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Research Article

FIRST RECORD OF *Micrasema cinereum* Mosely (TRICHOPTERA, BRACHYCENTRIDAE) IN TURKEY AND A LIST OF THE CADDISFLY FAUNA IN ARAÇ CREEK

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Abstract: Systematic studies on Trichoptera larvae in Turkey are very limited. The larval stage of the endemic species remains especially unknown. This study was carried out in Araç Creek between April and October 2013 and it is the first study to determine the caddisfly fauna in Araç Creek. Fourteen caddisflies taxa belonging to nine genera within eight different families (Brachycentridae, Hydropsychidae, Hydroptilidae, Lepidostomatidae, Leptoceridae, Limnephilidae, Psychomyiidae and Rhyacophilidae) were identified. Four of these taxa have previously been recorded from the creek and the other ten taxa are new records for the study area while *Micrasema cinereum* Mosely is the first record for the Trichoptera fauna of Turkey. As a result of the study, the number of caddisfly taxa in Turkey is now 501, with the addition of *M. cinereum*.

Key words: New record, larvae, description, benthic macroinvertebrates, Araç creek, Turkey.

Özet: Türkiye'de Trichoptera larvaları üzerindeki sistematik çalışmalar çok sınırlıdır ve özellikle endemik türlerin larva evresi bilinmemektedir. Çalışma Araç Çayı'nda 2013 yılı Nisan-Ekim ayları arasında gerçekleştirilmiştir ve Araç Çayı'nın Trichoptera faunasını belirleyen ilk çalışmadır. Sekiz farklı familyadan (Brachycentridae, Hydropsychidae, Hydroptilidae, Lepidostomatidae, Leptoceridae, Limnephilidae, Psychomyiidae ve Rhyacophilidae) 9 cinse ait 14 Trichoptera taksonu belirlenmiştir. Bu taksonlardan dördü daha önce Araç Çayı'ndan kaydedilmiş, diğer 10 takson ise Araç Çayı için yeni kayıttır. Buna ek olarak, *Micrasema cinereum* Mosely ise Türkiye Trichoptera faunası için ilk kayıttır. Çalışmanın sonucunda, Türkiye'deki Trichoptera takson sayısı yeni bir kayıtla birlikte 501 olmuştur.

Introduction

Brachycentridae larvae are characterized and distinguished from larval stages of other caddisfly families by (i) the absence of lateral and dorsal humps in the first abdominal segment, (ii) possessing transverse groove at midlenght of pronotum, (iii) a pair of plates (that are often divided longitudinally by narrow sutures) on mesotonum, and (iv) either the absence of primary setal area or the presence of sclerotized setae on metanotum (Flint 1984, Vieira-Lanero *et al.* 1998, Pescador *et al.* 1995). Larvae, in general, live in cold lotic fresh-water springs, streams and rivers while some species can also live in the shores of lakes, as in the case of the genus *Micrasema* McLachlan (Pescador *et al.* 1995). *Micrasema* consists of only 23 species in the West Palearctic Region fauna (Malicky 2004) and their larvae, whose inhabitant area is mostly surrounded by mosses and other plants, can resist and therefore inhabit shallow streams (Hilsenhof 1985). The caddisfly fauna of Turkey is represented by 500 taxa (461 species and 39 subspecies) (Darılmaz & Salur 2015, Küçükbasmacı & Kıyak 2017, Sipahiler 2016, Sipahiler 2017a, b, Sipahiler 2018a, b) Most of the studies on caddisflies have been performed based on their adults, but taxonomic studies on their larval stages are limited in Turkey. In particular, there is a lackabout descriptive studies on larvae of endemic species. The lack of taxonomic and ecological data on benthic macroinvertebrates has been concluded to negatively affect their reliable use in biomonitoring related studies in Turkey, and studies at species level on key taxons one of which is Trichoptera in addition to Ephemeroptera, Odonata, Plecoptera, Simuliidae and are believed to contribute to improvement of such studies (Kazancı & Ertunç 2010). From this point of view, this study was performed in order to determine caddisfly fauna of Araç Creek (Kastamonu) as a contribution to Turkish caddisfly fauna.

