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***Correspondence Address***
Trakya Üniversitesi Fen Bilimleri Enstitüsü Binası, Balkan Yerleşkesi – 22030 Edirne / TÜRKİYE
e-mail: tujns@trakya.edu.tr
Tel: +90 284 2358230
Fax: +90 284 2358237

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**Research Article**

# MICROWAVE IRRADIATION SYSTEM FOR A RAPID SYNTHESIS OF NON-TOXIC METALLIC COPPER NANOPARTICLES FROM GREEN TEA

Israt JAHAN*, Ibrahim ISILDAK

Department of Bioengineering, Yıldız Technical University, TURKEY

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***Edited by:***
*Yeşim Sağ Açıkel*

***Corresponding Author:**
*Israt Jahan*
*sonia.israt@yahoo.com*

***ORCID ID:***
*orcid.org/0000-0003-4166-1617*

***Key words:***
*Green tea*
*Microwave-assisted green synthesis*
*Bioreduction*
*Non-toxic metallic CuNPs*

**Abstract:** This paper presents a rapid protocol of microwave-assisted green synthesis of non-oxidized metallic copper nanoparticles (CuNPs) using green tea (*Camellia sinensis* (L.) Kuntze) extract. Following the successful biosynthesis, characterization techniques such as UV–vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM) associated with Energy Dispersive X-ray analysis (EDX), X-ray Diffraction (XRD) and Zeta analysis were employed to confirm the presence of metallic CuNPs and reveal their morphology. UV–vis spectrum of fabricated CuNPs indicated its characteristic maximum absorbance at 570 nm. Synthesized CuNPs were found to be round to globular in shape, with average size of 45.30 nm, and showed excellent stability without any aggregation for several months. EDX graph confirmed the highest amount of copper atoms (77.96%) along with carbon and oxygen with the percentage of 17.17% and 4.87%, respectively. The non-toxic nature of the phytosynthesized CuNPs was further established by using healthy mouse fibroblast L929 cell line, which showed their potentiality for biological research and many other applications.

**Özet:** Bu makale, yeşil çay (*Camellia sinensis* (L.) Kuntze) ekstraktsiyonu kullanılarak oksitlenmemiş metalik bakır nanopartiküllerinin (CuNPs) mikrodalga destekli yeşil sentezinin hızlı bir protokolünü sunmaktadır. Başarılı biyosentezi tamamladıkten sonra, bakır nanopartiküllerin varlığını doğrulamak ve morfolojilerini ortaya çıkarmak için UV–vis absorpsiyon spektroskopi, Fourier Dönüşümlü Kızıl Ötesi Spektrometresi (FTIR), Enerji dağıtıcı X-ışını analizi (EDX) ile ilişkili Taramalı elektron mikroskobu (SEM), X-ışını Kırınımı (XRD) ve Zeta analiz gibi karakterizasyon teknikleri uygulanılmıştır. Üretilmiş CuNP'lerin UV-vis spektrumu, 570 nm'de karakteristik maksimum absorbansını göstermiştir. Sentezlenen CuNP'lerin, ortalama 45,30 nm büyüklüğünde yuvarlaktan küre şeklinde, birkaç ay boyunca herhangi bir agregasyon olmadan mükemmel stabilite sergilediği bulunmuştur. EDX grafiği, karbon ve oksijenın sırasıyla %17,17 ve %4,87'lik oranlarla birlikte en yüksek miktarda bakır atomunu (%77,96) doğrulanmıştır. Son olarak, sağlıklı fare fibroblast hücreleri (L929 hücre çizgisi) üzerindeki biyosentezlenmiş bu CuNP'lerin non-toksik özelliği doğrulanmıştır ve bu durum, bunların biyolojik araştırmaların yanı sıra geniş kapsamlı uygulamalarda potansiyellerini de göstermektedir.

## Introduction

Noble metallic nanoparticles (NPs) have been attracted by the scientists of different fields, as a result of their unique magnetic, optical, catalytic, and electrical properties, and wide range multidisciplinary applicability (Tsuji *et al*. 2005). Concerning eco-safety, it is necessary to expand environmental friendly approaches without using toxic and hazardous chemicals. Use of plant-based extractions for the synthesis of metallic nanoparticles is advantageous, which is convenient, cost effective and non-hazardous for the environment (Jha *et al*. 2009, Annamalai *et al*. 2011). Moreover, synthesis of nontoxic metallic nanoparticles is also necessary since these particles are utilized extensively in the areas of human contact.

Copper nanoparticles have earned more importance because of their historical usages as coloring agents, as well as their broad-spectrum bioactivity and biomedical application in contemporary time. In recent times, they are taken as one of the most applied disinfectants for drinking-water purification due to their strong antimicrobial potential (Ruparelia *et al*. 2008). Besides, catalytic activities, high thermal and electrical conductivities of CuNPs facilitate their suitability for developing different biosensors and electrochemical sensors (Wei *et al*. 2010). Moreover, copper is most abundant naturally occurring metallic element; therefore, this metal is cheaper than other metals, i.e. platinum, gold and silver; therefore, CuNPs synthesis is

more profitable than other metallic nanoparticles. Nonetheless, the biggest problems for synthesizing stable CuNPs are their susceptibility to aggregation and tendency to oxidize easily during NPs production (Lee *et al*. 2013, Dang *et al.* 2011). For this reason, a rapid green synthesis method with suitable plant extract for the production of non-oxidized metallic copper nanoparticles is able to resolve this problem. Aiming this, plant mediated synthesis with microwave irradiation could be the fast and facile option for copper nanoparticle production. Microwave irradiation provides a fast and homogeneous heating system which confirms consistent nucleation and growth of nanoparticles in the reaction medium within a short period of time, increase the rate of capping by plant extracts, and speed up the stabilization process of NPs; and thereby, reduce oxidation and aggregation rate of CuNPs (Joseph & Mathew 2015, Nasrollahzadeh & Sajadi 2015). Previous studies have suggested that the microwave-assisted synthesis scheme is the effective approach for producing highly stable metallic CuNPs (Yallappa *et al.* 2013, Nasrollahzadeh & Sajadi 2015, Sreeju *et al.* 2016, Tanghatari *et al.* 2017). For instance, microwave irradiation was utilized in a study to synthesize zero valent metallic copper (Cu0) nanoparticles from *Terminalia arjuna* (Roxb.) Wight & Arn. bark extract (Yallappa *et al.* 2013). Similarly, highly stable metallic copper nanoparticles (CuNPs) were synthesized by using *Psidium guajava* L. leaf extract and hydrazine through microwave-assisted one-pot method (Sreeju *et al.* 2016). On the other hand, metallic non-oxidized CuNPs were successful synthesized separately from both potato starch and polyvinylpyrrolidone (PVP) after using microwave heating system (Tanghatari *et al.* 2017).

Based on the above mentioned reasons, this study was designed to establish a suitable rapid and facile green synthesis of metallic copper nanoparticles (CuNPs) by using green tea (*Camellia sinensis* (L.) Kuntze) extract under microwave irradiation. Choosing green tea for synthesizing CuNPs in this study was due to the fact that its extract is rich in different biologically active polyphenols, bioflavonoids, alkaloids, caffeine, volatile compounds, amino acids, glucides, proteins, reducing sugars, etc., which could be very effective reducers and stabilizers during the synthesis process (Reto *et al.* 2007). Previously, green tea leaves extract has been utilized for synthesizing copper oxide (Sutradhar *et al.* 2014), iron (Gottimukkala *et al.* 2017, Lourenço *et al.* 2019), zinc oxide (Irshad *et al*. 2018) and silver (Rolim *et al.* 2019) nanoparticles; and different bioactive polyphenols of the green tea extract were found to be common functional compounds that worked as reducing agent as well as capping agent for synthesizing these NPs.

Aiming for a rapid and simplistic synthesis using green tea extract therefore, two very basic parameters i.e., time and temperature of the microwave system were chosen for the production of metallic non-oxidized CuNPs. Afterwards following the successful biosynthesis, various characterization techniques were employed to confirm the presence of CuNPs as well as reveal their shape, size and other morphologic features. Furthermore, cytotoxic effect of phytosynthesized copper nanoparticles was also examined to ensure their safe usages of nano-based researches and application in biological science.

## Materials and Methods

### *Plant Extract Preparation and Fabrication of Cu-Nanoparticles*

In this study, copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) and other chemicals needed were analytical grade, which were acquired from Sigma-Aldrich (St. Louis, MO, USA). Instruments were properly dried and autoclaved before use. 10 gm dried green tea leaves were taken into 250 ml Erlenmeyer flask with 100 ml ultra-purified deionized water, place onto as electric heater (lab-grade) at 80°C for 20 min with continuous rousing using a magnetic stirring bar. Afterwards, Whatman No. 1 filter paper was employed for removing debris from the extract solution was then filtered using and kept at 4°C.

Different ratios of plant extract and salt solution were used to determine the optimum parameters for copper nanoparticle synthesis, and the successful synthesis was obtained when 50 ml fresh green tea extract and 50 ml 1 mM $CuSO_4.5H_2O$ solutions were mixed together and then, heated in the microwave for 2 minutes at 700 W with continuous stirring by magnetic stirrers. Afterward, 5 ml L-ascorbic acid (10%) solution was mixed within the synthesis medium, and then again, placed into microwave for 15 min at 700 W, which provided constant homogenous heating of 160-170°C (power setting: P80). L-ascorbic acid at very low concentration was used as an additional precursor for preventing the oxidation of biosynthesizing nanoparticles especially after post-synthesis phase until purification process since metallic copper nanoparticles are very sensitive to aqueous solution, and tend to oxidized easily (Cheng *et al.* 2006, Suresh *et al*. 2014). After forming a dark blackish brown colloid inside the synthesis medium, Whatman Grade No. 5 filter paper with 2.5 µm pore size was used to eliminate large discarded particles from the sample solutions; then centrifuged 3-4 times at 5000 rpm for 15 minutes at 4°C. Finally, the purified precipitate was dried under vacuum condition; and powdered CuNPs stocked in a dark colored vial, which was stocked up at 4°C further experiments.

### *Characterization of Synthesized CuNPs*

Shimadzu UV-1700 spectrophotometer was used for revealing the optical property of synthesized copper NPs. Using UV-vis quartz cell, powdered nanoparticles suspended in deionized water was used to collect spectral peaks at the rage of 200-800 nm wavelengths where ultra-purified $H_2O$ was taken as blank. Using FT/ IR-6300 (JASCO, Tokyo, Japan) spectrometer, potassium bromide (KBr) pellet (FTIR grade) method was applied to read IR

spectra in the range of 4000-400 $cm^{-1}$. XRD patterns with a step size of 0.02 was taken at the range of 2 h from 10° to 90° via X-ray diffraction (PANalytical Empyrean model) plan for recognizing crystalline nature of synthesized NPs; XRD graph was regenerated by the Origin 8.5 software. Scanning electron microscope adjusted with an EDX analyzer (SEM, SU-1510, Hitachi High-Technologies Corp., Tokyo, Japan) was used in order to identify morphological features and chemical composition of developed nanoparticles. Lastly, particle average size distribution, and potential value were determined using Zeta sizer (model name: Zetasizer nano ZS, Malvern Instruments Ltd., UK).

*Cytotoxicity Study of copper nanoparticles*

The cytotoxicity of biosynthesized CuNPs was evaluated on L929 mouse fibroblast cell lines. Using XTT assay [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] the percentage of viable cells in culture media was determined by observing optical intensity of these viable cells. For maintaining the culture of cell line, DMEM-F12 medium was utilized supplemented with 10% fetal bovine serum and penicillium-streptomycin, which was incubated at 37°C with 5% $CO_2$ air flow. After incubation, completely affluent cells were detached from the upper layers of the cell containing vessels using Trypsin. Afterward, by staining with Trypan blue, the viable cells were identified, and counted from the detached cultured cells. Prior to applying nanoparticle into cell medium, 1 mL medium was used to adjust the density of obtained viable cells to $10^6$. Aiming this adjustment, 100 µL of cell suspension was plated in every well of sterile 96-well flat bottom microplate (BD, Biosciences) within a short period of time. Before incubating at 37°C, the biosynthesized CuNPs was added to cultured cells with an increasing concentration (0, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL). After 24 hours of incubation, old medium was removed, and 100 µL XTT solution (with 0.5 mg/ml DMEM, which was adjusted to Phenazine methosulfate (7.5 µg/mL)) containing 100 ml fresh medium was added, and again incubated for 4 hours at the same temperature. Later on, a multiplate reader (model: Lab-Line Instruments, Melrose Park, IL, USA) at 450 nm was employed for measuring optical density (OD) of active viable cells from the suspension. Lastly, the cell viability was calculated in percentage (%) following this equation (Sahu *et al*. 2016):

$$\text{Cell viability (\%)} = \frac{\text{OD of specimen}}{\text{OD of control}} \times 100$$

## Results

In this study, the production of copper nanoparticles was accomplished using green tea (*Camellia sinensis*) extract, which played the vital role as reducer and stabilizer during the synthesis. Microwave-assisted synthesis system was adjusted based on temperature and time that provided high reaction kinetics in the reaction medium, and confirmed higher yield within a shorter period of time. Initially, the reduction of ionic copper to nanoparticles was confirmed by colloidal formation and color changing in the reaction medium (Fig. 1). Moreover, UV-Vis absorbance of reaction medium after synthesis showed a peak of λmax at 570 nm (Fig. 2) indicating the presence of stabilized non-oxidized copper nanoparticles (Dang *et al*. 2011, Hassanien *et al*. 2018).

Furthermore, XRD analysis confirmed the crystalline nature of phytosynthesized copper nanoparticles. Fig. 3 exhibits the XRD pattern of biosynthesized CuNPs using green tea extract. Five strong diffraction peaks were centered at 43.47°, 50.61°, 74.32°, 90.28° and 95.40°, which according to the standard database of the JCPDS card no: 04-0784, correspond to the planes of (111), (200), (220), (311) and (222) corroborate the presence of face-centered cubic crystalline structures metallic non-oxidized copper particles (Otte 1961).

Fourier transform infrared (FTIR) spectroscopy of fabricated nanoparticles revealed diverse functional groups of biomolecules that coated with the nano-scaled particles by creating a layer, and worked as reducer and stabilizer agents during the development of CuNPs (Usha *et al*. 2017). Fig. 4 represents FTIR spectra of CuNPs phytosynthesized via green tree extract. Peaks were observed mainly at 3,560.89 $cm^{-1}$ for O-H stretching; 2,917.42 $cm^{-1}$ for medium alkane C-H stretching; 1,631.24 $cm^{-1}$ for strong alkene monosubstituted bond; 1,094.80 $cm^{-1}$ for strong C-O stretching alcohol bond; 613.40 $cm^{-1}$ for -C-X bond (X=bromide) and 426.90 $cm^{-1}$ for metal ligand bond. Considering the existence of these functional groups with biosynthesized nanoparticles, FTIR spectra therefore have confirmed that nanoparticles obtained in this study were enclosed, capped, and stabilized by some amino acid residues, proteins, reducing sugars, polyphenols, flavanones, and terpenoids available in green tea extract (Usha *et al*. 2017)

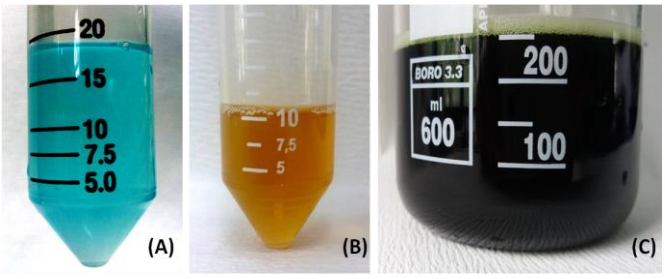


**Fig. 1.** (A) Copper (II) sulphate pentahydrate solution; (B) aqueous extract of green tea; and (C) color changed after the synthesis of copper nanoparticles.

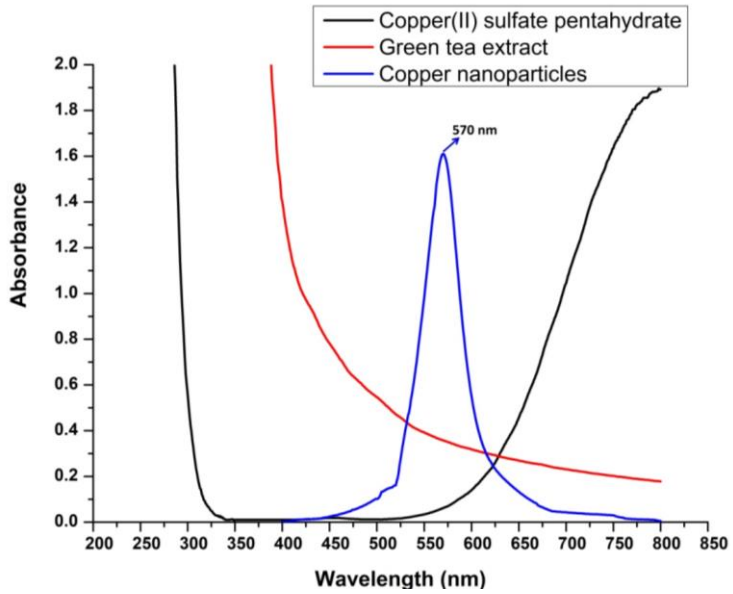


**Fig. 2.** UV-Vis spectra of biosynthesized copper nanoparticles using green tea extract.

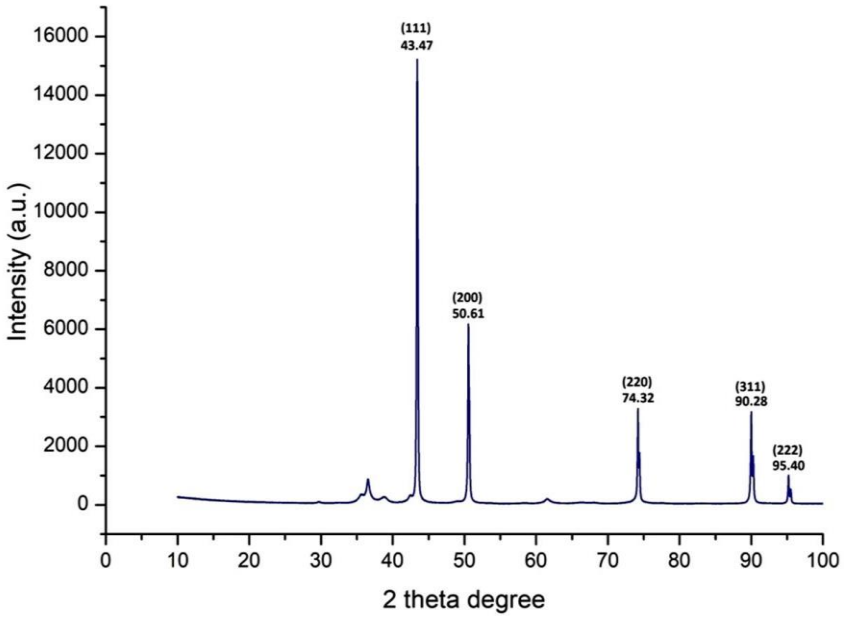


**Fig. 3.** XRD graph of phytosynthesized copper nanoparticles.

Scanning Electron Microscopy (SEM) at 5 µm scale indicated the presence of round shaped copper nanoparticles (Fig. 5A) phytosynthesized from green tea extract. Besides, Energy-dispersive X-ray spectrometry (EDX) was employed to determine both qualitative and quantitative analysis of nanoparticles. EDX graph confirmed the highest amount of copper atoms (77.96%) along with carbon and oxygen with the percentage of 17.17% and 4.87%, respectively (Table 1). EDX study (Fig. 5B) also indicated the presence of non-oxidized metallic copper nanocrystals by giving characteristic peaks at 1, 8 and 9 keV (Aziz 2017). Moreover, visible peaks of carbon and oxygen atoms were also followed the result of FTIR analysis.

Zetasizer provides the information about size distribution of nano-sized particles in terms of average particle diameter whereas the net surface charge of nanoparticles is measured by zeta potential value, which helps to understand the stability of the colloidal particles (Kaviya *et al*. 2011). The particle size distribution (Fig. 6A) and of potential value (Fig. 6B) the phytosynthesized CuNPs using green tea extract were revealed in Fig. 6. Particle dimension distribution by number has revealed the z-average of CuNPs as 45.30 nm with the mean potential value of -19.0 mV. Higher negative value of NPs proved their better stability as a result of possible capping of the biomolecules available in green tea extract (Edison & Sethuraman 2012).

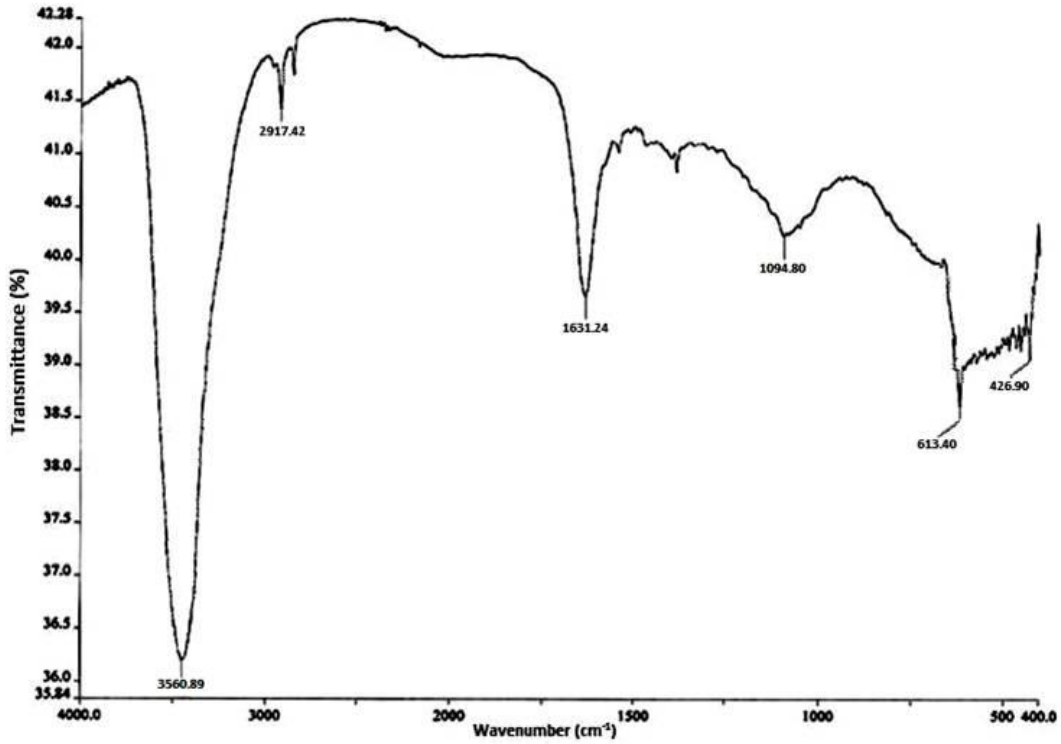


**Fig. 4.** IR spectra of biosynthesized copper nanoparticles using green tea extract.

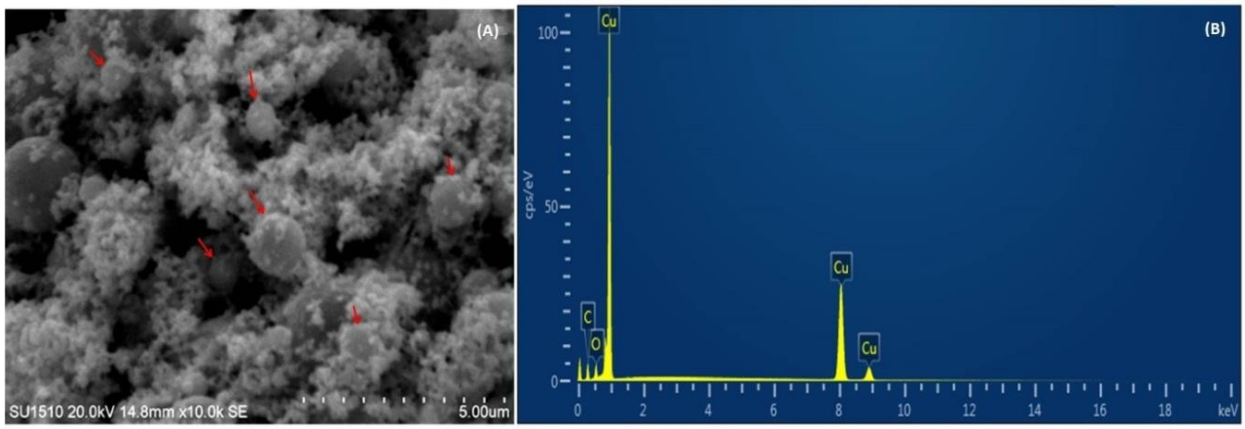


**Fig. 5.** (A) SEM image; (B) EDX graph of synthesized copper nanoparticles.

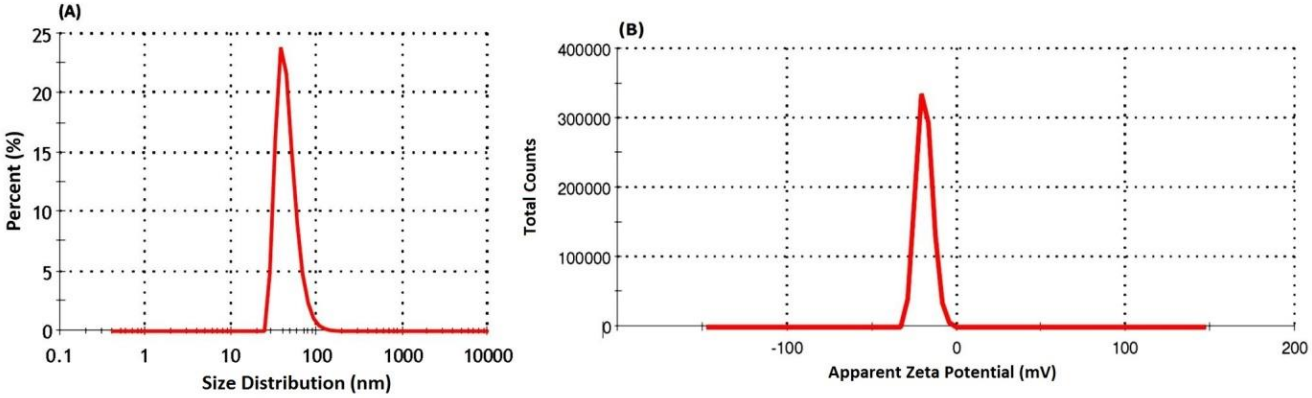


**Fig. 6.** (A) Particle size distribution; (B) zeta potential value of fabricated copper nanoparticles.

**Table 1.** The weight percentage (%) of different elements present in biosynthesized CuNPs from green tea.

| Element | Weight % | Weight % Sigma | Atomic % |
|---|---|---|---|
| **C** | 17.17 | 0.23 | 48.29 |
| **O** | 4.87 | 0.09 | 10.27 |
| **Cu** | 77.96 | 0.23 | 41.44 |
| **Total** | 100.00 | | 100.00 |

To estimate biocompatibility of biosynthesized CuNPs, cytotoxic effect of nanoparticles was studied using healthy regular mouse fibroblasts cell line (L929). By applying XTT reagent, pigmentation rate of functional mitochondrial enzymes of viable cells after treated with different concentrated (0, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL) copper nanoparticles was measured as the absorbance of optical density, which is directly proportional to the cell viability (Jahan *et al*. 2019). Fig. 7 showed the *in vitro* cytotoxic effects of copper nanoparticles phytosynthesized using green tea extract. Based on the absorbance values and by following the formula, the percentage of cell viability is more than 90% in each concentration of nanoparticles, which is considered as non-toxic (López-García *et al*. 2014).

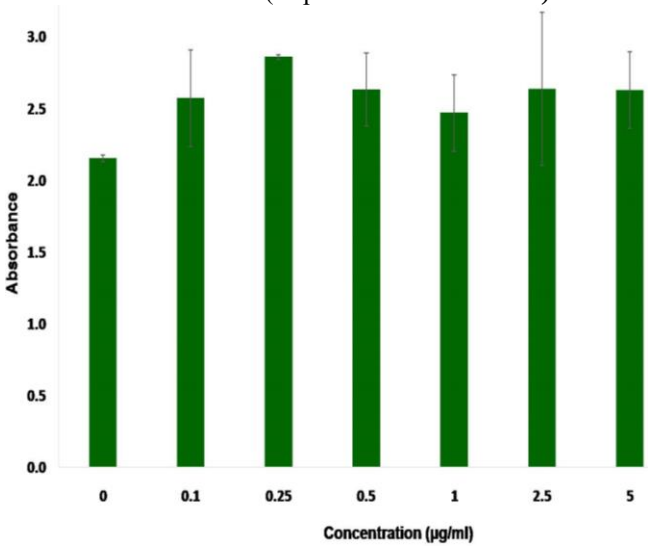


**Fig. 7.** Cytotoxic effects of copper nanoparticles on healthy regular mouse fibroblasts cell line (L929).

## Discussion

A rapid and facile microwave-assisted green synthesis for fabricating non-oxidized metallic cooper nanoparticles (CuNPs) was established in this study. Regarding the development of non-oxidized metallic CuNPs, it is always challenging to synthesize CuNPs without any oxidation since metallic copper has a high tendency to oxidize easily during NPs production process if the synthesis process takes longer period of time (Lee *et al*. 2013). During the synthesis, microwave irradiation played as the driving force by providing a rapid and homogeneous heating system that sped up the synthesis process and accelerated the rate of capping by plant extracts which promoted a faster stabilization of biosynthesized CuNPs, and thus, produced non-oxidized copper without any aggression. Presence of non-oxidized CuNPs was further detected by UV-vis spectroscopy and XRD analysis.

Previous studies have utilized green tea and black tea extract as reducing and stabilization agents for metallic nanoparticle synthesis. But, they were applied mainly for synthesizing copper (II) oxide nanoparticles (CuONPs). For instance, tea leaf extract and copper nitrate at the ratio of 3:1 was subjected to microwave-irradiated heating for fabricating copper (II) oxide nanoparticles, which showed their characteristic absorption peak at 271 nm (Sutradhar *et al.* 2014). In an another study, copper nitrate and black tea powder extract (1:2 ratio) were used for synthesizing CuONPs which were attained after placing the reaction medium at 300°C for 3 hours (Mathew 2018). The presence of copper (II) oxide nanoparticles (CuONPs) was further confirmed by XRD analysis based on their diffraction peaks (Mathew 2018). Moreover, fresh tea (*Camellia sinensis*) leaves were applied in a study to reduce non-oxidized metallic CuNPs by using copper (II) chloride ($CuCl_2$) salt (Keihan *et al.* 2016), where 10 ml aqueous extract of fresh tea leaves was inserted drop-wise into 100 ml of 1 mM copper (II) chloride salt solution and the system was refluxed at 100°C for 3 h. UV–visible spectra confirmed the development of metallic nano-copper by providing surface plasmon resonance at 560 nm (Keihan *et al.* 2016).

Nevertheless, in a comparison with the abovementioned literatures, this study utilized the microwave-heating system at 700 W with homogenous heating of 160-170°C just for 15 minutes for CuNPs synthesis, which was an expeditious, facile and time saving approach for creating non-oxidized metallic CuNPs. Moreover, copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) crystal salt was utilized for supplying $Cu^{2+}$ ions into the synthesizing medium, which is more economic since crystal copper (II) sulfate pentahydrate salt is comparatively cheaper than copper (II) chloride ($CuCl_2$) salt. Therefore, this protocol can also produce cheaper metallic CuNPs, and can be more profitable and convenient compared to other methods. In addition, different nanomaterials especially the metallic NPs themselves can often be toxic which produces risk to human body or other mammal cells because of their remarkable chemical, physical and biochemical properties (Dizaj *et al*. 2014, Phull *et al*. 2016). However, the result of this study presents non-toxic NP which can be more novel and risk free for evaluating their potentiality particularly in medical and beverage applications.

## Conclusion

Unlike the synthesis of Copper (II) oxide nanoparticles (CuONPs), this study has been a successful protocol of microwave-assisted synthesis of non-oxidized metallic copper nanoparticles (CuNPs) by using green tea (*Camellia sinensis*) extract. Besides, compared to previously applied methods, the protocol establishes a faster, facile and more time saving approach for creating metallic copper nanoparticles. Synthesized CuNPs obtained in this process also showed excellent stability

without any aggregation for several months. Moreover, non-toxic nature of these synthesized CuNPs on healthy mouse cells, which further signify their potential in a broad range applications including agriculture, medical and biological research.

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**Research Article**

# NOTES ON *Arabis kaynakiae* Daşkın (Brassicaceae), A CRITICALLY ENDANGERED SPECIES ENDEMIC TO TURKEY

Mehmet Cengiz KARAİSMAİLOĞLU[1*], Emrah ŞİRİN[2]

[1] Department of Biology, Faculty of Arts and Sciences, Siirt University, Siirt, TURKEY
[2] Department of Biology, Faculty of Science, Selçuk University, Konya, TURKEY

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**Abstract:** This study reports on the evaluation of the taxonomical, macro- and micro morphological, anatomical, palynological and cytological features, together with the geographical distribution of *Arabis kaynakiae* Daşkın (Brassicaceae), a Critically Endangered (CR) species from Turkey. The description of the species was updated following detailed studies on new specimens found in Karaman/Ermenek. The surface microphotographs of seeds and pollen were obtained using Scanning Electron Microscopy. The seed surface ornamentation is papillate and its margins minutely tuberculate. Pollen grains appear tricolpate, isopolar, subprolate in shape, with polar axis of 19.62 ± 0.33 µm and equatorial axis of 12.94 ± 0.17 µm. The exine thickness ranges from 1.68 to 1.87 µm; however, the intine thickness ranges from 0.21 to 0.39 µm. *Arabis kaynakiae* has pollen of coarse reticulate type surface ornamentation with slightly undulated muri. Anatomical characters of root, stem and leaf of the species were also given. The cytological investigations revealed that *A. kaynakiae* is diploid with 2*n* = 2x = 16 chromosomes. The discovered population of *A. kaynakiae* increased the known distribution range of the species in Turkey, and an updated evaluation of the species considering the IUCN criteria was provided.

**Özet:** Bu çalışma, Türkiye'den kritik olarak tehlike altındaki endemik (CR) bir tür *Arabis kaynakiae* Daşkın'ın (Brassicaceae) taksonomik, makro ve mikro morfolojik, anatomik, palinolojik ve sitolojik özelliklerinin coğrafi dağılımıyla birlikte değerlendirilmesini rapor etmektedir. Türün deskripsiyonu Karaman/Ermenek'te bulunan yeni örnekler üzerinde yapılan detaylı çalışmaların ardından güncellenmiştir. Tohum ve polenlerin yüzey mikrofotoğrafları Taramalı Elektron Mikroskobu kullanılarak elde edilmiştir. Tohum yüzey ornamentasyonu papillattır ve kenarları az çok tuberkulattır. Polen taneleri trikolpattırlar, 19,62 ± 0,33 µm polar ekseni ve 12,94 ± 0,17 µm ekvator ekseni ile izopolar ve subprolat şeklindedirler. Eksinin kalınlığı 1,68 ila 1,87 µm arasında değişmiş; buna karşın, intin kalınlığı 0,21 ila 0,39 µm arasında değişmiştir. *Arabis kaynakiae* hafif dalgalı muri ile kaba ağsı tip yüzey ornamentasyonuna sahiptir. Türün kök, gövde ve yaprak anatomik karakterleri de verilmiştir. Sitolojik araştırmalar *A. kaynakiae*'nin 2n = 2x = 16 kromozomlu diploit olduğunu ortaya koymuştur. *A. kaynakiae*'nin Keşfedilen popülasyonu, Türkiye'de türün bilinen dağılım aralığını artırmış ve türün IUCN kriterleri dikkate alınarak güncellenmiş bir değerlendirmesi sağlanmıştır.

***Edited by:***
*Regina Karousou*

****Corresponding Author:***
*Mehmet Cengiz Karaismailoğlu*
*cengiz.karaismailoglu@siirt.edu.tr*

***ORCID ID:***
*orcid.org/0000-0002-6856-2742*

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*Chromosome*
*Distribution*
*IUCN*
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## Introduction

The genus *Arabis* L. (Brassicaceae) includes about 118 species distributed in all warm areas of the Northern hemisphere (Warwick & Al-Shehbaz 2006, Koch *et al.* 2010, Mutlu & Erik 2015). Koch *et al.* (2010) suggested that this number may be higher since exhaustive investigations of the Central Asian and Russian taxa are still lacking (Mutlu & Erik 2015). Turkey is an important diversity center for *Arabis* since 24 taxa are distributed in the country, 10 of which are endemic (Cullen 1965, Davis 1988, Parolly & Hein 2000, Duman 2001, Duman & Duran 2001, Mutlu & Dönmez 2003, Mutlu 2004, Mutlu & Erik 2012, Daşkın 2013, Mutlu & Erik 2015).

*Arabis* was investigated both from a morphological point of view (Cullen 1965, Duman & Duran 2001, Mutlu 2002, Mutlu & Erik 2012, 2015), and a biogeographical and phylogenetic point of view (Karl & Koch 2013). Micromorphological and palynological characteristics of *Arabis* in Turkey were reported by İnceoğlu & Karamustafa (1975), Bıçakçı & Güleryüz (1998), Mutlu (2002), Mutlu & Erik (2012), and Karaismailoğlu (2019b).

*Arabis kaynakiae* Daşkın is a recently described local endemic of Gülnar district of Mersin in southern Anatolia

(Daşkın 2013). The anatomy, palynology, cytology, and micromorphology of this rare species have not been studied so far and they remain largely unknown. We, therefore, aimed in the present study to present a detailed examination of *A. kaynakiae* and evaluate taxonomic significance of obtained data. New data on distribution of the species and its updated evaluation considering the International Union for Conservation of Nature (IUCN) criteria are also presented.

## Materials and Methods

*Arabis kaynakiae* was collected from Karaman/Ermenek by the second author and collected specimens are deposited in the Herbarium of the Science Faculty of Selçuk University (KNYA). The morphometric analyzes were performed with 10 specimens in total according to Daşkın (2013).

Ten to twenty seeds and fifty pollen grains from ten different specimens were dehydrated in alcohol series (70%, 80%, 96% and 100%) for SEM analyses. Samples were covered with gold and microphotographs were taken with a Scanning Electron Microscope (ZEISS EVO LS-10). Terminology of SEM microphotographs is in accordance to Stearn (1992) and Koul *et al*. (2000). Images of the seeds were taken with a LEICA DFC295 digital camera attached to a LEICA S8AP0 stereo microscope.

Pollen preparations for light microscopy were performed according to the method of Wodehouse (1935). Pollen grains were stained with safranin, coated with a cover slip, analyzed with an Olympus CX21FS1 light microscope, and photographed by Kameram Imaging Software (Karaismailoğlu 2017, Karaismailoğlu & Erol 2019).

For anatomical investigations, cross-sections of root, stem and leaves for which different alcohol and xylene series were applied were obtained using a microtome (Thermo Shonda Met Finesse). Preparations were dyed with hematoxylin (ASC 720 Medite) and coated with Entellan to assess their anatomical characters (Karaismailoğlu 2015a, 2015b, 2016, 2019a, Karaismailoğlu *et al.* 2018). The Olympus CX21FS1 microscope and the Kameram Imaging Software were used for anatomical investigations.

Root tips obtained after seed germination were used for chromosome examinations. The protocol of Karaismailoğlu (2018) was followed with some modifications for slide preparations. The roots were pre-treated in a 5% a-bromonaphthalene solution for 4 h, kept in Carnoy's fixative (3:1 = ethyl alcohol: glacial acetic acid) for 24 h, hydrolyzed in 1 N HCl for 8-10 min at 60°C and dyed with aceto-orcein for 3 h. In the last step, preparations were coated with Entellan to make them permanent. The best metaphase plate was photographed under an Olympus CX21FS1 light microscope with a digital camera attached.

## Results

The updated description of *Arabis kaynakiae* is as follows;

Perennial herb with a woody stock. Flowering stem erect, 5−8 cm long, canescent with stellate hairs, 2−4 leaved and unbranched. Rosette leaves widely obovate 8−15 × 2−8 mm, densely silvery-canescent with 2−4-furcate and stellate hairs, few toothed to almost entire, apex obtuse, attenuate at base to a 5-7 mm petiole, with conspicuous midvein. Stem leaves oblong-lanceolate, 8−15 × 2−5 mm, sessile, tomentose with 2−4- furcate and stellate hairs. Inflorescence 1.5−3 cm long, 7−10-flowered, covered with 2−4- furcate and stellate hairs, without bracts. Pedicels 2−6 mm long, glabrous, ascending-erect. Sepals 3−4 × 0.8−1.0 mm, ovate-lanceolate, greenish white, margins membranous, sparsely to densely 2−4-furcate to stellate hairy. Petals white or white-lilac, 7−10 × 2.5−3.0 mm, oblong to spathulate, obtuse. Filaments yellow, cylindrical, long ones 6.8–7.0 mm long, short ones 3.5−4.0 mm long. Anthers 1.5 mm long, yellow, ovate, apex apiculate. Fruiting pedicels green, glabrous, spreading to reflexed, 10−12 mm long. Siliques brownish-green, 25−30 × 0.9−1.0 mm, flattened, glabrous, the valves without median nerve. Style 0.9−1.0 mm long; stigma capitate. Seeds 1.4−1.7 × 1.1−1.2 mm, ovate to orbicular, pale brown to black, surface papillate and margins minutely tuberculate, unwinged; radicle accumbent (Figs. 1-2).

*Arabis kaynakiae* is morphologically similar to *A. androsacea* Fenzl, *A. carduchorum* Boiss. and *A. alanyensis* H. Duman. However, it mainly differs from these species by its canescent flowering stems, rosette leaves and flowering stem leaves with 2−4-furcate and stellate hairs, sparsely to densely hairy sepals with 2−4-furcate and stellate hairs, and papillate seeds (Daşkın 2013). The results obtained from morphological studies are mainly consistent with the description given in the protologue (Daşkın 2013), except some features of rosette leaves, inflorescence, flowering and fruiting pedicel, filament, anther, silique, seed, and radicle that are presented in this study (Table 1).

The morphological characteristics of *A. kaynakiae* pollen were evaluated in detail (Fig. 3). Pollen grains are tricolpate, and isopolar and subprolate (P/E=1.28) in shape, with polar axis (P) in size 19.62 ± 0.33 µm and equatorial axis (E) in size 12.94 ± 0.17 µm. They have an oval appearance in equatorial and elliptical appearance in polar axes (amb) (Fig. 3). Colpi dimensions are varying from 14.72 µm to 15.33 µm in length, and from 1.41 to 2.13 µm in width. The edges are regular. The exine thickness ranges from 1.68 to 1.87 µm and the intine thickness ranges from 0.21 and 0.39 µm. Pollen grains have coarse reticulate type surface ornamentation with slightly undulated muri. The lumina is consisted of polygonal cells and its diameter varies between 0.59 and 1.91 µm.

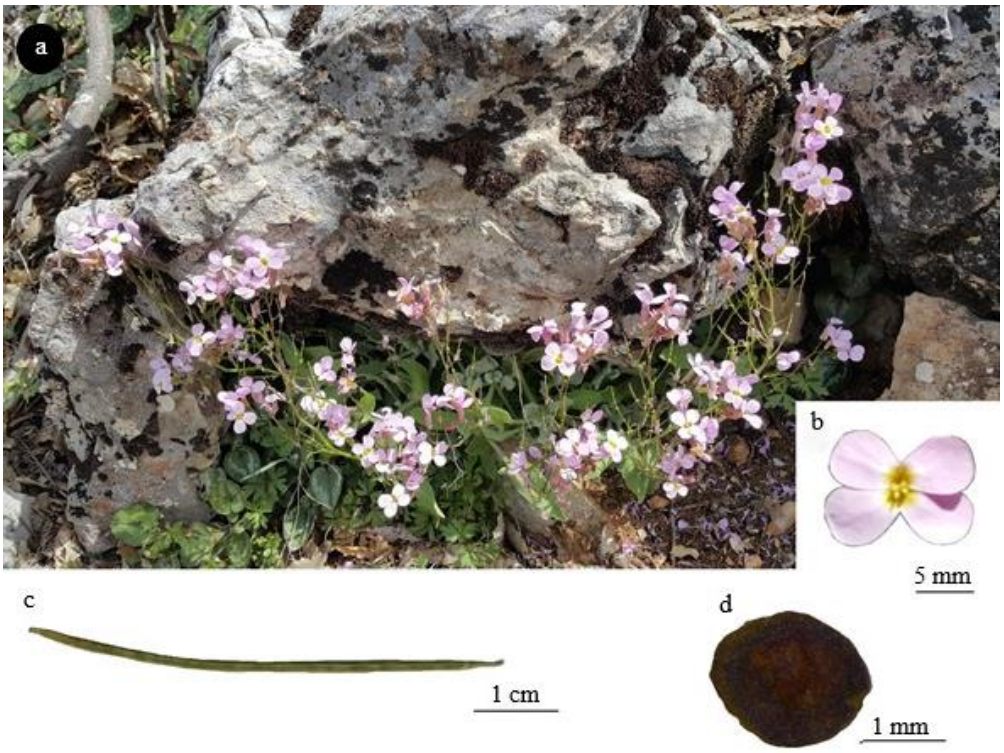


**Fig. 1.** *Arabis kaynakiae*. **a:** general appearance, **b:** flower, **c:** fruit, **d:** seed (Locality: Karaman, Ermenek, Tekeçatı high plateau, stony places, 1450 m, 8 April 2018, E. Şirin 684).

