HortiS (2022) 39(3):107-112
http://doi.org/10.16882/HortiS.1200592

RESEARCH PAPER

HORTICULTURAL STUDIES

Investigation of *In vitro* Propagation Possibilities of Endemic *Campanula phitosiana* Yıldırım & Şentürk

Ademi Fahri PIRHAN[1] Ebru SEVİNDİK[1] Başar SEVİNDİK[2]

[1]Ege University, Faculty of Science, Department of Biology, 35040, İzmir, Türkiye
[2]İzmir Demokrasi University, Vocational High School, 35140, İzmir, Türkiye

Article History
Received 22 September 2022
Accepted 10 November 2022
First Online 15 November 2022

Corresponding Author
E-mail: ademi.fahri.pirhan@ege.edu.tr

Keywords
Campanula
Endemic
Plant growth regulator
Regeneration
Tissue culture

Abstract

Campanula phitosiana Yıldırım & Şentürk, is a local endemic to the Aydın Mountain range, which distribute across in Western Anatolia (both İzmir and Aydın). This species belongs to the Mediterranean basin floristic region. According to the International Union for Conservation of Nature's criteria, *C. phitosiana* is classified as "Critically Endangered" (CR). The aim of the study is to develop *in vitro* regeneration protocol for critically endangered endemic *Campanula phitosiana*. To investigate the efficient medium and plant growth regulator combinations for callus initiation and shoot proliferation, petiole and leaves were used as explant and explants cultivated on MS medium including NAA (1-Naphthylacetic acid) (0.3 mg L^{-1}), TDZ (Thidiazuron) (0.5, 1.0, 2.0, and 3.0 mg L^{-1}), BA (6-Benzylaminopurine) (0.5, 1.0, 2.0, and 3.0 mg L^{-1}) and Gibberellic Acid (GA_3) (0.5, 1.0, 2.0, and 3.0 mg L^{-1}). Explants subcultured 3 times. Experiments were conducted according to completely randomized design repeated with 5 replicates and each replicates including 5 explants. As a result of the experiment, callus initiation and shoot proliferation were investigated. Efficient callus initiation was observed petiole explants as 100% rate. Shoot proliferation was observed on MS medium including 0.3 mg L^{-1} NAA + 2.0 mg L^{-1} GA_3.

1. Introduction

The genus *Campanula* is known as one of the crowded family in Campanulaceae family with 420 species. This genus separated to 6 subgenus such as Megalocalyx, Damboldt, Rapunculus (Fourr) Charadze, Roucela (Dumort.) Damboldt, Brachycodonia (Fed.) Damboldt, Sicyodon (Feer) Damboldt and Campunala (Özdöl et al., 2022). Additionally, 138 of this species naturally grown in Türkiye (Fedorov and Kovanda, 1976; Contandriopoulos, 1984; Lammers, 2007; Alçıtepe et al., 2011; Yıldırım, 2018; Yıldırım and Özdöl, 2019; Özdöl et al., 2022). These species are distributed across eastern Mediterranean region, Balkans, Caucasia, and Türkiye. A total of 140 taxa, 68 of them are endemic to Türkiye. These species are known as annual, biennial, and perennial shrubs which has high ornamental potential due to shape of its flowers and compact plant structures. Campanula species has been used in ornamental plant industry and landscape architecture as outdoor, indoor pot plant (Scariot et al., 2008). Most of the campanula species is grown naturally in Türkiye and Yıldırım et al. (2019) defined the Türkiye as a key point of the Campanula species. *C. phitosiana* is one of the endemic species and it is classified as critically endangered according to the International Union for Conservation of Nature's (IUCN) criteria. This species distributed in a limited area (0.76 km^2) in Western Anatolia especially in İzmir and Aydın province. *Campanula phitosiana* is known as chasmophyte and flowers of this species blooms in June and July. Leaves of this species is tomentose, rosette leaves are lyrate shape, petiolate or sessile, inflorescence is racemose,

