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RESEARCH ARTICLE

Callus induction and secondary metabolite production studies in *Centaurea tchihatcheffii* Fisch. & C.A.Mey.

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Summary

Centaurea tchihatcheffii Fisch. & C.A. Mey. (Mediterranean knapweed, in Turkish yanardoner) is an annual, appealing endemic species, which grows only very little location in the Golbasi district of Ankara-Turkey. Therefore it is very important to produce and multiply valuable endemic species biotechnologically. In order to induce callus, pappus of a group of the seeds was cut (pappus-cut-off seeds) and the other group of the seeds was left intact. Pappus-cut-off seeds germinated in vitro in 3 days whereas intact seeds germinated in 7 days. The best calli were induced from the cotyledons of aseptic seedlings on MS media with the application of cytokinins (benzyl adenine, kinetin). Phytochemicals of the calli and the flowering aerial parts of C. tchihatcheffii were evaluated by thin layer chromatography (TLC). Our studies demonstrated that both the calli and the plant have a secondary metabolite groups, flavonoids and terpenoids. Phytochemical profiles of the callus cultures changed by subculturing. Less polar substances were produced in subcultures I and polar substances in subcultures II. Phytochemical profiles of callus extracts were similar to the plant extracts. Callus induction and secondary metabolite production of C. tchihatcheffii have been studied for the first time.

Key Words: *Centaurea tchihatcheffii, in vitro germination, callus, secondary metabolite, endemic.*

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Centaurea tchihatcheffii Fisch. & C.A.Mey. bitkisinde kallus oluşturulması ve sekonder metabolit üretimi çalışmaları

Özet

Centaurea tchihatcheffii Fisch. & C.A. Mey. (Yanardöner) Gölbaşı bölgesinde (Ankara-Türkiye) çok küçük bir alanda yetişen tek yıllık, lokal endemik bir türdür. Değerli endemik türlerin çoğaltılması ve üretimi biyoteknolojik olarak çok önemlidir. Kallus üretimi için bu tohumların bir grubun pappusları kesilirken diğer grubun tohumları sağlam bırakılmıştır. Pappusu kesilen tohumlar 3 gün içinde çimlenirken sağlam bırakılan tohumlar 7 gün içinde çimlenmişlerdir. En iyi kallus oluşumu MS ortamlarında sitokinin uygulamasıyla (benzil adenin, kinetin), aseptik fidelerin kotiledonlarında uyarılmıştır. C. tchihatcheffii'nin kallus ve çiçekli örneklerinin fitokimyasalları ince tabaka kromatografisi ile değerlendirilmiştir. Çalışmamızda kallus ve bitkinin sahip olduğu sekonder metabolit grupları flavonoidler ve terpenoidler olarak tespit edilmiştir. Kallus kültürlerinin fitokimyasal profili alt kültüre gore değişmektedir. Az polar maddeler 1. alt kültürde, çok polar olanlar ise ikinci alt kültürde üretilmiştir. Kallus ekstrelerinin fitokimyasal profili bitki ekstrelerine benzerdir. C. tchihatcheffii'de kallus uyarımı ve sekonder metabolit üretimi ilk kez çalışılmıştır.

Anahtar Kelimeler: *Centaurea tchihatcheffii, in vitro çimlenme, kallus, sekonder metabolit, endemik.*

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INTRODUCTION

Exceeding 700 of *Centaurea* L. species are recorded in Mediterranean Region wherein many of them are endemic in their region. This genus is represented with 177 species in Turkish flora in which 109 (61.6%) is endemic to Turkey (1, 2). One of these is an annual endemic *Centaurea tchihatcheffii* Fisch. et C.A. Mey. (In last literature (3) *Centaurea tchihatcheffii* (Fisch.&C.A.Mey.) Wagenitz & Greuter), Mediterranean Region knapweed, yanardoner, growing in the Golbasi district of Ankara-Turkey with appealing flowers had been facing extinction. According to IUCN (The World Conservation Union) criteria, the plant is considered as Critically Endangered (CR) and Bern Convention on the Conservation of European Wildlife. Moreover, Natural Habitats listed name in absolute protection (4). Therefore the studies focused on the germination of the seeds and then easy regeneration for protection of the name from extinction. Since the seeds have dormancy, chilling was applied to the seeds. Despite the short germination rate that was obtained in 7 to 10 days with the rate of 90%, hyperhydricity caused to seedlings to become abnormal (5).

