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

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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 (GA₃) (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 GA₃.

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; Alçitepe 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 km²) 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-second or second, flower colour is pale purplish blue (Yıldırım and Özdoğru, 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* 'Aqualis' (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, Dalık 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, Dalık 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 °C, 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 GA₃ (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 containing 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.

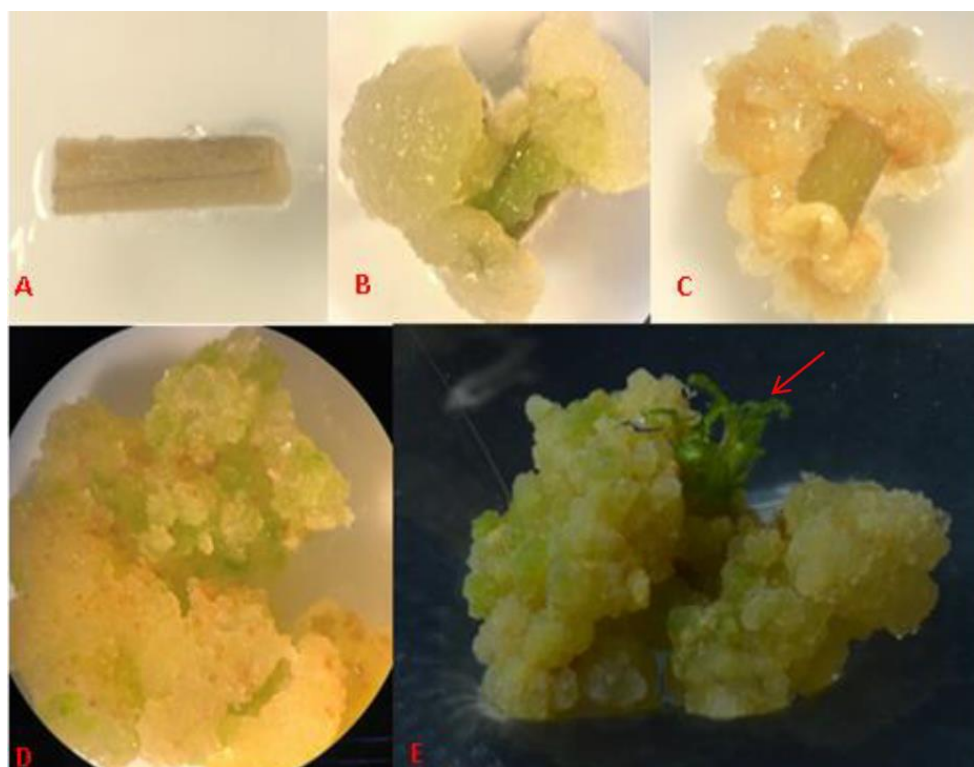


Figure 1. *In vitro* shoot regeneration from the petiole explants of *C. phitosiana* (A: First week of the culture, B-C: Callus at 4th week of the experiments, D: Shoot like formations, E: Shoots from petiole explants at 12th week).

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 GA₃. Shoot formation was obtained only from the MS medium supplied with 0.3 mg L⁻¹ NAA + 2.0 mg L⁻¹ GA₃ 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 microporpagation of *Campanula* species but microporpagation of this endangered *Campanula*

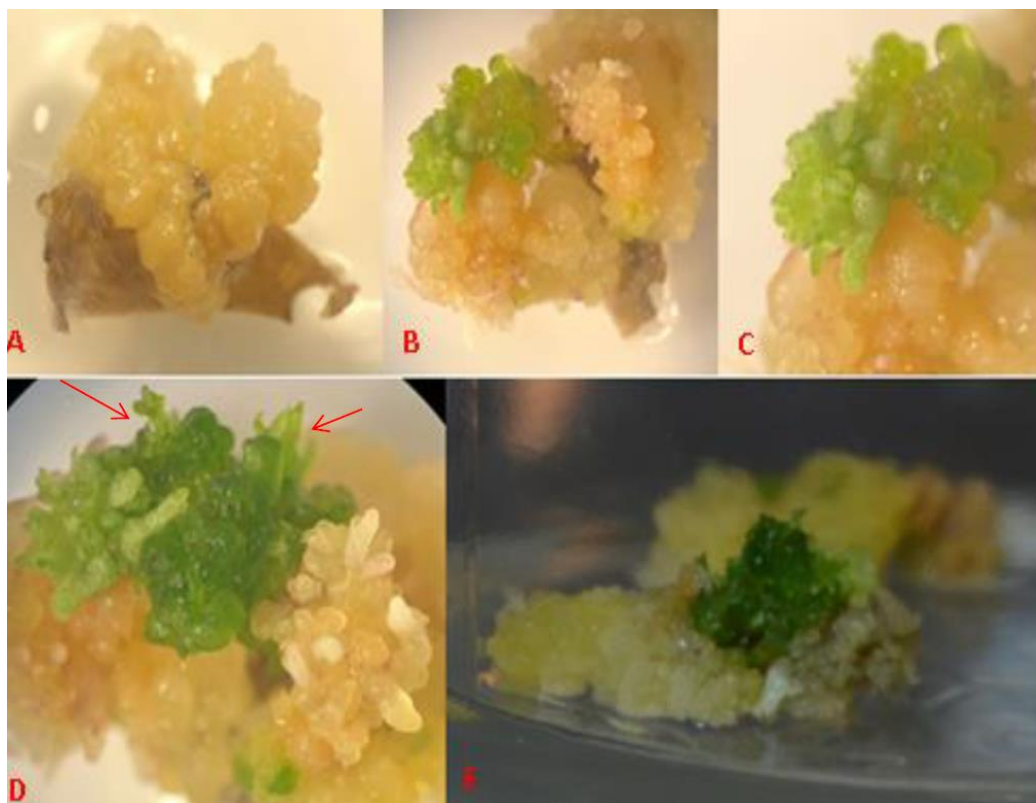


Figure 2. *In vitro* shoot regeneration from the leaf explants of *C. phitosiana* (A: Organogenic callus at the 8th week of the culture, B: Callus colours turned to the green at 10th week, C: Shoot like formations, D-E: Shoots from leaf explants at 12th week).

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 manipulate 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* 'Aqualis' 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 GA₃. Sivanesan et al., (2011) germinated the somatic embryos on MS medium including 1.0 mg L⁻¹ GA₃. Sevindik et al. (2017) cultured the shoots including nodes of *Origanum sipyleum* on MS medium BA+GA₃ combinations for multiplication. Ullah et al. (2011) reported that addition of GA₃ to the medium promotes the shoot elongation and plant growth (Roest and Bokelmann, 1976; Muller and Lipschutz, 1984). In our study, addition of the GA₃ 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 mass production of the endemic genetic resources. Micropropagation is one of the important techniques to multiply 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 endangered 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 GA₃ 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*.

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