Materials and Methods

Study area

The study was performed in Araç Creek located in northern part of Turkey (Fig. 1). Araç Creek, originating from the northern slope of Ilgaz Mountains and passing through İhsangazi and Araç districts of Kastamonu province, merges with Soğanlı Creek to form Yenice River (Filyos River) with other tributaries. The creek is affected by various human activities including city sewage systems, organic wastes, agricultural activities, hydroelectric power plants and sand quarries. Macroinvertebrate samplings were performed at 6 stations along the creek in April, August and October 2013 (Fig. 1). The coordinates and altitudes of the sampling stations as well as sampling dates and short descriptions of the localities where the stations were selected are given in Table 1.

Fig. 1. (A) Map of Turkey. (B) The general view of study area. (C) Sampling stations in Araç Creek (Google Earth 2019). The numbers denote the stations whose details are given in Table 1.

Table 1. Details of the stations from where the samples were collected.

Station No	Sampling date	Sampling station	Coordinate	Altitude (m)
1	28.04.2013 15.08.2013 27.10.2013	Kastamonu, İhsangazi, beyond 1 km from the Örencik village road junction	41°10′39.60″N 33°34′16.30″E	920
2	28.04.2013 15.08.2013 27.10.2013	Kastamonu, İhsangazi, İhsangazi–Araç road 7th km, Akkaya village road junction, the bridge vicinity	41°13′6.70″N 33°28′57.50″E	784
3	28.04.2013 15.08.2013 27.10.2013	Kastamonu, Araç, end of the Araç county, vicinity to the gas station	41°14′21.13″N 33°19′19.80″E	632
4	28.04.2013 15.08.2013 27.10.2013	Kastamonu, Araç, Araç–Karabük road 7th km, Tatlıca village road junction, the bridge vicinity	41°14′8.76″N 33°15′21.67″E	594
5	28.04.2013 15.08.2013 27.10.2013	Kastamonu, Araç, Araç–Karabük road 26th km, Yeşilova village	41°13′52.24″N 33°00′22.95″E	432
6	28.04.2013 15.08.2013 27.10.2013	Karabük, Safranbolu, Navsaklar village	41°12′56.85″N 32°44′14.81″E	301

Sampling method

Benthic macroinvertebrates in each station were collected between the stones, pebbles and plants on the ground using a standard dip net with a transecting area of 100 m^2. Each sampling lasted 5 minutes. All samples were fixed in 80% ethanol immediately after collection, and then taken to the laboratory. The sampled material was investigated in the laboratory under a binocular stereomicroscope (Leica APO S8) and caddisfly larvae among the collected macroinvertebrates were transfered into tubes containing fresh 80% ethanol. The study material is deposited in the Entomology Laboratory of Biology Department of Faculty of Sciences and Arts, Kastamonu University, Turkey.