flowers are sub-secund or secund, flower colour is pale purplish blue (Yıldırım and Özdol, 2019). This species has a great potential for the landscape design of rocky gardens, and it is one of the important endemic genetic resources for Türkiye. To protect and propagate the critically endangered species, tissue culture techniques are very significant methods. Development of efficient regeneration protocol is the first step for *in vitro* conservation of the genetic resources. There have been too many reports about *in vitro* propagation of different *Campanula* species such as *C. isophylla* (Brandt, 1994), *C. glomerata* (Tanaka et al., 1999), *Campanula glomerata* 'Acualis' (Joung et al., 2002), *Campanula carpatica* Jacq. (Frello et al., 2002), *Campanula rotundifolia* (Mørk et al., 2005), *Campanula punctata* (Shim et al., 2005), *Campanula punctata* var. Rubriflora Makino (Sivanesan et al., 2007), *C. sabatia* (Airo et al., 2009), *C. polymorpha* Witas (Paunescu, 2010), hybrid Campanula (Röper et al., 2015), *Campanula incurva* (Grigoriadou et al., 2014), *Campanula rotundifolia* (Maria, 2014) *C. sclerophylla* (Kolomiets et al., 2016). However, there is no report about the *Campanula phitosiana* Yıldırım & Şentürk.

In this study, developing efficient *in vitro* regeneration protocol for critically endangered *Campanula phitosiana* Yıldırım & Şentürk were investigated. Plants were collected from flora of İzmir, Tire, Dallık province. Leaf and petiole were cultivated on MS medium including NAA with two different cytokinin (TDZ and BA).

2. Material and Method

2.1. Plant material

Campanula phitosiana Yıldırım & Şentürk plants were collected from the natural flora of İzmir, Tire, Dallık province in Türkiye, in June 2020. Fifteen *C. phitosiana* genotypes were cultivated in greenhouse in Research and Application Centre of Botanical Garden and Herbarium of Ege University. One of the plants was sampled for Herbarium coded with AD3761. Plants were cultivated in 10 cm diameter pots containing sand / turf (1:1; v/v). Plants were irrigated once a week in the greenhouse. Leaf and petioles were used as explant and explants were chosen healthy donor plants.

2.2. Method

In vitro experiments were set up in the tissue culture laboratories of Research and Application Centre of Botanical Garden and Herbarium of Ege University. All materials used in the tissue culture studies (Pince and lancet) were sterilized with autoclave. Medium used in the *in vitro* regeneration studies and distilled water used in the sterilization were autoclaved (121℃, 1.05 ATM pressure for 15 minutes).

2.3. Surface sterilization

Randomly selected leaves and petioles of the healthy donor plants were washed under tap water during 30 min. Explants were immersed in 70% EtOH in the sterile laminar flow cabinet for 1-2 minutes and rinsed with sterilised distilled water. Then explants were soaked into 30% Domestos (NaOCl, 4.5% v/v) for 20 minutes and washed four or five times with sterile distilled water in the laminar flow cabinet.

2.4. Explant preparation and regeneration medium

Sterile petiole and leaves were used as explant. Fresh leaves were selected and cut into two pieces due to the limited explant number. Petioles were cut into equal parts and each petiole explant was 0.5 cm length. To obtain organogenic callus explants were cultured on MS medium including 30.0 g L^{-1} sucrose, 4.0 g L^{-1} gelrite and 0.3 mg L^{-1} NAA and different concentrations of TDZ and BA (0.5, 1.0, 2.0, and 3.0 mg L^{-1}) as plant growth regulator. Medium pH was adjusted to 5.6-5.8 with 1 N HCl and 1 N KOH. Callus were subcultured to the MS medium containing 30.0 g L^{-1} sucrose, 4.0 g L^{-1} gelrite and 0.3 mg L^{-1} NAA and GA_3 (0.3, 0.5, 1.0, and 2.0 mg L^{-1}) to induce shoot proliferation. Shoots and shoot like structures were transferred to the hormone free MS medium containig 30.0 g L^{-1} sucrose, 4.0 g L^{-1} gelrite to root formation.

2.5. Experimental design and statistical analyses

In vitro regeneration experiments were conducted as completely randomised design. Each concentration included 5 replicates (5 petri dishes) each petri dishes contains 5 explants. Observations was carried out each 4 weeks and data were analysed with JMP 8 programme. Experiment was repeated two times. Percentage values were arcsine transformed. LSD test was performed to separate the means at the 0.05 level of probability.

