. Biologically active natural products from Mongolian medicinal plants *Scorzonera divaricata* and *Scorzonera pseudodivaricata* (Asteraceae). *Journal of Natural Products*, 70: 962-967 (2007).
9. M. TURAN, S. KORDALI, H. ZENGIN, A. DURSUN, Y. SEZEN. Macro and Micro Mineral Content of Some Wild Edible Leaves Consumed in Eastern Anatolia. Acta Agriculturae Scandinavica, Section B - *Soil & Plant Science*, 53: 129-137, (2003).
10. C. ZIDORN, E.P. ELLMERER-MULLER, H. STUPPNER. Sesquiterpenoids from *Scorzonera hispanica* L. *Pharmazie*, 55: 550-551 (2000).
11. C. ZIDORN, E.P. ELLMERER-MULLER, H. STUPPNER. Tyrolobibenzyls-novel secondary metabolites from *Scorzonera humilis* L. *Helvetica Chimica Acta* 83: 2920-2925 (2001).
12. C. ZIDORN, E. P. ELLMERER, S. STURM, H. STUPPNER. Tyrolobibenzyls E and F from *Scorzonera humilis* and distribution of caffeic acid derivatives, lignans and tyrolobibenzyls in European taxa of the subtribe Scorzonerinae (Lactuceae, Asteraceae). *Phytochemistry*, 63: 61-67 (2003).
13. S. MAKBUL, K. COSKUNCELEBI, M. GULTEPE, S. OKUR, M.E. GUZEL. *Scorzonera ahmet-duranii* sp. nov. (Asteraceae) from southwest Anatolia and its phylogenetic position. *Nordic Journal of Botany*, 30: 2-11 (2012).
14. P.H. DAVIS. Flora of Turkey and the East Aegean Islands. Vol. 1-9. Edinburgh: Edinburgh University Press (1965-1985).
15. IUCN Red List, http://www.iucnredlist.org, 03 January (2012).
16. P. PROFUMA, P. GASTALDO, L. CAFFARO, R.M. DAMERI, G.R. MICHELOZZI, A. BENNICI. Callus induction and plantlet regeneration in *Cichorium intybus* L.: II. Effect of different hormonal treatments. *Protoplasma*, 126: 215-220 (1985).
17. D. KLAVINA, A. GAILĪTE, G. IEVINSH. Initial responses of explants from rare and endangered coastal plant species during initiation of tissue culture. *Acta Universitatis Latviensis*, 710: 81-91, (2006).

18. T. MURASHIGE, F. SKOOG. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures, *Physiologia Plantarum*, 15/3: 473-497, (1962).
19. D.B. DUNCAN, Multiple range and multiple F tests. *Biometrics* 11: 1-42 (1955).
20. G.N. SNEDECOR, W.G. COCHRAN. Statistical methods. 6th ed. Iowa State Univ. Press, Ames, IA, (1967).
21. S. GRANICA, U. LOHWASSERC, K. JOHRERD, C. ZIDORNA. Qualitative and quantitative analyses of secondary metabolites in aerial and subaerial of *Scorzonera hispanica* L. (black salsify) *Food Chemistry*, 173: 321-331 (2015).
22. Y. XIE, Q.S. GUO, G.S. WANG. Flavonoid Glycosides and Their Derivatives from the Herbs of *Scorzonera austriaca*, Molecules, 21/6: 803 (2016).