Identifications of the caddisflies

Caddisfly species, based on larvae, were identified under the binocular stereomicroscope using Trichoptera Families 2007 and Trichoptera 2005 package programmes (Lechthaler & Stockinger 2005, Lechthaler 2007) and literature (Brohmer 1979; Edington & Hildrew 1981, 1995; Hickin 1942, 1943, 1948, 1952, 1954, 1967, Ulmer 1909, Zamora-Muñoz *et al.* 1995). A general knowledge of morphological structures associated with caddisfly larvae is necessary for ease of identification. The head is dorsally divided by a Y-shaped ecdysial line referring to the frontoclypeal and coronal sutures, the frontoclypeus is bordered laterally by the frontoclypeal sutures, and the parietals extend posteromesally along the coronal suture. The anterolateral portion of the head contains the eyes and antennae, whose locations vary among the families. Mouthparts are among the essential structures for the identification of larvae, which include the labrum and labium that contain mandibles and maxillae. The position and view of these parts carry distinctive characteristics at family or genus levels. The posterior portion of the head often has a number of muscle scars which appear as dark or light spots. The shape and structure of the thorax segments are also important for identification of the larvae. The thorax segments -prothorax, mesothorax and metathorax- contain a dedicated pair of legs that are often a sclerotized notum. The prothorax generally has a finger-like prosternal horn and a lateral pair of trochantins, which can be distinctive for several families. The prothorax is always covered by dorsal sclerotized plates while the meso- and metathorax do not always have sclerotized notal plates but possess notal subdivisions. Setae, if arising on the meso- or metanota, reside in characteristic areas termed setal area 1 (sa1), setal area 2 (sa2) and setal area 3 (sa3). Arrangement of both setal areas and sclerites can be of taxonomic importance. Thoracic legs have a tarsal claw apically. Each tarsal claw usually has a basal seta, the size of which can be of taxonomic value. The first abdominal segment often bears a dorsal hump and a pair of lateral humps, which provide circulation of water through the case as well as in securing the larva in the case. Some families have abdominal segments with numerous tracheal gills that facilitate gas exchange. The tracheal gills may be single or more filamentous, finger-like, comb-like, etc. The shape of the abdominal gills provide important taxonomic characters in diagnosis. Each of the anal prolegs has an anal claw, which can be simple or complex, that bear accessory spines. The anal prolegs, in terms of sclerites and nature and extension of setation, vary from family to family in degree of separation from the body. The case structure and form are among the distinguishing characters for the larvae of caddisflies. Some larvae have a case while others can survive without. The case can be made out of a variety of materials including sand grains, small pebbles, vegetal fragments and secretions. The shape of the larval case and the type of the materials used are utilized for taxonomic characterization.

Results

A total of 1223 larvae belonging to the order of Trichoptera were collected from the Araç Creek. 14 taxa belonging to nine genera within eight families (Brachycentridae, Hydropsychidae, Hydroptilidae, Lepidostomatidae, Leptoceridae, Limnephilidae, Psychomyiidae and Rhyacophilidae) were identified (Table 2). The systematic list of the identified trichopterans is given in Table 2. Among the determined taxa *Micrasema cinereum* is the first record for Trichoptera fauna of Turkey.

Morphometric, biological and ecological characteristics and the distribution of new record for the fauna of Turkey are as follows:

Brachycentridae Ulmer

Micrasema cinereum Mosely, 1930

Description of the larva

Materials examined: 1 larva, (Station 1); 1 larva, (Station 2).

Head capsule: Mean head width 0.67 mm, mean head length 0.7 mm. Head round (Fig. 2A); chestnut in color with Y-shaped patterned in yellowish along dorsal ecdysial line.

Head with a dorsolateral supraocular ridge are clear on each side, and one extra lateral infraocular ridge present that is shorter and less conspicuous. The frontoclypeal apotome (Fig. 2A) follows broadening view from the starting point.

Profile view of the head capsule (Fig. 2B) has a curved dorsal edge view. Periocular area is light yellowish. Ventral side of the head capsule (Fig. 2C) has similar color-toned to dorsum; ventral apotome is more of rectangular viewed with a narrowing end towards the anterior section that separates the genae completely. Sclerites of cardo brown in color, median margin with a slightly sharp anterior process. Submental sclerites have extended view, possessing seta as the ventral side of the head along with possessing long seta on each (Fig. 2C).

Legs (Figs. 2D, E, F) have same but paler colored as seen for meso- and metanotum. Prothoracic legs are

shorter and stouter (Fig. 2D). Meso- and metathoracic legs are approximately same in size (Fig. 2E, F). Distal part of tibia of leg II and leg III show thin small tubercles (Fig. 2G). Tarsal claws of 3 legs have similar structure.

Thorax (Fig. 2H): One half of the posterior pronotum is uniformly brown, and anterior section is obviously paler. Pronotum has an obvious curved transverse ridge view, one half of anterior and ridge does not show sparse view. Mesonotum has greenish islands in brown colored surface while the metanotum has bluish view. Tergite on each half of mesonotum is consisting of one single plate with the total number of tergite plates 2 (Fig. 2H). Metanotum is membranous, and has 2 pairs of small sclerites.

Abdomen is nearly cylindrical and posterior section is gradually tapered while segment IX is clearly thinner (Fig. 2K). Dorsal hump is absent and lateral humps are indistinct in segment I. Abdominal gills and lateral lines are absent.