3. Result and Discussion

There was no regeneration for both explant type in control group. Each explant from different genotypes showed different *in vitro* regeneration capacity in all regeneration medium except leaf explants cultured on MS medium including 0.3 mg L^{-1} NAA + 3.0 mg L^{-1} TDZ. Efficient callus regeneration was obtained from the petiole explants cultured on MS medium containing 0.3 mg L^{-1} NAA + 1.0 mg L^{-1} BA, 0.3 mg L^{-1} NAA + 3.0 mg L^{-1} BA, 0.3 mg L^{-1} NAA + 1.0 mg L^{-1} TDZ, 0.3 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ as 100% (Table 1, Figure 1). Petiole was determined as efficient explant type for

Table 1. Regeneration results of the *C. phitosiana* leaf and petiole explants.

Plant growth regulator concentration	Leaf	Petiol
0.3 mg L^{-1} NAA + 0.5 mg L^{-1}BA	13.33 cd (17.71)	86.66 a (76.92)
0.3 mg L^{-1} NAA + 1.0 mg L^{-1} BA	46.66 ac (43.07)	100.00 a (90.00)
0.3 mg L^{-1} NAA + 2.0 mg L^{-1} BA	60.00 a (56.15)	80.00 a (68.07)
0.3 mg L^{-1} NAA + 3.0 mg L^{-1} BA	20.00 bd (26.57)	100.00 a (90.00)
0.3 mg L^{-1} NAA + 0.5 mg L^{-1} TDZ	46.66 ac (43.07)	66.00 a (60.00)
0.3 mg L^{-1} NAA + 1.0 mg L^{-1} TDZ	33.33 ac (30.00)	100.00 a (90.00)
0.3 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ	60.00 ab (51.14)	100.00 a (90.00)
0.3 mg L^{-1} NAA + 3.0 mg L^{-1} TDZ	0.00 e (0.00)	20.00 b (16.92)

LSDleaf = 27.62, LSDpetiole= 32.31 (p>0.05). All percentage values, indicated in parentheses, were arcsine transformed.
Different letters show significant differences.

Table 2. Shoot formation results of the *C. phitosiana* leaf and petiole explants.

Plant growth regulator concentration	Leaf	Petiol
0.3 mg L^{-1} NAA + 0.5 mg L^{-1} GA_3	0.00 b (0.00)	0.00 b (0.00)
0.3 mg L^{-1} NAA + 1.0 mg L^{-1} GA_3	0.00 b (0.00)	0.00 b (0.00)
0.3 mg L^{-1} NAA + 2.0 mg L^{-1} GA_3	100.00 a (90.00)	100.00 a (90.00)
0.3 mg L^{-1} NAA + 3.0 mg L^{-1} GA_3	0.00 b (0.00)	0.00 b (0.00)

LSDleaf = 0, LSDpetiole=0 (p>0.05). All percentage values, indicated in parentheses, were arcsine transformed.
Different letters show significant differences.

