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A Study on Endogenous Hormone Levels and Phenolic Profiles in Embryogenic and Non-Embryogenic Calli of Endemic Plant *Campanula tomentosa* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Author MG designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MIC managed the analyses of the study. Author HY managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A micropropagation method was developed for the first time in *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%) media for callus initiation, leaf explants taken from sterile seedlings were cultured after they were transferred to the MS, ½ MS, N6 and B5 media. The effect of the auxin/cytokine ratio in media 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 media of the total phenolic content (TPC), and condensed tannins content (CTC) in embryogenic,

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non-embryogenic callus and in vitro germinated roots. The results showed that the 50% acetone extracts exhibited the highest TPC and CTC for both1/2 MS type II. The 80% acetone extracts exhibited the highest TPC for N6 root and CTC for 1/2 MS root.

Keywords: Campanula tomentosa L.; endemic; callus; endogenous hormones; phenolic; tannin.

1. 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 International Union for Conservation of Nature (IUCN) criteria [1]. The majority (about 66%) of Campanula taxa are chasmophytic species. The rate of endemism is 52% [2,3].

Chasmophytic habitats are specific and local shelters but restricts endemic species spreading therefore it is considerably important to understand and investigate seed germination and conservation of endangered endemic species [4]. In vitro seed germination and other in vitro techniques can also be an effective way. Tissue culture research has also been done in some species of this genus.

Specially in propagation of endemic species but most Campanula species are propagated by conventional vegetative propagation methods [5].

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

In the present study we aimed to measure the effect of the callus type of *C. tomentosa*, on MS and B5 culture media on visible morphological characteristics and endogenous abscisic acids and indole-3-acetic acids levels. Also, it was intended to find differences in the acetone extract of the total phenolic content (TPC), and condensed tannins content (CTC) in ½ MS and N6 root, embryogenic and non-embryogenic callus. This research will provide other researchers to investigate phenolic composition included total phenolic content (TPC) and condensed tannin contents (CTC).

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

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

2.2 Plant Material

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

2.3 Obtainment of the Explants

Before the *Campanula tomentosa* seeds were sown on the nutrient media, seeds whose surface sterilization was performed with 2.5% sodium hypochlorite (NaOCl) and 2-3 drops of Tween 20 and germinated in four different nutrient media without including a plant growth regulator. After the completion of the dormancy period, seeds stayed in the nutrient media for about 8-10 weeks, radicles and leaf development were observed. Petiole segments were placed in erlenmeyer flasks containing 30 ml of the MS- ½ MS [7], N6 [8] and B5 [9] medium supplemented with 0,1 mg/l NAA and 4 mg/l BAP, 3 mg/l NAA and 1 mg/l BAP. Different media effect on petiole explants of the induction and development. In the experiments repeated 3 times with 100 seeds per medium, germination was obtained at different rates.

2.4 Establishment of Callus Culture

Using MS, ½ MS, N6 and B5 media combinations supplemented with 0,1 mg/l NAA and 4 mg/l BAP, 3 mg/l NAA and 1 mg/l BAP, g/l

sucrose and g/l agar whose amounts vary depending on the content of the medium added were dissolved in 1 liter of distilled water, the pH was adjusted to 5, 7 and autoclaved at 121°C under a pressure of 1 atmosphere for 15 minutes.The Erlenmeyer flasks were incubated 20-22°C in a growth room under 16 h photo-periods with the light intensity of 30-60 µm m-2 s-1. After 1 month of culture, induced calli were transferred to the same mediums.

2.5 Cell Suspension Cultures

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

About 5 g of E and NE calli of *C. tomentosa* divided into small clumps were shaken for 2h in 100 ml of liquid MS and B5 media. The calli were then separated from the media according to the procedure, the cell suspensions were drained through filter paper and sampled for hormone analysis. Each experiment was performed in 3 replications, where 1 replication consisted of a flask with 5 g of either embroygenic or non-embryogenic calli. The highest type I was observed in the MS medium (85.71%) and type II was in the B5 medium (71.43%) for this reason these media chosen for endogenous abscisic acids and indole-3-acetic acids in embryogenic and non-embryogenic calli induced from leaf explants.

2.6 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.

2.7 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 12 h. The extracts were centrifuged at 3000 rpm for 15 min, 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.

2.8 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 reagents 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 µg/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), hydrochloricacid (1.5 mL) and 4% methanol vanillin solution (3 mL) were added. The mixture stood for 15 min, and the absorbance was measured at 500 nm against acetone/ waterblank. The amount of condensed tannin was expressed as mg catechin equivalents. Linearity range of the calibration curve of catechin was 50 to 1000 µg/mL (r = 0.99).

2.9 Statistical Analysis

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

3. RESULTS

3.1 Germination

Due to *Campanula tomentosa* (Fig. 4A-B) critically endangered chasmophyt endemic and low germination potential motivated us to undertake the present study. Optimal conditions for seed germination and propagation was successfully established. Results showed germination differences among the effects of nutrient media in response to the medium, where the highest germination (Fig. 4C-D) was observed in the absence of phytohormone was the ½ MS (33% germination) the worst, very low rate germination seen was MS media.