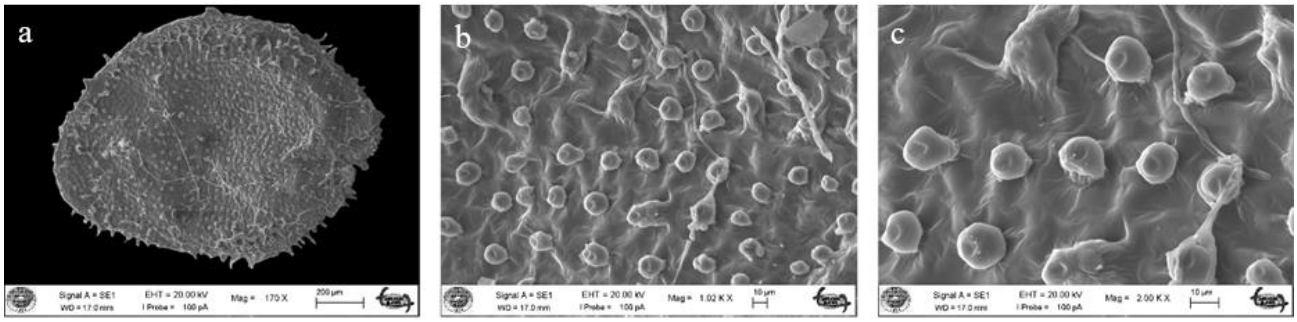


**Fig. 2**. Overview (**a**) and surface (**b-c**) SEM images of *A. kaynakiae* seeds.

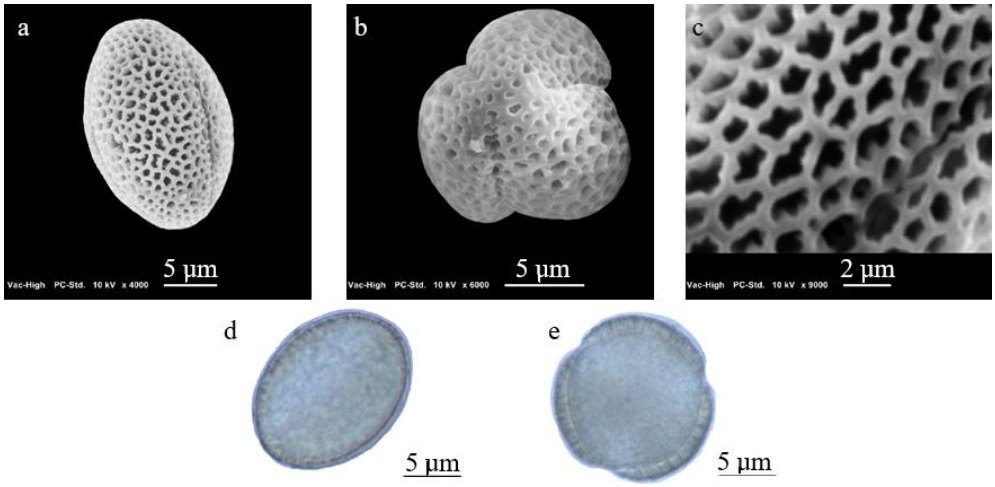


**Fig. 3.** SEM images of **a:** polar axis, **b:** equatorial axis and **c:** surface and light microscopy images of **d:** polar axis and **e:** equatorial axis of pollen grains.

**Table 1.** New characters of *A. kaynakiae* in addition to those reported by Daşkın (2013).

| Characters | The obtained outcomes |
|---|---|
| **Inflorescence** | 3-5 furcate to stellate hairy |
| **Pedicel** | glabrous |
| **Seed and radicle** | unwinged and accumbent |
| **Rosette leaves** | conspicuous midvein |
| **Filaments** | yellow and cylindrical |
| **Anthers** | ovate, apex apiculate |
| **Fruiting pedicels** | green, glabrous |
| **Siliques** | green - brownish |

The anatomical structures of the root, stem, and leaf for *A. kaynakiae* are given for the first time in this study (Fig. 4). The outermost surface of the root consists of a multi-layered periderm. The cortex under periderm comprises multi-layered parenchymatic cells and its thickness is 194.71 ± 5.44 µm. The endodermis layer is not clear. There is no obvious cambium amid phloem and xylem. The largest part of the roots is composed of secondary xylem elements. Pith rays are prolonged from large parenchymatic cells. The stem cross-sections showed that there is 1-layered epidermis containing flat cells in outermost (Fig. 4a). Its thickness varies from 30.71 µm to 43.65 µm. There is a cortex consisting of 3-4 layers of flat cells underneath epidermis. The thickness of this layer ranges from 149.78 µm to 181.14 µm. Under cortex, there is a sclerenchymatic layer, surrounding the side facing the cortex of the vascular bundles. This structure consists of a 3-4-celled layer and its thickness varies between 103.15 µm and 121.43 µm. Phloem components are not clear. The types of vascular bundles are closed collateral. The vessel member diameters range from 10.92 to 38.17 µm (Fig. 4b). The abaxial and adaxial surfaces of the leaves consist of a 1-layered epidermis cells, irregularly flat or polygonal in shape. The leaves are equifacial. The mesophyll layer is noted as a 2-4 layered palisade parenchyma 145.13-169.87 µm thick on both sides and a 2-3 layered spongy parenchyma 41.66-65.19 µm thick in the middle region. Leaves have collateral vascular bundle type. Vascular bundles are enclosed with parenchymatic cells (Fig. 4c-e).

The chromosome number of *Arabis kaynakiae* was determined as 2n = 16 in root tips (Fig. 5). The basic chromosome number of was found as x = 8.

*Arabis kaynakiae* is an endemic distributed in a limited area, and is only known from two populations in Mersin/Gülnar in southern Turkey (Daşkın 2013). In this study, we found a new population consisted of less than 50 individuals in Karaman/Ermenek (Fig. 6).

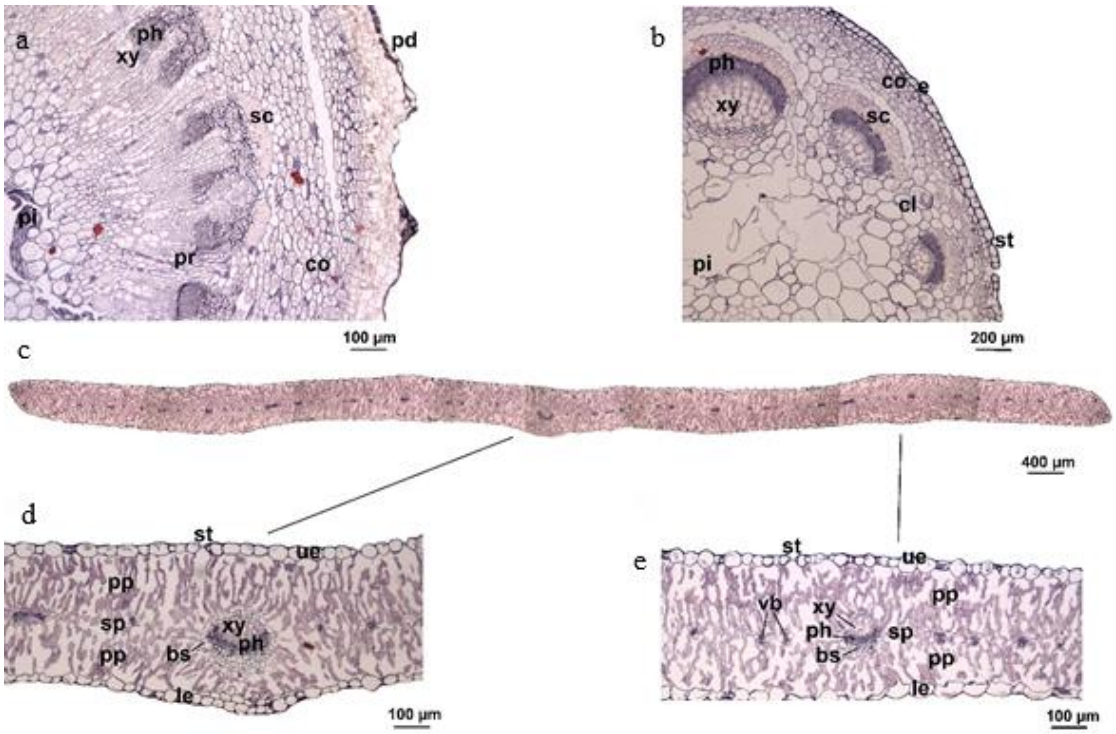


**Fig. 4.** The anatomical cross-sections of *A. kaynakiae*; **a:** root, **b:** stem, **c-e:** leaf (pd: periderm, co: cortex, ph: phloem, xy: xylem, pi: pith region, pr: pith ray, e: epidermis, cl: chlorenchyma, ue: upper epidermis, le: lower epidermis, pp: palisade parenchyma, sp: spongy parenchyma, vb: vascular bundle, sc: sclerenchyma, st: stoma, bs: bundle sheath).

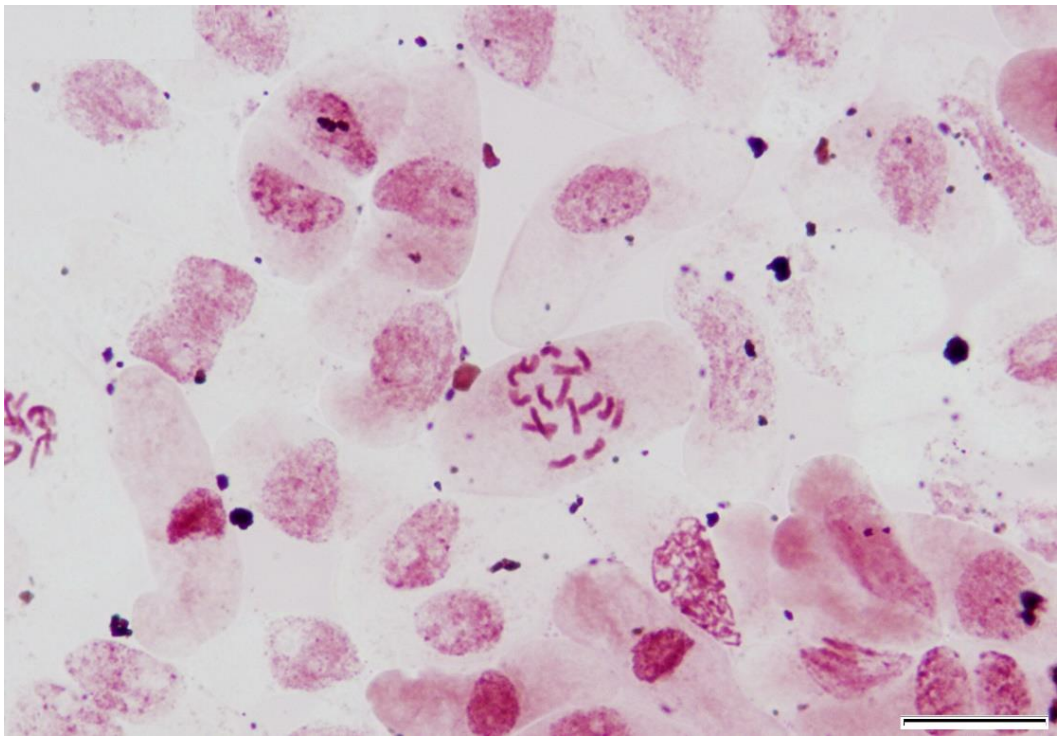

**Fig. 5.** The chromosomes of *A. kaynakiae* in a metaphase plate (2n=16, scale bar=10 µm).

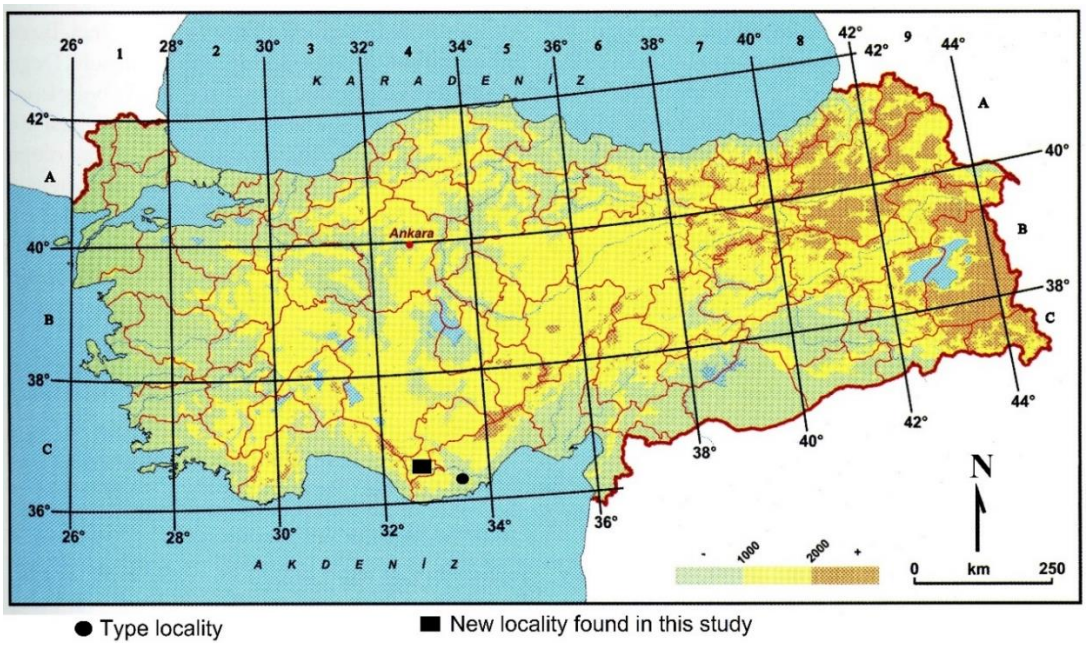


**Fig. 6.** The distribution map of *A. kaynakiae.*

## Discussion

The results of the present study expanded the former morphological description of some parts (flower, seed, leaves, fruits) of *Arabis kaynakiae* and provided the first data on anatomy, palynology, micromorphology, and karyology of the species.

Seed coat structure is considered as an important character in systematic studies of Brassicaceae. It is mostly used to establish taxonomic and evolutionary relationships (Khalik & Maesen 2002, Tantawy *et al.* 2004, Kaya *et al.* 2011, Bona 2013, Karaismailoğlu 2016, 2019a, Karaismailoğlu & Erol 2018). Seed micromorphology studies conducted so far on *Arabis* were based on 6 taxa in Mutlu (2002) and 2 taxa in Khalik & Maesen (2002). Our study is the first report on seed micromorphology of *A. kaynakiae*. The surface ornamentation of the species is recorded as papillate and

tuberculate, although the previously reported types of other species of the genus are reticulate and alveolate. This unique character easily distinguishes *A. kaynakiae* from other studied species. Also, this type is rarely observed in other genera of the family (Murley 1951, Koul *et al.* 2000, Zeng *et al.* 2004, Moazzeni *et al.* 2007).

The pollen features may present useful information to better understand the benefits of pollen studies in discrimination of closely related taxa. *Arabis kaynakiae* pollen has not been studied so far. The pollen shape was defined in this study as subprolate, which is one of the most common types in the family, and is consistent with the results of Khalik & Maesn Van Der (2002), Mutlu & Erik (2012), and Karaismailoğlu (2017, 2019b), who studied pollen grains of different genera in Brassicaceae. The pollen surface ornamentation type is coarse reticulate, similar to several other previously studied Brassicaceae taxa (İnceoğlu & Karamustafa 1975, Anchev & Deneva 1997, Bıçakcı & Güleryüz 1988, Mutlu & Erik 2012, Karaismailoğlu 2017, 2019b, Şirin & Karaismailoğlu 2020).

Mutlu & Erik (2012) studied palynomorphological features of 22 *Arabis* taxa from Turkey. They have described three different pollen morphotypes as Alpina (Polar axes equal or longer than 25 µm, equatorial axes longer than 23 µm, abm axes longer than 23 µm), Nova (Polar axes 18–23 µm, equatorial axes shorter than 23 µm, exine thickness longer than 1.80 µm) and Hirsuta (Polar axes shorter than 18 µm, equatorial axes shorter than 23 µm, exine thickness shorter than 1.80 µm) based on polar axes and exine thickness. Accordingly, *A. kaynakiae* is of Nova type, which has polar axes between 18 and 23 µm, and exine thickness longer than 1.80 µm.

The taxonomic application of anatomical characters is useful for discrimination of closely related taxa within Brassicaceae (Metcalfe & Chalk 1957). The anatomical features of root, stem, and leaf of *A. kaynakiae* were presented here for the first time. The roots have a multi-layered periderm in outermost part, with large pith rays arranged together with xylem components and sclerenchymatic structures. The cortex cells are below an epidermis layer (5-45 µm) in the stem and seem similar to the descriptions of some *Alyssum* L. and *Aubrieta* Adans. species (Orcan & Binzet 2003), and *Pachypragma macrophyllum* (Karaismailoğlu 2019a). The leaves are of the equifacial type. The palisade parenchyma in the mesophyll covers more space than the spongy parenchyma, which widely happens within the Brassicaceae family (Orcan & Binzet 2003, Cansaran *et al.* 2007, Karaismailoğlu 2016, 2019a).

Chromosome number in Brassicaceae is one of the most important characters in the evaluation of systematic relationships in the family (Karaismailoğlu 2018). The genus *Arabis* is represented by 118 species worldwide and chromosome numbers of 59 of these have been studied so far (Warwick & Al-Shehbaz 2006). Chromosome numbers of 14 *Arabis* species from Turkey have previously been reported (Mutlu 2002). The chromosome number of *A. kaynakiae* was found as $2n = 16$ ($\times=8$). The basic chromosome number differs from those of other studied *Arabis* taxa (6 in *A. glabra* (L.) Bernh., 9 in *A. drabiformis* Boiss. and 11 in *A. laxa* L.) (Mutlu 2002).

According to IUCN (2010), the extent of occurrence and the area of occupancy of *A. kaynakiae* are less than 100 $km^2$ and 10 $km^2$, respectively. The known populations and their localities are destroyed by road construction activities (Mutlu & Erik 2015). According to the criteria of geographic scale dimension (criteria B) in IUCN classes (Mutlu & Erik 2015), *A. kaynakiae* is assessed as CR; B1ab (i,v), B2ab (i,v). Although we found a new population consisted of less than 50 individuals in Karaman/Ermenek, this species is currently known from 3 localities occupying less than 100 $km^2$ (Fig. 6). According to IUCN 2010 criteria [B1ab (i,v), B2ab (i,v)], the CR status is still recommended.

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**Research Article**

# HABITAT PREFERENCES, DISTRIBUTION AND ANATOMY OF THE CLASPING-LEAVED PONDWEEDS OF TURKEY

Necati BAYINDIR, Nursel İKİNCİ*

Bolu Abant İzzet Baysal University, Department of Biology, 14280 Bolu, TURKEY

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**Abstract:** Clasping-leaved *Potamogeton* L. species growing in Turkey are *P. praelongus* Wulfen and *P. perfoliatus* L. There exists no detailed study about distribution, habitat requirements, and anatomical properties of the Turkish populations of the two species. *Potamogeton perfoliatus* is widespread throughout the country but *P. praelongus* was recorded only from a single locality. Therefore, *P. praelongus* is rare and endangered in Turkey. In this study, we recorded presence of *P. perfoliatus* in 54 wetlands based on examination of 86 herbarium specimens. Physical and chemical parameters of the water bodies where the two species occur were measured from 24 sites for *P. perfoliatus* and from one site for *P. praelongus.* According to our findings, *P. praelongus* grows in an alpine lake with oligotrophic, calcareous and alkaline water. *Potamogeton perfoliatus* occupies diverse habitats but prefers deep lentic water bodies with high pH and low salinity levels. Stem anatomy of the species were studied based on three individuals for *P. praelongus* and 35 individuals for *P. perfoliatus*. Morphological features of the species were also investigated and descriptions based on Turkish material were prepared. We provided the distinguishing anatomical and morphological characters between the species. Our anatomical findings showed that *P. praelongus* specimens have eight vascular bundles in contrast to previous reports on the species. Our results can be used for future monitoring of the two submerged *Potamogeton* species as we provide detailed information about their current distribution pattern and habitat features.

**Özet:** Türkiye'de yetişen gövdeyi saran yapraklı *Potamogeton* L. türleri *P. praelongus* Wulfen ve *P. perfoliatus* L.'tur. Bugüne kadar bu türlerin dağılımı, habitat tercihleri ve anatomik özellikleriyle ilgili detaylı çalışmalar yoktur. *Potamogeton perfoliatus* ülke çapında yaygın bir türdür ancak *P. praelongus* sadece bir lokaliteden kaydedilmiştir. Bu nedenle *P. praelongus* Türkiye'de nadir ve tehdit altındadır. Bu çalışmada 86 herbaryum örneğine dayanarak *P. perfoliatus*'u 54 sulak alandan kaydettik. Türlerin yetiştiği suların fiziksel ve kimyasal parametreleri *P. perfoliatus* için 24 noktadan, *P. praelongus* için bir noktadan ölçülmüştür. Bulgularımıza göre, *P. praelongus* oligotrofik, kalkerli ve alkali alpin bir gölde yetişmektedir. *Potamogeton perfoliatus* çok farklı habitatlarda bulunmakla birlikte, yüksek pH, düşük tuzluluk değerlerine sahip, derin ve durgun suları tercih etmektedir. Türlerin gövde anatomileri *P. praelongus* için 3 birey, *P. perfoliatus* için ise 35 bireyden örnek alınarak incelenmiştir. Türlerin morfolojik özellikleri de araştırılmış ve Türkiye'den toplanan materyallere dayalı olarak betimler hazırlanmıştır. Türler arasındaki ayırt edici anatomik ve morfolojik karakterler verilmiştir. Anatomi bulgularımız *P. praelongus*'un önceki bazı çalışmalara aykırı olarak sekiz iletim demetine sahip olduğunu göstermektedir. Bu çalışmada türlerin güncel dağılım ve habitat tercihleriyle ilgili sunduğumuz kapsamlı bulgular iki batık *Potamogeton* türünün gelecekte izlenmesinde faydalı olacaktır.

***Edited by:***
*Cem Vural*

***Corresponding Author:***
*Nursel İkinci*
*nursel.ikinci@gmail.com*

***ORCID ID:***
*orcid.org/0000-0002-6435-5869*

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## Introduction

Aquatic plants are primary producers and provide habitat and food to different organism groups, like algae, zooplankton, invertebrates, and different vertebrate taxa, such as fish and frogs (Bornette & Puijalon 2011). Additionally, they are very important for establishment and maintenance of healthy ecosystems as they improve water quality due to their filtering capacity of excessive nutrients. They also affect water flow and sediment properties. Nutrient content of waters is very important for the diversity of macrophytes. It is known that the highest macrophyte diversity can be seen under moderate nutrient levels, and extreme nutrient levels favor only certain species. Under very low nutrient levels, stress tolerant species manage to survive and under eutrophic conditions, very competitive species grow in high densities and replace other species (Bornette & Puijalon 2011). Therefore, macrophytes are very sensitive to habitat deteriorations mainly caused by anthropogenic

alterations. Aquatic plants have faced an increasing extinction risk in the last decades due to eutrophication, water regime changes, pollution and introduction of invasive exotic species (Guo *et al.* 2019).

Potamogetonaceae is one of the largest aquatic plant families of the world with approximately 100 species growing in very diverse habitats. *Potamogeton* L., one of the most ecologically important genera of all aquatic plants (Haynes 1985), has the highest number of species in the family with species having solely floating or submerged leaves and species with both submerged and floating leaves (Wiegleb & Kaplan 1998). The genus contains approximately 72 species and 99 hybrids (Kaplan *et al.* 2013). Taxonomy of the genus is quite difficult due to high number of hybrids and availability of limited reliable morphological characters (Wiegleb 1988). Additionally, *Potamogeton* species are known to exhibit extensive phenotypic plasticity, contributing to the difficulty of species delimitation.

First detailed taxonomical revision of *Potamogeton* was carried out by Ascherson and Graebner (1907) who divided *Potamogeton* into five sections and 13 subsections. Hagström (1916) published the most comprehensive study about *Potamogeton* including anatomical characters. Treatment of Hagström revealed five sections and 26 subsections. *Potamogeton praelongus* Wulfen (long-stalked or white-stemmed pondweed) belongs to the clasping leaved *Potamogeton* species group. Ascherson and Graebner (1907) evaluated *P. praelongus* and *P. perfoliatus* L. with submerged and clasping-leaved species in the subsection *Perfoliati* Graebner of section *Heterophylli* K. Koch. However, Hagström (1916) separated the clasping-leaved species into two subsections: Subsection *Perfoliati* (Graebner) Hagström and subsection *Praelongi* Hagström. Haynes (1985) followed Hagström's (1916) taxonomical treatment. More recently, Wiegleb (1988) recombined the two species in *P. perfoliatus* group.

Potamogetonaceae is the largest aquatic plant family in Turkey with 20 species and three hybrids belonging to five different genera (Uotila 1984, Aykurt *et al.* 2017, Bayındır 2018, Bayındır & İkinci 2020a, 2020b). As the largest genus within the family, *Potamogeton* is represented by 14 species and three hybrid in Turkey. *Potamogeton praelongus* was included in the Flora of Turkey (Uotila 1984) based on a single record in the Flora of Caucasus (Grossheim 1928) from Kars province of north eastern Turkey. Since then, it could not be collected again and was thought to be extinct in Turkey. Although we visited several herbaria to examine *Potamogeton* species, we could not find any *P. praelongus* specimens collected from Turkey. However, during our fieldworks in 2016, we collected *P. praelongus* from a second locality in southern Turkey (Fig. 1, Bayındır 2018). According to Vöge (1992), world distribution of *P. praelongus* is Nordic, weakly suboceanic, and circumpolar. In Europe, its distribution extends from northern Scandinavia to south of France in the Alps and in Pyrenees. The species is also distributed in similar latitudes of Asia and North America. Even though the species is distributed in a wide geographical area, it is still a rare species (Prausová *et al.* 2011). It is considered as endangered in Switzerland and in Germany (Vöge 1992) and as critically endangered in the Czech Republic (Prausová *et al.* 2011). In the UK it is considered as near threatened and it is a protected species in France (Julve 2017). Prausová *et al.* (2014) stated that *P. praelongus* is endangered in all Central Europe. However, IUCN assessed it as Least Concern (Lansdown 2014).

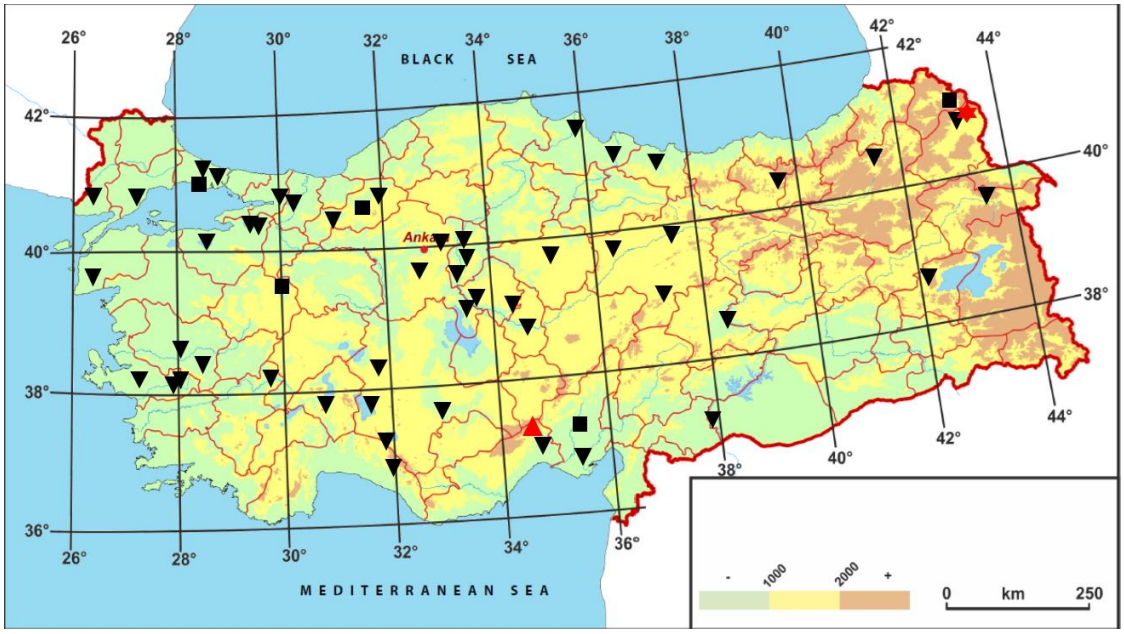


**Fig. 1.** Distribution map of *P. praelongus* (▲current location, ★ historical location) and *P. perfoliatus* (▼according to Bayındır (2018) and ■ new collection sites added with this study) in Turkey. Revised from Bayındır (2018).

*Potamogeton perfoliatus* is the other clasping leaved species in Turkey and is widespread throughout the country except the southeastern Anatolia. This region of Turkey has a hot and semi-arid climate with semi-arid steppe vegetation different from the rest of the country (Ergüner *et al.* 2019). According to Gupta & Lansdown (2013), *P. perfoliatus* can be considered a cosmopolitan species as it has a distribution throughout Eurasia (from the Mediterranean to northern Scandinavia and Iceland, Siberia), in North America and Greenland, in North and Central Africa and in Australia. It is assessed as Least Concern (LC) by IUCN (Gupta & Lansdown 2013). Haynes (1985) stated that *P. perfoliatus* is a morphologically very variable species. Therefore, several subspecific categories were defined for the species. However, these variations are mainly on vegetative characters and do not require distinct taxonomic divisions (Haynes 1985).

Studies on *P. perfoliatus* are mainly restricted to floristic records and altitudinal distribution analysis (Bayındır 2018, İkinci & Bayındır 2019, İkinci & Bayındır 2020) and there exist no study about the anatomy and habitat requirements of the species in Turkey. Therefore, we aimed in this study i) to provide detailed information about the geographical distribution of the clasping-leaved *Potamogeton* species in Turkey, ii) to determine the habitat features of the species including water chemistry and other environmental parameters, iii) to prepare new morphological descriptions of the species based on Turkish material and v) to provide morphological and anatomical comparisons between *P. praelongus* and *P. perfoliatus*.

## Materials and Methods

### *Plant* Materials

The plant material was collected from the field between 2014 and 2017 as a part the project on the revision of Potamogetonaceae family in Turkey. Entire plants were taken from fresh material in the field and preserved in 70% alcohol solution for anatomical studies which are stored at Bolu Abant İzzet Baysal University Herbarium (AIBU) (Table 1). Herbarium specimens were also prepared for each sample and stored in AIBU. Additionally, the following herbaria were visited to study the *Potamogeton* species: ANK, ISTE, ISTF, HUB, ISTO, ADA, K, E, L, LINN, VANF (acronyms according to Thiers 2016).

### *Environmental* Parameter Measurements

Physical and chemical parameters of the water bodies where the two *Potamogeton* species were sampled were measured from 25 different wetlands during the growth season of the plants in 2016 and 2017. We sampled the plants and physical and chemical properties of the water from the littoral zone of water bodies. In deeper sites, we gathered macrophytes with the help of a rake. Single measurements were made per site for physical and chemical variables. We measured seven environmental variables with a portable multi probe YSI-Professional Plus in situ. The environmental variables were dissolved oxygen concentration (DO, mg $l^{-1}$), water temperature (Tw, °C), electrical conductivity (EC, µS $cm^{-1}$), pH, total dissolved solids (TDS, g $l^{-1}$), salinity (ppt) and ammonium (mg $l^{-1}$). The altitudes of the sampling sites were recorded by using Magellan eXplorist 610. Environmental variables and abundances were measured at random locations where the species were collected. We estimated abundance visually and recorded the percentage area covered by each species. Abundance percentage measurements were based on ACFOR scale [abundant (>80% cover), common (61–80%), frequent (31–60%), occasional (5–30%), and rare (<5%)] (Crisp & Southward 1958, Dunham et al. 2018). The estimated values were converted to numerical scores from 1 to 5. We also recorded the accompanying aquatic plant species (Table 1).

**Table 1.** List of our collections for *P. perfoliatus* with information about other accompanying Potamogetonaceae species.

| Locality | Coordinates | Altitude (m) | Date | Anatomy material | *P. perfoliatus* | *P. lucens* | *S. pectinata* | *G. densa* | *P. natans* | *P. crispus* | *P. berchtoldii* | *P. gramineus* | *Z. palustris* | *P. nodosus* | *P. pusillus* | *P. trichoides* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Adana: Seyhan Dam Lake | 37.04921 N, 35.31672 E | 59 | 27.vi.2016 | √ | + | + | + | | | | | | | | | |
| Antalya: Gündoğmuş, Lake Eğil | 36.93217 N, 32.19954 E | 2078 | 25.vi.2016 | √ | + | + | | + | + | | | | | | | |
| * Ardahan: Güvenocak, Çıldır Lake | 41.09930 N, 43.24034 E | 1959 | 11.vii.2017 | - | + | | + | | | + | | | | | | |
| * Bolu: Gölköy Lake | 40.42'06 N, 31.31'09 E | 770 | 02.ix.2014 14.vii.2014 | - | + | + | + | + | | + | + | + | + | | | |
| Bolu: Yeniçağa Lake, S. of the lake | 40.77353 N, 32.02366 E | 989 | 13.vii.2015 | √ | + | | | | | | + | | | | | |

*Table 1. continued.*

| Locality | Coordinates | Altitude (m) | Date | Anatomy material | *P. perfoliatus* | *P. lucens* | *S. pectinata* | *G. densa* | *P. natans* | *P. crispus* | *P. berchtoldii* | *P. gramineus* | *Z. palustris* | *P. nodosus* | *P. pusillus* | *P. trichoides* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bursa: İznik Lake, near wharf | 40.47785 N, 29.66242 E | 85 | 29.vii.2015 | √ | + | | | | | | | | | | | |
| Bursa: İznik Lake | 40.44677 N, 29.71397 E | 87 | 29.vii.2015 | √ | + | | + | | | + | | | + | | | |
| Bursa: İznik Lake, Boyalıca | 40.48302 N, 29.56499 E | 83 | 29.vii.2015 | √ | + | | | | | | | | | | | |
| Bursa: Ulubat Lake, bird watching pier | 40.19893 N, 28.44291 E | 1 | 30.vii.2015 | √ | + | | | | | + | | | | | | |
| Denizli: Çivril, Işıklı Lake, entrance to Beydilli | 38.25761 N, 29.93300 E | 827 | 19.vi.2016 | √ | + | + | + | | | + | | | | | | |
| Edirne: Enez, W. of Gala Lake | 40.75159 N, 26.16873 E | 34 | 12.vi.2016 | √ | + | | + | | | + | | | | + | | |
| Erzurum: Uzundere, 2 km to Tortum Dam | 41.57944 N, 41.60204 E | 1018 | 19.viii.2016 | √ | + | | + | | | | + | | | | | |
| Gümüşhane: Erzincan-Bayburt Road, Salyazı Dam | 40.24449 N, 39.81048 E | 1684 | 20.viii.2016 | √ | + | | + | | | | | + | | | | |
| İstanbul: Arnavutköy, Terkos Lake | 41.37284 N, 28.56756 E | 2 | 10.viii.2016 | √ | + | + | | | | | | | | | + | |
| * İstanbul: Büyükçekmece, Büyükçekmece Lake | 41.06745 N, 28.57156 E | -1 | 10.vi.2016 | √ | + | | | | | | | | | | + | |
| İstanbul: Eyüp, Göktürk, Göktürk Dam Lake | 41.19319 N, 28.87544 E | 41 | 10.vi.2016 | √ | + | | | | + | + | + | | | | | + |
| İzmir: Torbalı, Torbalı Dam Lake | 38.17277 N, 27.16007 E | 63 | 20.vi.2016 | √ | + | | | | | + | + | | | | | |
| Kars: Doğruyol, Çıldır Lake | 41.06916 N, 43.32759 E | 1960 | 11.viii.2017 | - | + | | + | | | | | | | | + | |
| Kırıkkale: Ankara border, Kızılırmak River | 39.93968 N, 33.41290 E | 661 | 22.viii.2016 | - | + | | + | | | | | | + | | | |
| Kırıkkale: Keskin, Köprüköy, Kapulukaya Dam | 39.57404 N, 33.43205 E | 719 | 05.ix.2016 | √ | + | | | + | | | | | + | | | |
| Kırşehir: Mucur, near Karkın Village, Kargın Dam Lake | 39.00823 N, 34.48361 E | 1086 | 05.ix.2016 | √ | + | | + | | | + | + | | | | | |
| Konya: Bozkır, Dipsizgöl | 37.10161 N, 32.04097 E | 1687 | 08.ix.2016 | √ | + | | | | | + | | | | + | | |
| Konya: Ilgın, Çavuşçu Lake | 38.37327 N, 31.89334 E | 1016 | 08.ix.2016 | - | + | | | | | | | + | | | | |
| Konya: Çumra, Türkmenkarahüyük Village | 37.61108 N, 33.02601 E | 992 | 08.ix.2016 | √ | + | | | | | | | | | | | |
| * Kütahya: Eskişehir-Kütahya Road, Sofça Village, Porsuk Dam | 39.60567 N, 30.14925 E | 895 | 18.vi.2016 | √ | + | | + | | | | + | | + | | | |

*Table 1. continued.*

| Locality | Coordinates | Altitude (m) | Date | Anatomy material | *P. perfoliatus* | *P. lucens* | *S. pectinata* | *G. densa* | *P. natans* | *P. crispus* | *P. berchtoldii* | *P. gramineus* | *Z. palustris* | *P. nodosus* | *P. pusillus* | *P. trichoides* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mersin: Tarsus Dam Lake | 36.95359 N, 34.89384 E | 35 | 27.vi.2016 | √ | + | | + | | | + | | | | + | | |
| Ordu: Fatsa, Sefaköy, Gaga Lake | 40.75159 N, 26.16873 E | 67 | 15.viii.2016 | √ | + | | | | | | | | | + | | |
| Sakarya: Sapanca Lake, Eşme Village shores | 40.73328 N, 30.23521 E | 26 | 29.vii.2015 15.viii.2015 | √ | + | + | | | | | | | | + | | + |
| Samsun: Ayvacık, Suat Uğurlu Dam Lake | 41.07370 N, 36.66831 E | 53 | 14.viii.2016 | √ | + | | + | | | | | | | | | |
| Samsun: Delta Kızılırmak, bird sanctuary | 41.67075 N, 36.03464 E | 3 | 14.viii.2016 | √ | + | | + | | | | | | | + | | |
| Sivas: Hafik, Hafik Lake | 39.87247 N, 37.38256 E | 1269 | 21.viii.2016 | √ | + | | + | | | | | | | | | |
| Sivas: Halkaçayır Village, Dam Lake | 39.80974 N, 36.34908 E | 1372 | 21.viii.2016 | √ | + | | | | | | | | | | | + |
| Tekirdağ: Yazır Dam Lake | 40.92561 N, 27.40341 E | 59 | 12.vi.2016 | √ | + | | + | | | + | | | | + | | |
| Yozgat: Entrance to Sorgun, Mükremin Pond | 39.80372 N, 35.21713 E | 1076 | 21.viii.2016 | √ | + | | + | | | | | | | | | |
| Total (34 wetlands) | | | | 29 | 36 | 6 | 18 | 3 | 2 | 12 | 7 | 3 | 5 | 7 | 3 | 3 |

* New wetlands added to the list in Bayındır (2018).

*Anatomical* studies

Plant samples were taken to represent all parts of the plants for morphological diagnosis and samples preserved in 70% alcohol solution were used for subsequent anatomical studies. Stem anatomy samples were prepared from three individuals for *P. praelongus* and from 35 individuals for *P. perfoliatus*. The internode areas of stems of the specimens were cut about 0.05 mm thick with the aid of razor blades. Samples were put into safranin or toluidine blue dye and transferred to distilled water. Stem fragments were examined under the light microscope at 4x, 10x and 40x magnifications. The stele types, shape of endodermal cells, pseudohypodermis, presence of subepidermal bundles and interlacunar bundles in the cortex were determined and photographed.

## Results

### *Potamogeton praelongus* Wulfen

*Description*

Stem slightly branched or unbranched with rhizome; terete; clearly zig-zag shaped; mostly white or pale green, up to 200 cm in length. Leaves sessile, all submerged, alternate, lax, mostly undulate or entire, pale green to olive green, lanceolate to broadly lanceolate, broadly ovate elliptic, 11-17 veined, midrib without lacunae, 52-171 x 14-27 mm, margin entire, at base cuneate and semiamplexicaule, at apex clearly cucullate, (splitting when pressed), obtuse. Stipules persistent, conspicuous, convolute, free from blade, white, 17-42 mm, fibrous.

*Locality*

TURKEY, Mersin, Çamlıyayla, Darboğaz, near Summit Medetsiz, Karagöl Lake, 2591 m, 37.404261 N, 34.559753 E, 07 September 2016, N. Bayındır 1360 (AIBU 12735) (Fig. 1).

**Table 2.** Water chemistry measurements for *P. praelongus* (see materials and methods for abbreviations).

| Tw (°C) | pH | DO (mg $l^{-1}$) | EC (µS $cm^{-1}$) | TDS (g $l^{-1}$) | Salinity (ppt) | Ammonium (mg $l^{-1}$) |
|---|---|---|---|---|---|---|
| 17.9 | 7.86 | 5.92 | 165.9 | 0.1248 | 0.09 | 0.02 |

Ecological notes

*Potamogeton praelongus* was collected from an oligotrophic, calcareous, snow-melting lake (Karagöl Lake) with alkaline water (Table 2). In addition to *P. praelongus*, *P. natans* L., *Stuckenia filiformis* (Persoon)

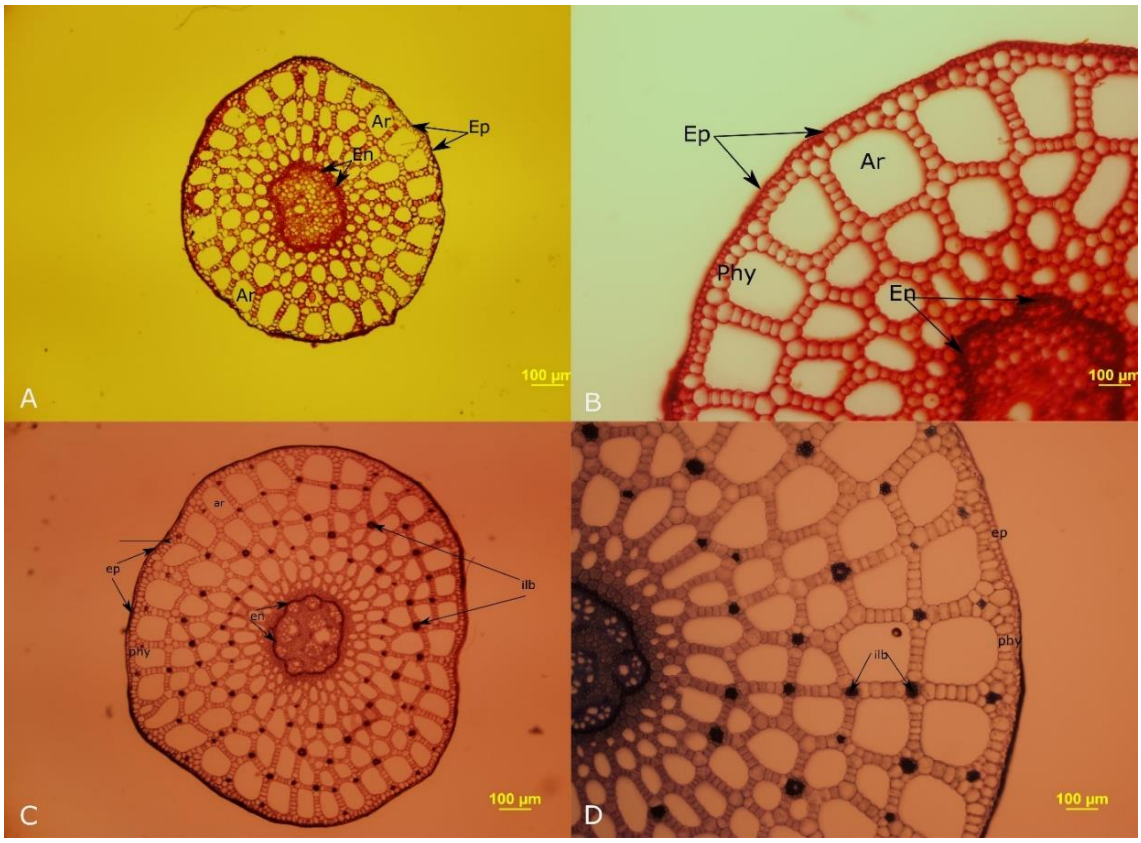


**Fig. 2.** Stem anatomy of *P. perfoliatus* (A, B) and *P. praelongus* (C, D). General view (A, C) (4×), Epidermis and cortex (B, D) (10×), (Ar: aerenchyma, En: endodermis, Ep: epidermis, Ilb: interlacunar bundle, Phy: pseudohypodermis).

