

Original Research Article

A Study on endogenous hormone levels and phenolic profiles in embryogenic and non-embryogenic calli of endemic plant *Campanula tomentosa* L.

Abstract

A micropropagation method was developed for *Campanula tomentosa* L. (Campanulaceae), adapted to chasmophytic habitats and colonized on calcareous rocky cliffs. Seeds of *C. tomentosa* were germinated on ½ MS (33%), N6 (30%), B5 (11%) and MS (4%) medias. For callus initiation, leaf explants taken from sterile seedlings were cultured after they were transferred to the MS, ½ MS, N6 and B5 medias. The effect of the auxin/cytokine ratio in medias and the quality of the callus varied depending on the nutrient medium made formation of a different callus type. The development of two callus types was observed, type I composed of globular embryos was observed in the MS medium (85,71%) and type II translucent, watery and lacking any sign of organisation was observed in the B5 medium (71,43%). The objective of this study was to investigate how acetone solvent systems affected the culture medias of the total phenolic content (TPC), and condensed tannins content (CTC) in embryogenic, non-embryogenic callus and in vitro germinated roots. The results showed that the 50% acetone extract exhibited the highest TPC and CTC for both ½ MS type II. The 80% acetone extract exhibited the highest TPC for N6 root and CTC for ½ MS root.

Keywords

Campanula tomentosa L., Endemic, Callus, Endogenous hormones, Phenolic, Tannin

Introduction

The members of this genus *Campanula*, containing the highest endemic species and endemic distribution of Turkey is categorized as CR (critically endangered) according to the

28 International Union for Conservation of Nature (IUCN) criteria[1]. Campanulaceae which is
29 In Turkey the majority (about 66%) of Campanula taxa are chasmophytic species. The rate of
30 endemism is 52% [2,3].

31 Chasmophytic habitats are specific and local shelters but restricts endemic species spreading
32 therefore it is considerably important to understand and investigate seed germination and
33 conservation of endangered endemic species[4].

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35 In vitro seed germination and other in vitro techniques can also be an
36 effective way. Tissue culture research has also been done in some species of this genus .Specially
37 in propagation of endemic species but
38 most Campanula species are propagated by conventional vegetative propagation methods[5].

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41 It is known that plants contain variety of phenols and tannins. Former phytochemical studies on
42 the genus *Campanula* taxa revealed the presence of
43 phenolic acids[6]. Different solvent systems such as methanol, aqueous mixtures of
44 acetone and water are commonly used to extract phenolics from plant materials. There is a lack of
45 literature to compare the effects of acetone solvent on the extraction of calli.

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47 In the present study we aimed to measure the effect of the callus type of *C. tomentosa*, on MS
48 and B5 culture medias on visible morphological characteristics and endogenous abscisic
49 acids and indole-3-acetic acids levels. Also, it was intended to find differences in the
50 acetone extract of the total phenolic content (TPC), and condensed tannin content (CTC) in ½MS
51 and N6 root ,embryogenic and non-embryogenic

52 callus. This research will provide other researchers to investigate phenolic composition included
53 total phenolic content (TPC) and condensed tannin contents (CTC).

54

55 **Materials and Methods**

56 **Chemicals and reagents**

57 Folin–Ciocalteu's phenol reagent, catechin, gallic acid,
58 sodium carbonate and vanillin were obtained from Sigma-Aldrich (St. Louis, Mo., U.S.A.).
59 Hydrochloric acid, acetone, ethanol and methanol were obtained from Merck (Darmstadt,
60 Germany).

61 **Plant material**

62 *Campanula tomentosa* L. seeds were used where gathered from the Selçuk district of İzmir
63 province in SW Anatolia by Dr. Ümit Subaşı. The plant material consisted of cell cultures of
64 embryogenic and non-embryogenic calli from *C. tomentosa* leaf explants obtained from sterile
65 seedlings for analyzing of endogenous hormone levels, total phenolic content (TPC),
66 and condensed tannins content (CTC).

67 **Obtainment of the explants**

68 Before the *Campanula tomentosa* seeds were sown on the nutrient medias, seeds whose
69 surface sterilization was performed with 2.5% sodium hypochlorite (NaOCl) and 2-3 drops of
70 Tween 20 and germinated in four different nutrient medias without including a plant growth
71 regulator. After the completion of the dormancy period, seeds stayed in the nutrient media for
72 about 8-10 weeks, radicles and leaf development were observed. Petiole segments were placed
73 in erlenmeyer flasks containing 30 ml of the MS- ½ MS [7] , N6 [8] and B5
74 [9] medium supplemented with 0,1 mg/l NAA and 4 mg/l BAP , 3 mg/l NAA and 1 mg/l
75 BAP. Different medias effect on petiole explants of the induction and development. In the

76 experiments repeated 3 times with 100 seeds per medium, germination was obtained at
77 different rates.