Besides appealing flowers, most of the *Centaurea* species (*C. phyllocephala* Boiss., *C. cyanus* L.) are known for their medical applications in folk medicine (6, 7, and 8). *C. solstitialis* L. is being used in treatment of ophthalmic symptoms, common cold, peptic ulcers, abdominal pains, malaria and herpes around lips (9, 10). Phytochemical studies showed that *Centaurea phyllocephala* contain tertiary and quaternary alkaloids, sesquiterpen lactones, methylated flavones and their glycosides (6). Sesquiterpene lactones and indole alkaloids from the aerial parts of *C. rupestris* L., *C. repens* L., *C. cineraria* L., *C. pseudoscabiosa* Boiss. & Buhse, *C. paniculata* L., *C. aspera* L., *C. moschata* L. exhibited cytotoxic, antitumor, antibacterial, antifungal and antiviral activities in different studies respectively (11, 12, 13, 14, 15, 16). In addition to sesquiterpens, flavonoid quercetagetin isolated from *Centaurea rupestris* succesfully inhibited tomato bushy stunt virus infection (12).

Despite the limited studies on the plant regeneration (5, 12) of *C. tchihatcheffii* there is no study on the callus production and comparative phytochemical analyzes of the plant and callus cultures. In the light of previous studies, the main goal of this study is to germinate the seeds of *C. tchihatcheffii* in a short time and to establish callus culture from the *in vitro* grown seedlings, additionally, evaluate the callus cultures by comparing their phytochemical content to the plant. Induction and production of callus is the first and the most fundamental step to produce secondary metabolites via biotechnological methods (17, 18, 19).

In this study, seeds of endemic species *C. tchihatcheffii* germinated *in vitro* rapidly and induction of the callus cultures for the first time was accomplished from those seedlings. Phytochemicals, of the callus subcultures and the aerial parts of the plant were compared via thin layer chromatography.

MATERIALS AND METHODS

Plant material

Aerial parts of *Centaurea tchihatcheffii* were collected from the Golbasi district of Ankara-Turkey in May-June 2006 and dried at room temperature for the phytochemical analysis (Figure 1A). Samples were identified by Prof.Dr.~<Mecit VURAL (Faculty of Science, Gazi University, Ankara, Turkey).

Seed germination

The seeds were germinated *in vitro* to obtain aseptic seedlings. The seeds were surface sterilized in 20% sodium hypochlorite (commercial ACE, Ankara, TR) for 10 min. then rinsed with autoclaved distilled water for 3 times. One part of the seeds were separated from their pappus by cutting, the other part was left intact, both pappus-cut-off seeds and intact seeds were placed in a solid MS media (Murashige, Skoog, 1962) (20) supplemented with 2.5% sucrose and 0.8% agar, pH was arranged to 5.8, prior to autoclave sterilization. The aseptic seedlings were incubated at 16/8h day/night photoperiod (irradiance of 42 µmol m^{-2} s^{-1} provided by cool-white fluorescent) at 23 ±1 ^{o}C in jars (100 mm x 200 mm).