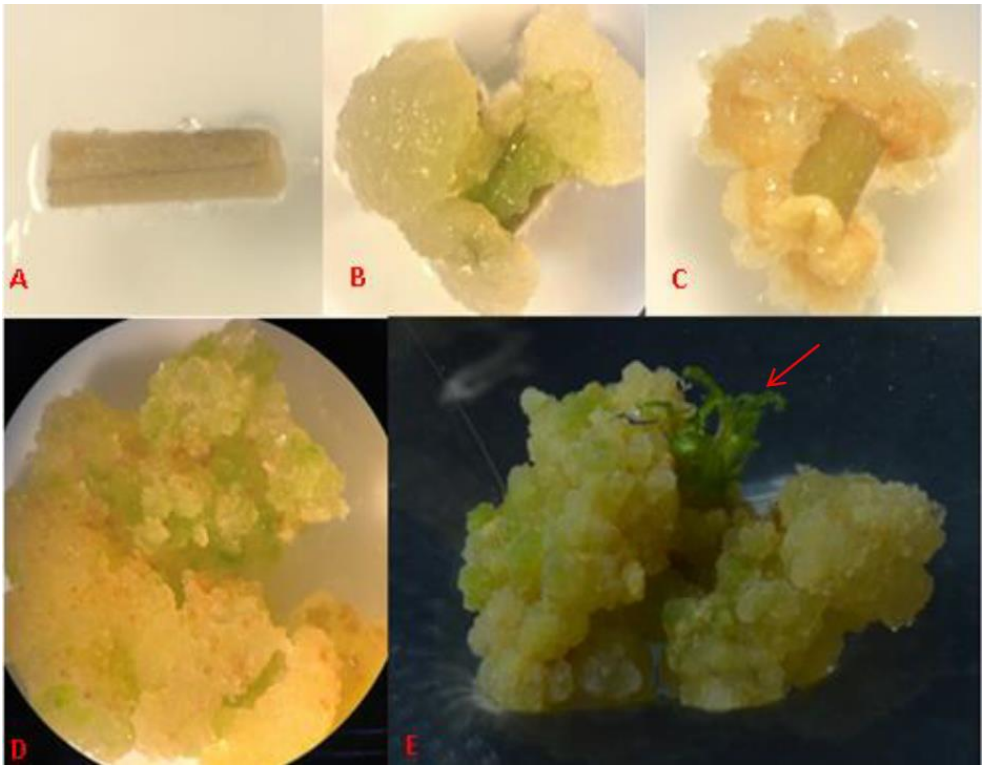

Figure 1. *In vitro* shoot regeneration from the petiole explants of *C. phitosiana* (A: First week of the culture, B-C: Callus at 4th week of the experiments, D: Shoot like formations, E: Shoots from petiole explants at 12th week).

Campanula phitosiana Yıldırım & Şentürk. Highest callus regeneration was obtained from leaf explants on the MS medium containing 0.3 mg L^{-1} NAA+ 2.0 mg L^{-1} BA and 0.3 mg L^{-1} NAA+ 2.0 mg L^{-1} TDZ as 60% (Table 1, Figure 2). These results showed that explant type was one of the important factors for *in vitro* regeneration of *Campanula phitosiana.* To transform the callus to the shoot, callus was cultured on MS medium containing 0.3 mg L^{-1} NAA and 0.5, 1.0, 2.0, and 3.0 mg L^{-1} GA_3. Shoot formation was obtained only from the MS medium supplied with 0.3 mg L^{-1} NAA + 2.0 mg L^{-1} GA_3 for both leaf and petiole explants (Table 2). There was no shoot formation on the other shoot proliferation medium. Shoots and shoot like structure were cultured on hormone free MS medium for root induction but root formation was not observed.

Protection and propagation of the genetic resource via *in vitro* technique has been developed for different species. Microporpagation provides the clonal multiplication of the rare endemic and endangered plants. In this study, *Campanula phitosiana* endemic to İzmir/Türkiye was efficiently propagated. There has been many reports on micropropagation of Campanula species but microporpagation of this endangered *Campanula*