Mid dorsal sclerite of segment IX (Fig. 2I) is weakly sclerotized, elliptical, with *ca.* 12 setae that are different in size along posterior margin.

Anal prolegs (Fig. 2I) are nearly sclerotized at dorsal view. Lateral sclerite is strongly sclerotized at dorsal view. Anal claw is stout and with 3 accessory hooks (Fig. 2J), for which one of them is poorly developed and often unobtrusive.

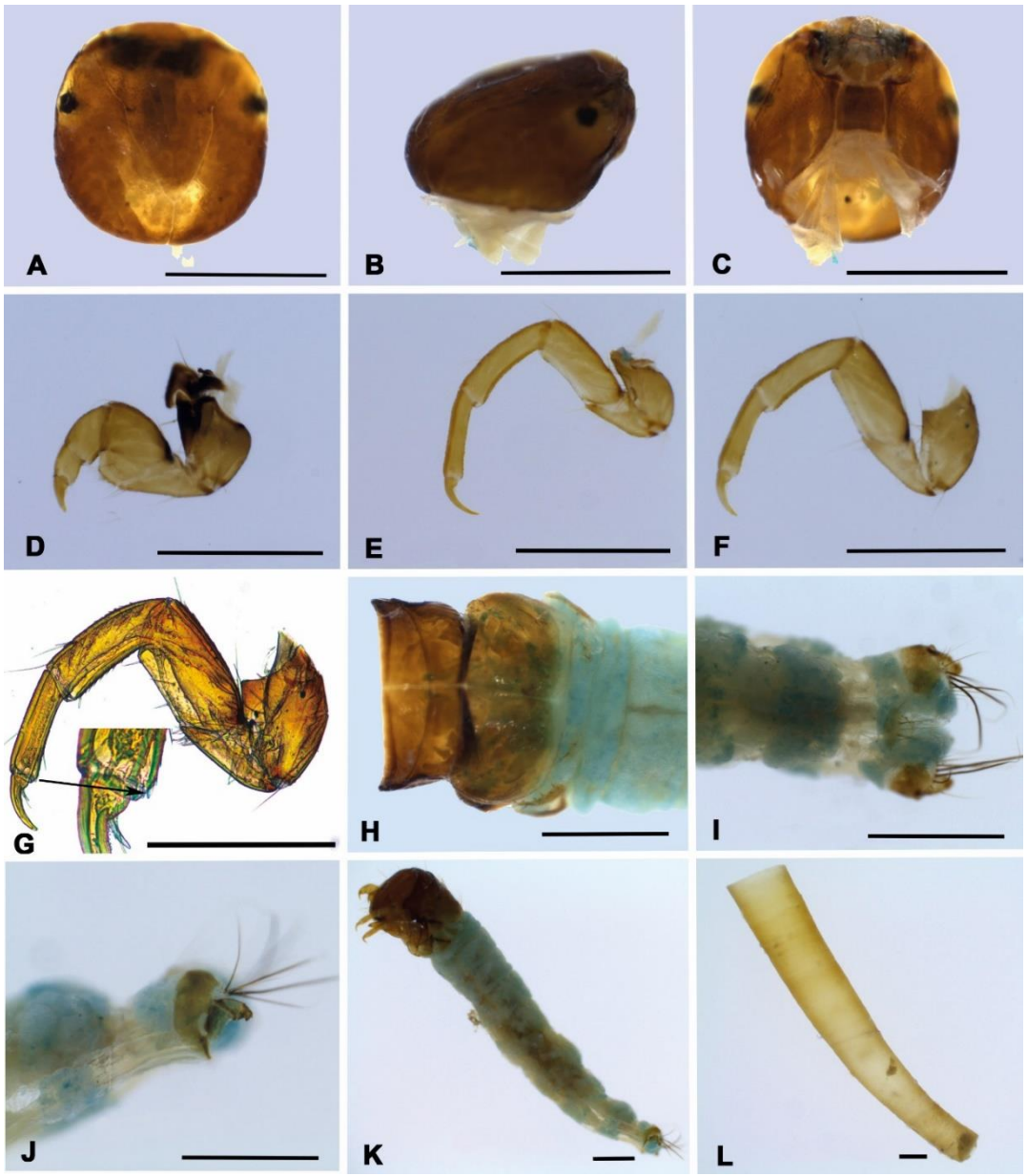

Fig. 2. Larva and case of *M. cinereum* (A) Head in dorsal view, (B) Head in lateral view, (C) Head in ventral view, (D) Prothoracic leg, (E) Mesothoracic leg, (F) Metathoracic leg, (G) Distal part of tibia of leg II and leg III, (H) Thorax in dorsal view, (I) 9th abdominal segment and anal prolegs in dorsal view, (J) 9th abdominal segment and anal prolegs in lateral view, (K) Larvae in lateral view, (L) Case in lateral view. Scale bars: 0.5 mm.

Table 2. Trichopteran taxa identified from the Araç Creek.

Family	Taxa	Number of larvae	Sampling Station 1	2	3	4	5	6
Rhyacophilidae	*Rhyacophila nubila* Zetterstedt	13	*	*			*	
	Rhyacophila sp.	2		*				*
Hydroptilidae	*Hydroptila* sp.	3					*	
Psychomyiidae	*Psychomyia pusilla* (Fabricius)	4					*	*
Hydropsychidae	*Cheumatopsyche lepida* (Pictet)	4				*	*	
	Hydropsyche botosaneanui Marinkovic-Gospodnetic	665	*	*	*	*	*	*
	Hydropsyche bulbifera McLachlan	250	*	*	*	*	*	*
	Hydropsyche exocellata Dufour	93				*	*	*
	Hydropsyche instabilis (Curtis)	64		*	*	*		
	Hydropsyche pellucidula	119	*		*	*	*	*
Brachycentridae	*Micrasema cinereum*	2	*	*				
Lepidostomatidae	*Lepidostoma hirtum* (Fabricius)	2		*				
Limnephilidae	*Limnephilus lunatus* Curtis	1					*	
Leptoceridae	*Setodes viridis* (Fourcroy)	1					*	

Table 3. The zoogeographical distribution of trichopteran taxa in Araç Creek. WP; West Palearctic, EP; East Palearctic, OL; Oriental, NA; Nearctic (Morse, 2018).

Family	Taxa	Zoogeographical distribution