Callus induction

MS media containing 2.5% sucrose and 0.8% agar supplemented with 0.5, 1, 1.5 and 2 mg/l in concentrations cytokinins (benzyl adenine -BA,

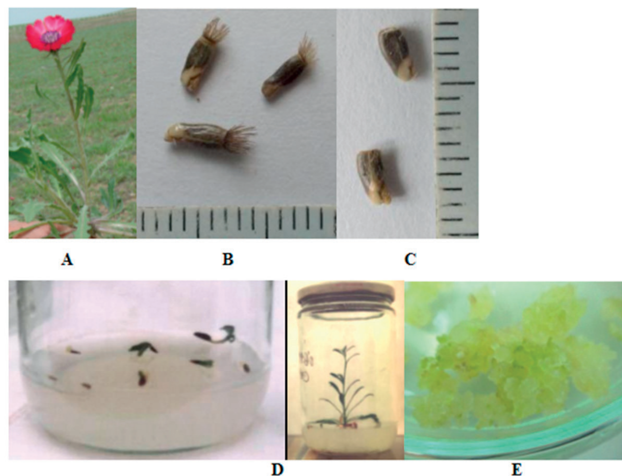

Figure 1. *Centaurea tchihatcheffii* Fisch et. Mey. in its natural habitat (A), intact seeds with acen (B), pappus-cut-off seeds (C), germinated seeds and an aseptic seedling (D), Cream-yellow subculture I, MS media supplemented with 2 mg/l BA (E)

kinetin-KIN) and auxin (2,4-D). Hypocotyl (0.5-1cm), cotyledon (as whole and as two fragments), apical meristem (1mm), young primary leaves (as whole and as two fragments) and stem (0.5-1cm) of 5-7 cm-long aseptic seedlings were used as explants to establish callus cultures (Figure 1B-D).Explants were incubated in solid media in dark for 4 weeks at 22-24°C. Callusing response (%) was determined in the first incubation period.

After 4 week incubation calluses were not grown enough so not harvested but weighted and transferred to the same medium. Than after 3 week we have enough calluses. These calluses weighted and separated two parts. One part were harvested (fist subculture) the other part were transferred to same media for 3 week. After second 3 week all the calluses were harvested (second subculture). First and second subcultures were collected, lyophilized and kept in -21ºC till the phytochemical analysis. Callus growth index (GI) was calculated by using below formula (21).

Callus growth index (GI) = Final Fresh Weight / initial FW

Extraction and TLC

Dried plants leaves, flowers (0.4 g) and lyophilized callus cultures (0.2 g) were ground and extracted with 10 ml methanol in an ultrasonic water bath for 10 minutes then left on water bath at 50°C for 1 h. Extraction method was modified from prior studies (22,23). The samples were applied on to 10x20 cm silica gel plates (Kieselgel 60 F254 DC-Plastikfolien, MERCK) with a micro capillary. Two mobile phase system were applied; System I; Chloroform: Methanol (90:10), System II; Ethyl acetate: Formic acid: Acetic acid: Water (100:11:11:26). The plates were sprayed with Naturstoff-reagent (Diphenylboric acid ethanolamine complex in methanol) for flavonoids and 5% H_2SO_4 followed by drying at 105 ºC for terpenic substances e.g. sesquiterpenes; the plates were investigated under UV (366 nm) (22,23).

Statistical analysis

Data were subjected to one- way analysis of variance (ANOVA) and the differences among means were compared by Duncan's multiple-range test. Each treatment was replicated three times and arranged in completely randomized design. Data given in percentages were subjected to arcsine transformation before statistical analysis.

RESULTS

Surface sterilization was successful with the 20% NaOCl for 10 min. Germination rate was 27.9% for intact seeds whereas 94.2% for the pappus-cut-off seeds. The intact seeds germinated in 7 days, while pappus-cut-off seeds germinated only in 3 days. *In vitro* germinated seedlings were used as an explant source to produce callus culture. The seeds were germinated *in vitro* in three days that is significantly short period compared to previous studies has been achieved *in vitro* in 7-10 days (5). Cutting the pappus of the seeds, most probably, removed the physical barriers (24) and enabled the seeds to germinate faster. Stratification is a well-known method with the hard coat seedlings, on the other hand pappus cutting is not very common method to allow the germinate seeds. Several plant parts such as apical meristem, cotyledons, hypocotyls, leaf, and stem were used as explant sources. The cotyledons were determined as the best source for the desirable callus culture (Table 1). Callus response in all the explants was much better with the media supplemented with BA and/or KIN compared to supplemented

Table 1. Callusing response (%) on MS medium supplemented with BA, Kinetin and 2,4-D.