78 **Establishment of callus culture**

79 Using MS, ½ MS, N6 and B5 medias combinations supplemented with 0,1mg/l NAA and 4
80 mg/l BAP, 3 mg/l NAA and 1mg/l BAP,g/l sucrose and g/l agar whose amounts vary
81 depending on the content of the medium added were dissolved in 1 liter of distilled water,the
82 pH was adjusted to 5,7 and autoclaved at 121°C under a pressure of 1 atmosphere for 15
83 minutes. The erlenmeyer flasks wereincubated20-22 °C in agrowth room under 16h
84 photoperiod with the light intensity of 30-60 µm m-2 s-1.After 1 month of culture,
85 inducedcalliweretransferredtothesamemediums.

86 **Cell suspension cultures**

87 To characterise the endogenous hormonal levels, cell suspension cultures were initiated
88 according to the procedure of Satoh et al. [10] was used to separate embryogenic(E) from
89 non-embryogenic(NE) cells. No level of organisation was observed in the cell clusters that
90 passed through the mesh that were marked as 'NE'. On the other side, the aggregates that 1
91 mm diameter mesh were marked as 'E' and were formed by large aggregates.

92 About 5 g of E and NE calli of *C.tomentosa* divided into small clumps, were shaken for 2h
93 in 100 ml of liquid MS and B5 medias.The calli were then separated from the
94 mediasaccording to the procedure, the cell suspensions were drained through filter paper and
95 sampled for hormone analysis. Each experiment was performed in 3 replications, where 1
96 replication consisted of a flask with 5 g of eitherembryogenic or non-embryogenic calli. The
97 highest type I was observed in the MS medium (85,71%) and type II was in the B5 medium
98 (71,43%) forthisreasonthesemediaschosenforendogenous abscisic acids and indole-3-acetic
99 acids in embryogenic and non-embryogenic calli induced from leaf explants.

100 **Extraction and Analysis of Endogenous IAA and ABA**

According to the methods Scott and Jacops, [11] and Gemici et. al. [12] were used with some modification in the internal hormone extraction in calli were frozen in liquid nitrogen and kept at -50°C , about 5 g of collected calli was extracted, the ethyl acetate phase was evaporated under vacuum to dryness. The dry residue was dissolved in 1 ml of pure methanol and after chromatography processes, significant value to be found on spectrophotometer in 224 nm and 263 nm.

Extraction of samples

The plant sample was ground to fine powder. A portion of 0.5 g of powder was extracted with 5 mL of solvents, including acetone/water (50:50, v/v), acetone/water (80:20, v/v). The mixture was shaken on shaker for 3h, was extracted for 12h. The extracts were centrifuged at 3000 rpm for 15min, and the supernatants were removed into new tubes for the determination of total phenolic contents and condensed tannins content (CTC) in embryogenic and non-embryogenic callus.

Determination of total phenolic and condensed tannin content

The total phenolic content (TPC) was determined by a Folin-Ciocalteu assay [13] using gallic acid as the standard. According to this method, sample solution (50 μL), 250 μL of Folin-Ciocalteu's reagent solution, distilled water (3 mL), and 7% NaCO_3 (750 μL) was vortexed and incubated for 8 min at room temperature. Later, distilled water (950 μL) was added. The mixture was stood for 2h at room temperature and the absorbance was measured at 765 nm against acetone/water as a blank. The total phenolic content was expressed as gallic acid equivalents. Linearity range of the calibration curve of gallic acid was 50 to 1000 $\mu\text{g/mL}$ ($r = 0.99$).

Analysis of condensed tannin content (CTC) was carried out according to the method of Broadhurst and Jones [14]. Sample solution (50 μL), hydrochloric acid (1.5 mL) and 4%

126 methanolvanillinsolution(3 mL)wereadded. Themixturestoodfor 15 min,
127 andtheabsorbancewasmeasured at 500 nmagainstacetone/waterblank. Theamount of
128 condensedtanninwasexpressed as mg catechinequivalents. Linearityrange of
129 thecalibrationcurveof catechinwas 50 to 1000 $\mu\text{g/mL}$ ($r = 0.99$).

130 **Statistical analysis**

131 Analyses were performed in triplicate.The data were analyzed by ANOVA using 2013 SAS
132 (Version 22). Duncan's multiple range test was used to determine significant differences in
133 endogenous hormone, phenol and tannin levels. Means and the germination rate (%) of the
134 seeds cultivated in different media was considered significant levels were defined using
135 $P < 0.05$.

