

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 organization 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 the genus *Campanula*, containing the highest endemic species and endemic distribution of Turkey are categorized as CR (critically endangered) according to the

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

Comment [D1]: In Turkey 66% of campanula taxa are of chasmophytic species and the rate of endemism is 52%.

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

34
35 *In vitro* seed germination and other *in vitro* techniques can also be an effective way. Tissue
36 culture research has also been done in some species of this genus. Specially
37 In propagation of endemic species but most Campanula species are propagated by
38 conventional vegetative propagation methods [5].

39
40
41 It is known that plants contain variety of phenols and tannins. Former phytochemical studies
42 on the genus *Campanula* taxa revealed the presence of phenolic acids [6]. Different solvent
43 systems such as methanol, aqueous mixtures of acetone and water are commonly used to
44 extract phenolics from plant materials. There is a lack of literature to compare the effects of
45 acetone solvent on the extraction of calli.

46
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 acetone
50 extract of the total phenolic content (TPC), and condensed tannins content (CTC) in ½MS
51 and N6 root, embryogenic and non-embryogenic callus. This research will provide other

52 researchers to investigate phenolic composition included total phenoliccontent (TPC) and
53 condensed tannin contents (CTC).

54

55 **Materials and Methods**

56 **Chemicals and reagents**

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

60 **Plant material**

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

66 **Obtainment of the explants**

67 Before the *Campanula tomentosa* seeds were sown on the nutrient medias, seeds whose
68 surface sterilization was performed with 2.5% sodium hypochlorite (NaOCl) and 2-3 drops of
69 Tween 20 and germinated in four different nutrient medias without including a plant growth
70 regulator. After the completion of the dormancy period, seeds stayed in the nutrient media for
71 about 8-10 weeks, radicles and leaf development were observed. Petiole segments were
72 placed in erlenmeyer flasks containing 30 ml of theMS- ½ MS [7] , N6 [8] and B5 [9]
73 medium supplemented with 0,1mg/l NAA and4 mg/l BAP , 3 mg/l NAA and 1mg/l BAP.
74 Different medias effect on petiole explants of the induction and development. In the
75 experiments repeated 3 times with 100 seeds per medium, germination was obtained at
76 different rates.

77 **Establishment of callus culture**

78 Using MS, ½ MS, N6 and B5 medias combinations supplemented with 0,1mg/l NAA and 4
79 mg/l BAP, 3 mg/l NAA and 1mg/l BAP g/l sucrose and g/l agar whose amounts vary
80 depending on the content of the medium added were dissolved in 1 liter of distilled water the
81 pH was adjusted to 5,7 and autoclaved at 121°C under a pressure of 1 atmosphere for 15
82 minutes. The erlenmeyer flasks were incubated 20-22°C in a growth room under 16h
83 photoperiod with the light intensity of 30-60 µm m⁻² s⁻¹. After 1 month of culture, induced
84 calli were transferred to the same mediums.

85 **Cell suspension cultures**

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

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

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

100 According to the methods of Scott and Jacops,[11] and Gemici *et. al.* [12] were used with
101 some modification in the internal hormone extraction in calli were frozen in liquid nitrogen

102 and kept at -50°C , about 5 g of collected calli was extracted, the ethyl acetate phase was
103 evaporated under vacuum to dryness. The dry residue was dissolved in 1 ml of pure methanol
104 and after chromatography processes, significant value to be found on spectrophotometer in
105 224 nm and 263 nm.

106 **Extraction of samples**

107 The plant sample was ground to fine powder. A portion of 0.5 g of powder was extracted
108 with 5 mL of solvents, including acetone/water(50:50,v/v), acetone/water (80:20, v/v).The
109 mixture was shaken on shaker for 3h ,was extracted for 12h. The extracts were centrifuged at
110 3000 rpm for 15min, and the supernatants were removed in to new tubes for the determination
111 of total phenolic contents and condensed tannins content (CTC) in embryogenic and non-
112 embryogenic callus.

114 **Determination of total phenolic and condensed tannin content**

115 The total phenolic content (TPC) was determined by a Folin Ciocalteu assay[13] using gallic
116 acid as the standard. According to this method, sample solution (50 μL), 250 μL of Folin-
117 Ciocalteu's reagents solution, distilled water (3 mL), and 7% NaCO_3 (750 μL) was vortexed
118 and incubated for 8 min at room temperature. Later, distilled water (950 μL) was added. The
119 mixture was stood for 2h at room temperature and the absorbance was measured at 765 nm
120 against acetone/water as a blank. The total phenolic content was expressed as gallic acid
121 equivalents. Linearity range of the calibration curve of gallic acid was 50 to 1000 $\mu\text{g/mL}$ ($r =$
122 0.99).

123 Analysis of condensed tannin content (CTC) was carried out according to the method of
124 Broadhurst and Jones[14]. Sample solution (50 μL), hydrochloric acid (1.5mL) and 4%
125 methanol vanillin solution (3 mL) were added. The mixture stood for 15 min, and the
126 absorbance was measured at 500 nm against acetone/water blank. The amount of condensed

127 tannin was expressed as mg catechin equivalents. Linearity range of the calibration curve of
128 catechin was 50 to 1000 µg/mL ($r = 0.99$).