Growth regulators (mg/l)	Hypocotyls	Cotyledons	Apical meristem	Leaf	Stem
BA					
0.5	43.85 ±0.45 a	**63.20 ±0.20 a**	35.80 ±0.85 a	12.29 ±0.25 a	34.36 ±0.95 a
1	45.38 ±0.56 a	**64.07 ±0.59 a**	**50.51 ±1.43 b**	21.39 ±0.15 b	28.48 ±1.97 b
1.5	44.83 ±0.49 a	**83.73 ±0.83 b**	36.52 ±1.07 a	21.69 ±0.26 b	19.36 ±0.51 b
2	45.38 ±0.19 a	**83.66 ±0.89 b**	26.50 ±0.75 c	21.47 ±0.18 b	23.11 ±1.45 b
Kinetin					
0.5	35.16 ±0.06 a	**44.99 ±0.04 a**	26.35 ±0.25 a	19.99 ±0.57 a	35.38 ±0.17 a
1	35.65 ±0.24 b	**52.55 ±0.29 b**	**54.44 ±0.36 b**	14.17 ±2.85 a	17.22 ±0.40 b
1.5	35.22 ±0.08 ab	**63.46 ±0.06 c**	35.32 ±0.18 c	13.50 ±1.07 a	21.78 ±1.17 b
2	35.65 ±0.24 b	**54.24 ±0.19 d**	35.55 ±0.36 c	14.83 ±0.19 a	24.99 ±2.41 b
2.4-D					
0.5	35.36 ±2.03 a	39.25 ±0.05 a	29.45 ±2.01 a	12.82 ±0.39 a	23.76 ±0.32 a
1	26.83 ±2.53 ab	26.56 ±0.21 b	26.50 ±2.28 a	14.53 ±0.29 b	25.13 ±0.09 b
1.5	27.78 ±2.67 ab	39.06 ±0.20 a	27.88 ±1.70 a	12.69 ±0.29 a	28.11 ±0.08 c
2	25.02 ±3.01 c	39.48 ±0.28 a	28.08 ±2.07 a	9.26 ±0.39 c	24.29 ±0.16 a

Means ±SE mean followed by the same letter are not significantly different using Duncan multiple comparison test within different concentrations of growth regulators

with 2,4-D. Creamy-yellow frangible, easily dispersible callus was obtained when the MS media supplemented with BA and KIN (Fig. 1E). On the other hand the creamy-white callus was obtained on MS media supplemented with 2,4-D. These calluses changed their color and became darker after 3 weeks of subculture.

In callus growth index of cotyledons, the best cell-mass increase was recorded at 1.5 mg/l of BA and 1 mg/l KIN in the 1st subculture, and at 1.5 and 2mg/l of BA and 1 mg/l KIN in the 2nd subculture. There was no significant difference between concentrations of 2,4-D and there was no difference between 1st and 2nd subcultures (Table 2).