136 **Results and Discussion**

137 **Germination**

138 Due to *Campanulatomentosa*(Fig 4A-B)critically endangered chasmophyt endemic and low
139 germination potential motivated us to undertake the present study.Optimal
140 conditionsforseedgerminationandpropagationwas successfully established.
141 Resultsshowedgerminationdifferencesamongtheeffects of nutrient media inresponsetothe
142 medium, where the highest germination (Figs4C-D) was observed in the absence of
143 phytohormone was the $\frac{1}{2}$ MS (33% germination) the worst,verylow rate germination seen
144 was MS media.Adaptation to chasmophytic calcareous habitats, endogenoushormonelevels,
145 physiologicalseeddormancyand increased mineral nutrient rate caused negative osmotic
146 potential effect in seeds germination rate.

147 **Total phenolicandcondensedtanin content**

148 The different ratio of auxin/cytokine in the nutrient medium is formed different callus types
149 as a result of embryogenicresponse.Comparison of the density,morphologic features of the
150 calli(Table 1)revealed that liketype I(Figs 4E-F)callus tight-granular, pale green structure is

the desired feature for the shoot regeneration from the tissue, but type II callus isn't suitable for the shoot regeneration which formed in the 1/2 MS and N6 medias are soft, spongy tissue and turned brown because of tannin accumulation. Tannins are formed via condensation of phenolics. As tannin synthesis proceeds, after its transposition within the vacuole glucose may be released and react with the phenolic acid to form the tannin molecule. The synthesis of tannin, may lead to cell death and turned brown.

The acetone extracts of TPC and CTC from 1/2 MS and N6 medias are represented in Fig 3. Our results clearly indicated that ratio of acetone for extracting phenolics and tannins from *C. tomentosa*'s non-embryogenic calli (type II) were different in all nutrient medias. The TPC extracted by the 50% acetone was in the following order from high to low: 1/2 MS type II, N6 root, N6 type II, 1/2 MS type I, N6 type I and 1/2 MS root. By the 80% acetone was in the following order from high to low: N6 root, 1/2 MS type I, 1/2 MS type II, N6 type I, 1/2 MS root and N6 type II. For CTC 50% acetone was in the following order from high to low: 1/2 MS type II, N6 type II, N6 type I, N6 root, 1/2 MS type I and 1/2 MS root. For the 80% acetone was in the following order from high to low: 1/2 MS root, N6 type I, 1/2 MS type I, 1/2 MS type II, N6 root and N6 type II.

These results showed that ratio of acetone had considerable effects. In *C. tomentosa* in vitro propagation protocol developed, germination, callus type, total phenolic contents, and condensed tannin contents is reported for the first time.

Endogenous IAA and ABA

Endogenous hormone levels related with morphogenetic capacity of the explants. The morphological characteristics of the two types of callus can be obtained from same explant [15]. The role of the auxin/cytokine ratio in different nutrient medias led to the formation of different quality of the callus types. The soft and pale green-brown callus structure (Type II callus = NE) and organogenic structure, shoot formations were observed on the calli (Type I

callus=E). To determine the effect of plant growth regulator on their further development and their endogenous hormone levels in E and NE calli are presented (Fig 2). ABA may have a significant role in vitro culture-raised plantlets [16]. Levels of ABA were not similar in both types of calli, whereas the ABA was higher in the E calli when compared to the NE ones, found a little difference in the endogenous IAA contents between E and NE callus lines of *C. tomentosa* cultured on B5 medium containing 0.1 mg/l NAA + 4 mg/l BAP. But in MS medium IAA levels were 2 times higher in NE callus cells than in the E ones. Levels of IAA reduced somatic embryogenesis and enhanced the formation of NE callus. These results indicate that endogenous IAA is one of the important factors controlling the embryogenic capacity of leaf explants in *C. tomentosa*.

Endogenous levels of ABA appear to be significant for initiation of E cultures, higher levels of ABA in E callus of *Pennisetum purpureum* Schum. and according to Kiyosue et al. [17]. Endogenous levels of ABA in E carrot cells were 67.2 times higher than that in NE cells per unit of fresh weight. ABA was 10 times more found in the E compared to the NE carrot callus cultures on a dry weight. They presumed that a high level of endogenous ABA may be necessary to induce E capacity in carrot culture systems. According to Lema-Rumińska et al. [18], adding a low amount of ABA rises up the elongation of embryos, while the high amount of ABA prevents growing. According to Pasternak et al. [19], in alfalfa, it has been even possible to compare evolution of endogenous hormone contents in E and NE cultures. These authors identified a peak in IAA synthesis in NE cultures, which showed a delay, when compared to the peak observed in E cells.