129 **Statistical analysis**

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

135 **Results and Discussion**

136 **Germination**

137 Due to *Campanulatomentosa* (Fig 4A-B) critically endangered chasmophyt endemic and low
138 germination potential motivated us to undertake the present study. Optimal
139 conditions for seed germination and propagation was successfully established.
140 Results showed germination differences among the effects of nutrient media in response to the
141 medium, where the highest germination (Figs 4C-D) was observed in the absence of
142 phytohormone was the ½ MS (33% germination) the worst, very low rate germination seen
143 was MS media. Adaptation to chasmophytic calcareous habitats, endogenous hormone levels,
144 physiological seed dormancy and increased mineral nutrient rate caused negative osmotic
145 potential effect in seeds germination rate.

146 **Total phenolic and condensed tannin content**

147 The different ratio of auxin/cytokine in the nutrient medium is formed different callus types
148 as a result of embryogenic response. Comparison of the density, morphologic features of the
149 calli (Table 1) revealed that like type I (Figs 4E-F) callus tight-granular, pale green structure is
150 the desired feature for the shoot regeneration from the tissue, but type II callus isn't suitable
151 for the shoot regeneration which formed in the ½ MS and N6 medias are soft, spongy tissue

152 and turned brown because of tannin accumulation. Tannins are formed via condensation of
153 phenolics. As tannin synthesis proceeds, after its transposition within the vacuole, glucose may be
154 released and react with the phenolic acid to form the tannin molecule. The synthesis of tannin,
155 may lead to cell death and turned brown.

156 The acetone extracts of TPC and CTC from 1/2 MS and N6 media are represented in Fig
157 3. Our results clearly indicated that ratio of
158 acetone for extracting phenolics and tannins from *C. tomentosa*'s non-embryogenic calli (type
159 II) were different in all nutrient media. The TPC extracted by the 50% acetone was in
160 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
161 and 1/2 MS root. By the 80% acetone was in the following order from high to low: N6 root, 1/2
162 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
163 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
164 and 1/2 MS root. For the 80% acetone was in the following order from high to low: 1/2 MS root,
165 N6 type I, 1/2 MS type I, 1/2 MS type II, N6 root and N6 type II.

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

169 **Endogenous IAA and ABA**

170 Endogenous hormone levels related with morphogenetic capacity of the explants. The
171 morphological characteristics of the two types of callus can be obtained from same explant
172 [15]. The role of the auxin/cytokine ratio in different nutrient media led to the formation of
173 different quality of the callus types. The soft and pale green-brown callus structure (Type II
174 callus = NE) and organogenic structure, shoot formations were observed on the calli (Type I
175 callus = E). To determine the effect of plant growth regulator on their further development and
176 their endogenous hormone levels in E and NE calli are represented (Fig 2). ABA

may 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 differences in the endogenous IAA contents between E and NE callus lines of *C. tomentosa* cultured on B5 medium containing 0,1 mg/l NAA+4mg/l BAP. But in MS medium IAA levels to be 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 was 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 low amount of ABA rise 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:

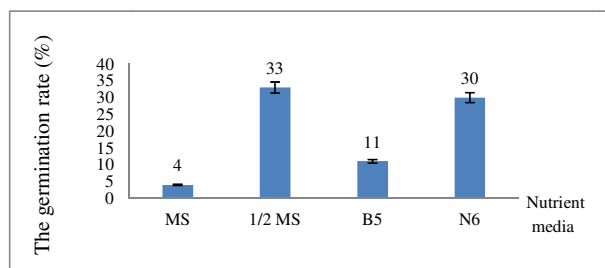


Figure 1. The germination rate (%) of the seeds cultivated in different media.

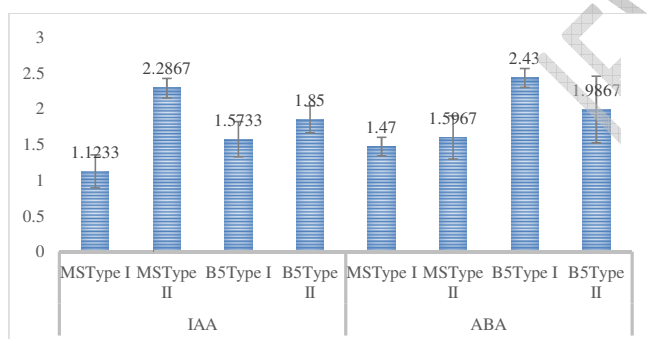


Figure 2. Endogenous hormone contents in Embryogenic (Type I) callus and Nonembryogenic (Type II) cultures on MS and B5 medias.

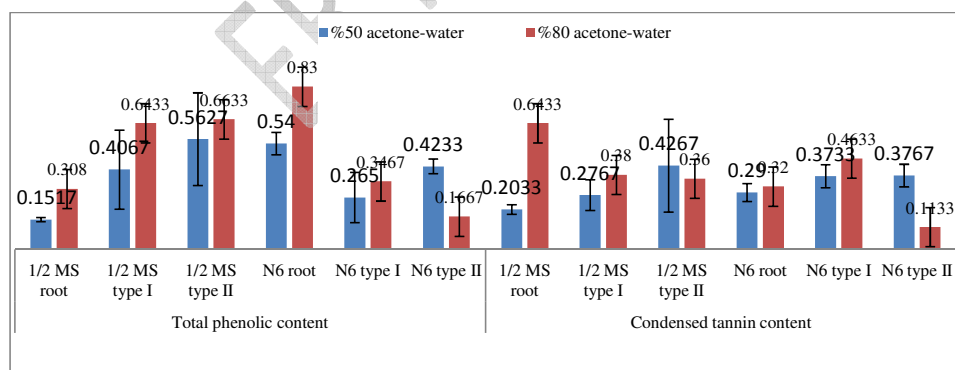


Figure 3. Differences in the acetone extract of the TPC and CTC in 1/2 MS and N6 root embryogenic and non-embryogenic callus.



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

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