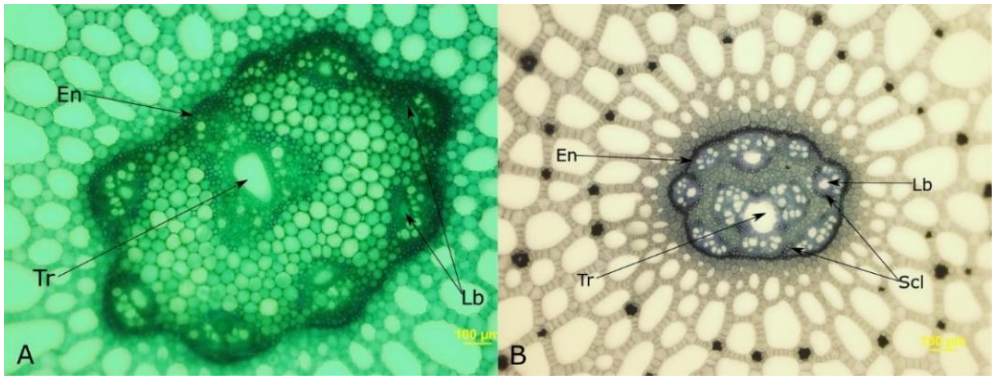


**Fig. 3.** Stele of *P. perfoliatus* (A) and *P. praelongus* (B) (10×), (En: endodermis, Lb: lateral bundle, Scl: sclerenchyma, Tr: trio-bundle).

Börner, *Persicaria amphibia* (L.) Gray were growing in the lake. Since the species is currently known only from this site in Turkey, we have single measurements of the physical and chemical parameters of the water body where the species was sampled.

*Anatomy*

Our anatomical findings for *P. praelongus* and *P. perfoliatus* are shown in Figs 2-3 and Table 3.

***Potamogeton perfoliatus L.***

*Description*

Stem slightly unbranched or branched at the top with rhizome; terete; dark green, pale green, whitish or brownish upto 200 cm length; without turion. Leaves sessile, all submerged, alternate, undulate or entire, usually olive-green, rarely dark green, rarely leaves with rusted colour, broadly lanceolate, broadly ovate or close to rounded, 5-25 veined, midrib without lacunae, 17-61 × 12-34 mm, margin slightly denticulate, at base cordate and amplexicaul, at apex mostly entire or slightly cucullate, obtuse, rounded or acute. Stipule free from blade, convolute, mostly deciduous rarely persistent, ca. 14 mm long, nonfibrous. Inflorescence spike, terminal, sometimes axillary, cylindrical, 19-27 mm long, continuous; flowers dense. Carpels 4. Fruits sessile, obovate, 2.5-3.5 × 1.8-2.25 mm; beak short, dark green to blackish, 0.3-0.5 mm long.

**Table 3.** Comparison of anatomical characters of *P. praelongus* and *P. perfoliatus* based on our collections.

| Character | *P. praelongus* | *P. perfoliatus* |
|---|---|---|
| **Stele types** | Trio type | Trio type |
| **Shape of endodermal cells** | U type | O type |
| **Pseudohypodermis** | 1 seriate | 1 seriate |
| **Presence of subepidermal bundles** | Present | Absent/rarely present |
| **Presence of interlacunar bundles in the cortex** | Present | Absent/rarely present |

*Additional herbarium specimens examined*

The information of the examined specimens at different herbaria are given in the below list. The list was organized in alphabetical order based on the names of the Turkish provinces (in bold). There are more than one herbarium specimens from the same wetland. The herbarium acronyms are in parenthesis.

Examined material: **Adana:** Yenice irrigation, main canal, G. Altınayar (DSI 1988). **Ağrı:** Balık Lake shores, 2210 m, 12 vi 1979, A. Baytop, B. Çubukçu (ISTE 42741). Balık Lake, 2261 m, 29 ix 2014, M.M. Uma, M. Kaya (AIBU). Suluçem (Musun), S. end of Balık Lake, 2300 m, shallow water, 23 vii 1967, P.H. Davis 47279 (K). **Ankara:** Bala, Kesikköprü Dam Lake, 790 m, 12 vii 2014, A.E. Yaprak, S.T. Körüklü, İ. Başköse, A. Güleryüz (AIBU, ANK). Elmadağ, 14 vii 1939, B. Kasaplıgil (ISTF 572). Eymir Lake, 08 vii 1933, W. Kotte (ANK). **Bitlis:** Nazik Lake shores, submerged, ca. 1870 m, 31 viii 1993, L. Behçet 4691 (VANF). **Bolu:** Abant Lake, 08 viii 1970, A. and T. Baytop (ISTE 18397). Yeniçağa Lake, near Gerede, 03 x 1961, A. and T. Baytop (ISTE 6882). **Bursa:** İznik, İznik Lake, ca. 85 m, 20 vii 1981, P. Uotila (EGE 24903). **Çanakkale:** Ayvacık, Creek Geme, 243 m, 03 ix 2014, E. Cabi, M.M. Uma, N. Albayrak (AIBU). **Denizli:** Çivril, W. of Işıklı Lake, Buca Village, ca. 850 m, 15 vi 1981, E. Leblebici (EGE 26217). **Edirne:** Enez, Gala Lake, G. Altınayar (DSI 1988). **Isparta:** N. of Eğirdir Lake, Hayran, ca. 940 m, 09 vii 1980, E. Leblebici (EGE 26841). **İstanbul:** Terkos Village, in the lake, 29 ix 1969, A. Baytop (E 00330333). Çatalca, Terkos Lake, 01 ix 1943, B. Kasaplıgil (ISTF 5282). Kurtköy, Terkos Lake shore, 04 x 1967, A. Baytop, G. Atila (ISTE 12146). Terkos Village, in the lake, 29 ix 1967, A. Baytop (ISTE). Terkos Lake, 29 vii 1952, A. Baytop (ISTE 2835). Terkos Lake, 02 vii 1969, A. Baytop (ISTE 15527). Terkos Lake, 17 vii 1970, A. Baytop (ISTE 18182). **İzmir:** Ödemiş, Bozdağ, Gölcük, 04 vii 1966, C. Regel (EGE 24864). Ödemiş, Gölcük, submerged, 07 vii 1962, C. Regel (EGE1851). Ödemiş, road to Gölcük, 08 vi 1946, A. Heilbronn, M. Başarman (ISTF). Ödemiş, Bozdağ Village, Kırkoluklar fountain, ca. 1000 m, 09 vi 1980, E. Leblebici (EGE 2680). **Kars:** Çıldır-Kars road, Lake Çıldır shore, 20 viii 1975, A. Baytop, E. Tuzlacı (ISTE 33416). **Kırıkkale:** Before Kızılırmak-Balaban stream mixing site, 678 m, 27 ix 2014, A.E. Yaprak, S.T. Körüklü, İ. Başköse, A. Güleryüz (AIBU, ANK). **Kırklareli:** City centre, Koyunbaba Village, Koyunbaba spring, submerged, 170 m, 24 v 1994, E. Üzen (ISTF). **Kırşehir:** Hirfanlı Dam, 875 m, 12 vii 2014, A.E. Yaprak, S.T. Körüklü, İ. Başköse, A. Güleryüz (AIBU, ANK). **Kocaeli:** Saracoğlu Motel at the Lake Sapanca, floating on the shore of the lake, 40 m, 01 ix 1972, P. Uotila 20153 (E). **Sakarya:** E. shores of Lake, near Arifiye, 04 vii 1976, A. Baytop, K. Alpınar (ISTE35123). N. shore of Sapanca Lake, 24 viii 1973, H. Güner (EGE12774). Sapanca Lake, 50 m, edge of lake, 30 vi 1962, P.H. Davis 36207, M.J.E. Coode (K). **Konya:** Akşehir, E. of Ilgın Village, Gedikören Village, ca. 1000 m, 08 vii 1980, E. Leblebici (EGE 26830). Ilgın, Çavuşçu Lake, submerged, 08 viii 1992, A.A. Dönmez, N. Emir (GAZI, HUB). Between Beyşehir-Şarkıkaraağaç, 12 km to Beyşehir, in water, 1100 m, 03 viii 1978, A. Baytop, E. Tuzlacı (ISTE41432). Beyşehir, Çiftlikköy, shores, 06 vii 2014, T. Körüklü (AIBU 12345). Beyşehir, 01 vi 1949, İ. Baykal (ISTF). Beyşehir (Isawia), Hoynan, in the lake, 05 viii 1949, P.H. Davis 16113 (K). Çumra irrigation canal, Yeni İsmil reserve irrigation canal, G. Altınayar (DSI 1988). **Malatya:** Karakaya Dam, 690 m, 19 vii 2014, M.M. Uma, N. Albayrak (AIBU). **Manisa:** Adala irrigation canal to Marmara Lake, G. Altınayar (DSI 1988). Salihli, N. of Gölmarmara, Sazköyü, ca. 90 m, 03 vi 1981, E. Leblebici (EGE26144). **Nevşehir:** Kızılırmak-Gülşehir Bridge, 888 m, 13 vii 2014, A.E. Yaprak, S.T. Körüklü, İ. Başköse, A. Güleryüz (AIBU, ANK). **Ordu:** Kaga Göl above Fatsa, 250 m, deep warm lake, 21 vii 1965, C. Tobey 1312 (E). **Samsun:** Bafra, Kızılırmak, 8 m, 08 vii 2014, A.E. Yaprak, S.T. Körüklü, İ. Başköse, A. Güleryüz (AIBU, ANK). **Sivas:** Kangal, Üçöz Dam Lake, 1573 m, 19 vii 2014, M.M. Uma, N.Albayrak (AIBU). **Şanlıurfa:** Birecik Dam Lake, 392 m, 24 vii 2014, M.M. Uma, N. Albayrak (AIBU). **Van:** Between Doğubeyazıt and Taşlıçay, Balık Lake, ca. 2450 m, 11 viii 1985, L. Behçet (VANF).

*Ecological notes*

Measurements of the physical and chemical parameters of the water bodies for *P. perfoliatus* were performed in 24 wetlands (Table 4). Unlike *P. praelongus*, *P. perfoliatus* is widespread and occurs in diverse habitats. The pH range of the water it occurs ranges from 7.52 to 9.01. Based on our collections from 34 wetlands, *P. perfoliatus* occurs together with other Potamogetonaceae species in 31 of these wetlands (Table 1). According to these findings, *P. perfoliatus* co-occurs in 18 wetlands with *Stuckenia pectinata* (L.) Börner, in 12 wetlands with *P. crispus* L., in 7 wetlands with *P. nodosus* Poir. and *P. berchtoldii* Fieber, in 6 wetlands with *P. lucens* L., and in 5 wetlands with *Zannichelia palustris* L., *Groenlandia densa* (L.) Fourr., *P. gramineus* L., *P. pusillus* L. and *P. trichoides* Cham. & Schltdl. were found to accompany *P. perfoliatus* in 3 wetlands. *Potamogeton natans* was found to grow together with *P. perfoliatus* at 2 sites and in 3 sites *P. perfoliatus* was the only species growing.

**Table 4.** Physical and chemical measurements for *P. perfoliatus* (NB stands for collector N. Bayındır. See materials and methods for additional abbreviations). Detailed information about each voucher is given in Table 1.

| Voucher | Sampling date | Tw (°C) | pH | DO (mg $l^{-1}$) | EC µS $cm^{-1}$ | TDS (g $l^{-1}$) | Salinity (ppt) | Ammonium (mg $l^{-1}$) | Abundance |
|---|---|---|---|---|---|---|---|---|---|
| NB 1178 | 10.6.2016 | 22.1 | 8.28 | 5.64 | 427.8 | 0.2944 | 0.22 | 0.50 | 3 |
| NB 1182 | 10.6.2016 | 23.6 | 8.49 | 8.48 | 309.9 | 0.2067 | 0.15 | 0.14 | 2 |
| NB 1185 | 10.6.2016 | 25.9 | 8.31 | 10.56 | 710.0 | 0.4615 | 0.34 | 0.38 | 4 |
| NB 1208 | 12.6.2016 | 27.6 | 7.53 | 3.98 | 2253 | 1.4040 | 1.10 | 1.11 | 1 |
| NB 1216 | 18.6.2016 | 24.7 | 8.86 | 9.03 | 550.0 | 0.2675 | 0.27 | 0.72 | 3 |
| NB 1228 | 19.6.2016 | 28.0 | 8.99 | 5.83 | 317.7 | 0.1911 | 0.12 | 0.32 | 4 |
| NB 1235 | 20.6.2016 | 31.2 | 8.82 | 12.31 | 369.3 | 0.2080 | 0.15 | 0.42 | 4 |
| NB 1261 | 25.6.2016 | 23.1 | 8.09 | 6.95 | 241.3 | 0.1631 | 0.12 | 0.26 | 4 |
| NB 1268 | 27.6.2016 | 18.4 | 7.57 | 8.99 | 259.0 | 0.1924 | 0.14 | 0.39 | 3 |
| NB 1273 | 27.6.2016 | 29.5 | 8.23 | 6.35 | 455.2 | 0.2723 | 0.20 | 0.21 | 2 |
| NB 1293 | 14.8.2016 | 23.9 | 7.73 | 7.53 | 1977 | 1.3130 | 1.03 | 0.59 | 2 |
| NB 1296 | 14.8.2016 | 24.1 | 8.24 | 7.64 | 415.5 | 0.2750 | 0.20 | 0.21 | 1 |
| NB 1301 | 15.8.2016 | 26.8 | 7.88 | 3.66 | 350.1 | 0.2223 | 0.16 | 0.14 | 1 |
| NB 1312 | 19.8.2016 | 17.1 | 7.52 | 6.40 | 470.0 | 0.3601 | 0.27 | 0.48 | 2 |
| NB 1318 | 20.8.2016 | 23.0 | 8.13 | 5.96 | 253.7 | 0.1716 | 0.12 | 0.27 | 3 |
| NB 1330 | 21.8.2016 | 23.2 | 7.77 | 3.52 | 2385 | 1.5065 | 1.27 | 0.11 | 3 |
| NB 1335 | 21.8.2016 | 22.7 | 8.90 | 3.35 | 411.0 | 0.2802 | 0.21 | 0.11 | 2 |
| NB 1337 | 21.8.2016 | 24.5 | 9.01 | 8.89 | 493.1 | 0.3237 | 0.24 | 0.22 | 2 |
| NB 1339 | 22.8.2016 | 21.2 | 7.82 | 6.78 | 1595 | 1.1180 | 0.87 | 0.66 | 1 |
| NB 1341 | 05.9.2016 | 17.4 | 7.60 | 6.37 | 974 | 0.7410 | 0.57 | 0.46 | 2 |
| NB 1345 | 05.9.2016 | 22.2 | 8.33 | 4.38 | 547 | 0.3770 | 0.28 | 0.21 | 2 |
| NB 1363 | 08.9.2016 | 21.9 | 8.03 | 6.55 | 432.3 | 0.2963 | 0.22 | 0.84 | 3 |
| NB 1367 | 08.9.2016 | 22.1 | 7.97 | 4.83 | 304.6 | 0.2100 | 0.15 | 0.04 | 2 |
| NB 1415 | 11.8.2017 | 22.0 | 8.65 | 7.91 | 133.3 | 0.0917 | 0.07 | 0.18 | 2 |

## Discussion

There are two clasping-leaved *Potamogeton* species growing in Turkey (Uotila 1984, Bayındır 2018). Until recently, *P. praelongus* was thought to be extinct in the country. However, we collected the species from a new location different from that of the historical site in 2016 (Bayındır 2018). Our field observations in the historical site showed that the *P. perfoliatus* is the only species growing here. We identified all recent collections from this historical locality cited in the Flora of Caucasus (Grossheim 1928) as *P. perfoliatus*. It can be considered that the specimens were incorrectly identified as *P. praelongus* in the past. The other possibility is the replacement of *P. praelongus* from the lake by *P. perfoliatus*. Our recent collection site for *P. praelongus* is from southern Turkey where *P. perfoliatus* does not co-occur (Fig. 1). As indicated previously, *P. praelongus* has a widespread distribution range throughout temperate Northern Hemisphere. However, it is a very rare species in several of the countries it occurs and listed as endangered, critically endangered or near threatened. In several countries, its populations are declining. In Turkey, the species grows in a Mediterranean mountain habitat in an oligotrophic alpine lake at c. 2600 m (Bayındır 2018). This is the single population for Turkey and the only record for the species in the Mediterranean basin. Distribution range of the population is less than 500 $m^2$. Around the lake where *P. praelongus* grows, there are camping areas for mountain climbers and other tourists. The nearest settlement is 300 m below the lake, which is used as a summer pasture by villagers for stock farming. Increasing visitors will lead to increased eutrophication of the lake. The species is known to be very sensitive to

eutrophication and was recorded to disappear in some lakes due to increased eutrophication. Therefore, its unique population is subjected to rapid deterioration due to eutrophication and future climate changes. *Potamogeton praelongus* is a possible candidate to be negatively affected by global warming due to increase in surface water temperatures since its population is already at 2600 m (İkinci & Bayındır 2020). Urgent conservation strategies should be developed for the species to protect its current habitat. In terms of water chemistry preferences, in a study performed throughout Japan, Kadono (1982) found that *P. praelongus* has a pH range between 7.1 and 7.8 and EC range between 77 and 112 ($\mu S\ cm^{-1}$). We recorded slightly higher EC levels but our pH measurements fall within the range of this study. *Potamogeton praelongus* grows in waters with low EC, low salinity and low ammonium concentrations (Table 2). These are expected results since the alpine lake is mainly fed by snowmelt and rainwater. Other macrophytes occurring in the same lake are *P. natans*, *Stuckenia filiformis* and *Persicaria amphibia*. Among these species, *S. filiformis* is also very rare in Turkey and has only one additional record from Eğrigöl Lake in Antalya at 2060 m altitude. Different studies classify Eğrigöl Lake as either oligotrophic or oligo-mesotrophic (Kaymakçı-Başaran & Egemen 2006, Aygen *et al.* 2009). On the other hand, *P. natans* is a widespread species throughout Turkey with a tendency of preferring higher altitudes (İkinci & Bayındır 2020). *Persicaria amphibia* is a very widespread species having natural distribution range as northern circumpolar temperate but it is naturalized in North and South America, and South Africa (Partridge 2001). It is also a very common species in Turkey (Seçmen & Leblebici 2008, İkinci & Bayındır 2019). It can be found in different habitats including disturbed ones. It is not found in fast flowing water bodies. The species has adaptation to water level fluctuations because it has both aquatic and terrestrial forms (Partridge 2001). *Persicaria amphibia* occurs in a wide range of altitude and it was also found at higher altitudes in the Himalayas similar to Turkish population mentioned in our current study (Ram *et al.* 1989).

*Potamogeton perfoliatus* has almost a cosmopolitan distribution in the world (Gupta & Lansdown 2013). In Turkey, we found records of the species from 54 wetlands based on examination of 86 herbarium specimens (Fig. 1). In addition to the recent Illustrated Flora of Turkey (Bayındır 2018), we added 5 new wetlands for the distribution of the species based on our collections (Table 1). Therefore, we can better understand the extent of occurrence of *P. perfoliatus* in Turkey. The species has a broad altitudinal distribution from sea level to 2450 m but majority of collections are below 1200 m (İkinci & Bayındır 2020). Water chemistry measurements were performed from 24 sampling sites for the species (Table 4). It grows in water bodies with high pH levels. Majority of the collection sites are inland freshwaters. However, we recorded the highest salinity from an inland lake (Hafik Lake) in Sivas. We found the species in lakes and canals close to the Black Sea and the Aegean Sea but still with low salinity levels (max. 1.10 ppt). These findings are parallel to previous reports about *P. perfoliatus* (Burns *et al.* 1995). According to Twilley *et al.* (1985), *P. perfoliatus* grows in oligohaline to mesohaline waters. We recorded *P. perfoliatus* mainly from lentic habitats, out of 54 wetlands only seven were fast flowing rivers and canals. Our collection sites for the species were not shallow waters because the submersed broad-leaved species generally cannot tolerate drawdowns. When we analyzed the other accompanying Potamogetonaceae species provided in Table 1, we see the highest co-occurrence with *Stuckenia pectinata* in 18 wetlands. *Stuckenia pectinata* is the most widespread Potamogetonaceae species in Turkey which we sampled from 65 wetlands (İkinci & Bayındır 2020). Other species with the highest number of co-occurrences with *P. perfoliatus* are *P. crispus*, *P. nodosus*, and *P. berchtoldii*, respectively. These are also common species which we collected from more than 30 wetlands in Turkey (İkinci & Bayındır 2020). On the other hand, *P. natans* was sampled only in two common wetlands with *P. perfoliatus* (Table 1). We collected *P. natans* from 11 wetlands in Turkey which are mainly above 1200 m, indicating a clear difference in altitudinal preference compared to *P. perfoliatus* (İkinci & Bayındır 2018). The other interesting result is that *P. perfoliatus* were not found in water bodies where seven rare Potamogetonaceae species are growing (Bayındır 2018, İkinci & Bayındır 2020).

There are clear morphological and anatomical differences between *P. praelongus* and *P. perfoliatus*. Zig-zag shaped stem, cucullate leaf apice, leaf base, leaf margins and conspicuous, long, persistent stipule, presence of interlacunar bundles, subepidermal bundles and shape of endodermal cells in stem discriminate two clasping-leaved *Potamogeton* species (Table 3 and 5). Persistence of the stipule is an important character in the identification of *Potamogeton* species. In the newly collected *P. praelongus* specimens, stipules are persistent until the late stages of plants life cycle. However, in *P. perfoliatus* specimens we collected stipules decay early during the developmental stages of the plants and therefore are not persistent (Table 5). Previous studies indicated that leaf length is clearly longer in *P. praelongus* (Hagström 1916, Ogden 1943, Haynes 1985, Wiegleb & Kaplan 1998). In our samples, we measured maximum leaf length as 170 mm for *P. praelongus* and as 61 mm for *P. perfoliatus* (Table 5).

**Table 5.** Comparison of morphological characters of *P. praelongus* and *P. perfoliatus* based on our collections and former reports on the two species.

| | | Character | | | | |
|---|---|---|---|---|---|---|
| | | **Stem** | **Leaf base** | **Leaf apice** | **Stipule** | **Maximum leaf lenght (mm)** |
| **This study** | *P. praelongus* | Zig-zag | Semiamplexicaule | Cucullate | Conspicuous, long, persistent | 170 |
| | *P. perfoliatus* | Straight | Amplexicaul | Rarely cucullate | Nonpersistent | 61 |
| **Hagström (1916)** | *P. praelongus* | - | Hooded | Always cucullate | Persistent | 200-250 |
| | *P. perfoliatus* | - | Cordate-amplexicaul | Slightly recurved | Fugacious | - |
| **Ogden (1943)** | *P. praelongus* | Often zigzag | Clasping about 1/4 or 2/4 | Cucullate | Persistent | 200 (-360) |
| | *P. perfoliatus* | - | Clasping about 3/4 | Noncucullate | Delicate, fugacious | 60 (-70) |
| **Haynes (1985)** | *P. praelongus* | Zig-zag | Semi-clasping | Cucullate | Persistent | 80-280 |
| | *P. perfoliatus* | - | Clasping | Non-cucullate (flattened) | Stipules delicate | 76 (-97) |
| **Kaplan & Wiegleb (1998)** | *P. praelongus* | - | Semiamplexicaul | Distinctly hooded | Long, persistent | 180 (-360) |
| | *P. perfoliatus* | - | Amplexicaul | Slightly hooded | Decaying early | 115 |

As regarding the anatomical differences of the two species, in majority of the previous studies (Raunkier 1903, Hagström 1916) and in our samples, interlacunar bundles and subepidermal bundles are present in *P. praelongus* specimens (Table 3). In *P. perfoliatu*s both interlacunar and subepidermal bundles are either not found or rarely occur in specimens with one or a few small bundles (Raunkier 1903, Wiegleb & Kaplan 1998). Our studies are in parallel to these findings (Figs. 2, 3). Hagström (1916) stated that *P. praelongus* have sclerenchyma cells which we also observed (Fig. 3). In contrast to previous studies (Raunkier 1903, Hagström 1916, Haynes 1985, Wiegleb 1990, Wiegleb & Kaplan 1998), we found trio type stele, not the proto type in *P. praelongus*. Such differences may occur among the populations of very widespread species. Our results showed that steles of our specimens are made up of four median bundles, three of which are united. We found that pseudohypodermis is uniseriate in both species. As a result, it can be stated that the presence interlacunar bundles and sclerenchyma cells are useful anatomical characters to distinguish these two species.

In this study, we present information about the physical and chemical properties of the water where *P. praelongus* and *P. perfoliatus* grow and their other habitat requirements. We give a detailed distribution of the two species based on a broad sampling covering entire Turkey. We also provide new morphological descriptions and the first anatomical studies of the species from Turkey. One of these species, *P. praelongus* is critically endangered (CR) in Turkey and was recorded only from a single site. The other species, *P. perfoliatus* is widespread throughout Turkey occurring under different environmental conditions. As we analyzed the habitat preferences of the species, our study provides useful information for future monitoring of the two species in terms of conservation strategies.

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**Research Article**

# AGE, GROWTH AND OTOLITH BIOMETRY-BODY LENGTH RELATIONSHIPS OF RED BANDFISH (*Cepola macrophthalma* L., 1758) IN THE SEA OF MARMARA, TURKEY

Ali İŞMEN[1], Mukadder ARSLAN İHSANOĞLU[1*], İsmail Burak DABAN[1], Haşim İNCEOĞLU[2]

[1] Çanakkale Onsekiz Mart University, Marine Science and Technology Faculty, Fisheries and Fish Processing Department, 17100, Çanakkale, TURKEY

[2] Republic of Turkey Ministry of Agriculture and Forestry, Sheep Research Institute, Fisheries Deparment, 10200, Bandırma, Balıkesir, TURKEY

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***Edited by:***
*Herdem Aslan*

***Corresponding Author:***
*Mukadder Arslan İhsanoğlu*
*mukadderarslan@gmail.com*

***ORCID ID:***
*orcid.org/0000-0003-0072-5848*

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**Abstract:** The age, growth, and the otolith biometry-total length relationships of *Cepola macrophthalma* (Linnaeus, 1758) were investigated. The individuals were caught with beam trawl in the Sea of Marmara from March 2012 to June 2014. The individual with 51.5 cm total length sampled in this study was recorded as the new maximum size of *C. macrophthalma* in the Marmara Sea. The length-weight relationship showed negative allometric growth with a 1.36 b value. Otolith length-otolith weight, otolith width-otolith weight, otolith length-total length, otolith width-total length, otolith length-otolith width and total length-otolith weight relationships were found as $OW=0.0002*OL^{2.6377}$ ($R^2=0.91$), $OW=0.001*OWi^{2.6215}$ ($R^2=0.94$), $O=0.057*TL+3.2087$ ($R^2=0.57$), $OWi=0.0316*TL+1.8511$ ($R^2=0.55$), $OWi=0.527*OL+0.2015$ ($R^2=0.86$), $OW=0.0004*TL+0.0029$ ($R^2=0.58$), respectively. Ages were estimated from 80 otolith readings and the minimum and maximum ages observed were 1 and 5 years, respectively. The growth parameters of the von Bertalanffy equation were calculated as $L_\infty=61.95$ cm, $K=0.19$ year$^{-1}$, $t_0 = -0.05$ years. A great majority of the stock (77%) consisted of younger individuals (1-2 age groups). *Cepola macrophthalma* stock consisted of mainly younger individuals which may indicate that an effective fishing pressure is effective on the stock.

**Özet:** *Cepola macrophthalma* (Linnaeus, 1758)'in yaş, büyüme ve otolit biyometri-total boy ilişkileri araştırılmıştır. Bireyler Mart 2012-Haziran 2014 tarihleri arasında Marmara Denizi'nde algarna ile yakalanmıştır. Bu çalışmada örneklenen 51,5 cm'lik *C. macrophthalma* bireyinin boyu, Marmara Denizi'nde bu tür için maksimum olarak kaydedilmiştir. Boy-ağırlık ilişkisi denklemine göre büyüme tipi negatif allometrik olarak belirlenmiştir (b=1,36). Otolith boyu-otolith ağırlığı, otolith genişliği- otolith ağırlığı, otolith boyu-total boy, otolith genişliği-total boy, otolit boyu-otolit genişliği, total boy-otolit ağırlığı ilişkileri $OW=0,0002*OL^{2,6377}$ ($R^2=0,91$), $OW=0,001*OWi^{2,6215}$ ($R^2=0,94$), $OL=0,057*TL+3,2087$ ($R^2=0,57$), $OWi=0,0316*TL+1,8511$ ($R^2=0,55$), $OWi=0,527*OL+0,2015$ ($R^2=0,86$) $OW=0,0004*TL+0,0029$ ($R^2=0,58$) olarak hesaplanmıştır. Yaşlar 80 adet otolit okumasından hesaplanmıştır; gözlemlenen en küçük ve en büyük yaşlar sırasıyla 1 ve 5'tir. von Bertalanffy denklemine göre hesaplanan büyüme parametreleri değerleri $L\infty=61,95$ cm, $K=0,19$ yıl$^{-1}$, $t_0 = -0,05$ yıl olarak belirlenmiştir. Stoğun büyük çoğunluğu (%77) genç bireylerden (1-2 yaş grubu) oluşmaktadır. Marmara Denizi'nde *C. macrophthalma* stoğunun daha çok genç bireylerden oluşması stokların av baskısı altında olduğunu düşündürmektedir.

## Introduction

The red bandfish, *Cepola macrophthalma* (Linnaeus, 1758) is a marine demersal fish species that inhabit soft and muddy bottoms at depths ranging from 15 to 400 m (Sanches 1991). It usually lives in vertical burrows and distributes singly or in groups. There is little information about early life stages of the species except that the eggs are pelagic. The main food source of the species are small crustaceans and chaetognaths (Whitehead *et al*. 1986). The maximum size of the species has been recorded as 80 cm TL in the Biscay Bay (Sanchez *et al*. 1995) but the mean length is at about 40 cm in Guiné-Bissau costs in the Atlantic Ocean (Sanches 1991). The details of ecology and life history of *C. macrophthalma* is poorly known. Although it is known as an eastern Atlantic origin species,

it has an extensive geographical distribution from the Strait of Gibraltar to northern Senegal; northward extending into the Canary Islands, the Mediterranean and north Atlantic up to Scotland and the Orkney Islands (Smith-Vaniz 2015).

In the Mediterranean, *C. macrophthalma* is present in the Catalan Sea (Coll *et al*. 2006), the Gulf of Lions (Gaertner *et al*. 1998), the Ligurian Sea (Molinari & Tunesi 2003), the Tyrrhenian Sea (Colloca *et al*. 2004, Carpentieri *et al*. 2005), the Cretan Sea (Kallianiotis *et al*. 2000), the Aegean Sea (Stergiou 1999, Lamprakis *et al*. 2003, Machias *et al*. 2004, Labropoulou & Papaconstantinou 2005, Gokce & Metin 2007, Özaydin *et al*. 2007) and the Sea of Marmara (Bok *et al*. 2011). It also is present in the Levantine Sea and along the North African coast. It does not occur in the Black Sea (Smith-Vaniz 2015). Due to the minor commercial interest and threats, *C. macrophthalma* is listed as Least Concern in the IUCN Red List of Threatened Species (IUCN 2015, Smith-Vaniz 2015).

The majority of the literature on the biology and ecology of the species is about its feeding, age, growth, reproduction, and length weight relationships (LWRs) (Stergiou *et al*. 1992, Kaya *et al*. 2001, Vallisneri *et al*. 2006, Dulčić *et al*. 2008, Özaydın *et al*. 2007, Bok *et al*. 2011). Some studies investigated the age, growth, and length-weight relationship parameters in Turkey seas (Kaya *et al*. 2001, Özaydın *et al*. 2007, Leblebici 2007, Bok *et al*. 2011) of which one study reported data on length-weight relationship parameters in the Sea of Marmara (Bok *et al*. 2011).

The aim of this study is to estimate the relationship between otolith size and fish size, age and growth parameters of *C. macrophthalma* in the Sea of Marmara. We reported the first results on the age and growth of *C. macrophthalma* in the Sea of Marmara and the first results about otolith and fish morphometric relationships in general.

## Materials and Methods

A total of 105 *C. macrophthalma* specimens were sampled in the Sea of Marmara with monthly samplings between March 2012 and June 2014 from, (Fig. 1). The samplings were carried out using a commercial beam trawl net. Total length (TL) and weight (W) values of the samples were determined to the nearest 1 mm and 0.01 g, respectively. The length-weight relationship was determined according to the formula of Le Cren (1951): $W=a*L^b$, where W is the total body weight (g), L is the total length (cm) while *a* and *b* are constants. To check whether fish growth is statistically different from isometric growth (b=3) Student's t-test was used by the equation according to Sokal & Rohlf (1987): ts=(b-3)/SE(b), where ts is the t-test value, b is the slope and SE(b) is the standard error of the b value.

The ages of 80 specimens were determined using the sagittal otoliths, while the rest 25 otoliths were not evaluated as they were broken to a degree which made it impossible to determine their age. The otolith is the most commonly used material for age estimation (Holden & Raitt 1974) and was commonly used for age determination of *C. macrphthalma* (Kaya *et al*. 2001, Leblebici 2007). The otolith of this species is easly readible. The nucleus is totally opaque, first translucent band starts after the opaque zone. Estimation of age was based almost exclusively on the interpretation of otolith structures for the presence of translucent and opaque zones which are assumed to represent winter and summer growth periods and the date of birth is assumed 1 January. Sagittal otoliths from each fish were removed and cleaned. The size of the otoliths was measured with Q-Capture Digital Imaging Software attached on an Olympus SZX-7 stereomicroscope with a camera sensitivity to 0.01 mm and weighed with the precision of 0.0001 g by scales (Fig. 2). Annual rings on the whole otoliths were counted in glycerin under a stereomicroscope. The translucent and opaque zones were counted for age determination. The otoliths were read by three different observers and age was determined when minimum two of the readings agreed.

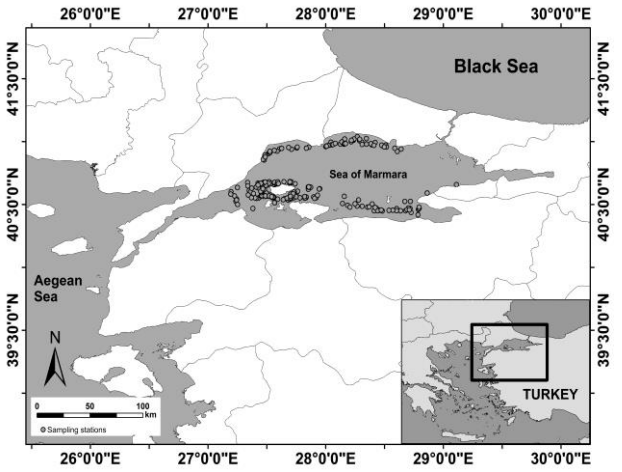


**Fig. 1.** Sampling stations in the Sea of Marmara, Turkey (İşmen *et al*. 2018). Each open dot represent a different station.

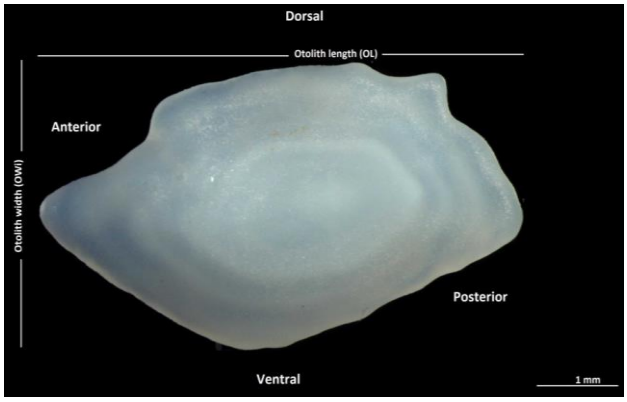


**Fig. 2.** Distal view and measurement axes of the sagittal otolith of *C. macrophthalma* in the Sea of Marmara (TL: 31.8 cm, W: 18.56 g, Age: 3).

Otolith length (OL), otolith width (OWi) and otolith weight (OW) were measured in all otoliths as shape parameters. For the analysis of relationships between OL-OW, OWi-OW, OL-TL, OWi-L, OL-OWi and TL-W, the equation y=ax+b was used where y is OW, OWi or OL, x is TL or OL, and equation $y=ax^b$ where y is OW, x is OL or OWi, and a and b are constant coefficients. The chosen regression model was decided based on the magnitude of the $R^2$ value.

Growth parameters were determined using the von Bertalanffy equation (Beverton & Holt 1957): $Lt=L_\infty (1-e^{-K(t-t_0)})$, where $L_\infty$ is the asymptotic total length, $L_t$ the total length at age t, K the growth curvature parameter and $t_0$ is the theoretical age when fish would have been at zero total length. Growth parameters were estimated using the "Analyze of length at age data" method in FAO-ICLARM Stok Assessment Tools (FISAT II) software. For the sake of comparison, the index of overall growth performance Φ proposed by Pauly & Munro (1984) was calculated. This test indicated the reliability of age estimates since it had been suggested that phi-prime test values were similar for the same species and genera. The test was based on $\Phi = \log K+2*\log L_\infty$ (Pineiro & Sainza 2003).

All statistics were analyzed using the MINITAB 16 program. The normality assumptions were checked with Kolmogorov-Simirnov test. Student's t-test was used for analyzing the fish growth type. Paired t-test was used to test whether there is a difference between right and left otoliths. The regression models were used to explain relationships between fish and otolith morphometry.

## Results

A total of 105 specimens ranged from 8.5 to 51.5 cm in TL and from 1.65 to 24.54 g in weight. A pair of 80 sagittal otoliths were measured. Otolith length, weight and width measurements are given in Table 1. There was no significant difference between right and left otoliths (paired t-test, P=0.63), therefore, only right otoliths (n=80) were used for further analysis.

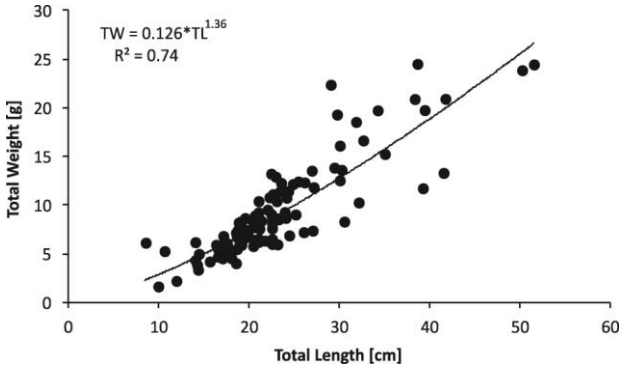


**Fig. 3.** Length - weight relationship of *C. macrophthalma* in the Sea of Marmara.

Length-weight relationship was calculated as $W=0.126*TL^{1.36}$ ($R^2=0.74$) and showed a negative allometric growth pattern (Fig. 3). Otolith length-otolith weight, otolith width-otolith weight, otolith length-total length, otolith width-total length, otolith length-otolith width and total length-otolith weight relationships were found as $OW=0.0002*OL^{2.6377}$ ($R^2=0.91$, $p<0.001$), $OW=0.001*OWi^{2.6215}$ ($R^2=0.94$, $p<0.001$), $OL=0.057*TL+3.2087$ ($R^2=0.57$, $p<0.001$), $OWi=0.0316*TL+1.8511$ ($R^2=0.55$, $p<0.001$), $OWi=0.527*OL+0.2015$ ($R^2=0.86$, $p<0.001$), $OW=0.0004*TL+0.0029$ ($R^2=0.58$, $p<0.001$), respectively (Fig. 4).

The von Bertalanffy growth parameters for *C. macrophthalma* were estimated as $L_\infty$=61.95 cm, K=0.19 $year^{-1}$, $t_0$ = -0.5 year and growth performance index was calculated as Φ=2.86 (Fig. 5).

The age interval ranged between 1 and 5. The highest represented age group was 2 (46%) and the age group 1 (30%) and age group 3 were considerably well represented (Table 2).

**Table 1.** Morphometric measurements of sampled *C. macrophthalma* and their and otolith length, weight and width.

| | TL (cm) | W (g) | OL (mm) | | OWi (mm) | | OW (g) | |
|---|---|---|---|---|---|---|---|---|
| | | | R | L | R | L | R | L |
| **Min. Max.** | 8.5-51.5 | 1.65-24.52 | 3.35-6.1 | 3.23-6.2 | 1.86-3.8 | 1.85-3.29 | 0.0051-0.0245 | 0.0053-0.0254 |
| **Mean** | 22.96±0.75 | 9.38±0.48 | 4.50±0.06 | 4.49±0.06 | 2.58±0.03 | 2.58±0.03 | 0.013±0.0004 | 0.012±0.0004 |
| **N** | 105 | 105 | 80 | 80 | 80 | 80 | 80 | 80 |

TL: total length, W: weight, OL: otolith length, OWi: otolith width, OW: otolith weight, R: right, L: left

**Table 2.** The age-length key of *C. macrophthalma.*

| Age | N | Total length (cm) | |
|---|---|---|---|
| | | Min-Max | Mean |
| 1 | 23 | 14.0-19.9 | 17.7±1.6 |
| 2 | 37 | 18.0-26.9 | 22.0±1.9 |
| 3 | 12 | 25.1-34.2 | 29.4±2.7 |
| 4 | 6 | 30.0-39.2 | 35.5±3.9 |
| 5 | 2 | 50.2-51.5 | 50.9±0.9 |

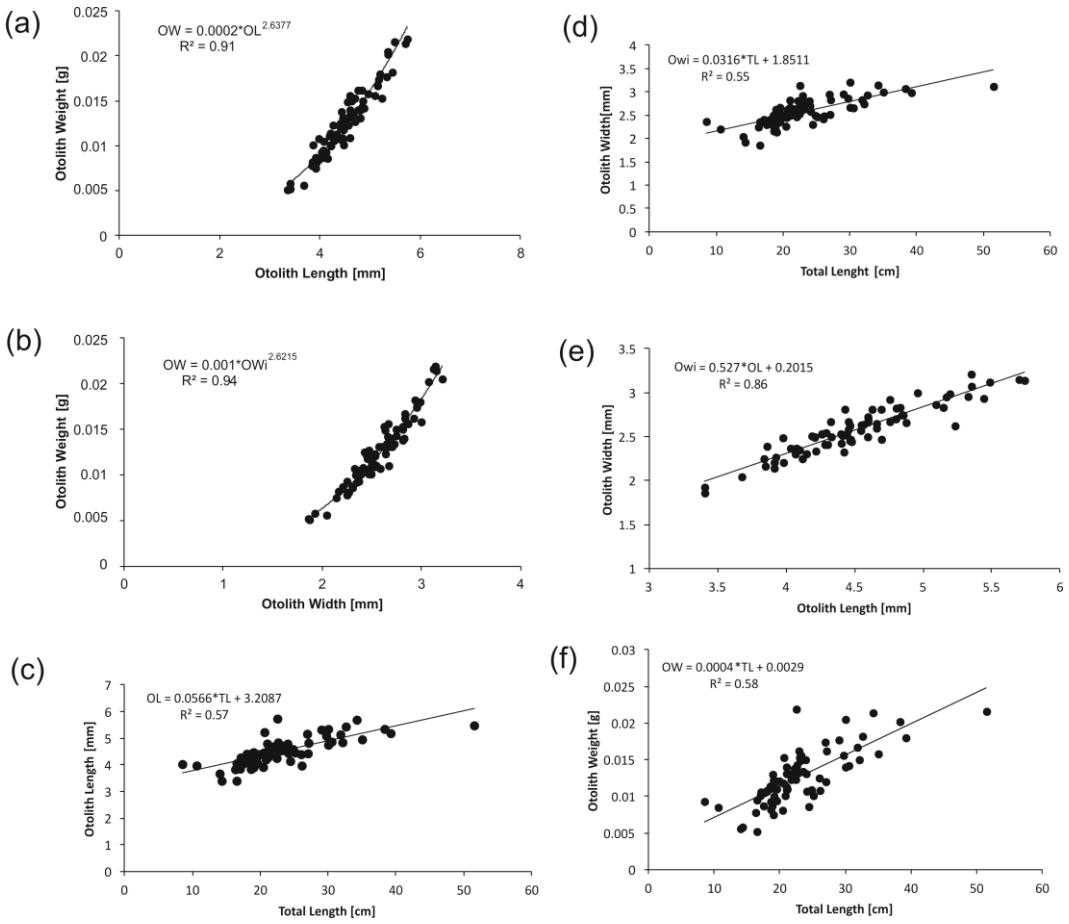


**Fig. 4.** OL-OW (a), OW-OWi (b), TL-OL (c), TL-OWi (d), OL-OWi (e) and TL-OW (f) relationships of *C. macrophthalma* in the Sea of Marmara.