3.2 Total Phenolic and Condensed Tanin Content

The different ratio of auxin/cytokine in the nutrient medium is formed different callus types as a result of embryogenic response. Comparison of the density, morphologic features of the calli (Table 1) revealed that like type I (Fig. 4E-F) callus tight-granular, pale green structure is the desired feature for the shoot regeneration from the tissue. The acetone extracts of TPC and CTC from1/2 MS and N6 medias are presented 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 media. The TPC extracted by the 50% acetone was in the following order from high to low: 1/2 MS type II, N6 root, N6type II, 1/2 MS type I, N6type 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, N6type 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.

3.3 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 explants [15]. The role of the auxin/cytokine ratio in different nutrient media 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 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 endogeneous 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 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.*

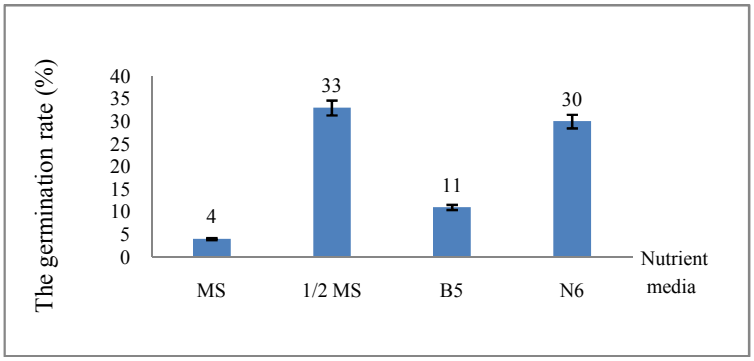

Fig. 1. The germination rate (%) of the seeds cultivated in different media

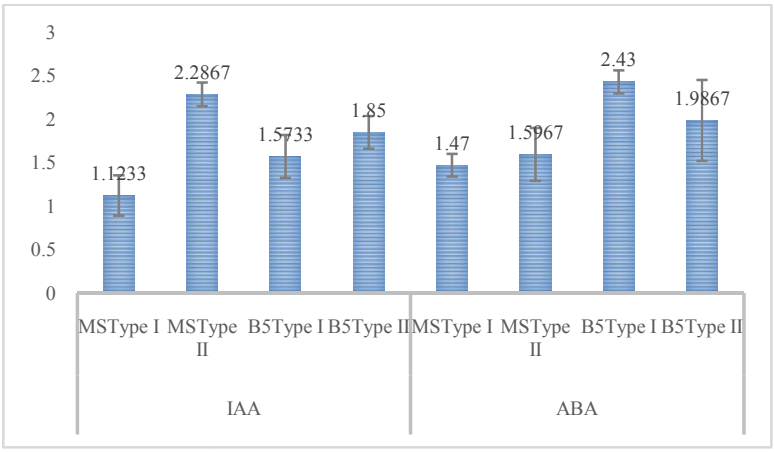

Fig. 2. Endogenous hormone contents in embryogenic (Type I) callus and non embryogenic (Type II) cultures on MS and B5 media

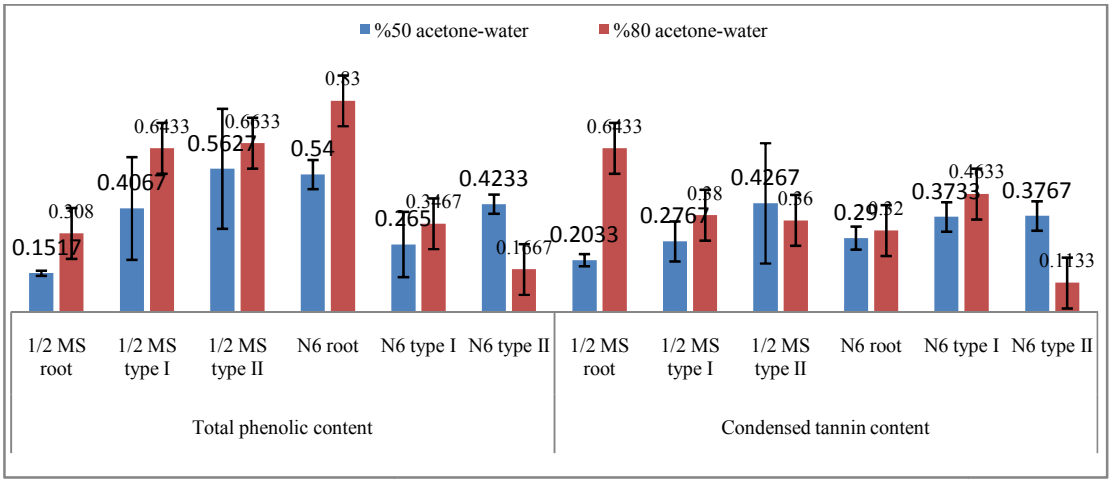

Fig. 3. Differences in the acetone extract of the TPC and CTC in ½MS and N6 root embryogenic and non-embryogenic callus

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

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

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

4. DISCUSSION AND CONCLUSION

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.

Adaptation to chasmophytic calcareous habitats, endogenous hormone levels, physiological seed dormancy and increased mineral nutrient rate caused negative osmotic potential effect in seeds germination rate.

Type II callus isn't suitable for the shoot regeneration which formed in the ½ MS and N6 media 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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