Table 2. Callus Growth Index of cotyledon in MS media supplemented with growth hormones in different concentrations

Plant Growth Regulators (mg/l)	First Callus	Subculture I	Subculture II
BA			
0.5	1.13 ±0.21	4.86 ±0.01	3.43 ±0.01
1	1.86 ±0.55	4.04 ±0.01	2.80 ±0.01
1.5	0.68 ±0.35	**7.79 ±0.01**	**3.59 ±0.01**
2	1.49 ±0.64	5.96 ±0.01	**3.87 ±0.005**
Kinetin			
0.5	1.82 ±1.26	3.14 ±0.01	3.20 ±0.01
1	0.79 ±0.59	**7.37 ±0.01**	**3.84 ±0.01**
1.5	0.43 ±0.12	6.58 ±0.01	2.36 ±0.01
2	0.92 ±0.47	3.57 ±0.01	3.16 ±0.01
2.4-D			
0.5	0.83 ±0.11	1.03 ±0.01	1.49 ±0.01
1	0.52 ±0.12	1 ±0.00	1.37 ±0.01
1.5	0.55 ±0.11	1.09 ±0.01	1.35 ±0.01
2	0.51 ±0.11	1.29 ±0.01	1.44 ±0.01

The studies revealed that MS media supplemented with different concentration of BA and KIN were the best choice to induce quality callus culture. Cell increase was also significantly greater than the MS media supplemented with 2.4D.

The extracts of the intact plant flowers, leaves and the calluses were comparatively analyzed by thin layer chromatography (TLC). Two different solvent systems were applied to observe the phytochemical profile of the plant and subcultures thoroughly. Luteolin and isoorientin standards, which have been found in some Centaurea species e.g *C. montana* and *C. calolepis*, were applied to evaluate the spots in the plants and the cultures (Fig.2B) (25,26). Solvent system I revealed less polar substances and were seen more spots in subculture I, whereas solvent system II revealed polar substances and better profile were seen in subculture II. The plant extracts were compared to subculture I and subculture II extracts at figure 2A-B.

DISCUSSION

Despite of the *in vitro* regeneration studies on the plants, no study has been report about the callus induction and comparison of phytochemical content of the plant and callus cultures (5, 23). Although auxins are known to be applied to induce callus, cytokinins can also induce callus in some other plants which give response to plant growth regulators (17, 27). Vidal *et al*. (2004) (28) reported that application of only auxins were not successful to induce callus from *C. solstitialis*, moreover, callus production was slowed down if there is no cytokinin was employed in the media. In this study, cytokinins were more effective on the induction and maintenance of the