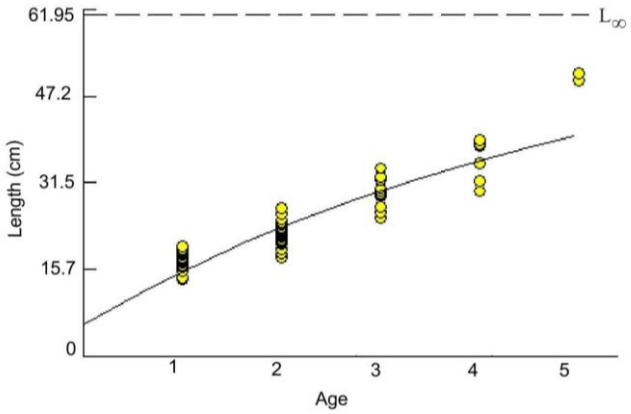


**Fig. 5.** The von Bertalanffy growth curve of *C. macrophthalma* in the Sea of Marmara.

## Discussion

The life span of the *C. macropthalma* is shorter than many demersal fish species (Dulčić *et al.* 2008). According to our results, the oldest individual was 5 years old. A great number of limiting factors may have contributed to the occurrence of a shorter life span, as high fishing pressure, nutritional inadequacy, morphological characteristics of the species, etc. The same results were observed in studies conducted in the eastern Adriatic (Dulčić *et al*. 2008), Adriatic (Vallisneri *et al*. 2006), Izmir Bay (Kaya *et al*. 2001) and Euboikos Gulf, western Aegean Sea (Stergiou & Papaconstantinou 1993). In contrast, relatively long life spans were determined in studies conducted in the British Isles (Atkinson 1976) and in Pagassitikos Gulf, western Aegean Sea (Stergiou & Papaconstantinou 1993). This may be a result of lower fishing pressure and availability of more sheltering areas for *C. macropthalma* in these areas. Its elongated, laterally compressed body shape [horizontal dimension 16 times the vertical one, Stergiou & Papaconstantinou

(1993)] may be advantageous for escaping from the mesh of fishing nets. Barely squeeze in the trawl and beam trawl bag prevents occurring of this advantage. It's slow, wavy mode of swimming (Wilson 1953) may make the species an open target for fishing vessels. The observed short life span in a great majority of studies caused us to focus on fishing pressure. Trawl fishing is the main reason for fishing pressure on demersal species. Demersal trawling is prohibited in the Sea of Marmara. Conversely, beam trawl fishery is legally allowed and extensively applied. The relatively lower growth rate and shorter life span may have resulted from the high fishing pressure of beam trawl fishery in the Marmara Sea. The age-frequency distribution also supports this finding. A majority of individuals were of age groups 1 and 2.

The *b* value shows negative allometry (Student t-test). Negative allometry is mandatory due to the physical nature of the species. The exponent *b* usually varies between 2.5-3.5 for other fish species. Merely, the lifestyle of *C. macrophthalma* may cause unproportional length increases according to growth in weight, and this may induce lower *b* values (Froese 2006). However, the calculated *b* value (1.358) in this study reveals the lowest value compared with the others (Table 3). Poor food availability and competition for food resources in the Sea of Marmara may have caused this situation. In a single study conducted in the Sea of Marmara on *C. macrophthalma*, Bok *et al*. (2011) calculated the *b* value as 1.510.

Due to the absence of studies on the age and growth parameters of *C. macropthalma* in the Sea of Marmara, growth parameters were compared with other studies conducted in adjacent waters. In almost all studies, the calculated $L_\infty$ and K values are greater than the ones in this study. The estimated $L_\infty$ and K values in our study are in agreement with the findings of Stergiou *et al*. (1992). Also, the same K value was obtained in the findings of Kaya *et al*. (2001), whereas our calculated $L_\infty$ value is lower. We think this may be due to the high number of smaller individuals in our data set (Table 4).

**Table 3.** Total length-total weight relationships of *C. macrophthalma* obtained by different researchers.

| Researcher(s) | Region | N | Sex | a | b | $R^2$ | Length |
|---|---|---|---|---|---|---|---|
| Stergiou 1991 | Euboikos and Pagassitikos Gulfs | 515 | Female | 0.0491 | 1.667 | 0.75 | 11.8-51.3 |
| | | 452 | Male | 0.0401 | 1.716 | 0.75 | |
| | | 967 | Mixed | 0.0456 | 1.683 | 0.75 | |
| Pereda & Villamor 1991 | Cantabrica | 103 | Mixed | 0.0128 | 2.169 | 0.98 | 11-65 |
| Stergiou *et al*. 1992 | Aegean Sea | 3351 | Mixed | 0.0166 | 2.03 | 0.93 | 10.4-58.7 |
| Kaya *et al*. 2001 | Aegean Sea | 131 | Female | 0.3288 | 1.270 | 0.79 | 11.5-45.6 |
| | | 144 | Male | 0.2154 | 1.384 | 0.81 | 19.8-47.1 |
| Lamprakis *et al*. 2003 | North Aegean Sea | 1021 | Mixed | 0.0863 | 1.543 | 0.80 | 10.3-53.2 |
| Özaydın & Taşkavak 2006 | İzmir Bay | 254 | | 0.0203 | 1.97 | 0.98 | |
| Özaydın *et al*. 2007 | İzmir Bay | 881 | Mixed | 0.0741 | 1.669 | 0.95 | 16.2-50.9 |
| Leblebici 2007 | İzmir Bay | 340 | Female | 0.0189 | 2.063 | 0.91 | 17.4-39.6 |
| | | 1450 | Male | 0.0405 | 1.823 | 0.94 | 16.3-54.0 |
| Türker Çakır *et al*. 2007 | Edremit Bay | 356 | Mixed | 0.1379 | 1.4421 | 0.88 | 12.3-43.7 |
| İşmen *et al*. 2007 | Saros Bay | 136 | Mixed | 0.03461 | 1.8533 | 0.92 | 19.1-49.6 |
| İlkyaz *et al*. 2008 | Aegean Sea | 635 | Mixed | 0.0716 | 1.65 | 0.97 | 16.4-51.6 |
| Bok *et al*. 2011 | Northern Sea of Marmara | 17 | Mixed | 0.0093 | 1.510 | 0.84 | 20.8-46.7 |
| Torres *et al*. 2012 | Gulf of Cadiz | 447 | Mixed | 0.0270 | 2.009 | 0.95 | 6.8-98.2 |
| Bilge *et al*. 2014 | Aegean Sea | 988 | Mixed | 0.0126 | 1.4421 | 0.88 | 7.5-51 |
| This study | Sea of Marmara | 105 | Mixed | 0.126 | 1.358 | 0.74 | 8.5-51.5 |

**Table 4.** Parameters of von Bertalanffy growth equation (K, $L_\infty$, $t_0$, Φ) obtained by different researchers.

| Researcher(s) | Region | N | Sex | L∞ | K | t0 | Φ |
|---|---|---|---|---|---|---|---|
| Stergiou *et al*. 1992 | South Evvoikos Gulf | | | 42.5 | 0.379 | -0.1 | 2.84 |
| Stergiou *et al*. 1992 | North Evvoikos and Pagassitikos Gulfs | | | 67.6 | 0.214 | 0 | 2.99 |
| Kaya *et al*. 2001 | İzmir Bay | | Female | 78.5 | 0.17 | 0.1 | 3.02 |
| | | | Male | 83.2 | 0.16 | 0.09 | 3.04 |
| Leblebici 2007 | İzmir Bay | 44 | Female | 48.78 | 0.354 | -0.39 | |
| | | 141 | Male | 56.82 | 0.393 | -0.45 | |
| This study | Sea of Marmara | 80 | Mixed | 61.95 | 0.19 | -0.50 | 2.86 |

Since otolith biometry (shape, size, etc.) varies according to to size of the fish species, the relationship between otolith biometry and fish length can be useful for species identification and prey length from the otoliths found in stomach content (Campana & Thorrold 2001). Besides, it can be used by archaeologists to reveal the mystery from excavation (Hajkova *et al*. 2003). Otolith biometry fish length relationship has been carried out for many species (Appelbaum & Hechte 1978, Hare & Cowen 1995, Hoşsucu *et al*. 1999, Bostancı 2009, Park *et al*. 2018). This study reveals the first results for otolith biometry-total length relationship approach of *C. macrophthalma*. The morphological difference was not found between the left and right otoliths (paired t-test, p=0.63). Therefore the researchers studying on *C. macrophthalma* can use both otoliths in their calculations.

Because of its minor commercial interest, *C. macrophthalma* is listed as Least Concern in the IUCN Red List of Threatened Species (IUCN 2015). According to IUCN, the species has been assessed lastly in 2013 and its population trend is identified as unknown. In addition, the stocks of mature individuals are stated to show a decline trend. *Cepola macrophthalma* is occasionally fished for utilizing fresh, fish soup, fish oil or fishmeal (Whitehead *et al*. 1986) especially around Spain, Portugal and along the West African coasts. Although it has no commercial value, it is known to have a high discard rate in trawling fishery in the Aegean and the Mediterranean Sea and the Sea of Marmara in Turkey. Knowledge of specific conservation measures for *C. macrophthalma* is unknown, and there exists not enough scientific work to take protective measures in the Sea of Marmara. More detailed further studies should be advised especially on the biology and population dynamics.

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**Research Article**

# BORON INCREASES THE VIABILITY OF HUMAN CANCER AND MURINE FIBROBLAST CELLS AFTER LONG TIME OF CRYOPRESERVATION

Taha Bartu HAYAL

Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul, TURKEY

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**Abstract:** Through the process of cryopreservation, cells are stored at very low temperature for a long time to decrease the biological and chemical reactions in viable cells. In this process, the administration of cryoprotective agents is crucial since cryopreservation is regarded as a leading process in various research fields such as biotechnology, clinical medicine and maintenance of both animal and plant cells. Even after a long time of storage in very low temperatures, a recovery is achieved by cryo-preservative agents that act on cellular metabolism and biophysiology of cells. In the current study, the effect of boron on cryopreservation of human lung cancer cell line, A549, and murine fibroblast cell line, L929, was investigated with the help of cell viability assay, colony forming unit assay and RT-PCR analysis. 15 µg/ml boron supplemented freezing medium was found to indicate a positive effect on cell viability. Moreover, gene expression profiles of A549 and L929 cell lines have been altered. The levels of apoptosis related genes decreased while proliferation related gene levels increased significantly after repeated freeze-thaw cycles or long period of freezing. As indicated through our results, sodium pentaborate pentahydrate, as a boron source, might be a crucial cryoprotective agent for cryo-protection and bio-banking of cancer and healthy cells while keeping their viability and functionality.

**Özet:** Canlı hücrelerin uzun süre boyunca çok düşük sıcaklıklarda saklanması işlemi kriyo-korunma olarak adlandırılır. Kriyo-koruma işlemi biyoteknoloji, klinik çalışmalar ve hayvan veya bitki hücreleriyle ilgili birçok çalışmada çok önemli bir rol oynadığından dolayı, kriyo-korumada kullanılan ajanların araştırılması son derece mühimdir. Kriyo- koruma ajanları, hücresel metabolizma ve biyofizyoloji üzerindeki etkileri nedeniyle uzun süreli kriyo-korumanın ardından hücresel canlılığın korunmasını sağlarlar. Mevcut çalışmada; hücre canlılık testi, koloni oluşturma testi ve gerçek zamanlı polimeraz zincir reaksiyonu tekniklerinden yararlanılarak, borun kriyo-koruma üzerindeki etkisi, insan akciğer kanser hücre hattı, A549 ve fare fibroblast hücre hattı, L929 kullanılarak araştırılmıştır. Hücre dondurma ortamını 15 µg/ml bor ile desteklemenin hücre canlılığı üzerine olumlu etki ettiği gözlemlenmiştir. Ayrıca, tekrar eden dondurma - çözme döngüleri ve uzun süreli kriyo-koruma sonucunda, gen anlatım profilleri değişen A549 ve L929 hücre hatlarının, bor takviyesi sonrasında, programlı hücre ölümüyle alakalı genlerinin anlatımında azalma, hücre çoğalması ile ilgili genlerinde de artış gözlemlenmiştir. Sonuçlarımız göstermiştir ki bor kaynağı olarak sodium pentaborat pentahidrat, kanser veya sağlıklı hücrelerin canlılıklarını kaybetmeksizin dondurulmalarını ve hücrelerin uzun süreli saklanmaları için son derece önemli bir kriyo-koruyucu ajan olarak kullanılabilir.

***Edited by:***
*Reşat Ünal*

****Corresponding Author:***
*Taha Bartu Hayal*
*bartu.hayal@yeditepe.edu.tr*

***ORCID ID:***
*orcid.org/0000-0003-1369-2715*

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## Introduction

Cryopreservation is a process that is used to store cells for a long period of time at very low temperature which reduces the biological and chemical reactions in viable cells (Pegg 2007). Cryopreserved cells are in the state called suspended animation which refers to decreasing biological functions of the cells to keep them in fine structure for desired period of time (Mazur 1970, Asfar *et al.* 2014, Sambu 2015). Nevertheless, due to water-to-ice transition, the formation of extra- and intracellular ice crystals can be mortal for living cells (Mazur 1970, Karlsson & Toner 1996, Pegg 2007). The freezing rate of the cells is important since slow cooling results in osmotic alterations caused by formed ice crystals or hypertonic medium. However, fast cooling causes an increase in intracellular ice formation (Jang *et al.* 2017). To overcome these limitations, freezing medium is supplied with cryoprotective agents to control water transport, nucleation and ice crystal formation (Jang *et al.* 2017).

Cryoprotective agents should possess some properties such as being biologically acceptable, being able to penetrate into the cell and being low toxic (Jang *et al.* 2017). With the purpose of maintaining the best survival rate of cells, the sample volume, cooling rate, warming rate and cryoprotective agent concentrations are required to be modified concerning different cell types (Yavin & Arav 2007). Therefore, optimizing the cryopreservation procedures by using effective cryoprotective agents is fundamental to reduce freeze-thaw stress.

Boron is an abundant mineral that plays a critical role in microorganisms (Tanaka & Fujiwara 2008), plants (Warington 1923), animals (Park *et al.* 2005, Hu *et al.* 2014) and humans (Clarke *et al.* 1987, Park *et al.* 2005). Previous studies showed that cell wall and membrane unity of plant cells is disrupted due to the lack of boron suggesting that boron is involved in membrane integrity (Dordas & Brown 2005). Furthermore, several studies demonstrated that boron is a key player in wound healing as well as in cellular proliferation (Blech *et al.* 1990, Dogan *et al.* 2014, Demirci *et al.* 2015, Demirci *et al.* 2016). Boron was also demonstrated as a potential cryoprotective agent in mesenchymal stem cells (Demirci *et al.* 2014) and semen (Yeni *et al.* 2018) culture systems. In this study, sodium pentaborate pentahydrate (NaB) was used as a boron source to investigate the effects of boron on cryopreservation of human lung carcinoma cell line, A549, and murine fibroblast cell line, L929. Thus, the use of NaB aims to develop an advanced method that offers a decrease in the adverse effects of long-term cryopreservation and freeze–thaw procedures.

## Materials and Methods

### *Cell Lines*

A549 and L929 cell lines were purchased from ATCC (American Type Culture Collection, Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK) which was supplemented with 10% fetal bovine serum (FBS, Gibco, UK) and 1% Penicillin/Streptomycin/Amphotericin (PSA, Gibco, UK), was used as a culture medium for both cell lines. Cells were incubated in humidified incubator at 37°C. Atmospheric conditions were adjusted as 5% $CO_2$. Cells were de-attached from the surface with the help of 0.25% trypsin-EDTA (Gibco, UK). Additionally, cells were kept in growth phase at all times by controlling the confluence less than 80%.

### *Freeze-Thaw Cycles*

Freeze-Thaw cycles were conducted to determine any potential cryoprotective effect of NaB on A549 and L929 cells. Freezing procedure was conducted as described previously with minor alterations (Demirci *et al.* 2014). Briefly, cells were trypsinized and counted with hemocytometer. $10^6$ cells were suspended in 1 ml freezing medium (90% FBS, 10% DMSO) with or without 15 µg/ml NaB supplementation. Cells were cryopreserved in 2 ml cryo-vials. The cryo-vials were frozen in Nalgene Mr. Frosty (Thermo Fisher, Waltham, MA, USA) to ensure gradually freezing at -80°C. After 24 hours, frozen cells were transferred to -196°C liquid nitrogen tank. Thawing was performed by increasing the temperature to 37°C immediately to reduce harmful effects of dimetilsulfoxide ($Me_2SO$, DMSO). Viable cells were counted after each thaw. Cell viability test was conducted by using trypan blue (T-8154, Sigma-Aldrich) and a hemocytometer. The cryo-preservative effect of NaB was determined by two different experimental designs, short-term and long-term cryopreservation. Routine cell culture conditions and cell banking for extended period of time were mimicked by short-term and long-term freeze-thaw cycles, respectively. Thawing procedure was performed after 1 day or 6 months after each cycle for short-term and long-term cryopreservation, respectively. Following each thawing, cells were cultured up to 80% confluency before they were subjected to another freezing cycle.

### *Colony Forming Unit (CFU) Assay*

Alterations in colony-forming capability caused by freeze-thaw cycle were determined via CFU assay (Digirolamo *et al.* 1999). After each thaw, A549 and L929 cells were plated in six-well plates (Corning, NY) as 300 cells/well. Culture medium was changed in every 48 hours with fresh growth medium (10% FBS, 1% PSA containing DMEM high glucose) for 15 days. Formed colonies were fixed by using 4% paraformaldehyde and stained with crystal violet. ZEISS microscopy system with AxioCam ICc 5 camera and ZEN2 (blue edition) application was used to take pictures.

### *Real Time PCR Assay*

Distinguishing primers for Beta actin (β-actin), Caspase-3, Caspase-7, B-cell lymphoma 2 (BCL2), Myelocytomatosis (C-MYC), Nuclear Factor Kappa B (NFKB), P53, Ubiquitin Specific Protease 7 (USP7) were designed by using the Primer-BLAST software (National Center for Biotechnology, USA) and Sentegen (Turkey) synthesized the primers. Relative gene expression levels were determined by SYBR Green method (Navarro *et al.* 2015). Briefly, 5 µl of PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher, USA) was supplied with 1.5 µl $dH_2O$, 1 µl primer and 2.5 µl cDNA for each well of 96-well PCR plate. RT-PCR conditions were applied by using RT-PCR system (Bio-Rad, Hercules, CA).

### *Statistical Analysis*

All experiments were conducted at least three times. Normality assumptions were checked using Shapiro-Wilk test by using GraphPad Prism 7 software. Statistical analyses were conducted using one-way ANOVA and Tukey's post hoc test by using GraphPad Prism 7 software. Statistical significance was determined as $P < 0.05$.

## Results

### *NaB supplementation protects A549 and L929 cells in cryopreservation process*

To identify the effect of NaB on repetitive freeze-thaw cycles, trypan blue staining was conducted. A549 and L929

cells were frozen and thawed for four times, repetitively, and viable cells were counted and compared. Results showed that after first freeze-thaw viable A549 and L929 cells were increased by 1.5 and 3 fold, respectively (Fig. 1A). Moreover, cryopreservation media supplemented with 15 µg/ml NaB increased the cell viability of A549 cells by 2.5 fold after second freeze-thaw cycle in which viability of L929 cells were observed to be increased by 3 fold (Fig. 1B). Similarly, increased profile of cell viability was noticed in both A549 (1.4 fold) and L929 (2 fold) cells after third cycle (Fig. 1C). However, the most remarkable results were obtained after fourth freeze-thaw cycle. Cell viability of A549 cells increased by 4 fold and L929 cells by 3.3 fold (Fig. 1D).

*NaB supplemented cryopreservation medium alters colony forming capacity of A549 and L929 cells*

CFU assay was conducted to determine the possible changes in colony forming capacity of A549 and L929 cells after short term freeze-thaw cycles. Obtained results demonstrated that the amount of A549 colonies doubled after NaB addition to cryopreservation media (Fig. 2A-B). Furthermore, dramatically bigger colonies were noticed in NaB included cryopreservation group (Fig. 2C). However, there was no significant change observed in number of L929 colonies between control and NaB groups (Fig. 2D-E). Also, no significant change was detected in diameter of L929 colonies after short term cryopreservation (Fig. 2F).

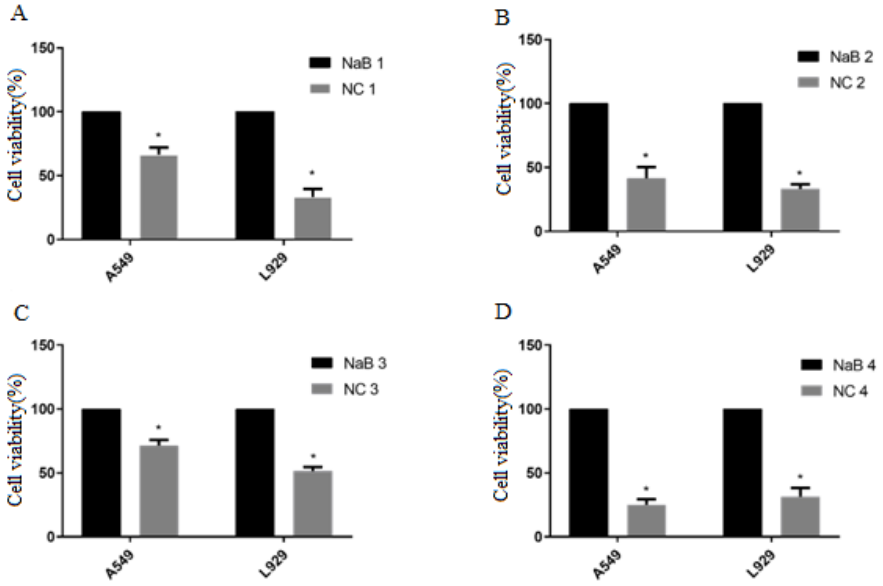


**Fig. 1**. NaB decreases the harmful effects of freeze-thaw cycles on viability of A549 and L929 cell lines. $10^6$ cells were suspended in 1ml freezing medium with or without 15 µg/ml NaB supplementation. Cell viability analysis was conducted after first (A), second (B), third (C) and fourth (D) freeze-thaw cycles. NC: Negative control, NaB: sodium pentaborate pentahydrate, $P<0.05$.

*NaB supplemented cryopreservation medium changes gene expression profiles of A549 and L929 cell lines*

To investigate the effect of NaB supplemented cryopreservation medium on gene expression profiles of A549 and L929 cell lines, RT-PCR assay was performed. A549 and L929 cells were frozen and thawed for four times and after each thaw, alterations in CASP3, CASP7, BCL2, C-MYC, NFKB and P53 gene expression levels were determined. Fig. 3 demonstrates that after the first freeze thaw cycle, expression levels of BLC2 gene dramatically increased in both A549 (12.6 fold) and L929 (3.5 fold). Approximately, 2-fold increase was observed in BCL2, C-MYC and NFKB expression levels in A549 cell line after the second freeze-thaw cycle. Similarly, expression levels of C-MYC (2 fold) and NFKB (2.3 fold) genes increased in L929 cells after the second freeze-thaw cycle. Additionally, after the second cycle, CASP3 gene expression level was found to be 4.6 and 5.99 fold decreased in A549 and L929 cells, respectively. After the third cycle, BCL2 and C-MYC expression levels of A549 cells increased by 10 fold and 8.9 fold, respectively. 5 fold increase in NFKB gene expression level was observed in L929 cell line after the third cycle. Last freeze thaw cycle with NaB supplemented media, resulted in a significant drop in P53 gene expression (27.7 fold) compared to the control group. Also increased C-MYC gene expression (2 fold) was observed in L929 cells after the fourth freeze-thaw cycle with NaB supplementation (Fig. 3).

*NaB supplemented freezing media prevents A549 and L929 cells from long term cryo-damage*

In addition to the short-term freeze-thaw cycles, the long term cryo-preservative effect of NaB supplementation was also investigated in A549 and L929 cells. Frozen cells were transferred into liquid nitrogen tank for 6 months and after that time, all experiments which were conducted for short-term freeze-thaw cycles, were replicated. Obtained results showed that, after long term cryopreservation, cell viability of A549 cells increased (5 fold) with NaB supplementation (Fig. 4A). Similarly, long term storage with regular freezing media resulted in a significant decrease (71.5%) in cell viability

of L929 cells compared to NaB including freezing media (Fig. 4B). After 6 months at -196°C, colony forming capacity of A549 cell line was found to be increased with NaB added freezing media (Fig. 4C). The amount of A549 colonies nearly doubled after NaB supplementation (Fig. 4D) and diameters of the colonies increased by 26.8 mm (Fig. 4E). Yet, there was no significant different observed in L929 colony forming capacity (Fig. 4F), the amount of colonies (Fig. 4G) and diameter of colonies (Fig. 4H). Furthermore, 4 fold increase in C-MYC gene expression was observed in A549 cell line (Fig. 4I). Additionally, the expression levels of CASP3 and CASP7 genes in A549 cell line decreased by 37-fold and 48-fold, respectively (Fig. 4I). A significant decrease in CASP3 (10-fold) and CASP7 (5.2-fold) gene expressions was also observed in L929 cell line (Fig. 4J). Moreover, after long-term cryopreservation of L929 cell line with the NaB supplemented freezing media, BCL2 and P53 expression levels were found to be increased by 2 fold and 3.5 fold, respectively (Fig. 4J).

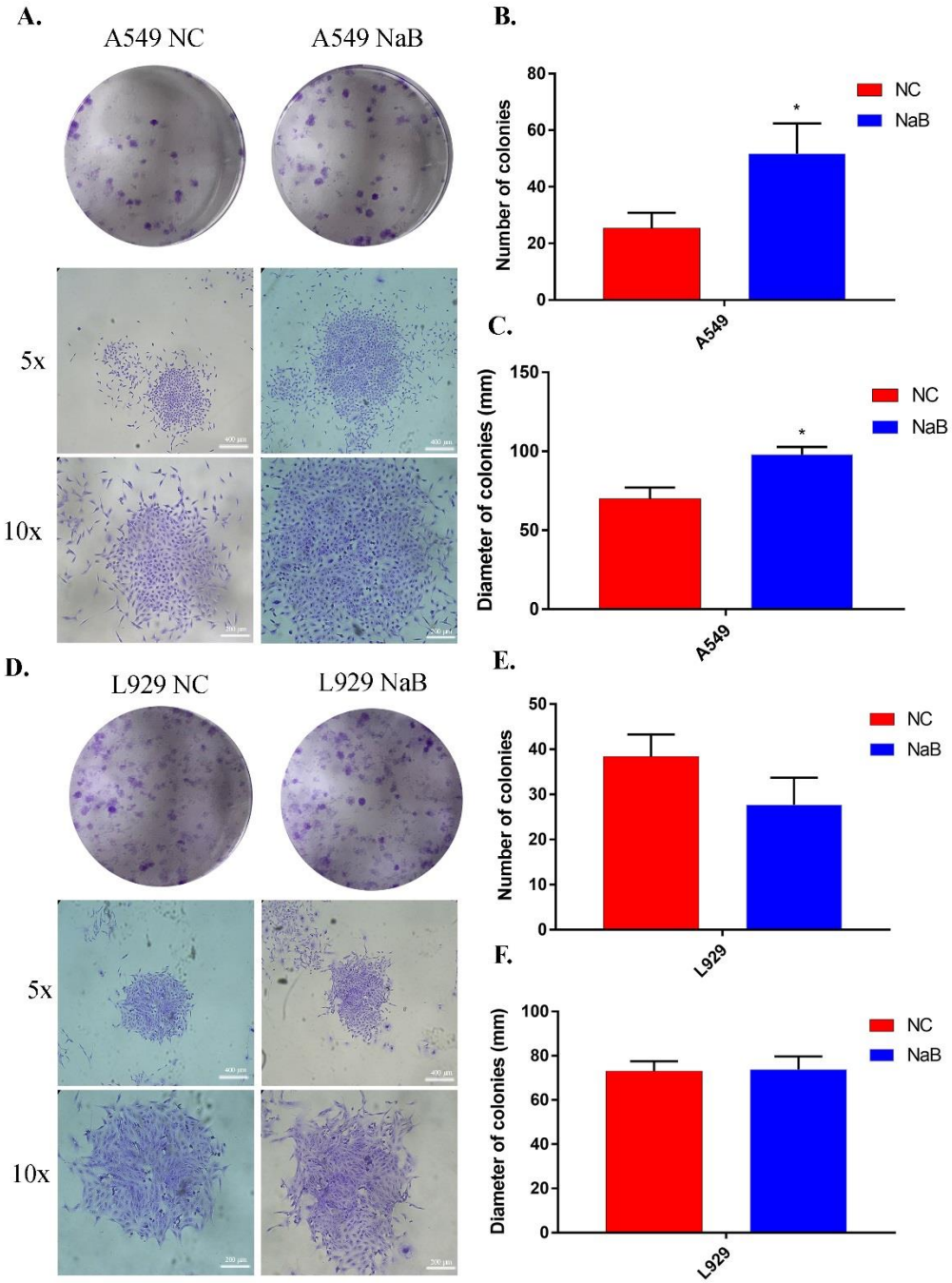


**Fig. 2.** NaB supplemented cryopreservation medium protects the colony forming ability of A549 and L929 cell lines. 15 µg/ml of NaB supplementation increased the capability of colony formation of A549 cell line (A) in terms of number (B) and diameter (C) of colonies. There was no significant change detected in the colony formation ability of L929 cell line (D) as similar amount (E) and size of colonies (F) was observed. NC: Negative control, NaB: sodium pentaborate pentahydrate, $P<0.05$.

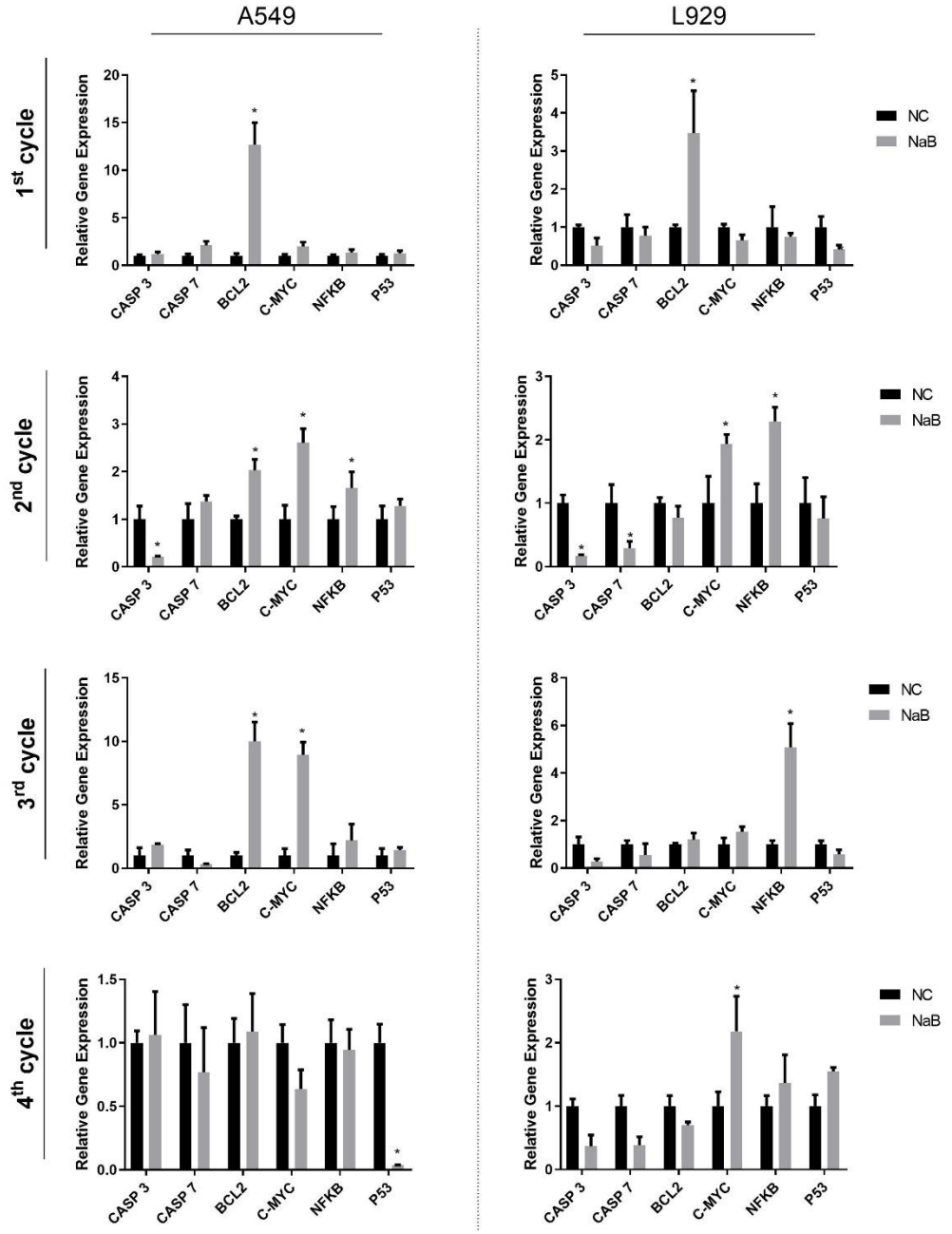


**Fig. 3.** NaB supplemented freezing medium alters the proliferation or apoptosis related gene expressions of A549 and L929 cell lines after each freeze-thaw cycle. NC: Negative control, NaB: sodium pentaborate pentahydrate, CASP3: Caspase 3, CASP7: Caspase 7, BCL2: B-cell lymphoma 2, C-MYC: Cellular myelocytomatosis, NFKB: Nuclear factor kappa B, $P<0.05$.

## Discussion

Cryoprotective agents have several vital roles in the cryopreservation process which can be considered as one of the most important technologies in different research areas such as biotechnology, clinical medicine and maintenance of animal and plant cells. The multiple effects of these agents on cellular metabolism and biophysiology of cells provide a recovery with functionality even after long time of cryopreservation in very low temperatures (Elliott *et al.* 2017). Previous studies identified boron as a possible cryoprotective agent for cryopreservation of stem cells (Demirci *et al.* 2014) and semen (Yeni *et al.* 2018). The cryoprotective effect of boron may be related to its ability to protect integrity and functionality of the membrane along with its interactions with ions to balance osmatic pressure (Park *et al.* 2004, Henderson *et al.* 2009). It is known that the type and the amount of cryoprotective agents might differ according to the cryopreserved cell type (Yavin & Arav 2007). Therefore, here we identify boron as a potential cryoprotective agent for human lung cancer cell line, A549, and murine fibroblast cell line, L929.

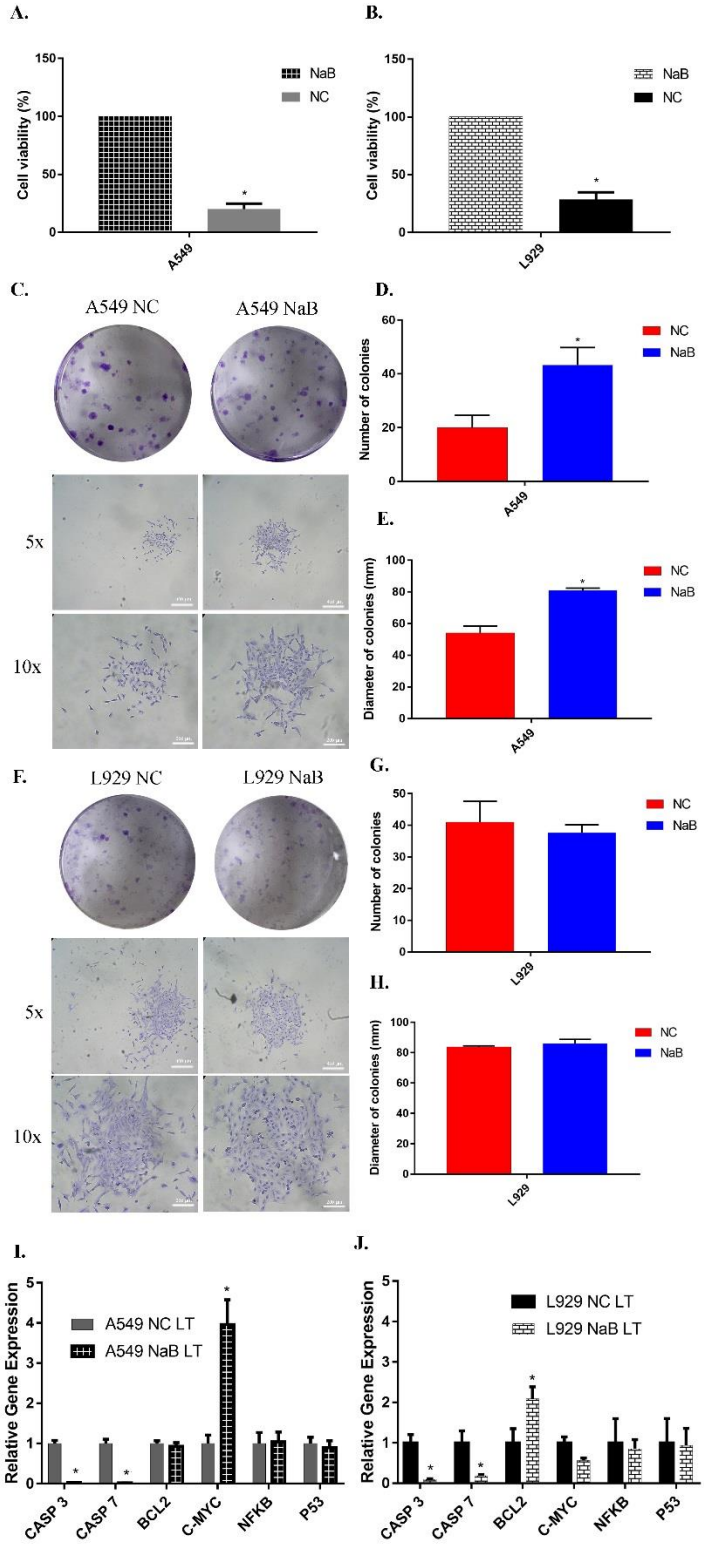


**Fig. 4.** As a cryoprotective agent, NaB protects A549 and L929 cell lines from harmful effects of long term freezing at -196°C. Cell viability of A549 (A) and L929 (B) cell lines was significantly increased by NaB supplementation after 6 months of cryopreservation. (C) Alterations in colony formation capacity of A549 cell line. The number (D) and diameter (E) of A549 colonies increased due to addition of NaB into cryopreservation medium. (F) Colony formation capacity of L929 cell line after cryopreservation with or without 15µg/ml NaB supplemented freezing medium. There was no significant change observed in the amount (G) and diameter (H) of L929 colonies after long-term cryopreservation. NaB supplemented freezing medium changes the proliferation/apoptosis related gene expressions of A549 (I) and L929 (J) cell lines after 6 months of freezing. NC: Negative control, NaB: sodium pentaborate pentahydrate, CASP3: Caspase 3, CASP7: Caspase 7, BCL2: B-cell lymphoma 2, C-MYC: Cellular myelocytomatosis, NFKB: Nuclear factor kappa B, $P<0.05$.

Previous studies demonstrated that boron supplemented cryopreservation medium significantly increases the cell viability of mesenchymal stem cells after freeze-thaw cycles (Demirci *et al.* 2014). Our results showed similar effects of boron on cell viability of A549 and L929 cell lines. The viable numbers of A549 and L929 cells were found to be significantly increased by the freezing medium supplemented with boron after short term freeze-thaw cycles. A similar effect of boron on cell viability was also observed even after long time cryopreservation of A549 and L929 cells as well. CFU result of A549 cell line also supports our results on cell viability. However, we observed no significant change in CFU assay of L929 cell line since CFU assay is used to detect stem cell products in the culture (Pamphilon *et al.* 2013). It is known that freeze-thaw cycles alter the gene expression profiles of cells (Caliskan *et al.* 2014). Our RT-PCR analysis revealed that NaB addition increases the expression levels of proliferation-related genes in both A549 and L929 cell lines. The expression levels of BCL2, C-MYC and NFKB were found to be increased in both A549 and L929 cell lines during freeze-thaw cycles. It is known that Bcl2, an anti-apoptotic protein, can interact with C-Myc and NFkB transcriptional pathways to increase proliferation and survival (Braun *et al.* 2013). Similarly, after long period of freezing, gene expression level of BCL2 was found to be increased in L929 cell line. Moreover, c-Myc as an essential protein for cell proliferation and reduction of apoptosis (Dang 1999) was found to be increased in A549 cell line after long term of cryopreservation with NaB supplemented medium. Furthermore, apoptosis related gene expression levels were found to be dramatically decreased in A549 and L929 cell lines after short term freeze-thaw cycles with boron supplementation. Gene expression level of P53 which is a vital protein for control of apoptosis (Fridman & Lowe 2003) is decreased after the fourth freeze-thaw cycle in A549 cell line. Additionally, expression levels of Caspase 3 and 7 that are identified as executioner caspases (Cardona *et al.* 2015) were found to be decreased dramatically in both A549 and L929 cell lines after short term freeze-thaw cycles. Long term cryopreservation of A549 and L929 with NaB supplemented freezing medium also resulted in a significant decrease of Caspase 3 and 7 expression profiles.

## Conclusion

Taken together, our results indicate that NaB, as a boron source, may be a very important cryoprotective agent for cryo-protection and bio-banking of cancer and healthy cells without losing their viability and functionality. The impact of boron on cryopreservation of various cell types should be further investigated to reveal the exact molecular mechanism of boron on cellular metabolism at low temperatures. Additionally, combination and comparison of different cryoprotective agents with boron should be investigated especially on highly sensitive cell lines such as embryonic stem cells and oocytes.

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**Research Article**

# THE COMMERCIAL AND DISCARD CATCH RATES OF THE TRAWL FISHERY IN THE İSKENDERUN BAY (NORTHEASTERN LEVANTINE SEA)

Cem DALYAN

İstanbul University, Faculty of Science, Depertment of Biology, Vezneciler, İstanbul, TURKEY

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***Edited by:***
*Herdem Aslan*

***Corresponding Author:***
*Cem Dalyan*
*cdalyan@istanbul.edu.tr*

***ORCID ID:***
*orcid.org/0000-0002-7386-5641*

***Key words:***
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*Landed Catch*
*Turkey*
*Levantine Sea*
*Fish species*

**Abstract:** The management of fishery is significant due to the sustainability of marine resources. Therefore, the fishing areas should be constantly monitored. In this study, the fishery data were collected from the Iskenderun Bay (Northeastern Levantine Sea) with a rented commercial trawl vessel. The fishery-dependent data was recorded during the 2012-2013 fishing season with the help of the crew. 33 tows were achieved using a trawl net (codend diamond mesh size of 44 mm) for 26 hours. Each tow was limited with 70 min and the towing speed varied between 2.5 to 3.0 knots. The depth contour ranged from 39 to 69 m. While 32 species were evaluated as discard, 35 species were included in the landed catch. The total catch consisted of 67.2% the landed and 32.8% of the discarded fish in terms of $CPUE_W$ (catch per unit effort by weight).