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CAPTIONS

Figures:

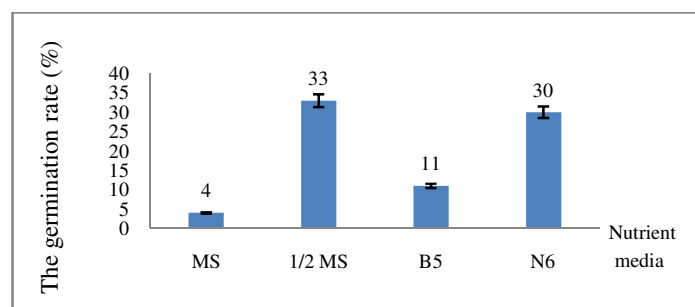


Figure 1. The germination rate (%) of the seeds cultivated in different media.

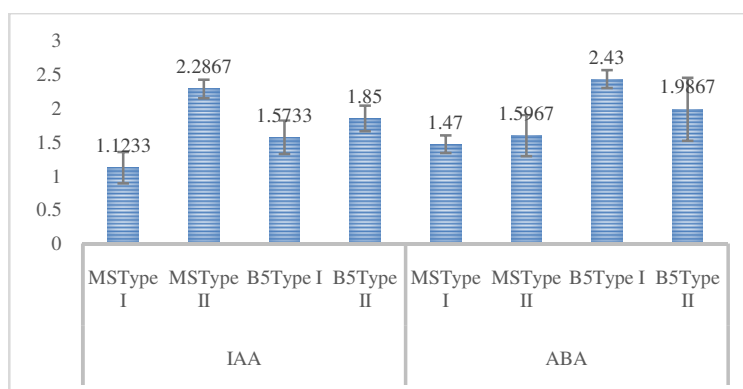
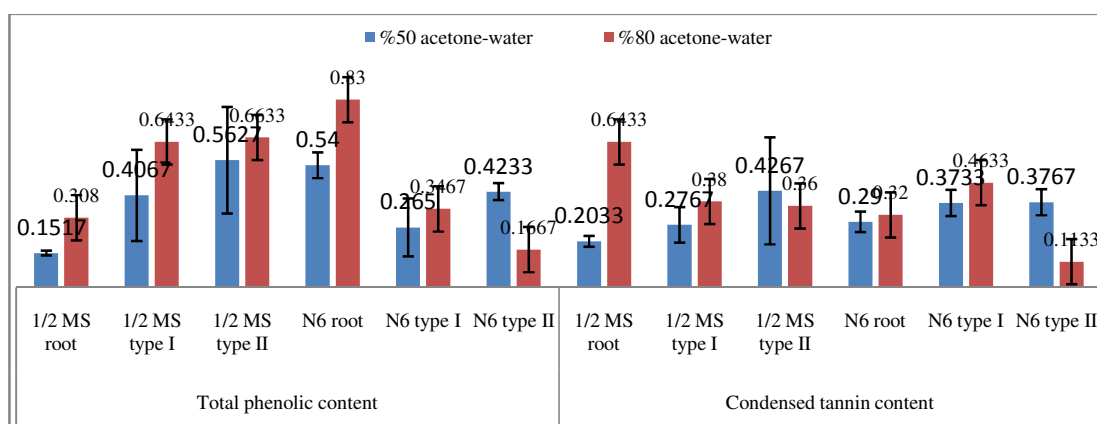


Figure 2.Endogenous hormone contents in Embryogenic (Type I) callus and Nonembryogenic (Type II) cultures on MS and B5 medias.



241 **Figure 3.**Differences in the acetoneextractof theTPC andCTCin $\frac{1}{2}$ MS and N6
242 rootembryogenic and non-embryogenic callus.

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245 **Figure 4.** *Campanula tomentosa*: A-B. in natural habitat (Photos taken by H.Yıldırım), C-D:
246 germinated seeds and sterile seedlings in $\frac{1}{2}$ MS, E. typeI callus(E), F. typeII callus (NE).
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Tables:

Table 1. Formation rates of Type I callus and Type II callus in terms of the features of the nutrient media.

Nutrient medias	Embryogenic (Type I) callus(%)	Non-embryogenic (Type II) callus(%)
MS (0,1mg/l NAA and 4 mg/l BAP)	85,71	14,29
MS (3 mg/l NAA and 1mg/l BAP)	75	25
½ MS(0,1mg/l NAA and 4 mg/l BAP)	62,50	37,50
½ MS (3 mg/l NAA and 1mg/l BAP)	45	55
N6 (0,1mg/l NAA and 4 mg/l BAP)	80	20
N6 (3 mg/l NAA and 1mg/l BAP)	44,44	55,56
B5 (0,1mg/l NAA and 4 mg/l BAP)	28,57	71,43
B5 (3 mg/l NAA and 1mg/l BAP)	42,86	57,14