Rhyacophilidae	*Rhyacophila nubila*	EP and WP
	Rhyacophila sp.	-
Hydroptilidae	*Hydroptila* sp.	-
Psychomyiidae	*Psychomyia pusilla*	WP
Hydropsychidae	*Cheumatopsyche lepida*	EP and WP
	Hydropsyche botosaneanui	EP and WP
	Hydropsyche bulbifera	WP
	Hydropsyche exocellata	WP
	Hydropsyche instabilis	WP
	Hydropsyche pellucidula	EP, WP, NA and OL
Brachycentridae	*Micrasema cinereum*	WP
Lepidostomatidae	*Lepidostoma hirtum*	EP and WP
Limnephilidae	*Limnephilus lunatus*	WP
Leptoceridae	*Setodes viridis*	EP and WP

Body (Fig. 2K) length up to 3.6 mm, mean body width up to 0.5 mm.

Case (Fig. 2L) is about 6.9 mm long with yellow to orange color. It has a narrowing tubular shape from anterior to posterior with a curved ending (1/3 of the size). The case is made of secretion by the larvae.

Habitat and distribution

Giudicelli & Orsini (1987) reported that *M. cinereum* inhabit one of the 3 sub-branches of Restonica river (Corsica), whose altitute is between 400-800 m. Décamps (1970) reported the presence of *M. cinereum* in low- and mid-altitudes of the rivers in moutainous areas, where *M. longulum* McLachlan, and *M. minimum* McLachlan were also found. We sampled *M. cinereum* at the proximity of the spring, where altitudes were recorded as 920 and 784 m for the stations 1 and 2, respectively. Station 1 resembles the area described elsewhere (Décamps 1970) and is located 1 km south of İhsangazi in a place relatively far from the settlements in the forest area. The depth is approximately 30 cm with sediment mostly composed of rubbles and pebbles that are 2–20 mm in size. Station 2, with a similar flow and habitat pattern as Station 1, is located at the 7th km of Kastamonu, İhsangazi, İhsangazi-Araç road, Akkaya village road junction, in the bridge vicinity. The depth is approximately 25-30 cm and with a base structure mostly composed of small rocks.