**Özet:** Deniz kaynaklarının sürdürülebilirliği için balıkçılık yönetimi son derece önemlidir. Bu nedenle balıkçılık alanları sürekli olarak izlenmelidir. Bu çalışmada kullanılan veriler İskenderun Körfezi'nde (Kuzeydoğu Levant Denizi) avlanan bir ticari trol teknesi ile toplanmıştır. Balıkçılığa bağlı veriler, 2012-2013 balıkçılık sezonunda tekne mürettebat yardımı ile kaydedilmiştir. 26 saat süren 33 trol çekimi 44 mm rombik ağ gözü boyutuna sahip torba kullanılarak gerçekleştirilmiştir. Trol çekim hızı 2,5 ila 3,0 knot arasında değişmiş ve en fazla 70 dakika sürmüştür. İskenderun Körfezi'nde 39-69 m derinlik konturu incelenmiştir. 32 tür ıskarta olarak değerlendirilirken, 35 tür ticari av içinde yer almıştır. Buna karşın, toplam av $CPUE_W$ (birim zamanda elde edilen biyokütle) açısından değerlendirildiğinde %67,2'sinin ticari ava %32,8'inin ise ıskarta ait olduğu saptanmıştır.

## Introduction

Discard is a part of the catch that is unwanted by fishers due to being too small, damaged, inedible, having a little or no economic value, or not being able to be retained with management or quota restrictions (Zeller *et al.* 2018). Data on discard rates plays a key role for understanding the energy flow of the marine ecosystem (Machias *et al.* 2001).

The bottom trawl makes the highest contribution to the 9.1 million tonnes annual discard levels worldwide with 4.2 million tonnes (Pérez Roda *et al.* 2019). Mitigation of discard catches is extremely decisive on biodiversity and ecosystem health. There are some ways like mesh size regulations, catch quotas or effort limitations, minimum landing sizes to reduce the amount of discard catch caught by trawls (Weissenberger 2014). In Turkish waters, the mesh size and minimum landing size regulations are preferred by the Ministry of Agriculture and Forestry.

Researchers performed so far in Turkish Seas have focused on discard catch in the last few decades and most of them used beam trawls (Bayhan *et al.* 2006, Demirci 2003, Gökçe & Metin 2006, Kınacıgil 1999a, b, Soykan *et al.* 2006, Yazıcı *et al.* 2006). However, conducting a long-term monitoring study to determine the discarded yield takes too much effort and it is financially compelling as well.

The present study was conducted in the Iskenderun Bay (Easternmost Levantine Sea) where a high biodiversity is present. Erythrean invasion is one of the main causes of this situation. Invasion is also the most important ecological process affecting fisheries in north-eastern Mediterranean Sea. The Red Sea immigrants are evaluated in trawl catch from the Northeastern Levantine Sea (Gücü *et al.* 1994, Gücü *et al.* 2010, Özyurt *et al.* 2018, Yemişken *et al.* 2014). Apart from this, the bay hosts many cartilaginous fishes and these fishes were previously investigated in terms of bycatch and discard. However, the majority of Elasmobranch species are threatened with extinction in the area (Yağlıoğlu *et al.* 2015, Yemişken *et al.* 2014).

This study aimed to define the catch composition and catch/discard rates of trawl fisheries in Iskenderun Bay.

## Materials and Methods

### *Study Area*

The Northeastern Levantine Sea has a wide continental shelf including the Iskenderun Bay (Fig. 1). The bay is preferred as a study area due to being an important fishing area in the Eastern Mediterranean (Yemisken *et al.* 2014). Its surface area is about 2275 km$^2$ with an average depth of 70 m, and its bottom is mainly covered with sand and mud. The range of sea surface water temperature was recorded between 16 and 30°C during 2012-2013 (Karpuz & Sakalli 2019) while salinity varied from 37.0 to 39.4 psu (Terbıyık Kurt 2018).

The regulations and restrictions of trawl fishery are determined by the Ministry of Agriculture and Forestry. The fishing season in the Iskenderun Bay is between September 15 and April 15 every year. Fishing in the bay is prohibited within the first 2 miles off the coast and 44 mm minimum mesh size for the diamond mesh can be used in the Turkish coasts of the Mediterranean Sea.

### *Sampling*

Fishery dependent data were collected by the commercial trawler (Faik Baba, 22 m, 400 HP), in December 2012, February and April 2013, during the legal fishing season. 33 tows with trawl net had achieved with a codend diamond mesh size of 44 mm. Each towing duration ranged from 30 to 70 min depending on topology, bottom type and vessel speed and the towing speed varied between 2.5 to 3.0 knots.

Fieldwork included recording the characteristics of the haul, the estimation of the total catch and landed catch. When the catch reached to the board, it was sorted out as commercial and discard by the crew. After the catch was sorted by the crew, the discard portion was put in boxes. Then the number of individuals of the discard catch was counted and the total weights of each species were noted. Weight was determined to the nearest 1 g. Sub-sampling was performed for the species with a high number of individuals. Species that are difficult to identify were identified in the laboratory.

### *Statistical Analysis*

The most reliable estimation used to determine negative or positive changes in stocks, is the CPUE (Catch Per Unit Effort) calculation (Bordalo-Machado 2006). The CPUE of the trawl was calculated and expressed as biomass/towing time or abundance/towing time (Eq. 1) (D'Onghia *et al.* 2003, Morgan & Burges 2005).

Eq. 1 $CPUE_W = Cw/t$ and $CPUE_A = C_A/t$

Cw: Biomass of the catch (g)

CA: Abundance of the catch (ind)

t: Towing time (h)

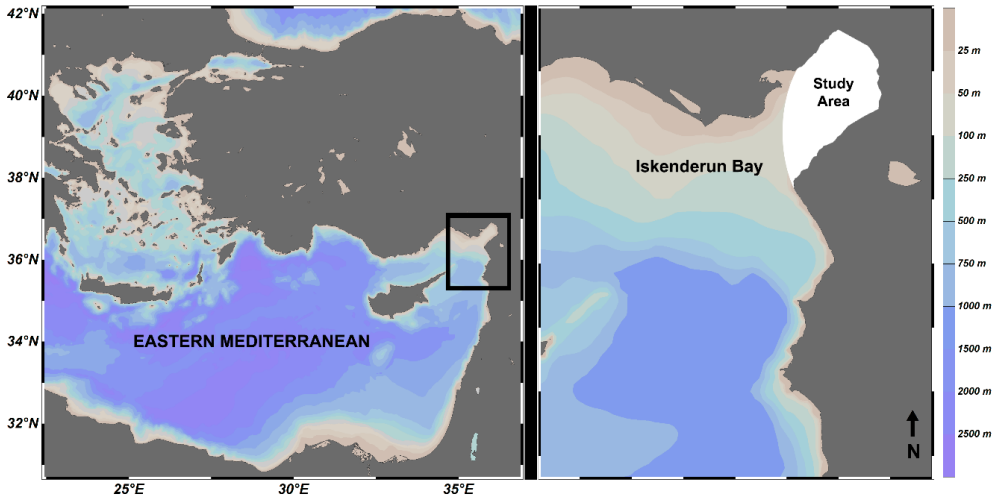


**Fig. 1.** Sampling area in the Iskenderun Bay, Northeastern Levantine Sea.

## Results and Discussion

The tows duration lasted 26 hours in 33 tows (Table 1). The tows were performed at 39-69 m (average depths) depth contour, which is the most visited by fishermen in fishing seasons. Among the tows, the highest abundance and biomass values belonged to the haul at 39 m, which is the shallowest depth. While the discard catch per haul was estimated between 0.25 and 25.5 kg/h, total fish catch varied between 2.3 and 41 kg/h during the fishing period (Table 1).

In 2012-2013 fishing season, a total of 67 species were evaluated by the crew, of which 32 were in discard and 35 were in the landed catch. *Glaucostegus cemiculus* (Geoffroy St. Hilaire, 1817), *Mustelus mustelus* (Linnaeus, 1758) and *Zeus faber* Linnaeus, 1758 were placed in discard catch due to avoided sizes of the individuals.

A total of 332 kg of the catch was obtained during the study. The total abundance of fishes was 7738. Data refers to 10506 individuals when the number of individuals in each tow is standardized by the swept area ($CPUE_A$), 60.5% of which are discarded (Table 2). In terms of $CPUE_W$ the trawl catches mainly consisted of 67.2% landed and 32.8% discarded fish.

**Table 1.** The informative data of the tows achieved in the Iskenderun Bay at 2012-2013 fishing season (Tow numbers 1-12 were carried out in Dec-2012, 13-25 in Feb-2013 and 26-33 in Apr-2013).

| Tows | Average Depth (m) | Towing Time (h) | Landed ($CPUE_A$)(ind/h) | Landed ($CPUE_W$) (g/h) | Discard ($CPUE_A$) (ind/h) | Discard ($CPUE_W$) (g/h) |
|---|---|---|---|---|---|---|
| 1 | 61 | 1.17 | 72 | 12228.2 | 182 | 3031.6 |
| 2 | 56 | 1.00 | 173 | 9323.0 | 149 | 5719.3 |
| 3 | 39 | 0.33 | 1060 | 21569.7 | 348 | 19416.0 |
| 4 | 46 | 1.17 | 142 | 7898.3 | 142 | 3544.3 |
| 5 | 62 | 0.70 | 222 | 12638.6 | 226 | 1732.3 |
| 6 | 62 | 1.00 | 541 | 7669.0 | 102 | 25407.4 |
| 7 | 56 | 0.65 | 154 | 22323.1 | 257 | 2776.9 |
| 8 | 57 | 1.00 | 98 | 17385.0 | 206 | 8030.0 |
| 9 | 62 | 0.70 | 101 | 26838.6 | 297 | 2634.3 |
| 10 | 67 | 0.75 | 227 | 9097.3 | 113 | 2542.7 |
| 11 | 69 | 0.77 | 173 | 7364.9 | 97 | 5037.7 |
| 12 | 61 | 0.77 | 140 | 17002.6 | 223 | 2619.5 |
| 13 | 63 | 0.72 | 183 | 4290.3 | 57 | 6486.1 |
| 14 | 61 | 1.30 | 20 | 2314.6 | 35 | 835.0 |
| 15 | 59 | 0.93 | 102 | 4146.2 | 67 | 2695.7 |
| 16 | 61 | 0.87 | 128 | 2973.6 | 40 | 1985.1 |
| 17 | 62 | 1.00 | 151 | 3396.0 | 47 | 1233.0 |
| 18 | 65 | 1.03 | 277 | 7538.8 | 104 | 6128.2 |
| 19 | 62 | 0.72 | 401 | 16966.7 | 257 | 5101.4 |
| 20 | 68 | 1.08 | 112 | 2621.3 | 40 | 866.7 |
| 21 | 67 | 0.58 | 114 | 4619.0 | 81 | 1524.1 |
| 22 | 60 | 0.72 | 78 | 7013.9 | 92 | 695.8 |
| 23 | 58 | 0.80 | 19 | 2988.8 | 38 | 258.8 |
| 24 | 55 | 0.58 | 57 | 2462.1 | 43 | 491.4 |
| 25 | 68 | 0.82 | 110 | 4573.2 | 62 | 704.9 |
| 26 | 68 | 0.55 | 275 | 4310.9 | 64 | 3930.9 |
| 27 | 53 | 0.75 | 99 | 13820.0 | 215 | 13917.3 |
| 28 | 53 | 0.83 | 359 | 9721.7 | 146 | 1394.0 |
| 29 | 57 | 0.62 | 179 | 12537.1 | 240 | 2293.5 |
| 30 | 55 | 0.48 | 169 | 4512.5 | 54 | 2447.9 |
| 31 | 57 | 0.58 | 76 | 3520.7 | 57 | 2334.5 |
| 32 | 60 | 0.50 | 84 | 1688.0 | 24 | 618.0 |
| 33 | 57 | 0.62 | 265 | 2766.1 | 42 | 2932.3 |

Five species were identified as cartilaginous fishes, among those *G. cemiculus* and *M. mustelus* are of limited economic value. These fishes take part in IUCN Red List as CR and VU, respectively. All chondrichthyes was sorted as discard and they covered 11% of the total catch. Cartilaginous fish were estimated to 33% of the total discard catch biomass. *Dasyatis pastinaca* covered the largest part of the total discard catch biomass but was represented by low numbers in the total abundance. Yemişken *et al.* (2014) mentioned that the species is among the vast majority of the discard catch biomass with *Gymnura altavela* in the area. Also, Yaglioglu *et al.* (2015) estimated that *D. pastinaca* constitutes 38% of the total elasmobranch biomass.

During the study period, 21 species (31%) were determined as Red Sea immigrants. These species were estimated as 57.5% of the total teleost catch biomass and the rest of the catch consisted of the Atlanto-Mediterranean species. Özyurt *et al.* (2018) mentioned that 35% of the teleost species are Red Sea species and percentage of them is 75% in the total biomass.

**Table 2.** The standardized values of the landed and discard catch as $CPUE_A$ (ind./h) and $CPUE_W$ (g/h) in the Iskenderun Bay. Status of the listed fishes were determined as D (Discard) and L (Landed) (Red Sea immigrants*; Cartilaginous$^{++}$).

| Species | Landed ($CPUE_A$) | Landed ($CPUE_W$) | Discard ($CPUE_A$) | Discard ($CPUE_W$) | Status |
|---|---|---|---|---|---|
| *Apterichtus caecus* (Linnaeus, 1758) | 0 | 0 | 6 | 37 | **D** |
| *Arnoglossus grohmanni* (Bonaparte, 1837) | 0 | 0 | 5 | 13 | **D** |
| *Arnoglossus thori* Kyle, 1913 | 0 | 0 | 149 | 644 | **D** |
| *Blennius ocellaris* Linnaeus, 1758 | 0 | 0 | 5 | 59 | **D** |
| *Boops boops* (Linnaeus, 1758) | 32 | 870 | 5 | 108 | **L** |
| *Bregmaceros nectabanus* Whitley, 1941* | 0 | 0 | 1 | 2 | **D** |
| *Caranx rhonchus* Geoffroy St. Hilaire, 1817 | 57 | 3536 | 43 | 1572 | **L** |
| *Cepola macrophthalma* (Linnaeus, 1758) | 0 | 0 | 9 | 30 | **D** |
| *Champsodon nudivittis* (Ogilby, 1895)* | 0 | 0 | 1478 | 13725 | **D** |
| *Chelidonichthys lastoviza* (Bonnaterre, 1788) | 2 | 124 | 22 | 272 | **L** |
| *Chelidonichthys lucerna* (Linnaeus, 1758) | 309 | 18396 | 17 | 3461 | **L** |
| *Chelon auratus* (Risso, 1810) | 1 | 157 | 0 | 0 | **L** |
| *Citharus linguatula* (Linnaeus, 1758) | 0 | 0 | 831 | 12368 | **D** |
| *Conger conger* (Linnaeus, 1758) | 1 | 411 | 0 | 0 | **L** |
| *Cynoglossus sinusarabici* (Chabanaud, 1931)* | 0 | 0 | 1 | 9 | **D** |
| *Dasyatis marmorata* (Steindachner, 1892)$^{++}$ | 0 | 0 | 1 | 230 | **D** |
| *Dasyatis pastinaca* (Linnaeus, 1758)$^{++}$ | 0 | 0 | 34 | 40168 | **D** |
| *Deltentosteus collonianus* (Risso, 1820) | 0 | 0 | 6 | 17 | **D** |
| *Deltentosteus quadrimaculatus* (Valenciennes, 1837) | 0 | 0 | 36 | 97 | **D** |
| *Diplodus annularis* (Linnaeus, 1758) | 38 | 2039 | 0 | 0 | **L** |
| *Dussumieria elopsoides* Bleeker, 1849* | 31 | 894 | 10 | 193 | **L** |
| *Echelus myrus* (Linnaeus, 1758) | 0 | 0 | 1 | 131 | **D** |
| *Echeneis naucrates* Linnaeus, 1758 | 0 | 0 | 1 | 30 | **D** |
| *Engraulis encrasicolus* (Linnaeus, 1758) | 0 | 0 | 42 | 173 | **D** |
| *Epinephelus aeneus* (Geoffroy St. Hilaire, 1817) | 2 | 135 | 0 | 0 | **L** |
| *Epinephelus costae* (Steindachner, 1878) | 2 | 304 | 0 | 0 | **L** |
| *Equulites klunzingeri* (Steindachner, 1898)* | 0 | 0 | 1681 | 19312 | **D** |
| *Etrumeus golanii* DiBattista, Randall & Bowen, 2012* | 2 | 146 | 0 | 0 | **L** |
| *Glaucostegus cemiculus* (Geoffroy St. Hilaire, 1817)$^{++}$ | 0 | 0 | 8 | 4951 | **D** |
| *Gobius niger* Linnaeus, 1758 | 0 | 0 | 3 | 67 | **D** |
| *Gobius paganellus* Linnaeus, 1758 | 0 | 0 | 1 | 8 | **D** |
| *Jaydia queketti* (Gilchrist, 1903)* | 0 | 0 | 26 | 105 | **D** |
| *Jaydia smithi* Kotthaus, 1970* | 0 | 0 | 49 | 381 | **D** |
| *Lagocephalus guentheri* Miranda Ribeiro, 1915 | 0 | 0 | 40 | 1355 | **D** |
| *Lagocephalus suezensis* Clark & Gohar, 1953* | 0 | 0 | 35 | 795 | **D** |
| *Lepidotrigla cavillone* (Lacepède, 1801) | 0 | 0 | 17 | 179 | **D** |
| *Lepidotrigla dieuzeidei* Blanc & Hureau, 1973 | 0 | 0 | 1 | 16 | **D** |
| *Merluccius merluccius* (Linnaeus, 1758) | 9 | 268 | 1 | 16 | **L** |

Table 2. Continued.

| Species | Landed ($CPUE_A$) | Landed ($CPUE_W$) | Discard ($CPUE_A$) | Discard ($CPUE_W$) | Status |
|---|---|---|---|---|---|
| *Mullus barbatus* Linnaeus, 1758 | 159 | 10800 | 4 | 1130 | **L** |
| *Mustelus mustelus* (Linnaeus, 1758)$^{++}$ | 0 | 0 | 2 | 750 | **D** |
| *Nemipterus randalli* Russell, 1986* | 1187 | 62856 | 1117 | 22317 | **L** |
| *Ostorhinchus fasciatus* (Shaw, 1790)* | 0 | 0 | 18 | 40 | **D** |
| *Oxyurichthys petersii* (Klunzinger 1871)* | 0 | 0 | 175 | 2673 | **D** |
| *Pagellus acarne* (Risso, 1827) | 168 | 5057 | 10 | 1032 | **L** |
| *Pagellus erythrinus* (Linnaeus, 1758) | 352 | 19220 | 13 | 677 | **L** |
| *Pelates quadrilineatus* (Bloch, 1790)* | 8 | 209 | 2 | 29 | **L** |
| *Pomatomus saltatrix* (Linnaeus, 1766) | 15 | 1767 | 0 | 0 | **L** |
| *Raja miraletus* Linnaeus, 1758$^{++}$ | 0 | 0 | 1 | 191 | **D** |
| *Sardina pilchardus* (Walbaum, 1792) | 8 | 271 | 0 | 0 | **L** |
| *Saurida lessepsianus* Russell, Golani & Tikochinski, 2015* | 822 | 85731 | 112 | 7575 | **L** |
| *Scomber colias* Gmelin, 1789 | 1 | 33 | 0 | 0 | **L** |
| *Serranus hepatus* (Linnaeus, 1758) | 0 | 0 | 100 | 1395 | **D** |
| *Siganus rivulatus* Forsskål & Niebuhr, 1775* | 2 | 54 | 0 | 0 | **L** |
| *Solea solea* (Linnaeus, 1758) | 50 | 6055 | 33 | 1676 | **L** |
| *Sparus aurata* Linnaeus, 1758 | 773 | 65683 | 0 | 0 | **L** |
| *Sphyraena chrysotaenia* Klunzinger, 1884* | 1 | 156 | 0 | 0 | **L** |
| *Spicara smaris* (Linnaeus, 1758) | 0 | 0 | 4 | 47 | **D** |
| *Synodus saurus* (Linnaeus, 1758) | 2 | 103 | 0 | 0 | **L** |
| *Torquigener flavimaculosus* Hardy & Randall, 1983* | 0 | 0 | 1 | 27 | **D** |
| *Trachinus draco* Linnaeus, 1758 | 0 | 0 | 6 | 343 | **D** |
| *Trachurus indicus* Nekrasov, 1966* | 28 | 1542 | 3 | 46 | **L** |
| *Trachurus mediterraneus* (Steindachner, 1868) | 2 | 16 | 0 | 0 | **L** |
| *Trachurus trachurus* (Linnaeus, 1758) | 3 | 40 | 87 | 294 | **L** |
| *Trichiurus lepturus* Linnaeus, 1758 | 27 | 2513 | 3 | 25 | **L** |
| *Upeneus moluccensis* (Bleeker, 1855)* | 50 | 653 | 99 | 570 | **L** |
| *Upeneus pori* Ben-Tuvia & Golani, 1989* | 4 | 81 | 0 | 0 | **L** |
| *Zeus faber* Linnaeus, 1758 | 0 | 0 | 3 | 6 | **D** |
| **Total** | **4148** | **290120** | **6358** | **141367** | |

Among Erythrean species, *Saurida lessepsianus* and *Nemipterus randalli* were the most important species that were obtained in the trawl fishery (Fig. 2). Also, these species constitute 41% of the landed catch. Gücü *et al.* (1994) estimated that about 30% of the Teleost catch from the bay belonged to *S. lessepsianus*. *Dasyatis pastinaca*, *Equulites klunzingeri* and *N. randalli* constituted 58% of the discard catch biomass while *E. klunzingeri*, *Champsodon nudivittis* and *N. randalli* were included in the abundance of discard catch with a percentage of 68%.

*Conger conger*, *Diplodus annularis*, *Epinephelus aeneus*, *E. costae*, *Etrumeus teres*, *Chelon auratus*, *Pomatomus saltatrix*, *Sardina pilchardus*, *Scomber colias*, *Siganus rivulatus*, *Sparus aurata*, *Sphyraena chrysotaenia*, *Synodus saurus*, *Trachurus mediterraneus* and *Upeneus pori* were not found in the discard catch. The local fishermen tend to evaluate all sizes of *E. aeneus*, *E. costae* and *S. aurata* in the landed catch. The intense trawl fisheries activity is the main threat for these fishes in the bay (Fig. 3).

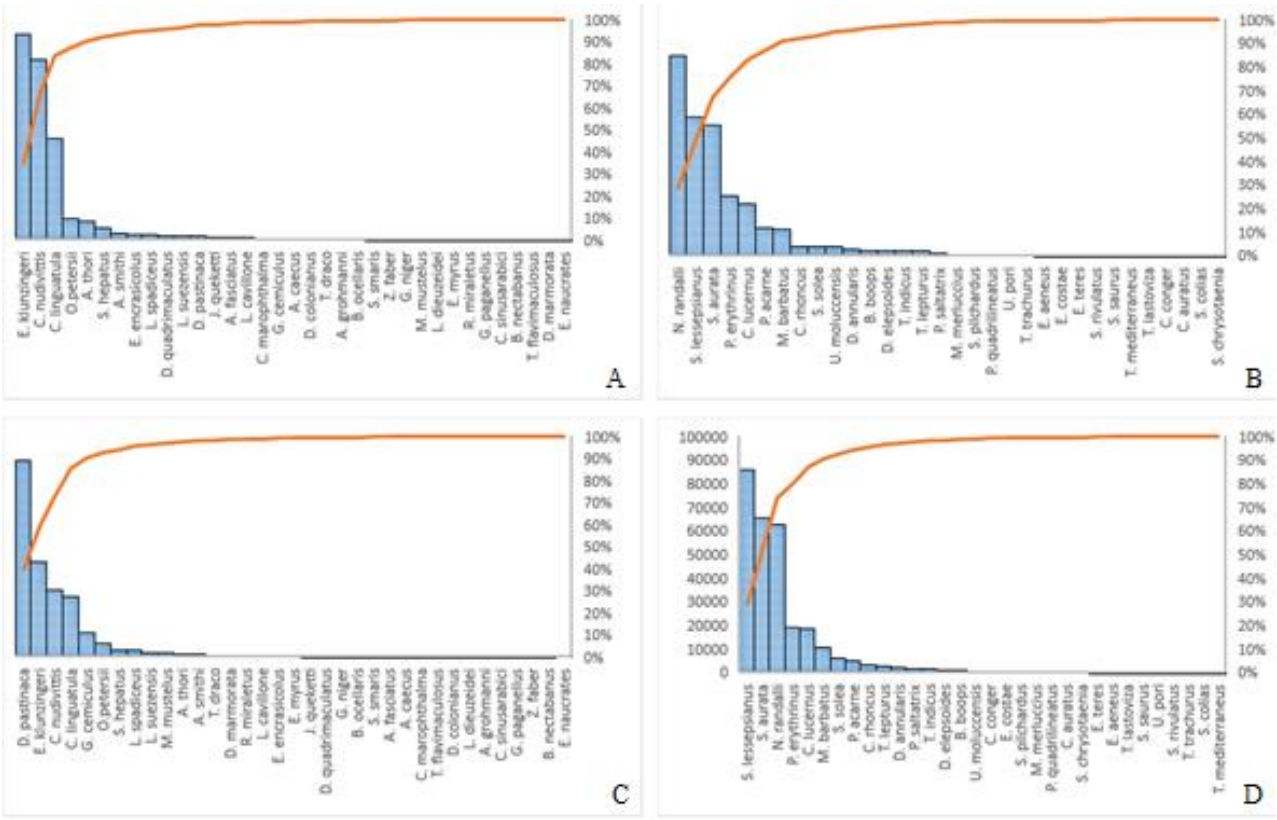


**Fig. 2.** The standardized values of the catch in the Iskenderun Bay; A) $CPUE_A$ of the discard catch, B) $CPUE_W$ of the discard catch, C) $CPUE_A$ of the landed catch, D) $CPUE_W$ of the landed catch.

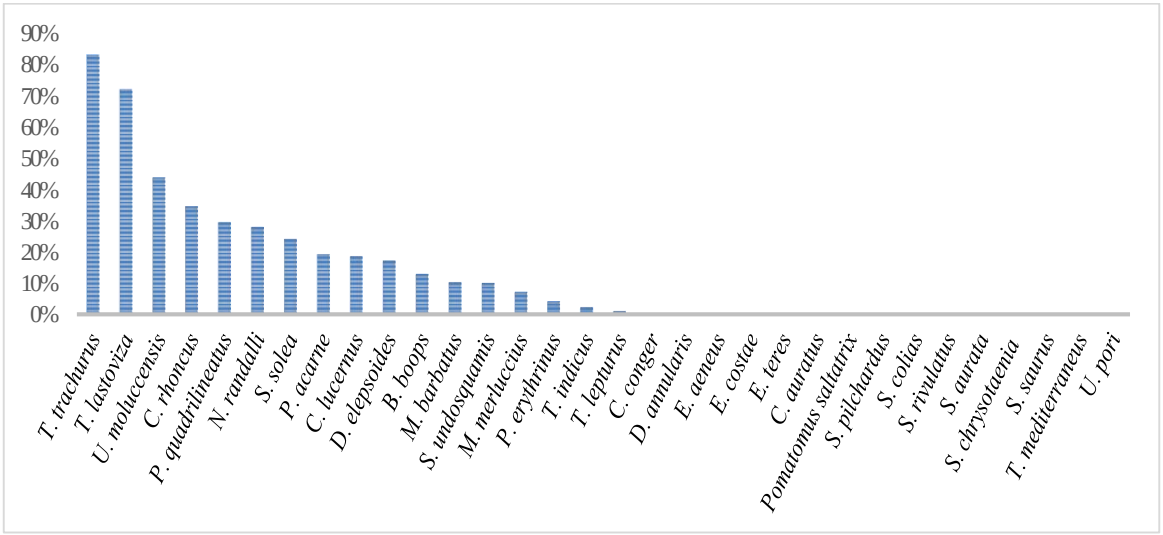


**Fig. 3.** The discard rates of fishes in the landed catch in the Iskenderun Bay at 2012-2013 fishing period.

In the Northeastern Levantine Sea, the catch and discard of the trawl fishery have a trend to decrease towards the end of the fishing season. The biomass value of seasonal catch declines sharply in the January-February period (Yemişken *et al.* 2014, Gökçe *et al.* 2016). The total catch biomass estimated in April was about 2 times higher than the February (Fig. 4). The depth range differences between the studies may have caused this mismatch.

Considering the values of trawl fishery of the bay, it appeared that most of the discard biomass consisted of chondrichthyes species that feed on carcasses formed as a result of intense fishing activity. Besides, two of the three species (*S. lessepsianus* and *N. randalli*) occupying the majority of the landed biomass belong to the Red Sea immigrants, indicating that the existence of these species in the region has irreversible effects. The dispersal success of the Red Sea immigrants plays a significant role

in the trawl fisheries and these species should be monitored with long-running studies.

Studies on discard catches are important in terms of fisheries management, especially for multispecies trawl fisheries. Additional comprehensive studies and long-term monitoring are needed in order to understand the population dynamics and to detect ultimate changes in the target area. Moreover, there is no study to reduce fishing pressure on the fish stocks, except Gücü (2012) who revealed that regulations of depth and fishing period had a positive effect to mitigate discard rates in the north-eastern Levantine Sea. Advanced studies should be encouraged in the area which has significant demersal resources that are under the pressure of intense fisheries activities.

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**Research Article**

# PIONEERING *in vitro* STUDIES FOR CALLUS FORMATION OF *Colchicum chalcedonicum* Azn.

Elif KARLIK[1*], Meltem DEĞER[2], Erdal ÜZEN[3], Nermin GÖZÜKIRMIZI[4]

[1] İstinye University, Department of Molecular Biology and Genetics, İstanbul, TURKEY
[2] HAN University of Applied Sciences, Department of Life Science, Nijmegen, NETHERLANDS
[3] İstanbul University, Faculty of Sciences, Department of Biology, İstanbul, TURKEY
[4] İstanbul University, Faculty of Sciences, Department of Molecular Biology and Genetics, İstanbul, TURKEY

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**Abstract:** *Colchicum calcedonicum* Azn is one of the endemic species distributed in Turkey, where many endemic plant species occur. It has long-oval shaped corm under the soil, and usually 3-4 leaves on it. *In vitro* production of endemic species using callus culture has become promising study for conservation. The aim of this study is to generate an efficient callus protocol for *in vitro* production of *C. chalcedonicum.* To sterilize the explants, 0.25% (w/v) mercuric chloride ($HgCl_2$) was used for 20 min. In addition to mercuric chloride, surface sterilization was conducted by using 6.5% NaCl with Tween 80 for 30 min. We used 19 different mediums and the primary callus formation was obtained in Murashige & Skoog's basal medium (MS) supplemented with 2,4-D (2 mg $L^{-1}$), 2IP (0.5 mg $L^{-1}$), 3% sucrose and 0.05% active carbon. Our study demonstrated the active carbon usage was effective for the primary callus formation. This study is the first report for primary callus formation of *C. chalcedonicum.* However, our work is a pioneering study to improve callus formation protocol system for *in vitro* conservation of endemic species *C. chalcedonicum.*

**Özet:** *Colchicum calcedonicum* Azn, pek çok endemik bitki türünün görüldüğü Türkiye'de yayılış gösteren endemik türlerden biridir. Toprak altında uzun-oval şekilli soğanı ile genellikle 3-4 yapraklıdır. Kallus kültürü kullanarak endemik türlerin in vitro üretimi, bu türlerin korunmasında umut verici bir çalışma haline gelmiştir. Bu çalışmanın amacı, in vitro C. chalcedonicum üretimi için verimli kallus protokolünün oluşturulabilmesidir. Explantların sterilizasyonunda, 20 dk %0.25 (w/v) cıva klorür ($HgCl_2$) kullanılmıştır. Cıva klorüre ilaveten, yüzey sterilizasyonunda 30 dk boyunca Tween 80, %6,5 NaCl ile birlikte kullanılmıştır. Bu çalışmada, 19 farklı besiyeri kullanılmış olup primer kallus oluşumu 2,4-D (2 mg L-1), 2IP (0,5 mg L-1), %3 sükroz ve %0,05 aktif karbon içeren Murashige & Skoog bazal besiyerinde elde edilmiştir. Çalışmamız, aktif karbon kullanımının primer kallus oluşumunda etkili olduğunu göstermiştir. Bu çalışma, C. chalcedonicum'un primer kallus oluşumu için ilk rapordur. Bununla birlikte, çalışmamız endemik tür olan C. chalcedonicum'un in vitro korunması ve kallus oluşum protokolünün geliştirilmesinde öncü bir çalışmadır.

***Edited by:***
*Mykyta Peregrym*

****Corresponding Author:***
*Elif Karlık*
*elif.karlik@istinye.edu.tr*

***ORCID ID:***
*orcid.org/0000-0003-0669-2725*

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*Tissue culture*
*Callus formation*
*Conservation*

## Introduction

The genus *Colchicum* L. within the family Colchicaceae have been known for more than 2000 years for their marked beneficial and poisonous effects (Brickell 1984). In Turkey, 50 *Colchicum* species were described of which 15 are endemic. Their limited distribution at a very high altitude and the restricted period of growth are the reasons for the low yield of members of the genus. *Colchicum* includes about 200 perennial flowering species growing from corms and their ovary flowers form underground. The important species of the genus, *C. luteum* Baker and *C. autumnale* L. contain 0.2-0.5g 100$g^{-1}$ dry wt. of colchicine, which is an antimitotic agent by preventing accumulation of microtubules and inhibiting the cell division in the metaphase (The Wealth of India 1962, Kapadia *et al.* 1972, Dumontet & Sikic 1999, Combeau *et al.* 2000, Pirildar *et al.* 2010).

*Colchicum chalcedonicum* Azn., which is also known as Kadıköy (Chalcedon) crocus, is one of the endemic species of *Colchicaceae* in Turkey. *Colchicum chalcedonicum* was collected in Kadıköy in İstanbul by Aznavour in 1897 (Aznavour 1897). It has usually 4 leaves and long-oval shaped corms under the soil. Their chromosome number is 2n=50. The plant grows in rich red soils in dry stony and rocky places. They are also found on rare peaks and shrubs. *Colchicum*

*chalcedonium* flowers from August to September, being out of leaves and fruits from February to April. Additionally, the plants only live underground for a part of the year, thus *in vivo* and *in vitro* micro-propagation and *in vitro* culture of this species are difficult making the species to be known as calcitrant (Brickell 1984, Persson 1988, 1998, 1999a, 1999b, 1999c, 2000, 2001, 2007, Akan & Eker 2005).

Several studies reported on production of colchicine alkaloid by plant tissue cultures (Hayashi *et al.* 1988, Yoshida *et al.* 1988). Callus tissues were first induced from flowering shoots of *C. autumnale* L. by using MS (Murashige & Skoog 1962) containing 2,4-Dichlorophenoxyacetic acid (2,4-D), while colchicine from callus tissue was produced by MS with indole butyric acid (IBA) and kinetin (Hayashi *et al.* 1988). Daradkeh *et al*. (2012) used *C. hierosolymitanum* Feinbrun for callus production on MS supplemented with 0.45 µM 2, 4-dichlorophenoxyacetic acid under dark conditions. To induce colchicine production, callus was sub-cultured every 27 days on the same liquid media supplemented with 0.54 µM 1- naphthaleneacetic acid. The researchers observed that higher cell fresh weight was resulted with 9 µM 6-benzyladenine with 0.45 µM 2, 4-dichlorophenoxyacetic acid. Additionally, the highest colchicine alkaloid (0.090 mg $g^{-1}$ DW) was obtained at 0.1 M sucrose after 4 weeks incubation (Daradkeh *et al.* 2012). Different parts of *C. chalcedonicum* and *C. micranthum* Boiss., which are endemic for Turkey, were also investigated for cytotoxic activities for future medical approaches. Daradkeh *et al.* (2012) managed to isolate colchicine, colchifoline, 2-demethylcolchicine, demecolcine, 4-hydroxycolchicine and *N-deacetyl-N*-formylcolchicine which showed high cytotoxicity. The main alkaloids of these two *Colchicum* species were found as colchicine and colchifoline. According to the results of this study, the greatest diversity in tropolone alkaloids were found in the seed of *C. chalcedonicum* (Gulsoy-Toplan *et al.* 2018). Despite its importance, no systematic attempt has been performed for mass propagation of *C. chalcedonicum*. The restricted distribution of this endemic plant has endangered its survival. Therefore, tissue culture approaches are required to get rapid propagation as *in vitro* protocol for micro-propagation, but there is no a practical protocol available for *in vitro* mass propagation of *C. chalcedonicum*. In the present study, we report the first and efficient protocol for callus generation of *C. chalcedonicum* using corms.

## Materials and Methods

### *Field Studies and the Plant Material*

*Colchicum chalcedonicum* corms used in this study were kindly provided by Erdal Uzen from Kadıköy. Field studies were performed from April 2017 to November 2017 at Çamlıca Hill (Ferah neighbourhood) (Fig. 1). The specimen was obtained from seed, leave, and corm during seeding, blooming and fruit time of *C. chalcedonicum* (Fig. 2). The specimens are deposited in the Istanbul University Alfred Heilbronn Botanical Garden.

Corms were long-ovaloid like egg-shaped, and corm shells mostly with several layers, the outer coriaceous and dark brown, the inner thin and reddish-brown. Leaves were 3-4, hysteranthous, patent, and oblong-lanceolate, light or dark-green hereinbefore mentioned by Küçüker (1984).

### *Explant Preparation*

The fresh corms, leaves and corm shells were used as explants. The explants were excised aseptically with sterile scissors and washed with running tap water for 30 minutes, washed with $dH_2O$ for three times for 5 min. The explants were then sterilized with 0.25% (w/v) mercuric chloride ($HgCl_2$) solution for 20 min. followed by surface sterilization with 6.5% NaCl with Tween 80 for 30 min. Then, the explants were rinsed thoroughly with sterile $dH_2O$ for three times for 5 min. followed by 70% ethanol for 10 min. The explants were again washed with $dH_2O$ three times and blotted dry on sterilized filter paper. Finally, ten sterilized explants were aseptically placed on tissue culture mediums.

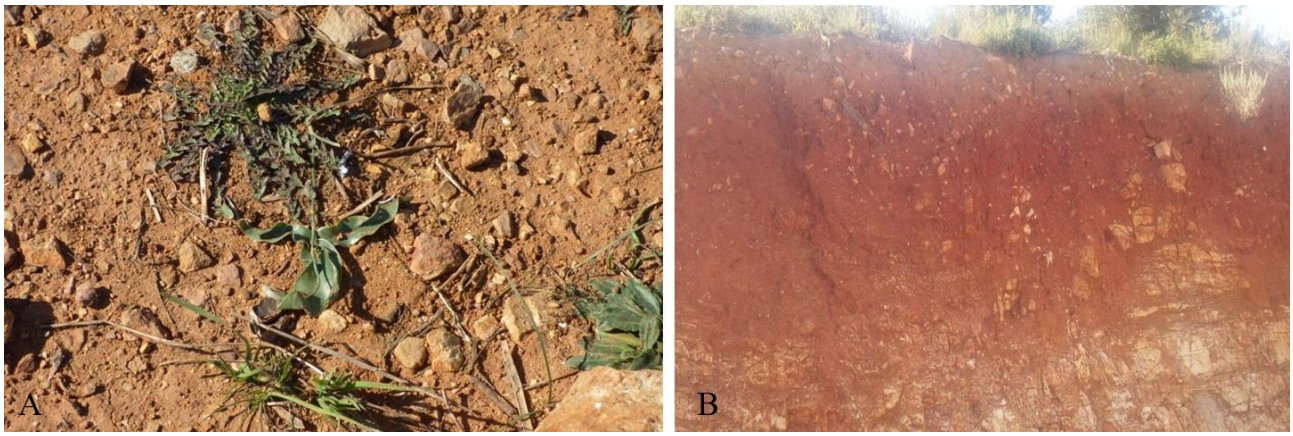


**Fig. 1.** The red and iron-rich Terra Rosa soil at Çamlıca Hill where *C. chalcedonicum* specimens were found during the study.


**Fig. 2.** The natural appearance of *C. chalcedonicum*' in field studies (A and B**)** and in The Istanbul University Alfred Heilbronn Botanical Garden (C and D).

*Inoculation and Incubation*

The disinfected explants were cultured on MS medium (Murashige & Skoog 1962) supplemented with different concentrations and combinations of sucrose and plant growth regulators (PGRs) [1-Naphthalene acetic acid (NAA), 6-Benzylaminopurine (BAP), Zeatin (ZEA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-(γ,γ-Dimethylallylamino) purine (2IP)]. To induce callus induction, all cultures were maintained in a growth chamber for two weeks to several months at 18-25 ± 2.0°C with dark according to used medium. After callus induction, the cultures were sub-cultured at 18-25 ± 2.0°C with the light intensity of 75 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps for a photoperiod of 16:8 hours of light and dark period in every 24-hour cycle. Relative humidity of the growth chamber was kept at 50%. Active carbon was also used for development of callus. MS medium was prepared with some modifications. The pH of the media was first adjusted to 5.8, then it was autoclaved for 15 min at 121°C. As the control group, MS medium supplemented with 2% Sucrose (Suc) were used. The explants were cultured on MS medium supplemented with 3, 8 and 10% (w/v) Suc and various plant growth regulators. For initiating callus, different treatment combinations including 2,4-D as an auxin (2 mg $ml^{-1}$) and BAP as a cytokinin (0.5 mg $ml^{-1}$); NAA (1 mg $ml^{-1}$) and ZEA (1 mg $ml^{-1}$); NAA (2 mg $ml^{-1}$) and BAP (0.5 mg $ml^{-1}$); 2,4 D (2 mg $ml^{-1}$) and BAP (0.5 mg $ml^{-1}$); 2,4 D (2 mg $ml^{-1}$) and ZEA (1 mg $ml^{-1}$); 2,4 D (2 mg $ml^{-1}$), ZEA (1 mg $ml^{-1}$) and BAP (0.5 mg $ml^{-1}$); 2,4 D (2 mg $ml^{-1}$) and 2IP (0.5 mg $ml^{-1}$) were applied to the corm slices as three replicates. The last medium was applied as $2^{-1}$ MS supplemented with 2,4 D (2 mg $ml^{-1}$), 2IP (0.5 mg $ml^{-1}$), 3% Suc and 0.05% active carbon according to the report of Yalcin Mendi *et al.* (2017).

## Results

Various explant types, PGRs, different sugar concentrations, and chemicals were tested for callus induction in *C. chalcedonicum*. Callus were only formed from corms (see Fig. 3). Leaves and corm shells as explant demonstrated no development for callus formation. Different researchers used different sterilization protocols which included different concentrations and combinations of NaOCl, NaCl, $HgCl_2$, Tween 20 and Tween 80 (Khan *et al.* 2011, Daradkeh *et al.* 2012, Wagh *et al.* 2015). In our sterilization protocol, we observed 0.25% (w/v) mercuric chloride ($HgCl_2$) and 6.5% NaCl with Tween 80 were required to sterile *C. chalcedonicum* corms. However, NaOCl is not necessary for sterilization of *C. chalcedonicum* corms.

After determination of the sterilization protocol, different sucrose concentrations were tested. At the first step, two sucrose concentrations (3% and 8%) were tested for all different PGRs combinations. Corms on 8% sucrose were formed as green and soft callus. Callus formation in 3% sucrose was found to be slower than 8% sucrose, thus corms in 3% sucrose were transferred to

mediums containing 10% sucrose to enhance callus formation.

Callus development was observed within a week after transferring medium to MS supplemented with NAA (1 mg $ml^{-1}$), ZEA (1 mg $ml^{-1}$) and 10% Suc, becoming partly green and soft. Callus formation ratio in this media was around 15%. Additionally, callus formation was observed on $2^{-1}$ MS supplemented with 2,4 D (2 mg $ml^{-1}$), 2IP (0.5 mg $ml^{-1}$), 3% Suc and 0.05% active carbon (see in Fig. 4). In this medium, callus formation ratio was around 75%. However, some cultures were transferred to $2^{-1}$ MS supplemented with 2,4 D (2 mg $ml^{-1}$), 2IP (0.5 mg $ml^{-1}$), 3% Suc, 0.05% active carbon medium after twelve weeks and the corms were observed to get a green colour and swelling. Eventually, after testing different PGRs and sugar concentrations, callus formations were only observed on MS supplemented with NAA (1 mg $ml^{-1}$), ZEA (1 mg $ml^{-1}$) containing different concentration of sucrose -%8 and 10% Suc- and $2^{-1}$ MS supplemented with 2,4 D (2 mg $ml^{-1}$), 2IP (0.5 mg $ml^{-1}$), 3% sucrose, 0.05% active carbon medium.