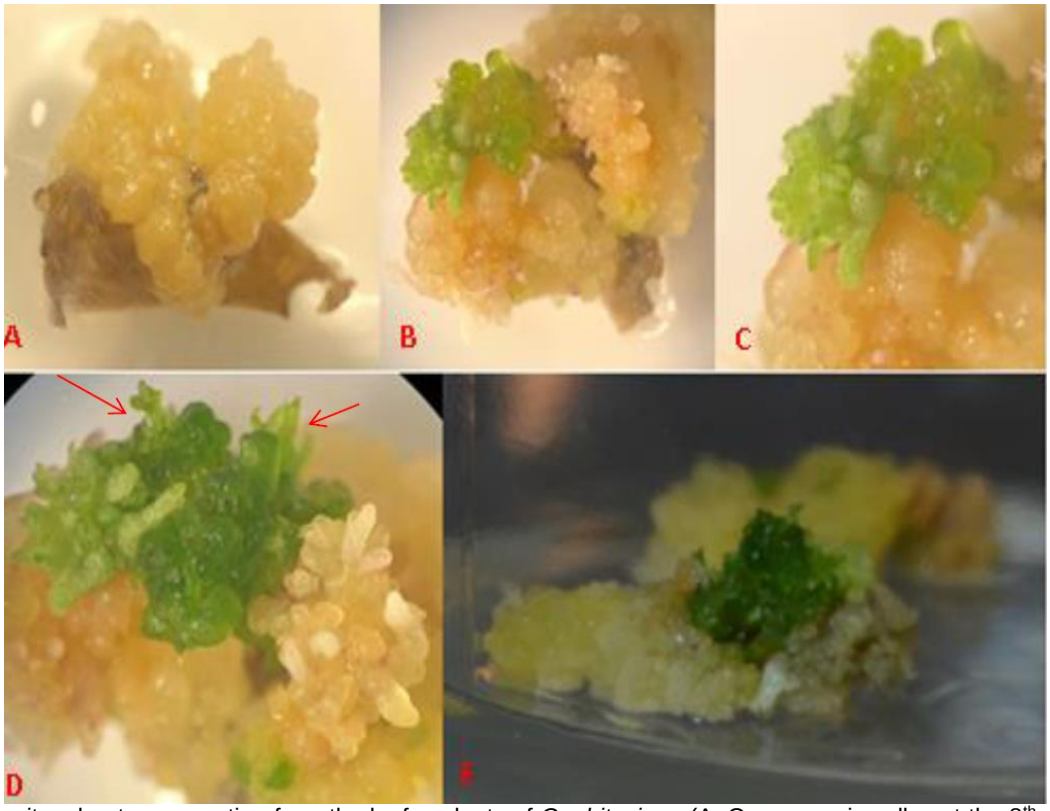

Figure 2. *In vitro* shoot regeneration from the leaf explants of *C. phitosiana* (A: Organogenic callus at the 8th week of the culture, B: Callus colours turned to the green at 10th week, C: Shoot like formations, D-E: Shoots from leaf explants at 12th week).

phitosiana was not performed. In this study, petiole explant showed efficient regeneration capacity according to leaf explants. On the other hand, leaf (Joung et al., 2002), cotyledon (Frello et al., 2002), buds (Maria, 2014), leaf (Tanaka et al., 1999) and petiole (Sivanesan et al., 2007; Sivanesan et al., 2011), ovule (Röper et al., 2015), hypocotil (Sriskandarajah et al., 2001; Mørk et al., 2005), nodes (Paunescu, 2010), shoots (Brandt, 1994), seedlings (Grigoriadou et al., 2014), seeds (Seglie et al., 2012) were used as explant for different Campanula species. Plant growth regulator is an important factor to manuplate the cell and tissue to obtain efficient regeneration. In this study, BA and TDZ were used as cytokinin and NAA used as auxin. Efficient callus regeneration was observed on MS medium 0.3 mg L^{-1} NAA + 1.0 mg L^{-1} BA, 0.3 mg L^{-1} NAA + 3.0 mg L^{-1} BA, 0.3 mg L^{-1} NAA + 1.00 mg L^{-1} TDZ, 0.3 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ. Joung et al. (2002) carried out micropropagation of *Campanula glomerata* 'Acualis' on MS medium and they cultured the leaves obtained from MS medium including 1.0 mg L^{-1} BA + 0.01 mg L^{-1} NAA with different cytokinins such as BA and 2iP. They reported that BA was the efficient cytokinin for the clonal uniform propagation. On the other hand, our result presented that there was no significant differences between BA and TDZ. Frello et al., (2002) cultured cotyledon explant of the two different genotype of *Campanula carpatica* on MS medium including 0.50 mg L^{-1} NAA + 0.25 mg L^{-1} 2.4-D + 0.75 mg L^{-1} BA. They reported that there was a significant difference between genotypes. In our study, genotypes were randomly selected and there were no differences between the genotypes. Kolomiets et al., (2016) cultured the *C. sclerophylla* species on MS medium including 3.0 mg L^{-1} BAP+ 1.0 mg L^{-1} IAA to preserve the genetic resource via slow growth preservation. BA was determined as efficient cytokinin for *in vitro* regeneration of different Campanula species (Brandt, 1994; Airo et al., 2009; Paunescu, 2010; Stamenković et al., 2012; Maria, 2014). Sriskandarajah et al., (2001) cultured the *Campanula* sp. on MS medium including 10.0 mg L^{-1} TDZ + 0.25 mg L^{-1} NAA for the regeneration before transformation experiments. According to our observations, TDZ can be used as alternative cytokinin for the *in vitro* micropropagation of *Campanula phitosiana*. Efficient shoot proliferation was obtained from MS medium including 0.3 mg L^{-1} NAA + 2.0 mg L^{-1} GA_3. Sivanesan et al., (2011) germinated the somatic embryos on MS medium including 1.0 mg L^{-1} GA_3. Sevindik et al. (2017) cultured the shoots including nodes of *Origanum sipyleum* on MS medium BA+GA_3 combinations for multiplication. Ullah et al. (2011) reported that addition of GA_3 to the medium promotes the shoot elongation and plant growth (Roest and Bokelmann, 1976; Muller and Lipschutz, 1984). In our study, addition of the GA_3 to the medium induce the shoot formation from callus. To obtain root formation shoots and shoot like formations was transferred to the hormone free MS medium but root formation was not occurred.