The distribution range of *M. cinereum* has been given from France (Corsica) and Spain (Mosely 1930, Décamps 1970, García de Jalón 1982, Casado *et al*. 1990).

From the zoogeographical point of view, the Trichoptera fauna of Turkey is strongly related to the European fauna. The Trichoptera fauna of northeastern

Turkey is related to the Caucasian/Transcaucasian fauna. The zoogeographical evaluation of caddisfly taxa in Araç Creek showed that 6 (42,85%) species belong to West Palearctic, 5 (35,71%) species belong to East-West Palearctic, and 1 (7,14%) species belongs to East-West Palearctic, Nearctic, and Orientalic (Table 3). The zoogeographic distribution of the two taxa which were identified as *Rhyacophila sp*. and *Hydroptila sp*. are not shown here.

Discussion

The present study has been performed as the first study aiming to determine the Trichoptera fauna in Araç Creek where 14 caddisfly taxa were identified (Table 2). The sampled material was found to be represented with 12 species while two taxa could be identified at genus level. *Rhyacophila sp*. is different from *R. fasciata* Hagen with that the muscle attachment spots within the dark triangular patch on the frontclypeus are paler than the surrounding pattern and clearly discernible. Besides, it is different from *R. nubila* Zetterstedt by possessing the dark pattern on frontclypeus that is not extended to the anterior margin. Although it may seem different from the previously recorded *R. nubila* and *R. fasciata*, identification of the species was confined to genus level of the *Rhyacophila sp*. samples due to the fact that the diagnostic characters were not completely clear in our samples. Hydroptilidae family is distinguished from other caddisfly families with its characteristic life cycle where the first four larval instars live freely without having case while the fifth larval instar builds a case to live in. Case type and the construction material are distinguishing parameter among Hydroptilidae members (Pescador *et al*. 1995). The Hydroptilidae larvae were not at their 5th instar, where the taxonomic characters are not clear enough, and we therefore preferred to use *Hydroptila sp.*

Although *M. cinereum* has been given as an endemic species from Corsica (Décamps 1970, Giudicelli & Orsini 1987, Botosaneanu & González 2006), its presence in Spain was also reported (García de Jalón 1982, Casado *et al.* 1990). According to the inventory given by Darılmaz & Salur (2015), three species of genus *Micrasema* (*Micrasema anatolicum* Botosaneanu, *M. bifoliatum* Martynov and *M. mencilis* Sipahiler) have been reported from Turkey. However, the larval stages of these three species are not known, because these species were reported as adult samples.

Micrasema cinereum, which is distinguished from the other species with the following key characteristics is given as a new record in this study. *Micrasema cinereum* and *M. longulum* possess fully secretion-based case structures which is not seen in other *Micrasema* species. The case is constructed from natural minerals for *M. moestum* (Hagen) and *M. minimum*. In contrast to this, the case is made of plant materials for *M. tristellum* McLachlan and *M. togatum* (Hagen) and of plant material and secretion for *M. morosum* (McLachlan). Structurally, the case of *M. cinereum* and *M. longulum* has curving view at posterior side (Décamps 1970). The structural characteristic of mesothorax is one of the key parameters used to distinguish these species. *Micrasema minimum*, *M. setiferum* (Pictet) and *M. moestum* have four sclerotized plates. In contrast to this, *M. longulum*, *M. cinereum*, *M. difficile* Mosely, *M. tristellum*, *M. togatum* and *M. morosum* possess two sclerotized plates (Decampas 1970; Vieira-Lanero *et al.* 1998). The distal point of the tarsial ventral side of meso- and metathoraxic legs possess conic tubercles in *M. longulum*, which are thinner for *M. cinereum*. While mesothorax has two chitinized plates with latero-anterior angles that are extending forward, those are not present in *M. cinereum*. Mesotonum is slightly less-colored in plain brown for *M. cinereum* in comparison to *M. longulum*. Dorsal view of cephalic capsule possesses protuberances in the eyes for *M. longulum* (Décamps 1970) while the protuberances are absent for *M. cinereum*.