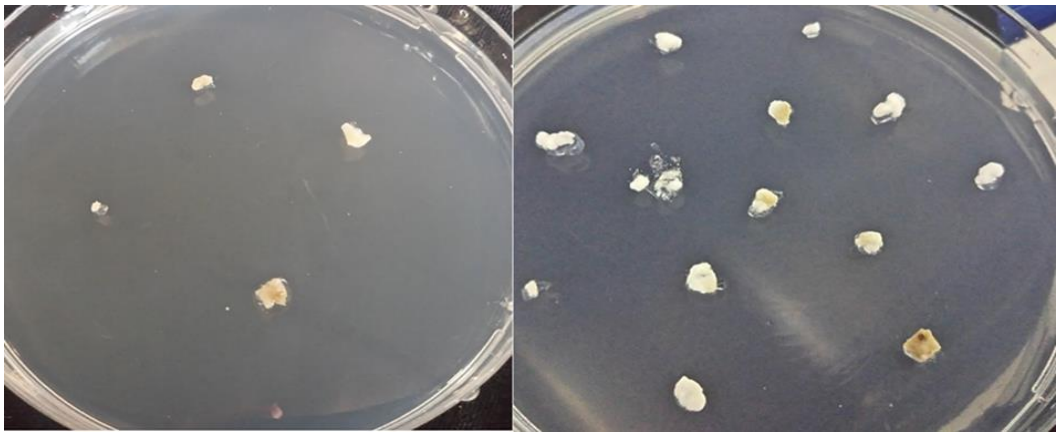

**Fig. 3.** Callus formation of *C. chalcedonicum* on [ZEA (1 mg $ml^{-1}$) + NAA (1mg $ml^{-1}$) + 10% Suc + MS basal medium]

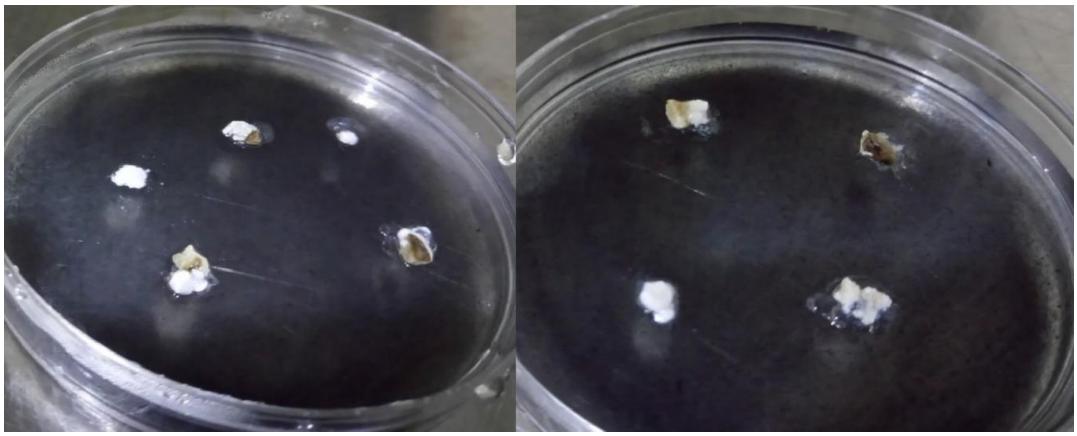

**Fig. 4.** Callus formation of *C. chalcedonicum* on [2,4-D (2 mg $L^{-1}$) + 2IP (0.5 mg $L^{-1}$) + 3% Suc + 0.05% active carbon + $2^{-1}$ MS basal medium]

## Discussion

The genus *Colchicum* in Turkey has a wide distributional range with 50 species of which 15 are endemic for the country (Dahlgren *et al.* 1985, Persson 1999a, Akan & Eker 2005). In addition to the relatively wide distribution range for the genus, Turkey is the richest country in terms of the species diversity. However, in the present study, only 61 *C. chalcedonicum*, known to be grown on rich red soils at dry stony and rocky places, were found only at Çamlıca Hill which is formed from the red, iron-rich Terra Rossa soil (see Fig. 2).

Limited number of experiments were performed about *Colchicum* tissue culture (Hayashi *et al.* 1988, Yoshida *et al.* 1988, Khan *et al.* 2011, Daradkeh *et al.* 2012, Wagh *et al.* 2015). In our sterilization protocol, we used 0.25% (w/v) $HgCl_2$ for surface sterilization, while Wagh *et al.* (2015) used 0.15% (w/v) $HgCl_2$ for surface sterilization of *C. luteum* Baker corms. The reason for the use of 0.25% (w/v) $HgCl_2$ in our study was that the soil where the corm explants obtained from Çamlıca Hill is composed of mineral particles, organic matter, water, air and living organisms.

In our study, we tested different sucrose concentrations -(3, 8 and 10%) and 8% and 10% resulted with better callus formation. Nagaraju *et al.* (2002) tested 30, 60, 90 and 120 g $L^{-1}$ sucrose concentrations and observed that sucrose showed a significant effect on corm size and weight, leaf

weight and root length. Additionally, studies revealed that *Colchicum* corms were more desirable for callus induction (Daradkeh *et al.* 2012, Wagh *et al.* 2015). Our study demonstrated that corm was better than leaves and corm shells as an explant for callus development. Callus formation rates for corm were observed around 15% and 75% for MS supplemented with NAA (1 mg $ml^{-1}$), ZEA (1 mg $ml^{-1}$) containing 10% sucrose- and $2^{-1}$ MS supplemented with 2,4 D (2 mg $ml^{-1}$), 2IP (0.5 mg $ml^{-1}$), 3% sucrose and 0.05% active carbon medium, respectively (see Figs 3, 4). However, no callus formation was observed from leaves and corm shells, indicating that they are not suitable for callus formation as explant. *Colchicum chalcedonicum* is known as a calcitrant species. Because of its advantages, micro-propagation of corm plants could be an alternative to the conventional techniques for vegetative propagation, increasing many times the multiplication level, enabling the plant materials to be freed from diseases especially for medicinal plants (Shibli & Ajlouni 2000, Chang *et al.* 2000).

Our efforts are continuing for developing callus formation protocol. Yalcin Mendi *et al.* (2017) reported micro-propagation of some endemic *Colchicum* species, but not on tissue culture for *C. chalcedonicum.* However, they used to active carbon to induce callus formation in *Colchicum* cultures. Active carbon is frequently used in tissue cultures to improve micro-propagation, orchid seed germination, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, rooting, stem elongation, corm formation etc (Thomas 2008). Studies with activated carbon in tissue culture demonstrated that activated carbon may be provide irreversible adsorption of inhibitory compounds in the culture medium significantly reducing the toxic metabolites, phenolic exudation and brown exudate accumulation (Thomas 2008). In our study, active carbon may enhance callus formation by limiting the brown exudate accumulation. We observed that green and soft callus formation resulted in medium containing active carbon. Also, callus formation in medium containing active carbon was faster than other mediums we tested.

*Colchicum* species comprise flavonoids, phenolic acids, tannin, fatty acids and colchicine is the major alkaloid isolated from *Colchicum* species such as *C. autumnale* and *C. luteum* (Kapadia *et al.* 1972, Levy *et al.* 1991, Evans 2002). Studies demonstrated that colchicine possesses antitumoral and anti-inflammatory activity and that it has a great potential for cancer treatment, making derivatives of colchicine, especially demecolcine and trimehyl colchicine acid methyl ester, to be evaluated as anti-cancer agent (Cocco *et al.* 2010, Bisi *et al.* 2015). However, no systematic attempt has been performed on micro-propagation for elite genotypes such as "Medicinal Plants" including *Colchicum* species. Our callus formation protocol has served promising results for future. Hayashi *et al.* (1988) used IBA and kinetin as PGRs in *C. autumnale* tissue culture for callus formation, and they managed to obtain colchicine by callus tissue culture system. However, production of secondary metabolites can be achieved by two main groups of *in vitro* cultures: organized cultures of differentiated tissues (i.e., organ cultures as root, shoot and embryo cultures) and unorganized cultures of undifferentiated cells (i.e., callus and cell suspension cultures). Although organized cultures of differentiated tissues produce the same secondary metabolites as the plant itself, which are relatively more stable than the undifferentiated cells, especially non-embryogenic plant callus cultures are mostly used for production of valuable secondary metabolites, including such as tropane alkaloids, hyoscyamine and scopolamine (Verpoorte *et al.* 2002, Filova 2014). Our present study is the pioneering study for tissue culture of *C. chalcedonicum.*, and tissue culture studies of *C. chalcedonicum* may also be applied for callus formation of important *Colchicum* species possessing colchicine.

An organism is identified as "endemic" which is native and has a restricted geographical region. Endemic species may be restricted due to physical barriers to dispersion, as in the case of many island faunas and flora, the barriers surround its area of origin, and consequently, they evolve within their limited distributional ranges (Masseti 2009). The extinction of plant and animal species, particularly with the ongoing climate change effects, has become an important issue, especially for endemic species. An alternative method of protection of endemic plant species is producing them *via* multiplying and conservation of plants by using *in vitro* culture techniques. The producing of endemic plants using tissue culture systems *via* multiplying is termed micro-propagation which has lots of advantages including high coefficient of multiplying, small needs on number of initial plants, small needs on space and reproducing of plants regardless seasons of the year, through multiplying intervals between subcultures in slow growing species (Kováč 1995, Engelmann 1997, 1998). We aimed to find the best nutrient media, PGRs and explants for micro-propagation and *in vitro* conservation of *C. chalcedonicum.* However, we were able to establish only efficient callus protocol were the pioneering of micro-propagation. This callus formation protocol will serve the improvement of tissue culture techniques to obtain more efficient callus formation protocols and to lead the micro-propagation of *C. chalcedonicum* in the future.

## Conclusion

The success of efficient callus protocol improvement for *in vitro* conservation of *C. chalcedonicum* relies on the optimal choice of the explants, on the efficiency of the sterilization method, and on the establishment of an *in vitro* culture protocol for these calcitrant species for the beginning of aseptic proliferative cultures and on the optimal choice of nutrient media and PGRs. According to our experimental data, the optimal media for efficient callus formation of *C. chalcedonicum* were MS supplemented with NAA (1 mg ml-1), ZEA (1 mg ml-1) containing 10% sucrose— and 2-1 MS supplemented with

2,4 D (2 mg ml-1), 2IP (0.5 mg ml-1), 3% sucrose and 0.05% active carbon medium. Additionally, supplying of active carbon in the media induced the callus formation. Our work is the pioneering study to obtain sophisticated callus formation protocol for *in vitro* conservation of *C. chalcedonicum.* Our study may help to save *C. chalcedonicum* which is endemic in İstanbul, Turkey.

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**Research Article**

# NOTES ON LEAF AND STEM ANATOMY OF *Thlaspi* sensu lato

Mehmet Cengiz KARAİSMAİLOĞLU[1*], Osman EROL[2]

[1] Department of Biology, Faculty of Arts and Sciences, Siirt University, Siirt, TURKEY
[2] Division of Botany, Department of Biology, Faculty of Science, İstanbul University, İstanbul, TURKEY

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****Corresponding Author:***
*Mehmet Cengiz Karaismailoğlu*
*cengiz.karaismailoglu@siirt.edu.tr*

***ORCID ID:***
*orcid.org/0000-0002-6856-2742*

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**Abstract:** In this study, anatomical characteristics of leaves and stems of 19 taxa of *Thlaspi* sensu lato, 9 of which are endemic to Turkey, were investigated in order to determine taxonomic relationships among the studied taxa. The anatomical characteristics of all taxa were determined and assessed using the cluster analysis and the principal component analysis. The transverse sections of the leaves and stems exhibited various diagnostic characters in terms of the pattern of epidermal cell and mesophyll layers, the number and size of vascular bundles, and the thickness of the cortex and endodermis. Important differences were detected in number, size, and index of stomata, and in epidermal cell wall structures in the adaxial and abaxial surfaces. The results showed that the compared anatomical characteristics among taxa are partially compatible with their sectional delimitation in their traditional rank in The Flora of Turkey and the East Aegean Islands. The results also point out that some taxonomic re-arrangements may be required.

**Özet:** Bu çalışmada, 9'u Türkiye için endemik olan 19 *Thlaspi* sensu lato taksonunun yaprak ve gövde anatomik karakterleri, taksonlar arasındaki taksonomik ilişkileri belirleme amacıyla tanımlanmıştır. Tüm taksonların anatomik karakterleri belirlendi ve kümeleme analizi ve temel bileşen analizi kullanılarak değerlendirildi. Yaprakların ve gövdelerin enine kesitleri epidermal hücre ve mezofil tabakalarının yapıları, vasküler demetlerin sayısı ve boyutu, korteks ve endodermisin kalınlığı açısından çeşitli teşhis karakterleri gösterdi. Ayrıca, adaksiyal ve abaksiyal yüzeylerde stomaların sayı, boyut ve indeksinde ve epidermal hücre duvarı yapılarında önemli farklılıklar saptandı. Bu sonuçlar taksonlar arasındaki karşılaştırılmış anatomik özelliklerin Türkiye Florası ve Doğu Ege Adaları'ndaki geleneksel sıralamasındaki seksiyon sınırlamaları ile kısmen uyumlu olduğunu göstermektedir. Elde edilen veriler ayrıca bazı taksonomik yeniden düzenlemelerin gerekli olabileceğine işaret etmektedir.

## Introduction

Brassicaceae is a large and important plant family in terms of economically significant species and model organisms such as *Arabidopsis* Heynh. and *Brassica* L. The family has approximately 338 genera and 3709 species worldwide, primarily in temperate zones of the Northern Hemisphere (Al-Shehbaz *et al.* 2006). In Turkey, Brassicaceae includes 571 species with 65 subspecies, 24 varieties and 660 taxa belonging to 91 genera (Al-Shehbaz *et al.* 2007). *Thlaspi* L. is known to be one of the largest genera of Brassicaceae and it has 75 species distributed mainly in Eurasia (Al-Shehbaz 1986, Appel & Al-Shehbaz 2003). The genus, represented by 36 taxa at various levels, is diverse in Turkey. Twenty-one of these taxa are endemic to Turkey, making the endemism rate of the genus 58% (Davis 1988, Karaismailoğlu & Erol 2019).

The classification in generic and subgeneric categories of *Thlaspi* sensu lato is quite confused with complex taxonomy and nomenclature. The traditional understanding of *Thlaspi* was radically changed with Meyer's works (1973, 1979, 1991, 2001), which were based on seed coat anatomy. Meyer divided the genus into 12 genera and maintained only six taxa in *Thlaspi* (*Thlaspi* sensu stricto). Many researchers (Greuter & Raus 1983, Greuter *et al.* 1986, Al-Shehbaz 1986, Artelari 2002, Appel & Al-Shehbaz 2003, Al-Shehbaz 2014, Karaismailoğlu & Erol 2018) opposed this classification by considering it as not practical for taxonomy and its extensive-scale use extremely limited (Al-Shehbaz 2014). In the following years, remarkable molecular phylogenetic studies such as Mummenhoff & Koch (1994), Zunk *et al.* (1996), Mummenhoff *et al.* (1997a, 1997b), Koch *et al.* (1998), Koch *et al.* (2001) clearly showed that the classification of Meyer (1973, 1979) was baseless (Al-Shehbaz 2014). However, despite the numerous works on the infrageneric and interspecific taxonomy of the genus, problems have yet to be elucidated.

Metcalfe & Chalk (1957) showed that the important distinctive anatomical characters of Brassicaceae include

epidermal cell type, stoma type and structures of the vascular bundles, which may provide insight into many taxonomical characters demonstrated to be significant in the species classification (Stace 1984). Many such data on these characters have been extensively used in taxonomical assignments (Liu & Zhu 2011, Selvi & Paksoy 2013, Ozcan *et al.* 2015, Karaismailoğlu 2016, 2019).

There is a lack of data in literature on anatomical features of *Thlaspi* taxa except those of seed anatomy (Karaismailoğlu & Erol 2019). This paper offers the first comprehensive assessment of the systematic importance of leaf and stem anatomy in the examined *Thlaspi* taxa.

## Materials and Methods

*Samples*

The examined taxa were collected from various phytogeographic regions of Turkey (Table 1). Voucher specimens are deposited in the Istanbul University Science Faculty Herbarium (ISTF).

*Anatomical preparations*

Anatomical studies were made using specimens preserved in 70% alcohol. Cross sections were taken with a fully automatic microtome (Thermo Shonda Met Finesse) from the stem and cauline leaves. Subsequently, they were taken through alcohol and xylene series, stained with hematoxylin (Harris-RRSP67-E) in a staining device (ASC 720 Medite) and covered with Entellan for examination of the anatomical structures (Karaismailoğlu 2015a, 2015b, Karaismailoğlu & Güner 2019). The stomatal density of leaf surfaces was enumerated using surface sections taken by hand. Anatomical characters were observed using an Olympus CX21FS1 microscope and Kameram Imaging Software.

The terminology used for anatomical characters of the leaves follows Wilkinson (1979) and Stace (1984).

**Table 1.** The locality and collection data details of the examined taxa.

| Section | Taxa | Locality | Collection number |
|---|---|---|---|
| *Nomisma* DC. | *T. arvense* L. (T1) | Samsun, Kavak-Akdağ, agricultural field edges, 766 m, 2.5.2015 | Karaismailoğlu 139 |
| | *T. huetii* Boiss. (T2) | Artvin, Şavşat, Ciritdüzü village, roadside, stony areas, 2050 m, 10.07.2014 | Karaismailoğlu 66 |
| *Thlaspi* L. | *T. orbiculatum* Stev. (T3) | Artvin, Ardanuç, above Peynirli village, steep slopes, 1663 m, 15.06.2015 | Karaismailoğlu 201 |
| | *T. kotschyanum* Boiss. & Hohen. (T4) | Kahramanmaraş, Göksun, Berit Mountain, humid areas, 2116 m, 19.06.2015 | Karaismailoğlu 202 |
| | *T. perfoliatum* L. (T5) | Tekirdağ, Tekirdağ-Kırklareli, roadsides and inclined slopes, 273 m, 21.03.2015 | Karaismailoğlu 109 |
| | *T. annuum* Koch (T6) | Amasya, Boraboy, village-lake, inclined slopes, 881 m, 02.05.2015 | Karaismailoğlu 143 |
| | *T. bulbosum* Spruner ex Boiss. (T7) | Kahramanmaraş, Andırın, Meryemçil plateau, grassland, 1633 m, 21.06.2015 | Karaismailoğlu 209 |
| | *T. leblebicii* Gemici & Görk (T8)* | Muğla, Köyceğiz, Sandras Mountain, Ağla village, roadsides, stony slopes, 1262 m, 05.06.2015 | Karaismailoğlu 192 |
| *Pterotropis* DC. | *T. ochroleucum* Boiss. (T9) | Hatay, Dörtyol, Topaktaş-Karamezra plateau, Fagus forest, stony slopes, 1752 m, 24.04.2016 | Karaismailoğlu 240 |
| | *T. violascens* Schott & Kotschy (T10)* | Osmaniye, Düziçi, Dumanlı Mountain, forest, 1259 m, 26.05.2015 | Karaismailoğlu 181 |
| | *T. densiflorum* Boiss. & Kotschy (T11)* | Kahramanmaraş, Ahir Mountain, Ulucak hill, stony slopes, 1751 m, 20.06.2015 | Karaismailoğlu 205 |
| | *T. cataonicum* Reuter (T12)* | Adana, Saimbeyli, Obruk plateau, grassland, 1472 m, 18.04.2015 | Karaismailoğlu 124 |
| | *T. elegans* Boiss. (T13)* | Osmaniye, Düziçi, near Haruniye, open fields and inclined slopes, roadside, 797 m, 19.04.2015 | Karaismailoğlu 130 |
| | *T. rosulare* Boiss. & Balansa (T14)* | Niğde, Çamardı, Yelatan village, stony slopes, 2085 m, 25.05.2015 | Karaismailoğlu 173 |
| | *T. praecox* Wulfen subsp. *praecox* (T15) | Kırklareli, Dereköy, roadsides, stony areas, 520 m, 09.06.2015 | Karaismailoğlu 197 |
| | *T. cariense* A. Carlström (T16)* | Muğla, Marmaris, Kırzeytin Mountain, serpentine rocks, 494 m, 05.06.2015 | Karaismailoğlu 190 |
| | *T. tatianae* Bordz. (T17) | Van, Güzeldere-Başkale, Güzeldere pass, summit, wetlands, 2651 m, 30.05.2015 | Karaismailoğlu 186 |
| | *T. aghricum* P.H. Davis & Kit Tan (T18)* | Ağrı, Hamur-Tutak, meadow, inclined slopes, 1605 m, 16.05.2015 | Karaismailoğlu 162 |
| | *T. watsonii* P.H. Davis (T19)* | Van, Güzeldere-Başkale, Güzeldere pass, summit, 2752 m, 02.07.2015 | Karaismailoğlu 210 |

* Endemic to Turkey

*Statistical analysis*

The data obtained from the examined parameters were evaluated with the SPSS (Statistical Package for the Social Sciences) and MVSP (Multi Variate Statistical Package) computer programs. The Duncan multiple-range test of the SPSS was used to determine the statistical importance of differences among the quantitative values obtained for different taxa in Tables 2-4 (SPSS 2006). Grouping of taxa was carried out utilizing the clustering analysis method (UPGMA) of the MVSP in accordance with the 36 characters in Tables 2-4 (Fig. 5). Principal component analysis (PCA) ordination of the MVSP was also performed (Fig. 6 and Table 5) (Kovach 2007).

**Results**

The comparative anatomical characteristics of the leaves and the stems are presented in Tables 2-4 and Figs 1-4, demonstrating important differences among the taxa. Tables 2-3 and Figs 1-3 show the anatomical features of the leaves and Table 4 and Fig. 4 show the anatomical characters of the stems of the examined taxa.

The cross-sections of the leaves feature a thin cuticle, which is more prominent in T4 and T6 than in other taxa, on the adaxial and abaxial epidermis. Epidermal cells are isodiametric and mostly range from square to rectangular in shapes, rarely polygonal. The thickness of the adaxial epidermis layer varies between 28.12 X 7.19 µm (T3) and 3.21 X 5.16 µm (T11), while the abaxial epidermis layer ranges from 69.77 X 6.14 µm (T4) to 4.08 X 2.98 µm (T1) (Table 2 and Fig. 1). The adaxial epidermis cells in most taxa are thicker than abaxial epidermis cells.

Most of the examined taxa have unifacial-type or two-layered, rarely three-layered (equifacial in T2 and T9) mesophyll with an arrangement of 2-4 layers of palisade parenchyma and 3-5 layers of spongy parenchyma with small intercellular gaps (Fig. 1). Mesophyll thickness ranges from 65.22 to 152.31 µm. It is widest in T17 and narrowest in T16 (Table 2). Most of the taxa appear to have straight anticlinal cell walls in epidermal layers, but T4, T7, T13, T17 and T19 have sinuous cell walls. The anticlinal cell walls of most of the taxa are sinuous on the abaxial surface, excluding T4, T15 and T16, which shows straight anticlinal cell walls.

Midrib dimensions varies from 50.12 (T7) to 95.91 µm (T8) in length, from 45.18 (T11) to 102.41 µm (T16) in width (Table 2). Midrib sizes are generally bigger in the taxa of the *Pterotropis* section than in the *Nomisma* and *Thlaspi* sections. The midribs are usually oval in shape; however, this varied, being circular to elliptical with a convex abaxial midrib surface in T10, T12, T16 and T18. Collateral vascular bundles are organized in a single row and enclosed with parenchymatic sheath cells.

Three different types of epidermal cells were observed on the leaf surfaces: irregular, polygonal, and rectangular (Figs 2-3). The stomatal index is between 12.37 (T3) and 63.01 (T5) on the adaxial surface, and 10.18 (T7) and 51.55 (T5) on the abaxial surface (Table 3). The stomata type of researched taxa were defined as anisocytic, and rarely anomocytic, which appeared to be on the same level as the epidermis. Stoma size differed substantially among the examined *Thlaspi* taxa. The largest stomatal size was found on the adaxial and abaxial surfaces of T18 and T17, whilst the smallest ones were observed on the adaxial and abaxial surfaces of T3 (Table 3, Figs 2-3)

The one-layer epidermis consisting of flat, square, or rectangular cells, with a thin cuticle (0.4-2 µm) on the outside was observed in stem cross sections. The surface is hairless, but there are protrusions in T1 and T2 (in the *Nomisma* section only) (Table 4 and Fig. 4). The epidermis layer is amphistomatic. The cortex consists of 3-12 parenchyma layers with thin- or thick-walled, regularly flat, or circular cells. Its thickness ranges from 14.46 (in T3) to 116.85 (in T11) µm (Table 4). Starch granules were observed in the cortex parenchyma. The endodermis layer located under the cortex consists of 1 or rarely 2 seriate flat-shaped cells, varying between 8.07 µm (T19) and 28.75 µm (T7) in length and between 3.84 (T10) and 12.87 µm (T11) in width. The number of bundles ranges from 5-6 (T5 and T7) to 14-16 (T4) (Table 4 and Fig. 4). This number is notably larger in taxa of the *Pterotropis* section compared to *Nomisma* and *Thlaspi*. The dimensions of the vascular bundles are 70.53-130.84 µm in length, and 40.62-156.06 µm in width (Table 4). The interfascicular region was found between vascular bundles in some of the examined taxa. Generally, cambium cells were not obvious. Some of the examined taxa also have a layer of scleranchymatic cells between 31.14 and 102.45 µm thick. The pith consists of polygonal or circular parenchymatic cells.

The unweighted pair group method with arithmetic mean (UPGMA) dissimilarity clustering dendrogram for 36 anatomical characters of leaves and stems of 19 *Thlaspi* taxa is presented in Fig. 5, and their infraspecific correlations in Tables (2-4). The dendrogram distinguishes the two major clusters and subsets from all other examined species. T11, T17, T14, T8, T13 and T7 form the first main cluster at a distance coefficient of about 25.0, while 13 other taxa make up the second main cluster at a distance coefficient of about 22.0. As indicated in the dendrogram, T11, T16, T5, T9 and T19 are markedly different from other taxa based on the examined characteristics (Tables 2-4). The clades included closely correlated taxa such as T3-T4, T1-T2 and T9-T19, consistent with the traditional taxonomic rank of *Thlaspi* taxa in Turkey.

The principal component analysis (PCA) ordination and dissimilarity matrix according to the anatomical characteristics of leaves and stems are given in Table 5 and Fig. 6. The closest and most distant taxa are defined. T9 and T19 are the most closely related taxa (dissimilarity percentage: 9.46), whereas T9 and T11 are the most distant taxa (dissimilarity percentage: 32.00) (Table 5 and Fig. 5). Additionally, the cumulative variance value of principal components reached 68.27% (Axis 1: 47.55%, Axis 2: 20.72%).

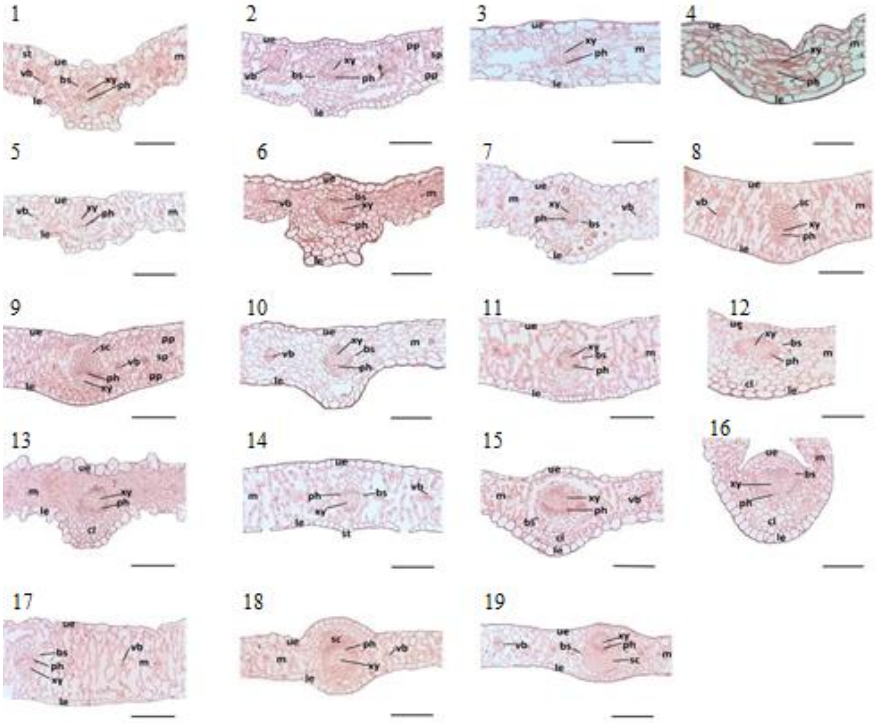

**Fig. 1.** Leaf cross sections of **1:** *T. arvense*, **2:** *T. huetii*, **3:** *T. orbiculatum*, **4:** *T. kotschyanum*, **5:** *T. perfoliatum*, **6:** *T. annuum*, **7:** *T. bulbosum*, **8:** *T. leblebicii*, **9:** *T. ochroleucum*, **10:** *T. violascens*, **11:** *T. densiflorum*, **12:** *T. cataonicum*, **13:** *T. elegans*, **14:** *T. rosulare*, **15:** *T. praecox* subsp. *praecox*, **16:** *T. cariense*, **17:** *T. tatianae*, **18:** *T. aghricum*, **19:** *T. watsonii*. (**ue:** upper epidermis, **le:** abaxial epidermis: **vb:** vascular bundle**, bs:** bundle sheath, **ph:** phloem, **xy:** xylem, **st:** stoma, **sc:** sclerenchymatic cells, **m:** mesophyll, **cl:** collenchyma, scale bars: 100 µm.)

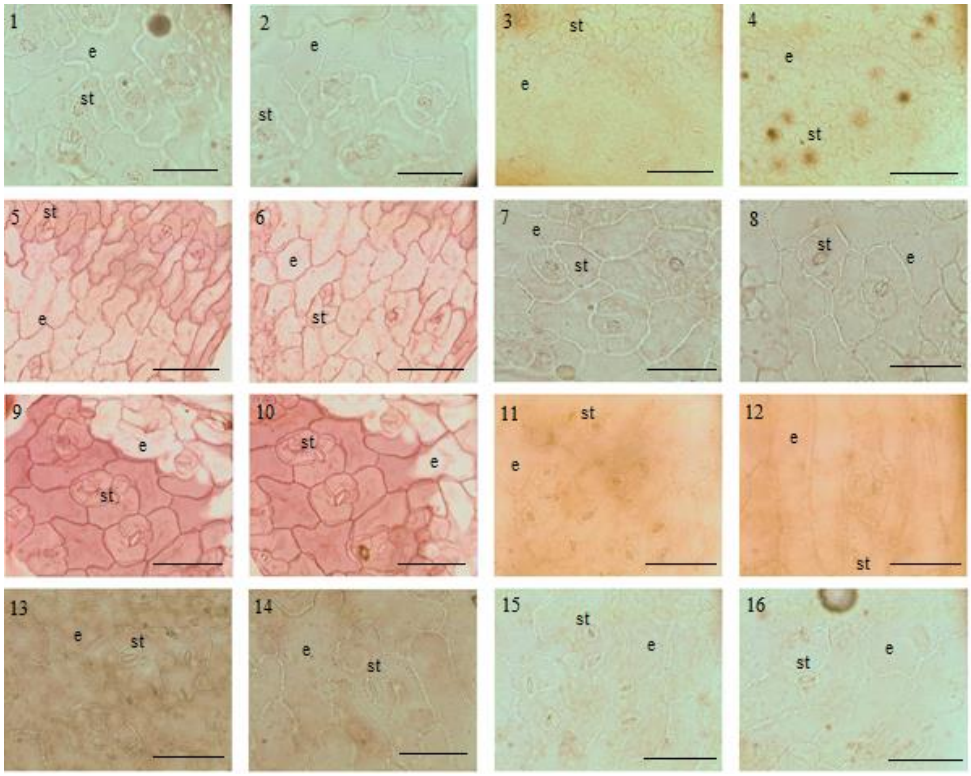

**Fig. 2.** Leaf surface patterns of **1-2:** *T. arvense*, **3-4:** *T. huetii*, **5-6:** *T. orbiculatum*, **7-8:** *T. kotschyanum*, **9-10:** *T. perfoliatum*, **11-12:** *T. annuum*, **13-14:** *T. bulbosum*, **15-16:** *T. leblebicii* (**e:** epidermis, **st:** stoma, scale bars: 100 µm, odd numbers show adaxial surfaces, even numbers show abaxial surfaces).

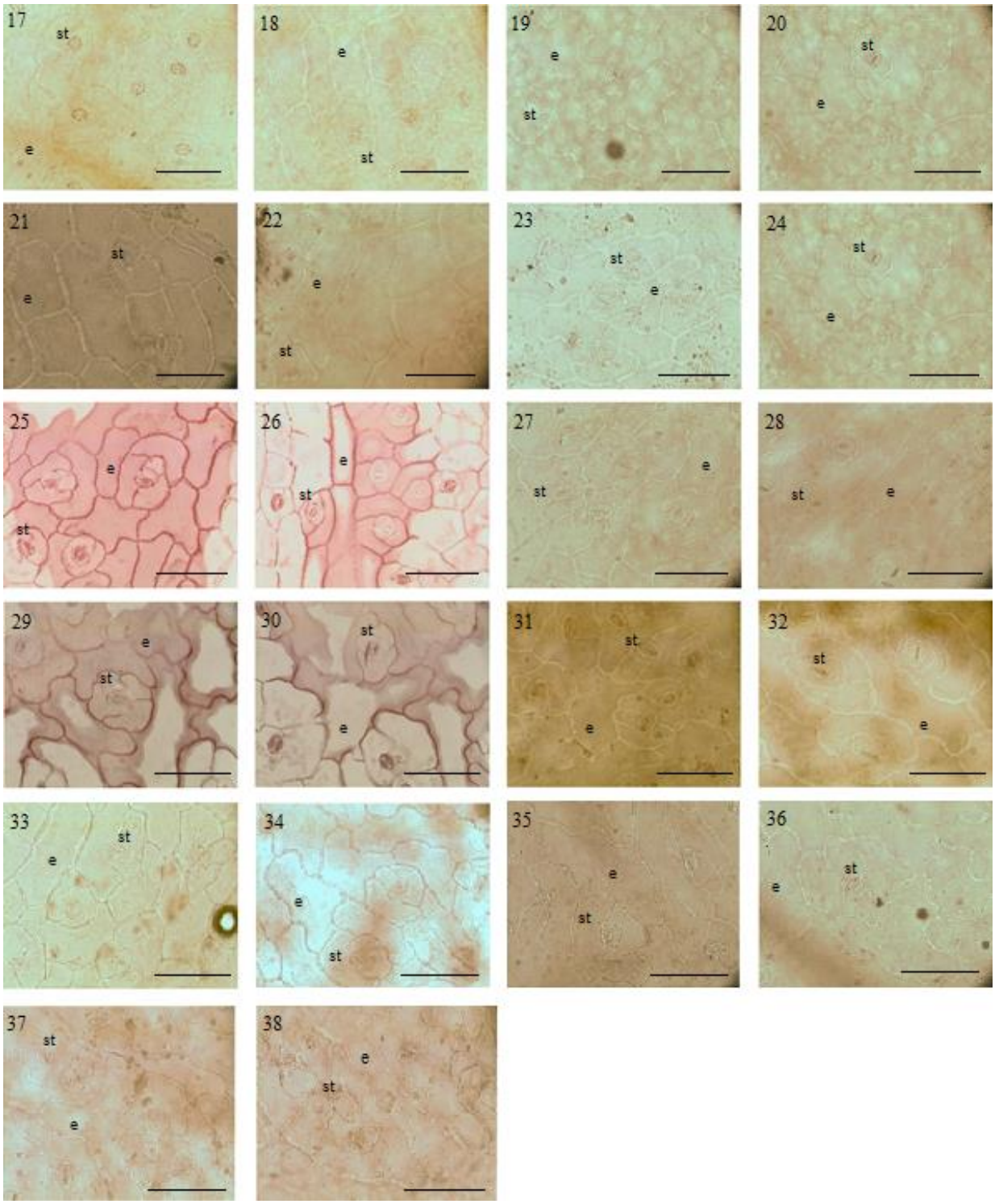


**Fig. 3.** Leaf surface patterns of **17-18:** *T. ochroleucum*, **19-20:** *T. violascens*, **21-22:** *T. densiflorum*, **23-24:** *T. cataonicum*, **25-26:** *T. elegans*, **27-28:** *T. rosulare*, **29-30:** *T. praecox* subsp. *praecox*, **31-32:** *T. Cariense*, **33-34:** *T. tatianae*, **35-36:** *T. aghricum*, **37-38:** *T. watsonii* (**e:** epidermis, **st:** stoma, scale bars: 100 µm, odd numbers show adaxial surfaces, even numbers show abaxial surfaces).

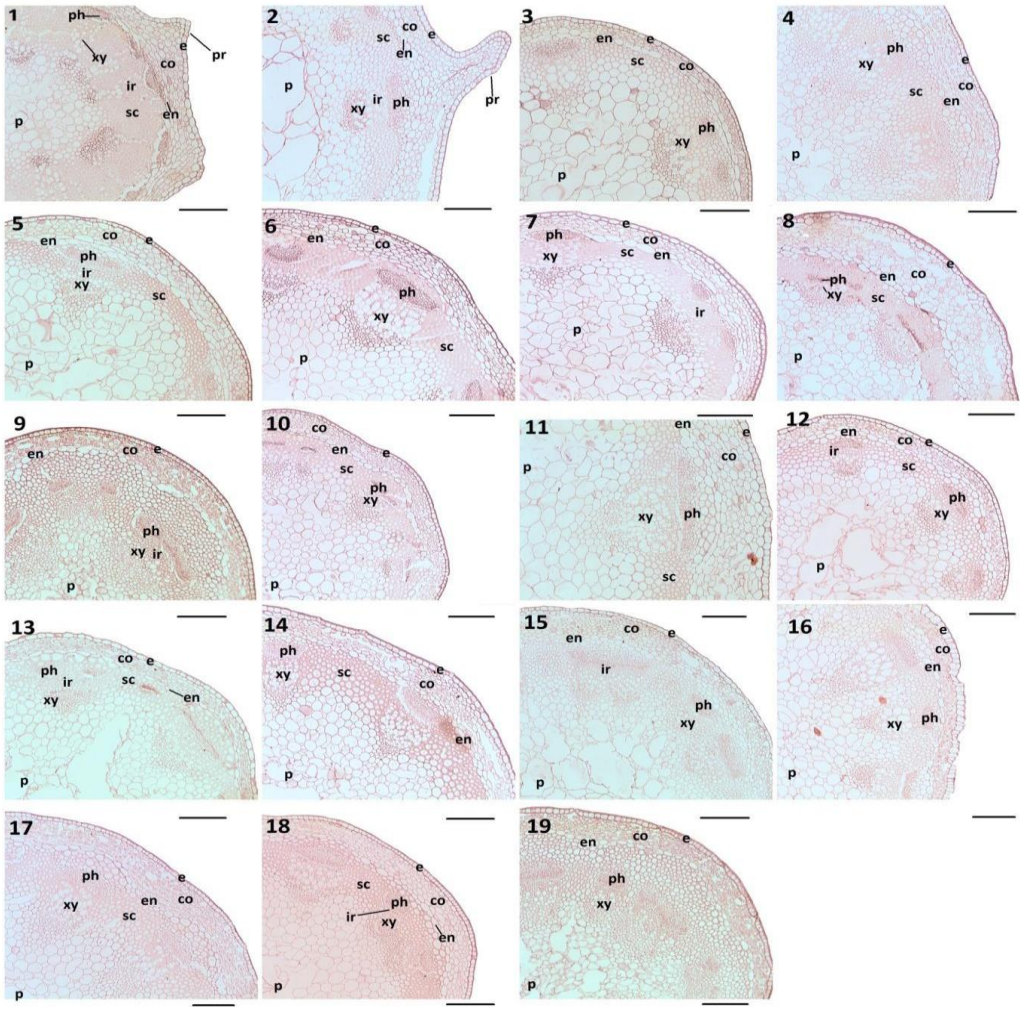


**Fig. 4.** Stem anatomical patterns of **1:** *T. arvense*, **2:** *T. huetii*, **3:** *T. orbiculatum*, **4:** *T. kotschyanum*, **5:** *T. perfoliatum*, **6:** *T. annuum*, **7:** *T. bulbosum*, **8:** *T. leblebicii*, **9:** *T. ochroleucum*, **10:** *T. violascens*, **11:** *T. densiflorum*, **12:** *T. cataonicum*, **13:** *T. elegans*, **14:** *T. rosulare*, **15:** *T. praecox* subsp. *praecox*, **16:** *T. cariense*, **17:** *T. tatianae*, **18:** *T. aghricum*, **19:** *T. watsonii*. (**e:** epidermis, **co:** cortex, **en:** endodermis, **ph:** phloem, **xy:** xylem, **ir:** interfascicular region, **sc:** sclerenchymatic region, **p:** pith, scale bars: 100 µm.)