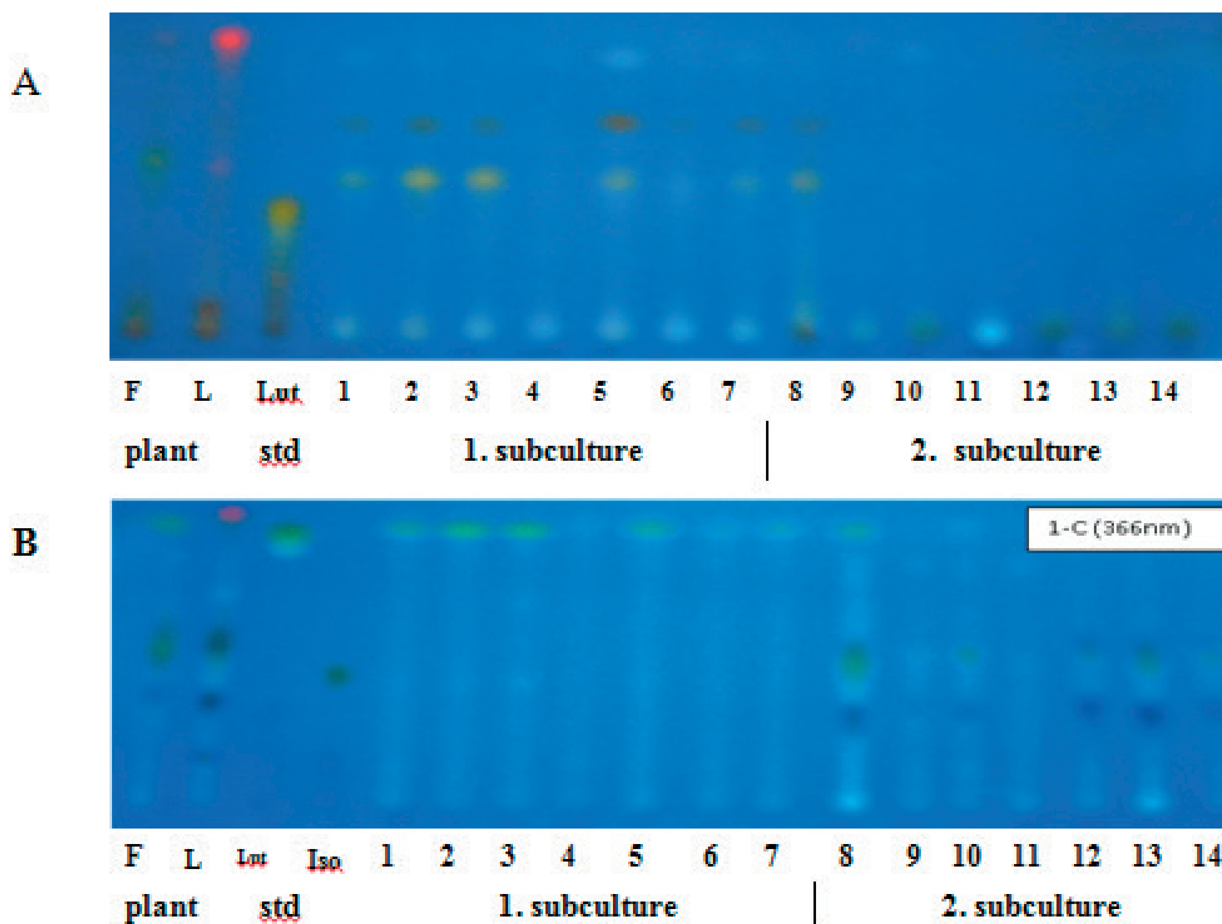

Figure 2. TLC analysis of the methanolic extracts at solvent system I (**A**) and solvent system II (**B**) with Naturstoff-reagent; F (Flowers), L (Leaves), Lut (luteolin), Iso (isoorientin), 1 (0.5BA), 2 (1BA), 3 (2BA), 4 (0.5 2,4D), 5 (0.5K), 6 (1K), 7 (2K), 8 (0.5BA), 9 (1BA), 10 (2BA), 11 (0.5 2,4D), 12 (0.5K), 13 (1K), 14 (2K)

callus form C. *tchihatcheffii* than auxins. Application of BA and KIN as auxins demonstrated productive results, on the other hand, application of 2,4-D did not display very pleasing results.

Less polar substances in plants and calluses were observed in the subculture I. The subculture II revealed more spots belongs to polar substances. As seen in figure 2, phytochemical profiles of the callus cultures changed by subculturing. Less polar substances were produced in subcultures I and polar substances in subcultures II. Phytochemical profiles of callus extracts were similar to the plant extracts.

Varieties of phytochemical studies have been completed on *Centaurea* species (12, 13, 28, 30, 31). Flavone C- glycosides like isoorientin were observed in other *Centaurea* species (32). *C. tchihatcheffii* might also contain Flavone C glycosides derivatives. According to our preliminary phytochemical data, plant and callus cultures might have flavonoids for naturstoff reagent revealed yellow colored spots on TLC plate. Also purple spots with H_2SO_4 reagent might be belong to terpenes e.g. sesquiterpenes. Phytochemicals of the plant and the callus cultures will be further analyzed and major ones will be identified.

CONCLUSIONS

Our studies have shown that C. *tchihatcheffii* does not just have alluring flowers; it may be potentially valuable phytochemical content. *In vitro* seed germination and propagation followed by plant tissue and cell cultures that have low impact on wild population with the use of minimum plant material can be very efficient to conserve the plant and to produce the groups of phytochemicals.

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