4. Conclusion

Protection and sustainability of the genetic recourse is very important issue. *In vitro* techniques provide important advantages to clonal and mess production of the endemic genetic resources. Micropropagation is one of the important techniques to multiplicate the plants by using different parts as explant. *Campanula phitosiana* is native to İzmir and Aydın Mountains and it is defined as critically endangered plant according to red list. Development of efficient micropropagation protocol for this critically endemic species is promising due to sustainability of this species. In a conclusion, to optimize the regeneration protocol, MS medium combined with NAA as auxin, BA and TDZ as cytokinin. Efficient callus regeneration was obtained from the petiole explants. NAA and GA_3 was the efficient combination for the shoot induction. This is the first study that shows the efficient organogenic callus obtention for the *C. phitosiana.* This protocol could be very important for *in vitro* propagation, cryopreservation, synthetic seed production and transformation experiments for *C. phitosiana.*

References

Airo, M., Savona, M., Mascarello, C., De Benedetti, L., Nicoletti, F., Casazza, G., Ruffoni, B., & Minuto, L. (2009). Analysis of *in vitro* performances and genetic variability of several *Campanula sabatia* populations in Italy. In IV International Symposium on Breeding Research on Medicinal and Aromatic Plants-ISBMAP2009 860 (pp. 109-112).

Alçıtepe, E., Everest, A., & Sungur, M.A. (2011). Some soil parameters in *Campanula* species (sect. Quinqueloculares) from Mediterranean climate areas in Turkey. *African Journal of Agricultural Research*, 6(7):1735–1743.

Brandt, K. (1994). Variation among and within clones in formation of roots and shoots during micropropagation of *Campanula isophylla*. *Plant Cell Tissue and Organ Culture*, 39(1):63-68.

Contandriopoulos, J. (1984). Differentiation and evolution of the genus Campanula in the Mediterranean region. In Grant, W.F. (Ed.). Plant biosystematics (ss 140–175). Ontario: Academic Press.

Fedorov, A.A., & Kovanda, M. (1976). *Campanula* L. In Tutin, T.G., Heywood, V.H., Burges, N.A., & Valentine, D.H. (Eds.). Flora Europaea, vol. 4 (ss 74–93). London: Cambridge University Press.

Frello, S., Stummann, B.M., & Serek, M. (2002). Shoot regeneration of *Campanula carpatica* Jacq. (Campanulaceae) via callus phase. *Scientia Horticulturae*, 93(1):85-90.

Grigoriadou, K., Krigas, N., & Maloupa, E. (2014). GIS-facilitated ex situ conservation of the rare Greek endemic *Campanula incurva* Aucher: Seed germination requirements and effect of growth regulators on *in vitro* proliferation and rooting. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, 148(6):1169-1177.

Joung, Y.H., Liao, M.S., Roh, M.S., Kamo, K., & Song, J.S. (2002). *In vitro* propagation of *Campanula glomerata*, 'Acaulis' from leaf blade explants. *Scientia Horticulturae*, 92(2):137-146.

Kolomiets, T.M., Malyarovskaya, V.I., & Samarina, L.S. (2016). *In vitro* conservation of Campanula sclerophylla Kolak endemic endangered species of Western Caucasus. *Plant Tissue Culture and Biotechnology*, 26(2):143-149.

Lammers, T.G. (2007). Campanulaceae Jussieu, Gen. Pl. 163 (1789), nom. Cons. In Kadereit, J.W. & Jeffrey, C. (Eds.). The families and genera of vascular plants, Vol. VIII, Asterales (ss 26-57). Springer.

Maria, A.E. (2014). Campanula rotundifolia L specie endangered with extinction, conserved through in vitro techniques. *Analele Universității din Oradea*, *Fascicula: Protecția Mediului*, 23:565-576.

Mørk, E.K., Sriskandarajah, S., & Serek, M. (2005). Influence of seed germination conditions on regenerative ability in *Campanula carpatica*. *European Journal of Horticultural Science*, 70(4):173.