Conclusion

In conclusion, 14 taxa belonging to nine genera within eight families (Brachycentridae, Hydropsychidae, Hydroptilidae, Lepidostomatidae, Leptoceridae, Limnephilidae, Psychomyiidae, Rhyacophilidae) were identified in six stations along Araç Creek. *Rhyacophila nubila, P. pusilla, H. bulbifera* and *H. pellucidula* have previously been recorded in the creek by Küçükbasmacı & Kıyak (2017), while *Rhyacophila sp., Hydroptila sp., C. lepida, H. botosaneanui, H. exocellata, H. instabilis, M. cinereum, L. hirtum, L. lunatus* and *S. viridis* are new records for the creek. Among *M. cinereum* was recorded for the first time in Turkey. The present records increased the number of caddisflies taxa in Turkey fauna to 501.

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Research Article

FUNCTIONAL CHARACTERIZATION OF SPERMINE FAMILY TRANSPORTER *caf5*$^+$ IN *Schizosaccharomyces pombe* (Lindner)

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Abstract: Polyamines are well conserved polycationic molecules that are known to interact with nucleic acids and contribute to multiple functions including cell cycle and stress response. The transport of polyamines in and out of the cell is driven by polyamine transporters that play a significant role in polyamine homeostasis. *Schizosaccharomyces pombe* (Lindner) *caf5*$^+$ gene codes for a spermine family transporter that is yet to be characterized functionally. This study aims to understand the contribution of *caf5*$^+$ on different processes previously associated with polyamines, by reverse genetics. Deletion mutants of *caf5*$^+$, which are viable in normal conditions, were scanned for multiple cellular processes. The results showed that *caf5*$^+$ deletion caused shorter cell length and slightly faster growth rate at the optimum conditions. *caf5Δ* cells also showed sensitivity to high doses of UV irradiation, while no sensitivity was observed against osmotic stress or another DNA damaging agent hydroxyurea. The mutants could successfully go through different phases of mitosis and meiosis as observed by DNA and septum staining. In summary, *caf5*$^+$ gene is involved in normal growth and cell cycle progression, as well as stress response upon UV irradiation.

Key words: *Schizosaccharomyces pombe*, cell size, cell cycle, stress response, polyamine.

Özet: Poliaminler, nükleik asitlerle etkileştiği ve hücre döngüsü ile stres tepkisi gibi pek çok hücresel işleve katıldığı bilinen, korunmuş polikatyonik moleküllerdir. Poliaminlerin hücre içine girişi ve hücre dışına çıkışı, poliamin homeostazında görevli olduğu bilinen poliamin taşıyıcı proteinleri tarafından yürütülmektedir. *Schizosaccharomyces pombe* (Lindner) *caf5*$^+$ geni de henüz işlevsel olarak karakterize edilmemiş bir spermin ailesi taşıyıcısıdır. Bu çalışmanın amacı ters genetik yöntemlerle *caf5*$^+$ geninin poliaminlerle ilişkili olduğu bilinen hücresel işlevler üzerindeki önemini anlamaktır. Normal koşullarda yaşayabilir durumda olan, *caf5*$^+$ geni delesyon mutantları (*caf5Δ*) pek çok farklı hücresel işlev üzerinden taranmışlardır. Sonuçlar, *caf5*$^+$ geni delesyon mutantlarının hücre boyunun daha kısa olduğunu ve optimum koşullar altında daha hızlı bölündüklerini göstermektedir. *caf5Δ* hücreler, aynı zamanda yüksek dozda UV ışınlarına karşı hassasiyet göstermişler ancak ozmotik stres koşullarında ve bir başka DNA hasarı ajanı olan hidroksiüre'ye karşı herhangi bir hassasiyet göstermemişlerdir. DNA ve septum boyamaları sonucunda bu mutantların mitoz ve mayoz bölünmenin farklı fazlarını başarılı bir şekilde tamamlayabilir olduğu bulunmuştur. Özetle, *caf5*$^+$ geni normal hücre büyümesinde, hücre döngüsünde ve UV ile indüklenen stres tepkisinde rol oynamaktadır.