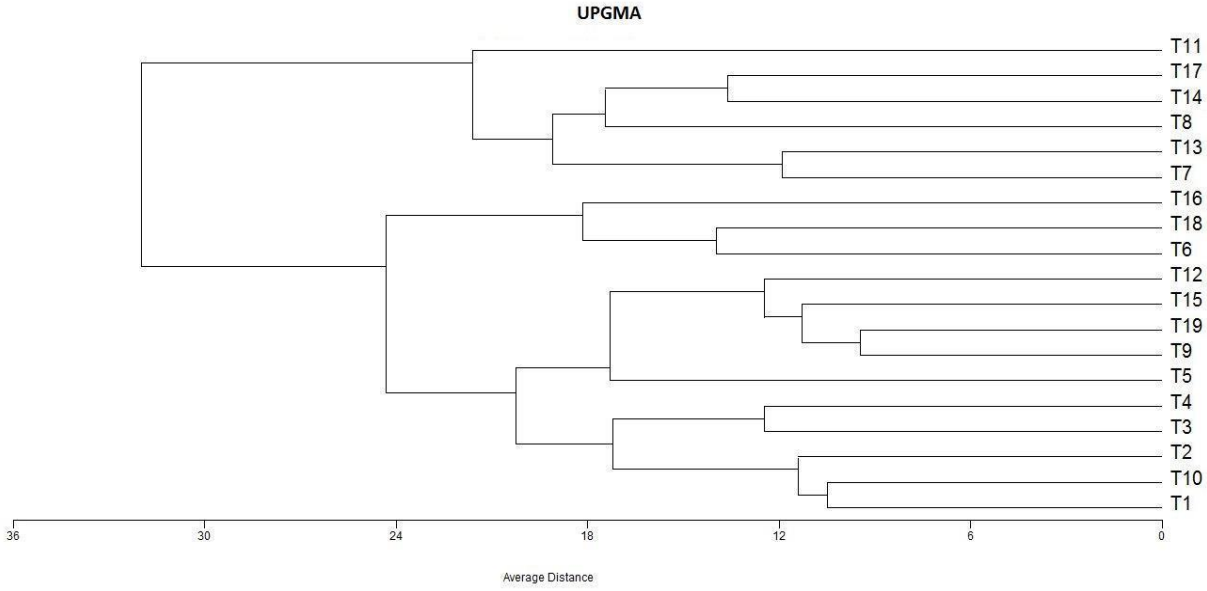


**Fig. 5.** The dendrogram obtained with UPGMA of the examined taxa (See Table 1 for taxa abbreviations).

**Table 2.** Anatomical characteristics of leaves of the examined taxa. Outcomes represent mean values ± standard deviation; means with different letters are significant at p = 0.05 level (Duncan's multiple-range test), for taxa abbreviations see Table 1 (L: Length, W: Width).

| Taxa | Leaf | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Adaxial Epidermis | Midrib | | | Xylem | Phloem | Trachea | Mesophyll | Abaxial Epidermis |
| | L (1) X W (2) (µm) | Bundle Sheath (3) | Sclerenchyma (4) | L (5) X W (6) (µm) | Height (7) (µm) | Height (8) (µm) | Diameter (9) (µm) | Thickness (10) (µm) | L (11) X W (12) (µm) |
| T1 | 9.13±0.16c X 5.02±0.22bc | present | absent | 67.15±2.45f X 59.48±3.18h | 44.16±2.08de | 5.16±0.54d | 3.17±0.12c | 99.23±4.04g | 4.08±2.32i X 2.98±1.02h |
| T2 | 6.71±2.14ef X 3.41±0.14c | present | absent | 64.05± 0.15g X 62.17±0.21h | 32.59±0.19h | 11.04±0.22a | 2.24±0.08d | 108.75±2.72e | 4.12±1.08i X 6.02±0.51e |
| T3 | 28.12±4.91a X 7.19±2.03b | present | absent | 59.74±0.26h X 47.25±1.09k | 20.42±0.37i | 9.05±0.33a | 4.12±0.22a | 115.32±0,29d | 18.26±4.97b X 4.35±0.24fg |
| T4 | 24.27±2.75b X 6.17±1.98bc | absent | absent | 61.47±2.15h X 65.79±1.35g | 29.27±0.98h | 7.16±0.28ab | 3.15±0.11c | 103.95±4.17f | 69.77±10.98a X 6.14±0.22de |
| T5 | 6.18±0.39e X 4.27±1.45bc | present | absent | 58.45±2.21h X 51.12±1.23j | 31.04±1.17h | 3.11±1.74de | 3.01±0,09c | 98.14±0.21g | 6.78±0.25h X 6.54±0.65de |
| T6 | 5.17±0.22ef X 7.11±0.19b | present | absent | 62.17±1.01h X 82.24±1.18c | 45.16±0.54d | 4.17±0.24e | 2.22±0.36d | 79.16±1.23hi | 4.98±0.33i X 7.11±0.31d |
| T7 | 5.42±0.16e X 5.21±0.11bc | present | absent | 50.12±1.07k X 46.22±0.89k | 42.07±0.56e | 5.63±0.21d | 3.07±0.15c | 105.18±0.21f | 9.44±0.12e X 6.25±0.16de |
| T8 | 10.22±2.01de X 5.08±0.25bc | absent | present | 95.91±2.16a X 68.24±0.98f | 20.16±0.15i | 4.01±0.12e | 1.15±0.04f | 108.11±0.34e | 6.35±0.29i X 2.56±0.14h |
| T9 | 7.72±2.03e X 3.28±1.35c | present | present | 91.14±2.54b X 70.46±1.14e | 36.15±0.44g | 5.18±0.10d | 1.19±0.06ef | 107.08±0.97ef | 5.04±0.22i X 3.03±0.18h |
| T10 | 8.91±0.26e X 3.25±0.12c | present | absent | 87.14±0.33c X 56.59±0.89h | 60.18±0.35a | 9.21±2.09a | 2.34±0.11d | 101.12±3.79fg | 8.72±0.07f X 3.21±0.08h |
| T11 | 3.21±0.21g X 5.16±0.17bc | present | absent | 54.45±0.14i X 45.18±0.27l | 39.47±2.15ef | 4.18±0.14e | 2.02±0.08d | 137.27±4.13b | 3.49±0.16i X 5.64±0.22e |
| T12 | 5.19±0.27f X 3.25±0.16c | present | absent | 51.17±1.17k X 81.14±0.21cd | 46.28±0.44d | 6.17±0.46bc | 1.39±0.18e | 126.39±2.12c | 10.69±0.27d X 6.75±0.17de |
| T13 | 4.71±0.26f X 7.49±0.22b | absent | absent | 69.27±0.59ef X 53.81±1.34i | 43.19±1.18de | 5.21±0.11d | 1.27±0.07e | 99.18±1.17fg | 4.27±0.12i X 6.51±0.14de |
| T14 | 6.28±0.21e X 4.57±0.16c | present | absent | 81.57±0.28d X 50.24±0.11j | 41.19±0.27f | 5.67±0.22d | 2.18±0.04d | 110.25±0.22e | 6.52±0.25i X 4.86±0.36f |
| T15 | 10.65±0.44de X 6.79±0.52b | present | absent | 70.56±0.87e X 88.95±1.96b | 48.56±1.85bc | 7.19±0.26ab | 2.38±0.06d | 102.91±0.98f | 21.16±0.08b X 16.35±2.32b |
| T16 | 7.12±.23e X 11.47±0.33a | present | absent | 68.79±1.45ef X 102.41±1.22a | 40.11±0.41f | 4.56±0.21e | 3.77±0.11b | 65.22±2.18i | 8.23±0.41g X 23.23±0.94a |
| T17 | 12.11±0.89d X 6.22±0.41b | present | absent | 90.53±2.11b X 51.48±0.99j | 45.29±1.33d | 5.11±0.33de | 2.56±0.29cd | 152.31±3.32a | 6.65±0.33hi X 5.14±0.17f |
| T18 | 20.15±0.77c X 7.14±0.30b | absent | present | 61.82±1.33h X 82.46±0.85c | 52.35±2.77b | 8.27±0.92a | 3.02±0.35c | 80.54±1.08h | 12.05±0.19c X 8.75±0.33c |
| T19 | 10.63±0.19de X 5.27±0.27bc | present | present | 53.13±0.39j X 46.29±0.45kl | 35.84±0.95g | 6.28±0.19b | 2.11±0.12d | 97.35±1.54g | 8.19±0.21g X 6.28±0.22de |

**Table 3.** Characteristics of leaf surface patterns of the examined taxa. Outcomes represent mean values ± standard deviation; means with different letters are significant at p = 0.05 level (Duncan's multiple-range test), for taxa abbreviations see Table 1 (L: Length, W: Width).

| Taxa | Adaxial epidermis | | | | | | Abaxial epidermis | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Anticlinal cell wall | Shape of epidermal cells | Stomata | | | | Anticlinal cell wall | Shape of epidermal cells | Stomata | | | |
| | | | L (µm) | W (µm) | Number per mm$^2$ | Index | | | L (µm) | W (µm) | Number per mm$^2$ | Index |
| | (13) | (14) | (15) | (16) | (17) | (18) | (19) | (20) | (21) | (22) | (23) | (24) |
| T1 | Undulated | Irregular | 30.76±1.21g | 21.22±0.76j | 81±2c | 33.19±0.15ef | Undulated | Irregular | 27.29±0.89i | 20.96±0.44h | 36±4f | 31.45±0.18de |
| T2 | Sinuate | Irregular | 30.22±0.37g | 19.27±0.22lm | 36±2g | 28.68±0.27h | Sinuate | Irregular | 25.05±0.33jk | 19.16±0.31j | 27±2g | 22.63±0.09i |
| T3 | Undulated | Irregular | 22.14±0.23k | 17.42±0.33n | 36±2g | 12.37±0.11p | Sinuate | Irregular | 21.23±0.26l | 16.98±0.22k | 27±4g | 10.89±0.32p |
| T4 | Undulated | Polygonal | 26.51±0.17j | 20.05±0.12jk | 45±2f | 34.62±0.15d | Undulated | Polygonal | 32.17±0.28e | 21.12±0.11h | 36±2f | 21.96±0.25j |
| T5 | Undulated | Irregular | 30.89±0.25g | 16.18±0.31o | 99±2b | 63.01±0.08a | Undulated | Irregular | 29.17±0.22g | 18.02±0.15k | 90±2a | 51.55±0.20a |
| T6 | Undulated | Polygonal | 31.15±0.22g | 24.28±0.35n | 135±5a | 48.76±0.24b | Straight | Rectangular | 27.62±0.37i | 20.19±0.23i | 36±4f | 27.16±0.12fg |
| T7 | Undulated | Irregular | 34.53±0.44f | 22.62±0.23i | 45±2f | 33.33±0.15ef | Straight or Undulated | Irregular | 26.44±0.18j | 16.48±0.29kl | 18±2h | 10.18±0.28r |
| T8 | Undulated | Irregular | 38.56±0.37d | 24.51±0.26h | 72±4cd | 44.15±0.33g | Undulated | Irregular | 31.28±0.51ef | 19.27±0.41j | 45±4e | 20.11±0.15l |
| T9 | Undulated | Irregular | 36.41±0.20e | 22.78±0.39i | 82±4c | 32.05±0.13e | Straight | Polygonal | 26.11±0.79ij | 21.03±0.38h | 65±3c | 21.21±0.18k |
| T10 | Undulated | Polygonal | 28.16±0.39hi | 25.67±0.20g | 74±5cd | 33.87±0.41e | Undulated | Polygonal | 30.14±0.25g | 23.09±0.30f | 40±4ef | 25.29±0.10h |
| T11 | Straight | Polygonal | 29.75±0.16g | 19.49±0.14l | 40±4efg | 18.41±0.30o | Straight or Undulated | Polygonal | 26.38±0.11i | 19.27±0.08j | 10±2i | 12.58±0.27o |
| T12 | Undulated | Irregular | 28.54±0.33h | 21.16±0.25j | 80±6c | 20.84±0.27mn | Sinuate | Irregular | 25.17±0.14j | 21.04±0.47h | 70±4bc | 32.29±0.41d |
| T13 | Undulated | Irregular | 25.89±0.11jk | 21.41±0.17j | 51±3e | 18.76±0.19no | Straight or Undulated | Polygonal | 27.09±0.23i | 29.45±0.51c | 75±5b | 27.61±0.24f |
| T14 | Undulated | Irregular | 40.16±0.25c | 28.14±0.26d | 74±2cd | 27.46±0.21i | Straight | Polygonal | 38.27±0.11d | 25.38±0.34d | 76±2b | 30.55±0.12e |
| T15 | Sinuate | Irregular | 38.41±0.35d | 27.36±0.23e | 51±4e | 23.19±0.16l | Sinuate | Irregular | 24.75±0.21k | 20.77±0.18h | 45±4e | 19.80±0.14m |
| T16 | Undulated | Irregular | 41.07±0.14bc | 26.61±0.35f | 100±4b | 38.59±0.33c | Undulated | Irregular | 38.99±0.14c | 25.23±0.39de | 36±4ef | 33.33±0.19c |
| T17 | Undulated | Irregular | 42.21±0.29b | 33.15±0.26b | 47±2ef | 20.98±0.22m | Sinuate | Irregular | 45.40±0.30b | 42.01±0.11a | 40±2ef | 21.08±0.27kl |
| T18 | Undulated | Irregular | 45.10±0.11a | 39.08±0.22a | 70±4cd | 24.51±0.08k | Undulated | Irregular | 46.34±0.33a | 41.24±0.35b | 24±2g | 15.52±0.35n |
| T19 | Undulated | Irregular | 31.79±0.18g | 31.17±0.15c | 72±2cd | 25.49±0.15j | Undulated | Irregular | 28.47±0.11h | 22.62±0.27fg | 60±4cd | 40.11±0.46b |

**Table 4.** Anatomical characteristics of stems of the examined taxa. Outcomes represent mean values ± standard deviation; means with different letters are significant at p = 0.05 level (Duncan's multiple-range test), for taxa abbreviations see Table 1 (L: Length, W: Width).

| Taxa | Epidermis cells | | Cortex Thickness (µm) | Endodermis cells | | Vascular bundles | | Interfascicular region | Scleranchymatic region thickness |
|---|---|---|---|---|---|---|---|---|---|
| | Structure | L(26) X W(27) (µm) | | Structure | L (30) X W (31) (µm) | Number | Sizes | | |
| | (25) | | (28) | (29) | | (32) | L(33) X W(34) | (35) | (36) |
| T1 | flat cells | 14.16±0.59b X 9.81±0.12b | 59.45±0.97e | large flat cells | 12.14±1.45h X 5.66±0.19fg | 10-12b | 104.13±0.78f X 41.22±0.44m | clearly | 102.45±1.56a |
| T2 | rectangular cells | 9.87±0.22d X 7.65±0.18d | 74.59±0.81c | flat cells | 9.88±0.55i X 4.99±1.11fg | 9-10bc | 95.18±2.07g X 48.46±1.22l | clearly | 60.48±1.97e |
| T3 | flat cells | 10.83±0.22d X5.96±0.21g | 14.46±0.16l | large flat cells | 15.12±0.21f X 8.26±0.14c | 6-8d | 107.19±2.23e X 60.85±1.54k | unclearly | 43.41±1.05i |
| T4 | square-shaped cells | 5.02±0.21k X 4.84±0.19ij | 48.74±2.10fg | large flat cells | 12.87±0.12h X 6.12±0.23f | 14-16a | 110.54±0.88d X 68.96±2.05j | unclearly | 48.91±0.88h |
| T5 | thick-walled flat cells | 8.77±0.22f X 4.09±0.37k | 39.10±1.14h | 1 or 2 seriate large flat cells | 24.05±1.22b X 8.74±0.44bc | 5-6de | 102.85±0.77fg X 83.24±1.33h | clearly | 31.14±0.63l |
| T6 | flat cells | 5.48±0.21j X 3.91±0.12k | 52.46±1.08f | large flat cells | 12.05±0.19h X 7.43±0.10d | 7-9cd | 106.11±1.54ef X 102.85±0.77de | unclearly | 82.41±3.46cd |
| T7 | thick-walled flat cells | 10.63±0.12d X 6.32±0.16f | 25.61±0.49k | flat cells | 28.75±0.86a X 6.99±0.21e | 5-6de | 108.77±1.21de X 112.86±0.86c | clearly | 34.78±2.46k |
| T8 | thick-walled flat cells | 7.55±0.86g X 5.31±0.12h | 105.46±0.27b | large flat cells | 14.73±0.92f X 6.02±0.12f | 8-9cd | 70.53±1.32k X 105.09±1.27d | unclearly | 48.97±2.13h |
| T9 | thick-walled square-shaped cells | 6.96±0.12hi X 6.59±0.24ef | 38.76±0.95hi | large flat cells | 18.65±0.26e X 7.83±0.15c | 12-14ab | 77.85±0.92j X 40.62±0.77m | clearly | - |
| T10 | flat cells | 8.07±0.22g X 4.88±0.08ij | 44.61±0.74g | flat cells | 9.41±0.17i X 3.84±0.34g | 10-12b | 81.50±3.17ij X 46.41±2.91l | unclearly | 54.64±0.89g |
| T11 | square-shaped cells | 9.21±0.22de X 8.94±0.11c | 116.85±2.38a | flat cells | 14.15±0.22fg X 12.87±0.25a | 5-6de | 124.41±1.47b X 156.06±2.85a | unclearly | 83.55±1.14c |
| T12 | flat cells | 10.23±0.84d X 7.49±0.13d | 37.45±0.51i | flat cells | 14.66±0.31fg X 6.54±0.21ef | 10-12b | 84.66±0.77i X 59.52±0.65k | clearly | 46.11±0.39hi |
| T13 | flat cells | 19.16±0.36a X 6.78±0.21ef | 42.75±0.36h | large flat cells | 25.16±0.24b X 7.42±0.21d | 6-8d | 104.11±2.51fg X 124.04±1.08b | clearly | 39.41±0.65j |
| T14 | thick-walled flat cells | 12.16±0.17c X 6.88±0.27e | 49.56±0.21f | large flat cells | 15.08±0.21f X 8.27±0.15c | 7-9cd | 123.81±1.24bc X 91.73±0.85g | unclearly | 97.44±0.86b |
| T15 | flat cells | 9.54±0.12d X 6.61±0.08f | 47.81±0.39f | flat cells | 10.85±0.33h X 7.21±0.24de | 10-12b | 95.12±0.89g X 74.18±0.65i | clearly | - |
| T16 | elongated rectangular cells | 7.15±0.25h X 15.36±0.16a | 29.96±0.37j | large flat cells | 18.49±0.22e X 9.17±0.21b | 10-12b | 130.84±0.86a X 102.39±0.77de | unclearly | - |
| T17 | rectangular cells | 8.47±0.12fg X 5.09±0.10hi | 71.25±2.52d | large flat cells | 20.35±0.12c X 7.84±0.29c | 9-10bc | 120.54±2.18c X 95.17±0.72f | unclearly | 56.22±0.29f |
| T18 | rectangular cells | 8.14±0.15g X 5.86±0.12g | 58.91±1.46e | large flat cells | 20.07±0.32cd X 8.19±0.39c | 7-9cd | 106.45±0.59e X 102.16±0.46de | clearly | 67.18±0.37d |
| T19 | thick-walled flat cells | 11.02±0.12d X 6.91±0.15e | 51.04±2.32f | flat cells | 8.07±0.12j X 5.47±0.12fg | 10-14ab | 88.43±0.18h X 46.63±0.32l | clearly | - |

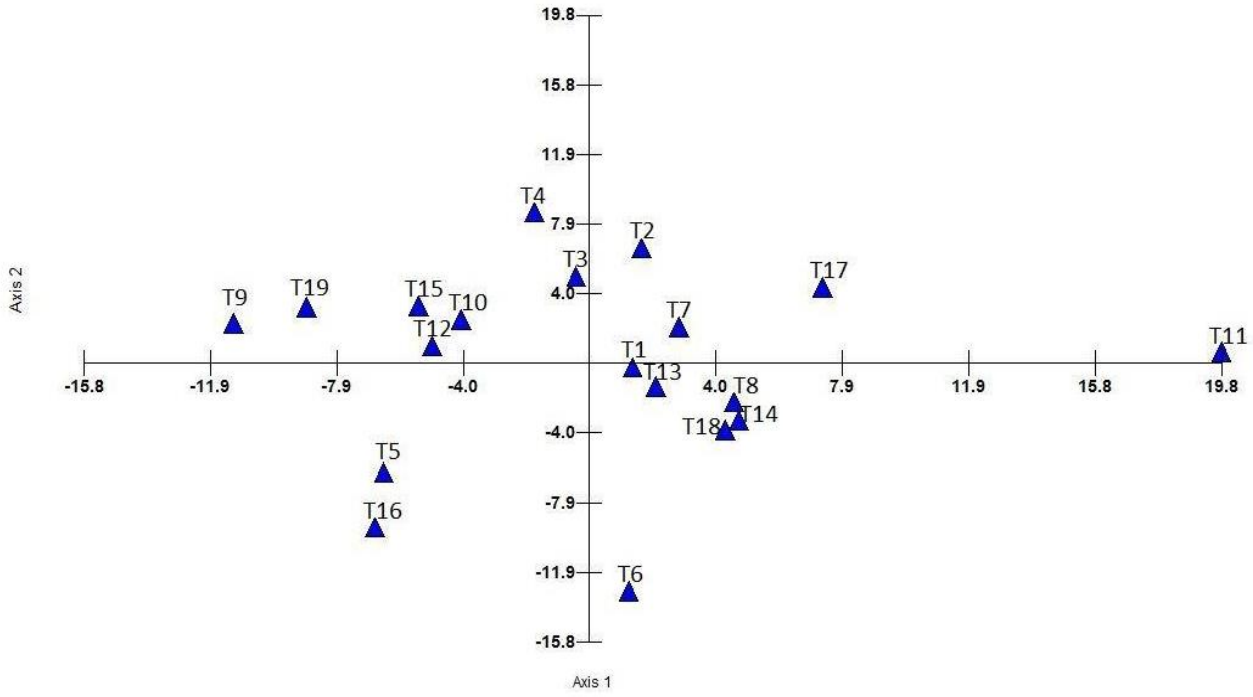


**Fig. 6.** Principal component analysis of the examined taxa (See Table 1 for taxa abbreviations).

## Discussion

*Thlaspi* s.l. is represented by 36 taxa, including species and subspecies, found in six sections in the Flora of Turkey and the East Aegean Islands (Flora of Turkey) (Hedge 1965, Davis 1988, Yıldırımlı 2001). The present work assesses the significance of anatomical characteristics and evaluates the correlations among leaf and stem anatomy. The anatomical structures of the leaves and stems of the examined *Thlaspi* taxa were investigated in detail for the first time in this study.

Generally, the examined taxa have a unifacial mesophyll, rarely bifacial and made up of 2-5 layers of palisade parenchyma and 3-6 layers of abaxial spongy parenchyma, or equifacial. Metcalfe & Chalk (1957) formerly found that bifacial mesophyll is most common in the Brassicaceae family, but unifacial, bifacial and equifacial types were commonly observed in the present study. These types of leaf mesophyll, except unifacial, are widespread in correlated genera, such as bifacial in *Alyssum* L. (Orcan & Binzet 2002), bifacial and equifacial in *Ricotia* L. (Selvi & Paksoy 2013) and bifacial in *Aubrieta* Adans (Karaismailoğlu 2016).

All but three (*T. huetii*, *T. densiflorum* and *T. praecox* subsp. *praecox*) of the examined taxa have undulated anticlinal cell walls (Fig. 1). These three taxa (straight or sinuate) grow at low altitudes and humid zones, while the 13 taxa with undulated anticlinal cell walls are found in higher altitudes and arid habitats. According to Stace (1984), epidermal cells with undulated or straight outlines are widespread in xeromorphic and mesomorphic plants. Another study reported that most epidermal cells of leaves in dicotyledons have sinuate anticlinal cell walls, which may be caused by pressure exerted on the walls during cell development (Orcan & Binzet 2002).

The studied *Thlaspi* taxa grow at various altitudes between 270 and 2750 m and in different ecological conditions. While the mesophyll layer in the taxa growing at low altitudes is quite loose, it is denser in appearance of the parenchyma cells at high altitudes. This shows that high altitude and inadequate water can trigger further development of the mesophyll parenchyma, and consequently enhance photosynthetic activity (Fahn 1990, Ozcan *et al.* 2015).

All the examined taxa are of the amphistomatic type. Stomatal density differs significantly between the abaxial and adaxial surfaces of leaves (Table 3). The density is clearly higher on adaxial surfaces than abaxial surfaces in most of the examined taxa. The exceptions are T12-T14, T17 and T18 taxa, which exhibited the opposite. This result is compatible with several previous studies, including Orcan & Binzet (2002), Arambarri *et al.* (2006), Ozcan *et al.* (2015) and Karaismailoğlu (2016). Stomata are mostly anisocytic (Cruciferae type) or rarely anomocytic (Ranunculaceae type) in the examined taxa. These types of stomata were formerly found in the family Brassicaceae by Metcalfe & Chalk (1957), Cansaran *et al.* (2007), Selvi & Paksoy (2013) and Karaismailoğlu (2016, 2019).

The stems of herbaceous *Thlaspi* taxa generally produce no secondary tissues. Cortex is composed of flat or circular parenchyma in 3-12 layers. A thick bundle sheath covers the adaxial side of the phloem and xylem.

All vascular bundles were of the collateral type. The inner part of the pith breaks down in the late or early phases of primary growth, like in some other Brassicaceae family members (Metcalfe & Chalk 1957).

Yentür (2003) showed that the arrangement of bundles provides useful information in comparative anatomical investigations. The number of vascular bundles in the stem is usually between 5 and 16 and the bundles are arranged in a single ring. Selvi & Paksoy (2013) and Karaismailoğlu (2016) reported that vascular bundles are distributed in a circular manner in one ring in stems of some Brassicaceae species. Clustered scleranchymatic cells were positioned on the adaxial and abaxial sides of the vascular bundles in most of the examined taxa (Fig. 4), except for T9, T15, T16 and T19 (Fig. 4 and Table 4). The presence or absence of secretory channels is quite significant in comparative anatomical studies (Makbul *et al.* 2011). Also, the content, distribution and presence or absence of secretory channels were shown to differ among the plant taxa (Milan *et al.* 2006). This work found that there are a few secretory networks in the stem cortex near the vascular bundles of the examined taxa.

A dendrogram was formed to assess the anatomical characteristics of the leaves and stems of the examined *Thlaspi* taxa using UPGMA cluster analysis. The dendrogram indicating two major clusters was partially compatible with Hedge (1965), Davis (1988) and Yıldırımlı (2001), where 36 taxa were placed into six sections. The anatomical differences between the leaves and stems were observed at the species level, but showed no correlation at section level. It seems that anatomical features are not in full agreement with the available classification, nevertheless it proves to be valuable data. That is, the anatomical characters of leaves and stems partially supported the features utilized in the distribution of *Thlaspi* species in The Flora of Turkey.

Principal component analysis can provide data about the variability of the used anatomical characters. The high cumulative variance values of principal components indicate that the characters investigated can be used to elucidate variances among the studied *Thlaspi* taxa. Dissimilarity ratios among the taxa were defined. Accordingly, the closest relationship was seen between T9 and T19, while the most distant relationship was found between T9 and T11.

## Conclusion

This study questions the usefulness of the studied characteristics in the infrageneric delimitation in *Thlaspi*. The compared anatomical characteristics among the examined *Thlaspi* taxa are partially in accordance with their sectional delimitation given in The Flora of Turkey. However, the findings showed that new arrangements may be necessary for the systematic positions of some taxa. The leaf and stem anatomical characteristics are helpful for distinctions based on morphology. This study is a preliminary study to determine the usefulness of the examined anatomical characters. Further investigations that include all the taxa of the genus are required to define all variations and obtain a better systematic understanding of the genus.

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**Research Article**

# CONSERVATION STATUS OF THREE RARE AND ENDEMIC SPECIES FROM TURKEY (*Kalidium wagenitzii, Muscari adilii* & *Verbascum gypsicola*)

Ali Murat KESER*, Gül AYYILDIZ, Merve YILDIRIM, Ahmet Emre YAPRAK, Gül Nilhan TUĞ

Ankara University, Faculty of Science, Biology Department, 06100, Tandoğan, Ankara, TURKEY

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**Abstract:** In this study, we aimed to determine the population size and distribution areas and to re-evaluate IUCN threat categories of *Kalidium wagenitzii* (Aellen) Freitag & G. Kadereit, *Muscari adilii* M.B. Güner & H. Duman and *Verbascum gypsicola* Vural & Aydoğdu, which are endemics of the Central Anatolia region of Turkey. The three species have limited distribution areas and high risk of extinction as a result of human impact. The perennial halophytic species *K. wagenitzii* was found in 5 localities around Salt Lake (Tuz Gölü) and a total number of 6458 mature individuals were determined. The area of occupancy of the species is 36 km$^2$ and the extent of occurrence is 213 km$^2$. Uncontrolled use of water resources for agricultural purposes is a serious threat factor for the species. *Kalidium wagenitzii* is listed in EN category according to the IUCN Red List criteria. *Muscari adilii* prefers marly soils and its habitat type is formed by secondary succession after the destruction of *Pinus nigra* and oak forests. It is known from 3 localities around Nallıhan-Beypazarı with a population size of 6144 mature individuals. The area of occupancy and the extent of occurrence of the species is 12 km$^2$ and 28 km$^2$, respectively. The pressures on the species are road construction and increase of farmland, afforestation and factory establishment. According to the IUCN Red List criteria, the species is listed in CR category. *Verbascum gypsicola* is distributed on marly soils. It is known from 3 localities around Nallıhan–Beypazarı and one locality in Sivrihisar-Eskişehir with 2755 mature individuals in total. The area of occupancy of the species is 16 km$^2$ and the extent of occurrence is 269 km$^2$. Overgrazing and expansion of agricultural land, together with factory establishment are the major threats for this species, which is listed as EN in IUCN Red List.

**Özet:** Bu çalışma ile Türkiye'nin İç Anadolu bölgesi için endemik olan *K. wagenitzii* (Aellen) Freitag & G. Kadereit, *M. adilii* M. B. Güner & H. Duman ve *V. gypsicola* Vural & Aydoğdu türlerinin popülasyon yapıları ve yayılış alanlarının belirlenmesi ve bunların sonucunda IUCN tehlike kategorilerinin tekrardan değerlendirilmesi amaçlanmıştır. Bu türlerin seçilmesinin nedeni sahip oldukları sınırlı yayılış alanları ile insan faktörü sonucu yüksek oranda yok olma riski taşımalarıdır. Çok yıllık halofitik *K. wagenitzii* türünün Tuz Gölü çevresinde 5 lokalitede yayılışı belirlenmiş ve toplam olgun birey sayısı 6458 olarak belirlenmiştir. Yaşam alanları toplamı 36 km$^2$ olmasına karşın yayılış alanı 213 km$^2$ dir. Tarım amaçlı kontrolsüz su kaynaklarının kullanılması önemli bir tehdit faktörüdür. *K. wagenitzii* türünün tehdit kategorisi IUCN Kırmızı Liste kriterlerine göre EN olarak değerlendirilmiştir. *M. adilii* marnlı toprakları tercih etmekte ve habitat tipi *P. nigra* ve meşe ormanlarının yıkımı sonucu oluşan steplerdir. Nallıhan-Beypazarı çevresinde 3 lokaliteden bilinmekte ve olgun birey sayısı 6144 olarak belirlenmiştir. Yaşam alanları toplamı ve yayılış alanı sırasıyla 12 km$^2$ ve 28 km$^2$ dir. Tür üzerindeki baskılar yol inşası ve tarım alanlarının büyümesi, ağaçlandırma çalışmaları ve fabrika kurulumu olup CR kategorisinde değerlendirilmiştir. *V. gypsicola* marnlı topraklarda yayılmaktadır. Beypazarı-Nallıhan çevresinden 3 lokalite ve Sivirihisar-Eskişehir'de bir lokalite yayılışı bulunmaktadır. Olgun birey sayısı 2755 olarak belirlenmiştir. Yaşam alanları toplamı 16 km$^2$ ve yayılış alanı 269 km$^2$ dir. Aşırı otlatma ve tarım alanlarının büyümesi ile birlikte fabrika kurulumu tür üzerindeki baskılar olup EN olarak değerlendirilmiştir.

***Edited by:***
*Ioannis BAZOS*

***Corresponding Author:***
*Ali Murat Keser*
*muratkeser05@hotmail.com*

***ORCID ID:***
*orcid.org/0000-0003-2245-3978*

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## Introduction

All organisms from microorganisms to plants and even to humans are influenced by global changes. Species with restricted distribution areas or requiring specialized habitat needs are much more vulnerable to changes than the others (Işık 2011). The number of plant species in Turkey that have faced threats of any kind due to local or global changes are high in number. As in most parts of the world, according to historical and present records, there

are many plant species in Turkey under the risk of extinction as a result of overgrazing, mining, erosion, expansion of agricultural areas and drought. According to the International Union for Conservation of Nature (IUCN), 2% of all species on earth are extinct, 7% are critically endangered, 10% are endangered, and 19% are threatened (Global Biodiversity Outlook-3 2010). The number of species facing the risk of extinction is increasing and about 1500 plant species in Turkey were reported to be under the risk of extinction by the year 2009 (Vural 2009). To protect these species, it is important to make them well-known by the public, to know their population status, ecological preferences etc. (Vural 2009). For the conservation of biodiversity, there are two methods as *in situ* and *ex situ* conservations widely accepted in the world. *In situ* conservation protects the organism in its natural habitat, whereas in *ex situ* method conservation processes are conducted in elsewhere other than the natural habitat of the organism (Dokuzoğlu 1990).

IUCN provides some criteria for the determination of the status of rare, endemic and threatened species with an easily understandable and applicable system called the "IUCN Red List Categories and Criteria" (IUCN 2012). For the global extinction risks of species, IUCN provides accepted standards (Lamoreux *et al*. 2003, Rodrigues *et al*. 2006). The IUCN Red list provides valuable information not only for the list of species that are under the risk of extinction or some kind of threat but also for the habitats that are important for the conservation of these species (Collar 1993, 1996). According to these criteria, the threat category for each species can be determined.

Three of the rare and endemic species, *Kalidium wagenitzii* (Aellen) Freitag & G. Kadereit, *Muscari adilii* M.B. Güner & H. Duman and *Verbascum gypsicola* Vural & Aydoğdu, of Central Anatolia (Turkey) were chosen for determination and re-evaluation of their IUCN threat categories. The most vulnerable taxa against habitat degradation and habitat fragmentation are the local endemics (Breggin *et al*. 2003) and are generally used for the determination of the presence of the protected areas of rare and endemic species (Bernardos *et al*. 2006). The criteria and categories provided by IUCN are important for the evaluation of the conservation status of threatened species and provide useful information for their conservation efforts (Vischi *et al*. 2004). Especially for rare and local endemics, it is important to have information about their life history, to predict their future trends and to plan conservation measures (García 2008).

The species provided below are mainly distributed around Ankara, except for *K. wagenitzii* which is distributed around Tuz Lake, within the borders of Konya and Aksaray provinces. All three species have special soil preferences, a factor that mainly restricts their distribution areas. *Kalidium wagenitzii* is perennial halophytic species distributed around Tuz Lake (Davis 1967, Sekmen *et al*. 2004). Tuz Lake and the surrounding areas were declared as an area of natural importance in 1992 and consequently as a Special Environmental Protection Area (SEPA) in 2000, the largest in Turkey with an area of 7.414 km² (Mergen & Karacaoğlu 2015).

*Muscari adilii* was found as three populations around Ankara where the soils are marly and the vegetation forms steps of secondary succession after the destruction of *Pinus nigra* J. F. Arnold and oak forests (Güner & Duman 1999). The richest location of *M. adilii* in terms of the number of individuals is in Nallıhan Bird Paradise wildlife protection and improvement area (Aslım *et al*. 2012).

*Verbascum gypsicola* is known from three localities in Ankara, where it grows on marly soils (Vural & Aydoğdu 1993), and one locality in Eskişehir (Öztürk *et al*. 2018). The locality from Eskişehir province was unintentionally given wrong, and according to our data and herbarium records the correct locality of Eskişehir province was determined and studied (Öztürk *et al*. 2018 & our data).

The aim of this study is to evaluate the present situation of population sizes, IUCN threat categories and distribution areas of *K. wagenitzii*, *M. adilii* and *V. gypsicola*.

## Materials and Methods

Through the review of literature and visits to the main herbaria of Turkey (ANK, GAZI, OUFFE, ESSE) the distribution areas of each species were determined and based on the distributional data, the potential distribution areas were visited between 2016 and 2019 searching for new populations. To calculate the distribution areas, minimum convex polygons were drawn on Google Earth by using the GPS coordinates of the locatilities. Also, the distances between population were measured.

For each species, flower-bearing mature individuals in the populations were counted. The reason to count floral or fruity specimens is that they are capable of continuing their generation and often it is not possible to separate these species from other similar species without these characters. To determine the threat category, the area of occupancy (AOO) and the extent of occurrence (EOO) were drawn using Geospatial Conservation Assessment Tool program (GeoCAT- http://geocat.kew.org) according to Bachman *et al*. (2011) and were calculated according to IUCN recommended grid cell size 2×2 km. The threat category of each species was re-evaluated by using all these data considering the categories and criteria of the IUCN Red List (IUCN 2012, IUCN Standards and Petitions Committe 2019).

## Results

The results about each taxon are given in an alphabetical order and each of the three species is evaluated according to IUCN Red List Categories and Criteria (IUCN 2012, IUCN Standards and Petitions Committee 2019).

*Kalidium wagenitzii*

*Kalidium wagenitzii* has five subpopulations in salt marshes around Tuz Lake with 6458 mature individuals (see Table 1 for detailed information about each subpopulation). The AOO and EOO are 36 km$^2$ and 213 km$^2$, respectively (Fig. 1).


**Fig. 1.** The extent of occurrence of *K. wagenitzii.*

**Table 1.** Number of mature individuals, distribution area, and threat factors of *K. wagenitzii.*

| Locality | Number of mature individuals | GPS data | The surface area occupied by each subpopulation | Threat factors |
|---|---|---|---|---|
| B4, Konya: Cihanbeyli, 4 km east of Gölyazı, 918 m | 1371 | N 38º36'46" E 33º11'34" | 7.3 km$^2$ | Illegal housing and legal wells, uncontrolled and illegal wells. |
| **A-** B4, Aksaray: Eskil, between Eskil and Yenikent, 910 m | 150 | N 38º27'10" E 33º29'60" | 0.036 km$^2$ | Legal wells and uncontrolled and illegal wells. |
| **B-** B4, Aksaray: Eskil, between Eskil and Yenikent, 910 m | 40 | N 38º25'05" E 33º30'13" | 0.001 km$^2$ | Legal wells and uncontrolled and illegal wells. |
| **C-** B4, Aksaray: Eskil, between Eskil and Yenikent, 910 m | 40 | N 38º24'29" E 33º34'14" | 0.001 km$^2$ | Legal wells and uncontrolled and illegal wells. |
| **D-** B4, Aksaray: Eskil, between Eskil and Yenikent, 910 m | 4857 | N 38º22'50" E 33º32'44" | 7.64 km$^2$ | Legal wells and uncontrolled and illegal wells. |
| **Total Number of Individuals** | **6458** | | | |

The subpopulations between Eskil and Yenikent were given by adding letters A to D because of the lack of discriminative definition of the area. GPS coordinates of each subpopulation were also given (Table 1). The distance between the two main subpopulations from Gölyazı and Eskil is about 35 km. The subpopulations at Eskil are close to each other and the distance between them are as follows; The distance between A and B is 3.93 km, A and C is 7.83 km. The distance between B and C subpopulations is 5.91, and that of B to D is 4.70 km and the distance between C and D subpopulations is 4.35 km.

*Muscari adilii*

*Muscari adilii* is known from three localities (Güner & Duman 1999) with a total population size of 6114 mature individuals. The detailed information about each subpopulation is given in Table 2. The AOO and the EOO are 12 km$^2$ and 28 km$^2$, respectively (Fig. 2).

The distance between the subpopulations of Nallıhan Bird Sanctuary locality and Hırkatepe locality is 16.55 km and between Nallıhan Bird Sanctuary locality and Çoban Ahmet Fountain locality is 22.25 km. The distance between the localities from Beypazarı, Hırkatepe and Çoban Ahmet Fountain is 5.78 km.

*Verbascum gypsicola*

There are four known localities of *V. gypsicola* (Vural & Aydoğdu 1993, Öztürk *et al.* 2018) with 2755 mature individuals in total. Detailed information about each subpopulation is given in Table 3. The AOO and the EOO are 16 km$^2$ and 269 km$^2$, respectively (Fig. 3).


**Fig. 2.** The extent of occurrence of *M. adilii.*

**Table 2.** Number of mature individuals, distribution area and threat factors of *M. adilii.*

| Location | Number of mature individuals | GPS data | The surface area occupied by each subpopulation | Threat factors |
|---|---|---|---|---|
| Ankara: Nallıhan, Nallıhan. SW of Nallıhan Bird Sanctuary, 500 m. | 5795 | N 40º06'17" E 031º35'29" | 0.0734 km$^2$ | Close to road construction |
| Ankara: Beypazarı, Hırkatepe. Uyku De. 900-950 m. | 119 | N 40º11'53" E 031º50' 20" | 0.001 km$^2$ | Erosion owing to road construction |
| Ankara: Beypazarı, Beypazarı-Sekli village. Doğandede Hill, above Çoban Ahmet Fountain, 990 m. | 200 | N 40º11'26" E 031º46'17" | 0.001 km$^2$ | Expansion of agricultural areas, afforestation, factory construction |
| **Total Number of Individuals** | **6114** | | | |

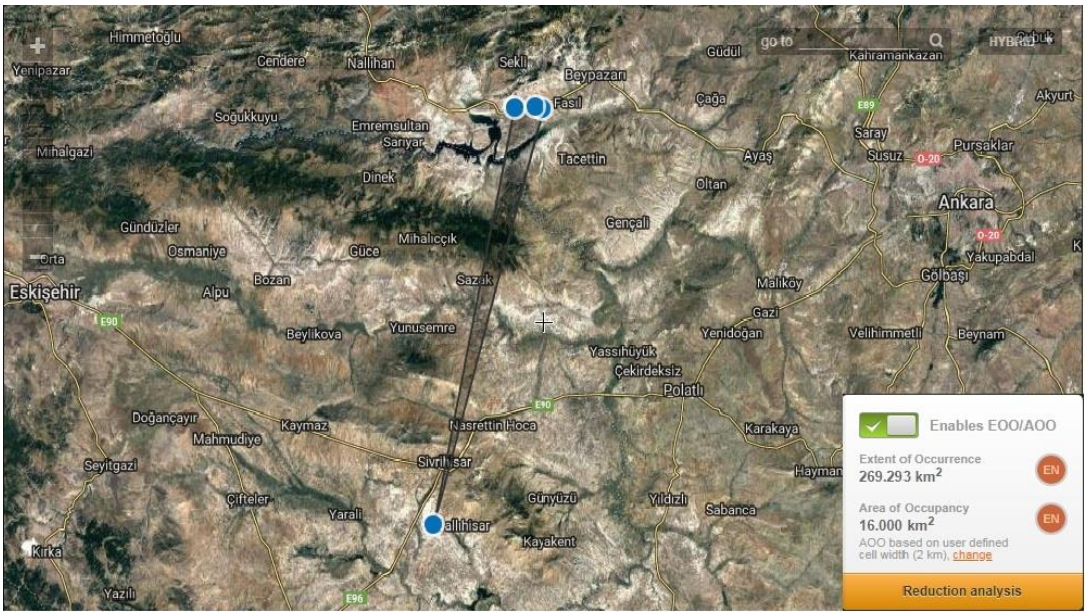


**Fig. 3.** The extent of occurrence of *Verbascum gypsicola.*

**Table 3.** Number of mature individuals, distribution area and threat factors of *Verbascum gypsicola.*

| Location | Number of mature individuals | GPS data | The surface area occupied by each subpopulation | Threat factors |
|---|---|---|---|---|
| Ankara: Beypazarı-Çayırhan, 2 km to Çayırhan, Solta Pass, 580 m. | 1535 | N 40º06'28" E 031º42'54" | 0.0088 km² | Gypsum quarry |
| Ankara: Beypazarı, Kösebükü village, 600 m | 700 | N 40º06'20" E 031º47'17" | 0.0285 km² | Expansion of agricultural areas and overgrazing |
| Ankara: Beypazarı, 15 km from Beypazarı to Çayırhan, 625 m. | 335 | N 40º06'20" E 31º46'30" | 0.0734 km² | Expansion of agricultural areas and overgrazing |
| Eskişehir: Sivrihisar, between Yeşilköy village- and Aşağıkepen, 947 m. | 185 | N 39º20'04" E 31º31'11" | 0.0011 km² | Expansion of agricultural areas and overgrazing |
| **Total Number of Individuals** | **2755** | | | |

**Table 4.** New populations/subpopulations found during the study.

| Species | Locality | Gps |
|---|---|---|
| *Kalidium wagenitzii* | **A**-B4, Aksaray: Eskil, between Eskil-Yenikent, 910 m. | N 38º27'10" E 33º29'60" |
| | **B**- B4, Aksaray: Eskil, between Eskil-Yenikent, 910 m. | N 38º25'05" E 33º30'13" |
| | **C**- B4, Aksaray: Eskil, between Eskil-Yenikent, 910 m. | N 38º24'29" E 33º34'14" |
| | **D**- B4, Aksaray: Eskil, between Eskil-Yenikent, 910 m. | N 38º22'50" E 33º32'44" |
| *Verbascum gypsicola* | Ankara: Beypazarı, 15 km from Beypazarı to Çayırhan, 625 m. | N 40º06'20" E 31º46'30" |

The mean distance between the locality at Sivrihisar and the 3 localities at Beypazarı-Nallıhan is 89 km. The distance between the localities at Beypazarı and Nallıhan is 5.10 km between Solta Pass and "15 km from Beypazarı to Çayırhan" 6.37 between Solta Pass and Kösebükü and is 1.48 km between "15 km from Beypazarı to Çayırhan" and Kösebükü.

According to the literature search, the distribution area for *K. wagenitzii* is the area between Eskil district and its dump area. After the field studies conducted within this study, new populations close to the known distribution area were found and the exact locations and areas of each subpopulation were defined. Also, one new locality for *V. gypsicola* is found.

## Discussion

*IUCN status of Kalidium wagenitzii*

*Kalidium wagenitzii* fulfils the below criteria:

Criterion B, Geographic range in the form of either B1 (EOO) or B2 (AOO) or both: Under this criterion, the species fulfils both 1a and 1b i, ii and iii. Even though there are 5 localities mentioned for *K. wagenitzii* for the determination of threat category in Fig. 1 and Table 1, these subpopulations are under the influence of the same risk factors so they were accepted as one locality according to IUCN (2012, Standards and Petitions Committee 2019).

The main difficulty for all the plant species around Tuz Lake is the water loss because of global climate change, wrong irrigation practices, and un-controlled and/or illegal wells. The groundwater level at Aksaray-Eskil, close to the distribution area of *K. wagenitzii*, has dropped about 4 meters between 2000 and 2004 and this dropping of water level is almost equal to the water level dropping in 25 years between 1975 and 2000 (Arslan & Göçmez 2007). The main threat for *K. wagenitzii* populations is the habitat loss, habitat fragmentation and lack of water. Tuz Lake water surface area decreased about 400 km² between the years of 2000-2015 (Orhan *et al*. 2017).

These water surface changes influence both terrestrial and marsh plants that need different levels of water alike, and the change in habitat structure causes shrinking of populations.

According to EOO and AOO values, the threat category should be EN [B1 ab (i,ii,iii) + B2 ab (i,ii,iii)]. However, the quantitative data from our field studies and the available literature data show that the species is under severe pressure.