Muller, S.A.,& Lipschutz, L. (1984). Potato. In: Ammirato, P.V., D.A. Evans, W.R. Sharp and Y. Yamada, (Eds.), Handbook of Plant Cell Culture, Collier Mcmillan Publishers, London, 3: 295.

Özdöl, T., Altıoğlu, Y., Deniz, İ.G., & Yıldırım, H.A. (2022). New variety of *Campanula myrtifolia* (Campanulaceae) from South Anatolia, Turkiye. *Herbarium Turcicum*, 1:37-43.

Paunescu, A. (2010). *In vitro* propagation of *Campanula polymorpha* Witas. An endemic plant of Carpathian Mountains. *Analele Universităţii din Oradea - Fascicula Biologie*, 17(2):235-238.

Roest, S., & Bokelmann, G.S. (1976). Vegetative propagation of *Solanum tuberosum* L. *in vitro*. *Potato Research,* 19:173-178.

Röper, A.C., Lütken, H., Christensen, B., Boutilier, K., Petersen, K.K., & Müller, R. (2015). Production of interspecific Campanula hybrids by ovule culture: exploring the effect of ovule isolation time. *Euphytica*, 203(3):643-657.

Scariot, V., Seglie, L., Caser, M., & Devecchi, M. (2008). Evaluation of ethylene sensitivity and postharvest treatments to improve the vase life of four Campanula species. *European Journal of Horticultural Science*, 73(4):166.

Seglie, L., Scariot, V., Larcher, F., Devecchi, M. and Chiavazza, P. M. (2012). *In vitro* seed germination and seedling propagation in Campanula spp. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, 146(1):15-23.

Sevindik, B., İzgü, T., Şimşek, Ö., Tütüncü, M., Çürük, P., Yılmaz, Ö., Kaynak, Y., Aka Kaçar, Y., Teixeira da Silva, J.A., Mendi, Y.Y. (2017). *In vitro* culture of Turkish *Origanum sipyleum* L. *American Journal of Plant Biology*, 2(5-1):32-36.

Shim, J.N., Kim, G.H., & Jeong, B.R. (2005). Effect of CO_2 concentration, NAEH and light intensity on the photoautotrophic growth of Campanula punctata'Rubriflora'plantlets *in vitro*. *Journal of Bio-Environment Control*, 14(4):233-238.

Sivanesan, I., Lee, Y. M., Song, J.Y., & Jeong, B.R. (2007). Adventitious shoot regeneration from leaf and petiole explants of *Campanula punctata* Lam. var. rubriflora Makino. *Propagation Ornamental Plants*, 7:210-215.

Sivanesan, I., Lim, M.Y., & Jeong, B R. (2011). Somatic embryogenesis and plant regeneration from leaf and petiole explants of Campanula punctata Lam. var. rubriflora Makino. *Plant Cell, Tissue, and Organ Culture*, 107(2):365-369.

Sriskandarajah, S., Frello, S., & Serek, M. (2001). Induction of adventitious shoots *in vitro* in Campanula

carpatica. *Plant Cell, Tissue and Organ Culture*, 67(3):295-298.

Stamenkovıć, V., Jelenčıć, B., & Tkalec, M. (2012). Propagation and antioxidative enzymes of *Campanula velebitica* Borbas grown *in vitro*. *Periodicum Biologorum*, 114(1):77-82.

Tanaka, N., Matsuura, E., Terahara, N., & Ishimaru, K. (1999). Secondary metabolites in transformed root cultures of Campanula glomerata. *Journal of plant Pphysiology*, 155(2):251-254.

Ullah, I., Jadoon, M., Rehman, A., Zeb, T., & Khan, K. (2012). Effect of different GA_3 concentration *on in vitro* propagation of potato variety Desiree. *Asian Journal of Agricultural Sciences*, 4(2):108-109.

Yıldırım, H. (2018). *Campanula leblebicii* (Campanulaceae), a new chasmophyte species from western Turkey. *Phytotaxa*, 376:114–122.

Yıldırım, H., & Özdöl, T. (2019). A new suggestion IUCN threat category for *Campanula peshmenii* Güner (Campanulaceae). *Acta Biologica Turcica*, 32(3):168–173.

Yıldırım, H., Şentürk, O., Özdöl, T., & Pirhan, A.F. (2019). A new bellflower, *Campanula phitosiana* sp. nov.(Campanulaceae) from Western Anatolia, Turkey. *Phytotaxa*, 399(1):25-36.