*IUCN status of Muscari adili*

*Muscari adilii* fulfils the below criteria:

Criterion B, which is the geographic range in the form of either B1 (EOO) or B2 (AOO) or both. The species is known from only three localities with 28 km$^2$ of EOO and 12 km$^2$ of AOO. These subpopulations are declining and it is estimated that they will continue to decline in the future. (i) extent of occurrence, (ii) area of occupancy and (iii) area, extent and quality of habitat are affected. So the IUCN status is CR [B1 ab (i,ii,iii)].

*Muscari adilii* is found in localities close to road construction areas and agricultural areas. Road construction cause habitat fragmentation, afforestation and the expansion of agricultural areas increase the habitat loss. The establishment of a factory close to Çoban Ahmet Fountain has put the relevant subpopulation at risk of extinction.

*Muscari adilii* is included in CR [B1 ab (i,ii,iii)] according to EOO values in IUCN Red List criteria and categories.

However, the number of mature individuals is high and the threats for this species are given in Table 2. The threats that affect and place this species at risk will continue to act in the future, and therefore, *M. adilii* is placed in the CR category.

*IUCN status of Verbascum gypsicola*

*Verbascum gypsicola* fulfils the following criteria:

Criterion B, which is the geographic range in the form of either B1 (EOO) or B2 (AOO) or both. The species is known from only 4 locations which are under severe pressure (Table 3), and the EOO is 269 km$^2$ and the AOO is 16 km$^2$. The decline of the subpopulations is estimated to continue in the future; (i) extent of occurrence (ii) area of occupancy (iii) area, extent and quality of habitat are affected. Therefore, the IUCN status is EN [B1 ab (i,ii,iii)+ B2 ab (i,ii,iii)].

The largest subpopulation of *V. gypsicola* is surrounded by fences, which protect the population against threats related to overgrazing and expansion of agricultural areas. However, the establishment of a new gypsum factory within 500 m of the population poses a serious danger to the population.

The threat category of *V. gypsicola* was initially determined as EN in the Red Data Book of Turkish Plants (Ekim *et al*. 2000) but afterwards re-evaluated as CR by Eker *et al*. (2015).

EOO and AOO values, the distribution area of each subpopulation, the total number of mature individuals and the number of mature individuals in each subpopulation, and the threat factors over this species are provided in Table 3. In the view of our findings and estimations, the species is here evaluated in the EN category, being in accordance with the first evaluation by Ekim *et al*. (2000).

The number of mature individuals of B and C subpopulations of *K. wagenitzii* at Eskil is 40 for both subpopulation. As a precaution against the extinction of these subpopulations, individuals from D subpopulation with a high number of individuals should be transferred.

Çoban Ahmet Fountain subpopulation of *M. adilii* is under the threat of factory construction, for the protection of the genetic diversity of this subpopulation, new potential habitats should be determined in areas very close to this subpopulation and in these potential habitats new populations should be grown from the seeds of Çoban Ahmet Fountain subpopulation. The number of mature individuals of all subpopulations of *V. gypsicola* is low and should be increased. The Yeşilköy subpopulation is relatively isolated from all other subpopulations and is under the threat of agricultural area expansions and overgrazing, so it is suggested to fence the area where the individuals occupy and use informative signs.

Certain education programs intended for local people may provide protection for these species. We also recommend that in-situ and ex-situ conservation actions should be started for the conservation of each species.

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**Research Article**

# USE OF MULBERRY POMACE AS SUBSTRATE FOR CITRIC ACID PRODUCTION BY *Aspergillus niger* MT-4

Rufina AIDYNOVA[1], Nazli Pinar ARSLAN[2], Mehmet Nuri AYDOĞAN[1*]

[1] Department of Biology, Science Faculty, Atatürk University, 25240 Erzurum, TURKEY
[2] Vocational School of Health Services, Bingöl University, Bingöl, TURKEY

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**Abstract:** Mulberry pomace (MP) is a waste material obtained after the production of pekmez, a traditional Turkish food. This study was performed to test the usability of MP as substrate for citric acid (CA) production by *Aspergillus niger* MT-4 for the first time. In the study, some culture conditions were also optimized to increase CA production in MP-based medium. Moisture, total carbohydrate, water-soluble carbohydrate, protein, lipid and ash contents of MP were determined as 31.1, 47.1, 1.3, 13.4, 1.8 and 1.4%, respectively. Experiments were carried out in 250 mL flasks containing 100 mL of production medium. Optimal MP concentration for both fungal biomass (FB) and CA production was determined as 120 g/L. All concentrations of $KH_2PO_4$ added to MP-based medium were found to decrease CA production but increase FB production. Optimal concentrations of $MgSO_4$ and $(NH_4)_2SO_4$ for CA production were found as 1 and 2 g/L, respectively. The other optimal parameters were determined as an initial pH of 7.0 and an incubation period of 5 days. Under the optimized culture conditions, the amount of CA produced was determined as 24.6 g/L. On day 5, Yp/s, Yp/x and Yx/s were calculated as 0.2 g CA/g MP, 1.43 g CA/g FB and 0.14 g FB/g MP, respectively.

**Özet:** Dut posası (DP), geleneksel bir Türk yiyeceği olan pekmez'in üretiminden sonra ortaya çıkan bir atık maddedir. Bu çalışma, *Aspergillus niger* MT-4 ile sitrik asit (SA) üretimi için DP'nin substrat olarak kullanılabilirliğini test etmek için gerçekleştirilmiştir. Çalışmada ayrıca, SA üretimini artırmak için bazı kültür koşulları da optimize edilmiştir. MP'nin nem, toplam karbonhidrat, suda-çözünür karbonhidrat, protein, lipid ve kül içerikleri sırasıyla %31,1; 47,1; 1,3; 13,4; 1,8 ve 1,4 olarak belirlenmiştir. Deneyler, üretim besiyerinin 100 ml'sini içeren 250 ml'lik erlenler içerisinde gerçekleştirilmiştir. Hem fungal biyokütle (FB) hem de SA üretimi için optimum DP konsantrasyonu, 120 gr/lt olarak belirlenmiştir. DP bazlı ortama eklenen tüm $KH_2PO_4$ konsantrasyonlarının SA üretimini azalttığı fakat FB üretimini artırdığı belirlenmiştir. SA üretimi için $MgSO_4$ ve $(NH_4)_2SO_4$'ün optimal konsantrasyonları sırasıyla 1 ve 2 gr/lt olarak bulunmuştur. Diğer optimal parametreler, başlangıç pH'sı 7,0 ve inkübasyon süresi 5 gün olarak belirlenmiştir. Optimize edilmiş kültür koşulları altında, üretilen SA miktarı 24,6 gr/lt olarak belirlenmiştir. Beşinci günde, Yp/s; Yp/x ve Yx/s sırasıyla 0,2 gr SA/gr DP; 1,43 gr SA/gr FB ve 0,14 gr FB/gr DP olarak hesaplanmıştır. DP'nin SA dahil mikrobiyal metabolitlerin üretimi için fermentasyon substratı olarak kullanılabilirliği ilk kez bu çalışmada test edilmiştir.

***Edited by:***
*Mustafa Yamaç*

****Corresponding Author:***
*Mehmet Nuri Aydoğan*
*mnaydogan@atauni.edu.tr*

***ORCID ID:***
*orcid.org/0000-0001-7518-4746*

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*Citric acid*

## Introduction

Mulberry belongs to the *Morus* L. genus of the Moraceae family. The genus includes 24 species and one subspecies. *M. alba* L., *M. nigra* L. and *M. rubra* L. are the main species grown in Turkey. Mulberry cultivation in Turkey has long been known dating back to 400 years ago (Ercisli & Orhan 2007).

Mulberry fruits in Turkey are consumed as fresh or fresh fruits are processed to prepare traditional products such as pekmez, pestil and köme. Pekmez, which is consumed mainly in breakfast is made from different fruits but grape and mulberry are the most common fruits used in the process (Gunes & Cekic 2004, Cakmakci & Tosun 2010). For pekmez production, mulberry fruits are boiled in water until the sugars and other organic substances are passed into the water. After the boiling process, the mixture is filtered and the liquid fraction obtained is used for pekmez production. The remaining non-degrading solid fraction is referred to as pomace. Mulbery pomace (MP) is used as animal feed additives and no other use in Turkey is known. MP has also no important use in any application in the world.

Citric acid (CA) is an organic acid commonly used in the food, pharmaceutical, cosmetic, detergent, chemical and textile industries (Dhillon *et al.* 2011, Torrado *et al.* 2011). Most of CA is used in food (about 70%) and pharmaceutical (12%) industries (Darouneh *et al.* 2009). The production of CA is carried out by extraction from plants or by chemical synthesis and microbial fermentation but about 99% of the production is achieved via microbial fermentation (Taskin *et al*. 2013, Arslan *et al*. 2016). Several microorganisms such as bacteria, filamentous fungi and yeasts are capable of producing CA. For example, filamentous fungi such as *Aspergillus niger* van Tieghem, *A. wenti* Wehmer, *A. foetidus* Thom & Raper and *A. carbonaries* (Bainier) Thom and *Trichoderma viride* Pers. and *Mucor pyriformis* Scop. have been reported to be good producers of CA. Similarly, yeast species belonging to different genera such as *Candida* Berkhout, *Yarrowia* Van der Walt & Arx, *Pichia* E.C. Hansen, *Hansenula* Syd. & P. Syd. have been documented to be potential producers of CA (Soccol *et al.* 2006, Show *et al*. 2015). Despite the variety of these different producers, microbial production of CA is mainly performed using *A. niger* since this filamentous fungus is able to use numerous cheap raw materials and to accumulate CA with higher yields (Soccol *et al.* 2006, Angumeenal & Venkappayya 2013, Taskin *et al*. 2013, Arslan *et al*. 2016). Microbial CA production is achieved by three different fermentation techniques as surface fermentation, submerged fermentation and solid-state fermentation. Submerged fermentation is performed using shaking flasks or fermenters. Shaking flask technique is usually used for the optimization of fermentation conditions (Show *et al*. 2015). CA production is affected by operational culture conditions such as pH, temperature, oxygen, incubation time, substrate concentration, minerals, carbon and nitrogen sources. In particular, high carbon source concentrations under the nitrogen and phosphorus-limited conditions are known to significantly increase CA synthesis (Soccol *et al.* 2006, Darouneh *et al.* 2009, Show *et al*. 2015, Arslan *et al*. 2016). Therefore, optimization of culture conditions in CA production is considered an important criterion.

Growth substrates make up the major part of the production costs in fermentation studies. Therefore, selection of a low-cost substrate is considered as a major aspect in microbial fermentation studies (Taskin *et al.* 2013). For example, with the aim of reducing the production cost in the production of CA, cheap agricultural wastes and/or byproducts such as beet molasses, black strap molasses, cane molasses, *n*-paraffin, glycerol, whey and waste oil are usually preferred as substrate (Soccol *et al*. 2006, Torrado *et al*. 2011; Show *et al*. 2015, Arslan *et al*. 2016). However, to our best knowledge, there is no study on the use of MP as a substrate in microbial fermentations. Therefore, the present study was performed to produce CA from *A. niger* MT-4 in shaking flask culture using MP as substrate and to optimize some culture conditions for enhancement of CA production.

## Materials and Methods

### *Microorganism, materials and chemicals*

*Aspergillus niger* MT-4 was obtained from Professor Mesut Taskin from the Department of Molecular Biology and Genetics, Atatürk University, Turkey (Taskin *et al.* (2013). MP was obtained from a commercial company producing mulberry pekmez in Erzurum province (Turkey). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Merck (Germany). The other chemicals (Tween 80, phenol, sulfuric acid, acetylacetone, Ehrlich reagent, N-acetylglucosamine, citric acid, pyridine and acetic anhydride) were purchased from Sigma (USA).

### *Preparation of spore suspension*

The fungal culture was left to sporulation at 30°C for 10 days on the slant containing PDA medium. At the end of this period, conidia were suspended in sterile physiological water including a surfactant (0.2 mL/L Tween 80). The slant was vortexed for approximately 5 min to distribute the spores homogeneously. The prepared suspension was then filtrated through three layers of muslin to eliminate hyphae and unsuspended conidia. The spore concentrations were determined by using a haemocytometer and the final spore count was adjusted to $10^6$ spores per mL. During the experiments, 1 mL of the prepared spore suspension was used for inoculation of the production medium containing MP.

### *Determination of chemical composition of MP*

The moisture content of MP was determined by the weight difference before and after drying in a oven for about 24 h at 100°C, up to constant weight. Total nitrogen content was determined using a micro-Kjeldahl apparatus, and the protein content was estimated by multiplying the nitrogen content by 6.25. Ash content was determined by combusting MP for 3 h in a muffle furnace (Thermolyne 62 700, Barnstead/Thermolyne Corp., Dubuque, IA, USA) at 550°C. The total lipid content was determined according to the Soxhlet extraction method using diethyl ether as solvent. Water-soluble carbohydrate and total carbohydrate contents were determined using phenol-sulfuric acid method (Dubois *et al*. 1956). For analysis of water-soluble carbohydrates, MP was boiled in water for about 5 min. The suspension was then centrifuged to remove solid particles. Finally, the analysis of water-soluble carbohydrates in the obtained liquid fraction (supernatant) was performed by the phenol-sulfuric acid method.

### *CA production in mulberry pomace-based medium*

CA production using *A. niger* MT-4 was performed in 250 mL flasks containing 100 mL of sterile production medium. The production medium (pH 6.0) was prepared by adding MP into distilled water. Optimization of fermentation parameters was performed by using one-factor-at-a-time method. Initial experiments were performed to determine the most favorable concentration of dried MP. For this purpose, different concentrations of

dried MP from 30 to 150 g/L were tested. The effects of different concentrations of $KH_2PO_4$ (0-1.5 g/L), $MgSO_4$ (0-2.0 g/L) and $(NH_4)_2SO_4$ (0-4 g/L) on CA production in MP-based medium were tested. Then, different initial pHs (pH 2-8) and incubation times (2-7 days) were tested to increase CA production in MP-based medium. All the experiments were performed at 30°C on a shaking incubator at 150 rpm.

*Analytical methods*

Final pH of the culture and the initial pH of the MP-based medium were measured using a pH meter (Ohaus Starter 3100). 10 mL sample taken from the culture after an appropriate cultivation was centrifuged at 5000 rpm for 5 min. The obtained supernatant was used for CA analysis. The concentration of CA was determined according to the acetic anhydride method (Marier & Boulet 1958). In brief, 1 mL of supernatant was first mixed with 1.3 mL of pyridine. Then, 5.7 mL acetic anhydride was added into the samples. After incubation at 32°C for 30 min, absorbance of CA was determined spectrophotometrically at 410 nm. Anhydrous CA (50-300 µg/mL) was used as standard. In case of very intense color formation, samples were diluted with distilled water. CA content of the samples was determined according to the standard graph prepared with anhydrous CA.

During the incubation period, some of the MP was not used as a substrate by the fungus. This part, which was not used as a substrate and remained in the culture, was named non-fermented MP. At the end of the cultivation period, it was observed that the non-fermented MP was attached to the fungal biomass (FB) and not separated from it by centrifugation. Therefore, the solid fraction (non-fermented MP + FB) consisting of non-fermented MP and FB was named as total biomass (TP).

For the determination of FB amount in TP, N-acetylglucosamine content in TP was measured. In brief, 1 mL of concentrated $H_2SO_4$ was added to 0.5 g of TP. Following this, acetylacetone reagent (1 mL) was added to the mixture. The mixture was incubated in a boiling water bath for 20 min and then cooled at room temperature. Then, 6 mL of ethanol and 1 mL of Ehrlich reagent were added to the cooled mixture. The final mixture was again left to incubation at 65 °C for 10 min and then cooled at the room temperature. Finally, optical density (OD) of the final mixture was determined at 530 nm (Velmurugan *et al.* 2011). The obtained absorbance was then used for the calculation of FB produced in MP-based medium. For this purpose, the following simple equation was used.

FB= (A1/A2) x TB

A1: The absorbance assayed for N-acetylglucosamine content in per g of the dried TB (TB; FB + non-fermented MP) in MP-based medium at the end of cultivation period.

A2: The absorbance assayed for N-acetylglucosamine content in per g of dried FB, which was produced in standard PDB medium at the end of cultivation period.

TB: Total biomass (FB + non-fermented MP) in MP-based medium at the end of cultivation period.

*Statistical analysis*

Each analysis was repeated at least three times in two replicates. Statistical difference was analyzed with one-way ANOVA in the SPSS 15.0 package program at $P < 0.05$ significance level.

## Results

*Chemical composition of MP*

The moisture content of raw MP was determined as 31.1%. Total carbohydrate, protein, lipid and ash contents of MP were determined as 47.1, 13.4, 1.8 and 1.4%, respectively. The ratio of water-soluble carbohydrates was found to be 1.3%.

**Table 1.** Chemical composition of MP.

| Components | Content (%) |
|---|---|
| **Moisture** | 31.1±1.90 |
| **Protein** | 13.4±1.04 |
| **Lipids** | 1.8±0.17 |
| **Ash** | 1.4±0.12 |
| **Total carbohydrate** | 47.1±2.72 |
| **Water-soluble carbohydrate** | 1.3±0.10 |

All measurements are mean ± standard deviations (±SD) of six determinations (n = 6).

*Optimization of CA production from A. niger in MP-based medium*

In the first step of the study, different concentrations of dried MP were tested for production of CA and FB from *A. niger* MT-4. Increased MP concentration increased both CA synthesis and fungal growth, and maximum concentrations of CA (10.6 g/L) and FB (14.3 g/L) were reached in medium containing 120 g/L MP (Fig. 1). Based on these results, the following experiments were performed on medium containing 120 g/L MP.

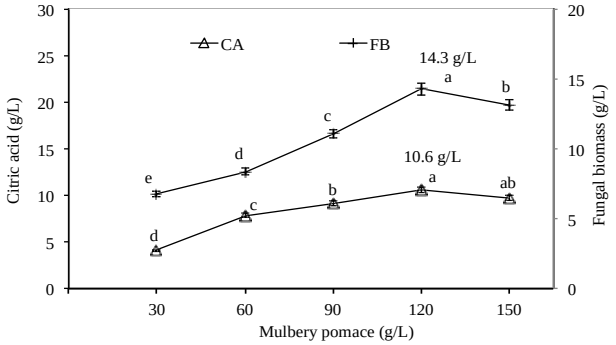


**Fig. 1.** Effect of MP on CA synthesis and fungal growth in *A. niger* MT-4. Culture conditions: initial pH 6.0, temperature 30°C, shaking speed 150 rpm and incubation time 4 days. All the measurements were mean ± standard deviations (±SD) of six determinations (n = 6). Different letters in the line of CA and FB indicate significant differences ($P < 0.05$).

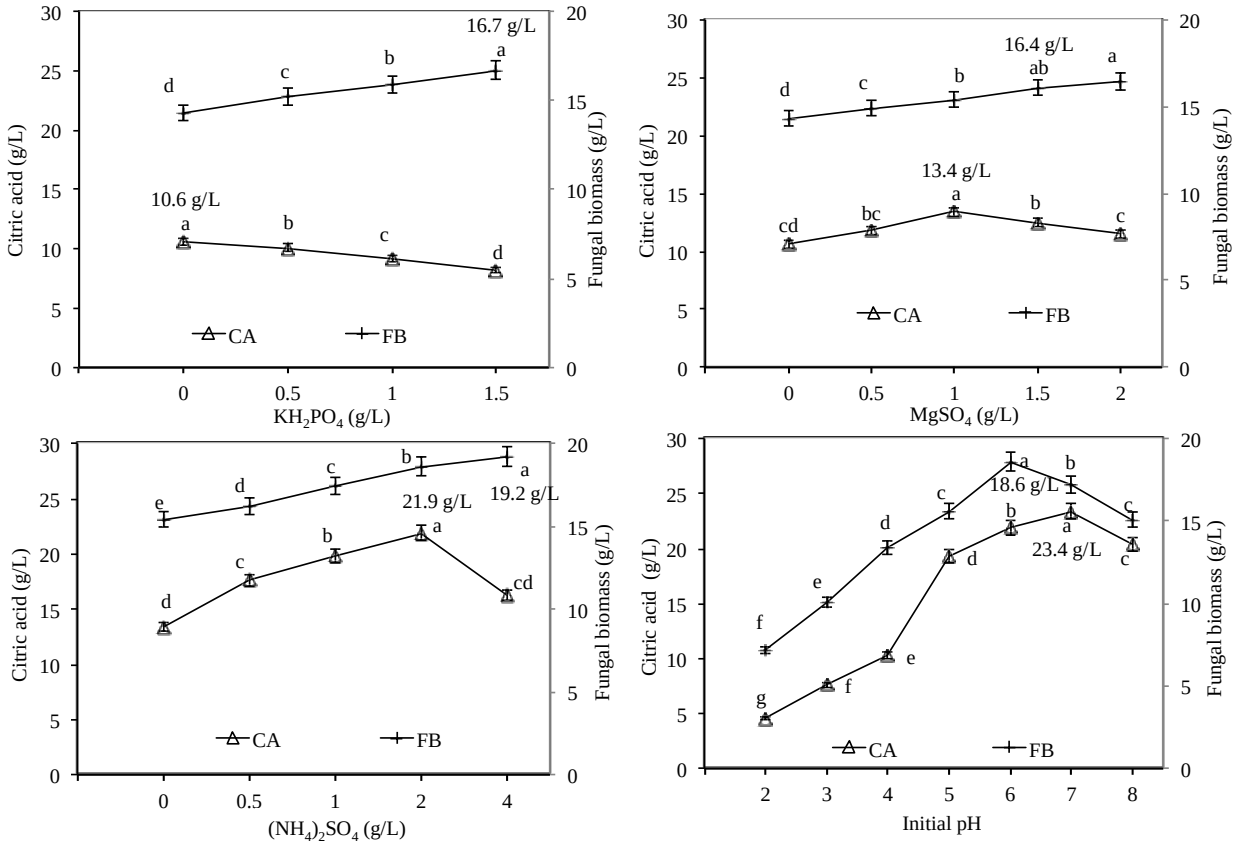


**Fig. 2.** Effect of $KH_2PO_4$, $MgSO_4$, $(NH_4)_2SO_4$ and initial pH on citric acid synthesis and fungal growth in *A. niger* MT-4. Culture conditions for optimization of $KH_2PO_4$ concentration: MP concentration 120 g/L and initial pH 6.0. Culture conditions for optimization $MgSO_4$ concentration: MP concentration 120 g/L, $KH_2PO_4$ 0 g/L and initial pH 6.0. Culture conditions for optimization of $(NH_4)_2SO_4$ concentration: MP concentration 120 g/L, $KH_2PO_4$ 0 g/L, $MgSO_4$ 1 g/L and initial pH 6.0. Culture conditions for optimization of initial pH: MP concentration 120 g/L, $KH_2PO_4$ 0 g/L, $MgSO_4$ 1 g/L and $(NH_4)_2SO_4$ 2 g/L. All experiments were performed at 30°C and 150 rpm for 4 days. All the measurements are mean ± standard deviations (±SD) of six determinations (n = 6). Different letters in the line of CA and FB indicate significant differences ($P < 0.05$).

After determination of the optimum concentration of MP, the effect of different concentrations of $KH_2PO_4$ on cell growth and CA synthesis was investigated. The maximum CA production was achieved in the control medium ($KH_2PO_4$-free medium), whereas FB reached to the maximum (16.7 g/L) when 1.5 g/L $KH_2PO_4$ was added to MP-based medium (Fig. 2). Considering these results, the following experiments were carried out in the medium, which was not supplemented with $KH_2PO_4$. All tested concentrations of $MgSO_4$ increased fungal growth (Fig. 2). In contrast to fungal growth, the maximum CA production (13.4 g/L) was achieved in the medium supplemented with 1 g/L $MgSO_4$. However, excessive concentrations of $MgSO_4$ gradually decreased CA production. For example, CA production decreased up to 11.5 g/L when 2 g/L $MgSO_4$ was added to the medium. Therefore, the subsequent experiments were performed in the medium supplemented with 1 g/L MgSO4. The experiments revealed that 2 g/L $(NH_4)_2SO_4$ caused maximum CA production (21.9 g/L) but higher $(NH_4)_2SO_4$ concentrations decreased CA production. In contrast to CA synthesis, all tested concentrations of $(NH_4)_2SO_4$ increased fungal growth and the maximum FB (19.2 g/L) was reached at the highest $(NH_4)_2SO_4$ of 4 g/L (Fig. 2). Taking into account these results, an ammonium sulfate concentration of 2 g/L resulting in the maximum CA production was chosen for subsequent experiments.

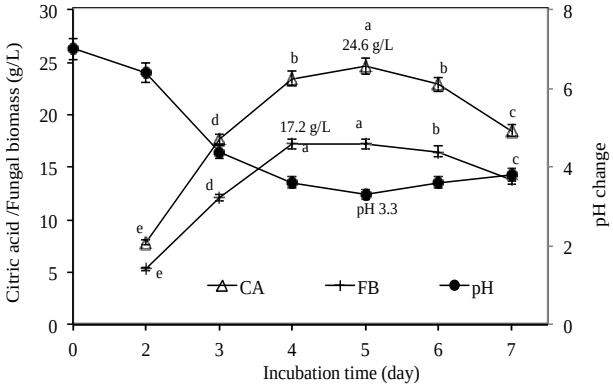


**Fig. 3.** Effect of incubation time on cell growth, CA synthesis and pH change in MP-based medium. Culture conditions: MP concentration 120 g/L, $KH_2PO_4$ 0 g/L, $MgSO_4$ 1 g/L, $(NH_4)_2SO_4$ 2 g/L**,** initial pH 7.0, temperature 30°C, shaking speed 150 rpm. All the measurements are mean ± standard deviations (±SD) of six determinations (n = 6). Different letters in the line of CA and FB indicate significant differences ($P < 0.05$).

When the effect of initial pH on CA synthesis and fungal growth was investigated, it was determined that maximum CA synthesis (23.4 g/L) occurred at an initial pH of 7.0, but FB reached to the maximum (18.6 g/L) at pH 6.0 (Fig. 2). Based on this result, the following experiments were carried out in the medium with an initial pH of 7.0.

The highest increase in CA production occurred in the first 4 days of incubation and CA concentration reached to the maximum (24.6 g/L) on day 5 (Fig. 3). FB reached the highest value (17.2 g/L) on day 4 and remained stable on day 5. But, reductions in both FB and CA concentrations were observed after the 5$^{th}$ day. For example, at the end of day 7, FB and CA concentrations were measured as 13.8 and 18.5 g/L, respectively. Since maximum productions of CA (24.6 g/L) and FB (17.2 g/L) were achieved at 120 g/L MP concentration, Yp/s (gram CA produced/gram substrate), Yp/x (gram CA produced/ gram FB produced) and Yx/s (gram FB produced/gram substrate) were calculated as 0.2 g CA/g MP, 1.43 g CA/g FB and 0.14 g FB/g MP, respectively. When initial pH was adjusted at 7.0, there was a continuous decrease in culture pH up to the end of 5$^{th}$ day, but an increase in culture pH was observed again after 5$^{th}$ day. Lowest pH value (pH 3.3) was detected on day 5, by which the maximum CA concentration was reached.

**Discussion**

The previous studies demonstrated that hydrolysates, which are prepared from agricultural wastes and/or byproducts using chemical or enzymatic hydrolysis processes can be utilized as substrates for CA production in the culture of *A. niger* strains (Watanabe *et al*. 1998, Hu *et al*. 2014, Faruk *et al.* 2014, Muna *et al*. 2018). But, it is well known that when chemicals and/or enzymes are used for the preparation of hydrolysates, the production cost of CA increases. Therefore, researchers suggested using *Aspergillus* strains in CA production, which are capable of secreting hydrolytic enzymes (amylase, cellulose, pectinase etc.) and thereby growing directly on agricultural wastes and/or by-products (Andersen *et al*. 2011, Afify *et al*. 2012, Rehman *et al*. 2014). However, the potential of *A. niger* to produce CA on MP, which is not subjected to a pretreatment process such as chemical or enzymatic hydrolysis, has not been studied yet.

The chemical analyses revealed that dried MP contained vey low amount of water-soluble carbohydrates. This situation can be attributed to the boiling of mulberry fruits at high temperature for a long time during the production of pekmez (molasses). In other words, since water-soluble carbohydrates such as sucrose and glucose in mulberry fruits passed into pekmez, there was rather low soluble carbohydrate in MP. On the contrary, the experiments showed that the total carbohydrate content of MP was high. These carbohydrates may include pectin, starch, cellulose and soluble sugars. Considering that *A. niger* strains have hydrolytic enzymes, it is possible that insoluble carbohydrates such as pectin, starch and cellulose in MP can be used as carbon source by *A. niger* MT-4. Besides, it was determined in this study that MP was rich in ash. Considering the knowledge that ash consists of macro and micro-elements, it is possible to say that MP can also be used as a mineral source by this filamentous fungus. The experiments also indicated that MP contained protein and therefore it may be used as a nitrogen source in the medium.

Usability of non-pretreated MP as a substrate for CA production from *A. niger* MT-4 was tested in this study. In the first stage, MP was used as a sole source of all nutritional factors (minerals, carbon and nitrogen source) for production of FB and CA. The maximum CA and FB production were achieved in medium containing 120 g/L MP, while higher MP concentrations decreased both CA and FB production. This result might be attributed to the increase in solid/liquid ratio of the production medium. Namely, high solid ratio (high MP concentration) might have prevented the homogeneous mixing of the substrate and sufficient oxygen intake into the culture. Since oxygen is particularly important for CA synthesis (Max *et al*. 2010), the decrease in oxygen concentration might have limited CA synthesis in MP-based medium.

The experiments revealed that when $KH_2PO_4$ was used as phosphorus (P) and potassium (K) source, all concentrations caused inhibition on CA synthesis but a continuous increase in fungal growth. These results should not be so surprising, because it has been reported that excessive P concentrations can limit CA synthesis but increase fungal growth in *A. niger* (Jernejc *et al*. 1982, Chen 1996, Vandenberghe *et al.* 1999, Max *et al*. 2010, Angumeenal & Venkappayya 2013). These results indicate that P content of MP was sufficient to promote CA synthesis and no additional P source is required in MP-based medium for CA synthesis. When $MgSO_4$ was added into the medium, its concentrations $\leq 1$ g/L promoted CA synthesis but higher concentrations caused a slight inhibition. This finding is good agreement with the fact that although $MgSO_4$ has a beneficial effect on CA synthesis in *A. niger*, its excessive concentrations can inhibit CA synthesis (Vandenberghe *et al*. 1999, Ikram-ul *et al*. 2004).

It has been documented that CA production is directly influenced by the nitrogen source, and $(NH_4)_2SO_4$ is a good nitrogen source for CA production (Vandenberghe *et al*. 1999, Max *et al*. 2010). Considering this knowledge, the present experiments also focused on investigating the effect of $(NH_4)_2SO_4$ as additional nitrogen source on CA production in MP-based medium. The experiments showed that CA production could be achieved in MP-based medium without $(NH_4)_2SO_4$ (additional nitrogen source). This can be explained by the use of proteinous compounds in MP as nitrogen source in the medium. However, it was seen that $(NH_4)_2SO_4$ concentrations $\leq 2$ g/L caused increases in CA synthesis. This result revealed that the nitrogen in MP was insufficient for the production of high amounts of CA and additional nitrogen was required in the medium. The results also showed that

$(NH_4)_2SO_4$ concentrations over 2 g/L decreased CA synthesis in the culture. Conversely, there was a continuous increase in FB even at the highest $(NH_4)_2SO_4$ of 4 g/L. This finding is in good agreement with the fact that excessive nitrogen concentration increases fungal growth but decreases the amount of CA produced (Hang *et al*. 1977, Vandenberghe *et al*. 1999, Soccol *et al*. 2006, Auta *et al*. 2014, Arslan *et al*. 2016).

The results showed that initial pH values between 5 and 7 were more suitable for CA and FB production. Especially, initial pH of 7.0 was the superior for CA production. It was reported that although acidic pHs such as pH 2 and 3 are more suitable for CA synthesis in *A. niger*, the germination of *A. niger* spores requires pH > 5 (Soccol *et al*. 2006). Namely, the initial pH of the culture for CA synthesis in *A. niger* should be adjusted to pH levels > 5. Therefore, it should not be surprising that initial pHs of 5-7 lead to more CA synthesis in *A. niger* MT-4.

The final experiments showed that FB and CA reached to maximum concentrations on days 4 and 5, respectively. The maximum increases in concentrations of both CA and FB were achieved in the first four days of fermentation. These results are similar to those reported in previous studies (Kareem *et al*. 2010, Taskin *et al*. 2013, Dienye *et al*. 2018) showing that incubation time affects cell growth and CA synthesis in *A. niger*. On day 5, CA concentration and CA yield (Yp/s) were determined as 24.6 g/L and 0.2 g CA/g MP, respectively. The CA yield achieved in submerged culture was similar to those reported in previous studies (Alben & Erkmen 2004, Guc & Erkmen 2017).

The reduction in fungal biomass after day 5 may be attributed to cell lysis due to the depletion of nutritional compounds. The most likely cause of the decrease in CA concentration after day 5 may be the degradation of CA by the enzymes released into the culture when the fungal cells are lysed. A continuous drop in culture pH up to the end of the 5$^{th}$ day could be ascribed to the production of organic acids, especially CA. Similar decreases in culture pH during CA production were also reported in the previous studies (Ali *et al.* 2002, Dienye *et al.* 2018). Increment in culture pH after the 5$^{th}$ day might be attributed to the releasing into the culture of some alkaline compounds due to fungal cell lysis.

In conclusion, this study revealed that MP alone could be used as a complex substrate for CA production with *A. niger* MT-4, but more CA production could be achieved when MP-based medium was supplemented with $(NH_4)_2SO_4$ and $MgSO_4$ at suitable concentrations. On the other hand, optimization of initial pH and incubation time could increase CA production in MP-based medium. When the optimal culture parameters are selected, 24.6 g/L CA could be produced in MP-based medium. The use of MP in CA production will contribute to the reduction of both fermentation cost and environmental pollution. In future studies, MP can be also tested as a substrate in the culture of microorganisms in the production of other substances such as lactic acid, acetic acid, single cell protein, ethanol, pigment and polysaccharide.

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**Özet ve Anahtar kelimeler:** Türkçe ve İngilizce özet 250 kelimeyi geçmemelidir. Özetin altına küçük harflerle anahtar kelimeler ibaresi yazılmalı ve yanına anahtar kelimeler virgül konularak sıralanmalıdır. Anahtar kelimeler, zorunlu olmadıkça başlıktakilerin tekrarı olmamalıdır. İngilizce özet koyu harflerle "Abstract" sözcüğü ile başlamalı ve başlık, İngilizce özetin üstüne büyük harflerle ortalanarak yazılmalıdır. Yazıdaki ana başlıklar ve varsa alt başlıklara **numara verilmemelidir.**

**Giriş:** Çalışmanın amacı ve geçmişte yapılan çalışmalar bu kısımda belirtilmelidir. Yazıda SI (Systeme International) birimleri ve kısaltmaları kullanılmalıdır. Diğer kısaltmalar kullanıldığında, metinde ilk geçtiği yerde 1 kez açıklanmalıdır. Kısaltma yapılmış birimlerin sonuna nokta konmamalıdır (45 m mesafe tespit edilmiştir). Kısaltma cümle sonunda ise nokta konmalıdır (… tespit edilen mesafe 45 m. Dolayısıyla…).

**Materyal ve Metod:** Eğer çalışma deneysel ise kullanılan deneysel yöntemler detaylı ve açıklayıcı bir biçimde verilmelidir. Yazıda kullanılan metod/metodlar, başkaları tarafından tekrarlanabilecek şekilde açıklayıcı olmalıdır. Fakat kullanılan deneysel yöntem herkes tarafından bilinen bir yöntem ise ayrıntılı

açıklamaya gerek olmayıp sadece yöntemin adı verilmeli veya yöntemin ilk kullanıldığı çalışmaya atıf yapılmalıdır.

**Sonuçlar**: Bu bölümde elde edilen sonuçlar verilmeli, yorum yapılmamalıdır**.** Sonuçlar gerekirse tablo, şekil ve grafiklerle de desteklenerek açıklanabilir.

**Tartışma:** Sonuçlar mutlaka tartışılmalı fakat gereksiz tekrarlardan kaçınılmalıdır. Bu kısımda, literatür bilgileri vermekten çok, çalışmanın sonuçlarına yoğunlaşmalı, sonuçların daha önce yapılmış araştırmalarla benzerlik ve farklılıkları verilmeli, bunların muhtemel nedenleri tartışılmalıdır. Bu bölümde, elde edilen sonuçların bilime katkısı ve önemine de mümkün olduğu kadar yer verilmelidir.

**Teşekkür:** Mümkün olduğunca kısa olmalıdır. Teşekkür, genellikle çalışmaya maddi destek sağlayan kurumlara, kişilere veya yazı yayına gönderilmeden önce inceleyip önerilerde bulunan uzmanlara yapılır. Teşekkür bölümü kaynaklardan önce ve ayrı bir başlık altında yapılır.

**Kaynaklar**: Yayınlanmamış bilgiler kaynak olarak verilmemelidir (*Yayınlanmamış kaynaklara örnekler: Hazırlanmakta olan veya yayına gönderilen yazılar, yayınlanmamış bilgiler veya gözlemler, kişilerle görüşülerek elde edilen bilgiler, raporlar, ders notları, seminerler gibi*). Ancak, tamamlanmış ve jüriden geçmiş tezler ve DOI numarası olan yazılar kaynak olarak verilebilir. Kaynaklar, yazı sonunda alfabetik sırada (yazarların soyadlarına göre) sıra numarası ile belirtilerek verilmelidir.

Yazıların ve kitapların referans olarak veriliş şekilleri aşağıdaki gibidir:

**Makale:** Yazarın soyadı, adının baş harfi, basıldığı yıl. Makalenin başlığı, *derginin adı*, cilt numarası, sayı, sayfa numarası. Dergi adı italik yazılır.

*Örnek:*

*Tek yazarlı Makale için*

Soyadı, A. Yıl. Makalenin adı. (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin açık ve tam adı*, Cilt(Sayı)**:** Sayfa aralığı.

Kıvan, M. 1998. *Eurygaster integriceps* Put. (Heteroptera: Scuteleridae)'nin yumurta parazitoiti *Trissolcus semistriatus* Nees (Hymenoptera: Scelionidae)'un biyolojisi üzerinde araştırmalar*. Türkiye Entomoloji Dergisi*, 22(4): 243-257.

*İki ya da daha çok yazarlı makale için*

Soyadı1, A1. & Soyadı2**,** A2. Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt(Sayı): Sayfa aralığı.

Lodos, N. & Önder, F. 1979. Controbution to the study on the Turkish Pentatomoidea (Heteroptera) IV. Family: Acanthasomatidae Stal 1864. *Türkiye Bitki Koruma Dergisi*, 3(3): 139-160.

Soyadı1, A1., Soyadı2, A2. & Soyadı3, A3**.** Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt (Sayı): Sayfa aralığı.

Önder, F., Ünal, A. & Ünal, E. 1981. Heteroptera fauna collected by light traps in some districts of Northwestern part of Anatolia. *Türkiye Bitki Koruma Dergisi***,** 5(3): 151-169.

**Kitap:** Yazarın soyadı, adının baş harfi, basıldığı yıl. Kitabın adı (varsa derleyen veya çeviren ya da editör), cilt numarası, baskı numarası, basımevi, basıldığı şehir,toplam sayfa sayısı.

*Örnek*:

Soyadı, A., Yıl. *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Önder F., Karsavuran, Y., Tezcan, S. & Fent, M. 2006. *Türkiye Heteroptera (Insecta) Kataloğu*. Meta Basım Matbaacılık, İzmir, 164 s.

Lodos, N., Önder, F., Pehlivan, E., Atalay, R., Erkin, E., Karsavuran, Y., Tezcan, S. & Aksoy, S. 1999. Faunistic *Studies on Lygaeidae (Heteroptera) of Western Black Sea, Central Anatolia and Mediterranean Regions of Turkey.* Ege University, İzmir, ix + 58 pp.

**Kitapta Bölüm:** Yazarın soyadı, adının baş harfi basıldığı yıl. Bölüm adı, sayfa numaraları. Parantez içinde: Kitabın editörü/editörleri, *kitabın adı*, yayınlayan şirket veya kurum, yayınlandığı yer, toplam sayfa sayısı.

*Örnek*:

Soyadı, A., Yıl. Bölüm adı, sayfa aralığı. In: (editör/editörler). *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Jansson, A. 1995. Family Corixidae Leach, 1815—The water boatmen. Pp. 26–56. In: Aukema, B. & Rieger, Ch. (eds) Catalogue of the Heteroptera of the Palaearctic Region. Vol. 1. Enicocephalomorpha, Dipsocoromorpha, Nepomorpha, Gerromorpha and Leptopodomorpha. The Netherlands Entomological Society, Amsterdam, xxvi + 222 pp.

**Kongre, Sempozyum:** Yazarlar, Yıl. "Bildirinin adı (Sözcüklerin ilk harfi küçük), sayfa aralığı". Kongre/Sempozyum Adı, Tarihi (gün aralığı ve ay), Yayınlayan Kurum, Yayınlanma Yeri.

*Örnek:*

Bracko, G., Kiran, K., & Karaman, C. 2015. The ant fauna of Greek Thrace, 33-34. Paper presented at the 6th Central European Workshop of Myrmecology, 24-27 July, Debrecen-Hungary.

**Internet:** Eğer bir bilgi herhangi bir internet sayfasından alınmış ise (*internetten alınan ve dergilerde yayınlanan yazılar hariç*), kaynaklar bölümüne internet sitesinin ismi tam olarak yazılmalı, siteye erişim tarihi verilmelidir.

Soyadı**,** A. Yıl. Çalışmanın adı. (Sözcüklerin ilk harfi küçük) (web sayfası) http://www.....) (Date accessed: 12.08.2009).

Hatch, S., 2001. Studentsperception of online education. Multimedia CBT Systems. (Web page: http://www.scu.edu.au/schools/sawd/moconf/papers2001/hatch.pdf) (Date accessed: 12.08.2009).

Kaynaklara metin içinde numara verilmemeli ve aşağıdaki örneklerde olduğu gibi belirtilmelidir.

Örnekler:
... x maddesi atmosferde kirliliğe neden olmaktadır (Landen 2002). Landen (2002) x maddesinin atmosferde kirliliğe neden olduğunu belirtmiştir. İki yazarlı bir çalışma kaynak olarak verilecekse, (Landen & Bruce 2002) veya Landen & Bruce (2002)'ye göre. ... şeklinde olmuştur; diye verilmelidir. Üç veya daha fazla yazar söz konusu ise, (Landen *et al.* 2002) veya Landen *et al.* (2002)'ye göre .... olduğu gösterilmiştir; diye yazılmalıdır.

**Şekil ve Tablolar:** Tablo dışında kalan fotoğraf, resim, çizim ve grafik gibi göstermeler "Şekil" olarak verilmelidir. Resim, şekil ve grafikler, net ve ofset baskı tekniğine uygun olmalıdır. Her tablo ve şeklin metin içindeki yerlerine konmalıdır. Tüm tablo ve şekiller yazı boyunca sırayla numaralandırılmalı (Tablo 1., Şekil. 1), başlık ve açıklamalar içermelidir. Şekillerin sıra numaraları ve başlıkları, alta, tabloların ki ise üstlerine yazılır.

Şekiller (tablo dışında kalan fotoğraf, resim, çizim ve grafik gibi) tek tek dosyalar halinde en az **300 dpi** çözünürlükte ve **tif** dosyası olarak şekil numaraları dosya isminde belirtilmiş şekilde ayrıca sisteme ek dosya olarak yüklenmelidir.

Sunulan yazılar, öncelikle Dergi Yayın Kurulu tarafından ön incelemeye tabii tutulur. **Dergi Yayın Kurulu, yayınlanabilecek nitelikte bulmadığı veya yazım kurallarına uygun hazırlanmayan yazıları hakemlere göndermeden red kararı verme hakkına sahiptir.** Değerlendirmeye alınabilecek olan yazılar, incelenmek üzere iki ayrı hakeme gönderilir. Dergi Yayın Kurulu, hakem raporlarını dikkate alarak yazıların yayınlanmak üzere kabul edilip edilmemesine karar verir.

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Tel : 0284 235 82 30
Fax : 0284 235 82 37
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Aybeke, M. 2016. The detection of appropriate organic fertilizer and mycorrhizal method enhancing salt stress tolerance in rice (*Oryza sativa* L.) under field conditions. *Trakya University Journal of Natural Sciences,* 17(1): 17-27.

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Balkan Yerleşkesi
22030 - EDİRNE-TURKEY

Phone : +90 284 235 82 30
Fax : +90 284 235 82 37
e-mail : tujns@trakya.edu.tr