Introduction

Polyamines are small, ubiquitious polycations that can be found in every organism except Archae, Methanobacteriales and Halobacteriales (Hamana & Matsuzaki 1992). Spermine and spermidine are the most common polyamines, but there are also polyamines in thermophilic organisms in the form of long or branched hydrocarbon chains (Fukuda *et al*. 2015).

Polyamines are involved in multiple functions in the cells such as stabilizing nucleic acids, regulation of gene expression, cell cycle, stress response and pathogenic activity. Polycationic nature of polyamines enables their binding to anionic molecules in the cells and most of the intracellular polyamines are found as polyamine-RNA complexes. The ability of polyamines to readily bind to DNA and RNA contributes to nucleic acid stability (Katz *et al.* 2017). Stabilization of DNA by polyamines is especially important for heat resistance, and hence survival of thermophilic microorganisms at extreme temperatures (Fukuda *et al.* 2015). Polyamines also contribute to the regulation of gene expression at the level of translation by different mechanisms including formation of the initiation complex, facilitating fMet-tRNA binding in bacteria or by supporting post translational modification of the translation factor eIF5A in yeasts (Gevrekci 2017). Among others, the role of polyamines in providing resistance to environmental stress is best characterized in a number of organisms including bacteria, yeasts, fungi and plants. Polyamine

mutants were shown to be sensitive to oxidative stress in *Ustilago maydis* (DC.) Corda and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Balasundaram 1993, Valdés-Santiago 2010), while putrescine level was shown to increase upon oxidative stress in *Escherichia coli* T. Escherich (Ucisik-Akkaya *et al.* 2014). Similar results were reported also for osmotic stress, showing that *U. maydis* polyamine synthase gene mutants (Valdés-Santiago 2009) and yeast polyamine uptake regulator gene (*pts2)* mutants were sensitive to osmotic stress (Erez & Kahana 2002). Moreover, *S. cerevisiae* and *Synechocystis* sp. were shown to respond to osmotic stress by regulating polyamine transport, showing the role of polyamines in osmotic stress response (Aouida *et al.* 2005, Jantaro *et al.* 2003, Lee *et al.* 2002).

Another significant role assigned to polyamines is the regulation of cell cycle progression. Cell cycle is a unidirectional series of events that initiates with monitoring the intracellular and extracellular environment and finally leads to the formation of two identical copies of the cell. The involvement of polyamines in cell cycle control is mostly characterized in higher eukaryotes although there are several examples among microorganisms. Inhibition of polyamine biosynthesis prolonged the S phase in the Chinese hamster (*Cricetulus griseus* Milne-Edwards) ovary (Fredlund & Oredsson 1996) and arrested the cell cycle at G1 phase in intestinal epithelial cells (Ray *et al.* 1996). Additionally, polyamine depletion caused T lympoblastic cells to arrest at the G1 phase (Choi *et al.* 2000). The significance of polyamines in cell cycle regulation was also shown in *Schizosaccharomyces pombe* (Lindner). Depletion of polyamines at the early phases of the cell cycle was shown to induce G1 arrest. Upon prolonged polyamine deprivation, however, *S. pombe* cells showed a number of cell cycle phenotypes such as disruption of the actin network, disintegration of the nucleus and absence of septum (Chattopadhyay *et al.* 2002).

Since polyamines are involved in multiple different functions in the cells, polyamine homeostasis should be well established. A number of mechanisms have evolved to keep the intracellular polyamine levels at optimum levels. Polyamine biosynthesis (*de novo* or by interconversion of polyamine molecules) and transport are the two major ways to supply the right amount of polyamine to the cells. The most common biosynthetic pathway initiates with conversion of L-ornithine into putrescine by ornithine decarboxylase (ODC), followed by spermidine and spermine formation by the consequitive activities of spermidine synthase (SpdSyn) and spermine synthase (SpmSyn) (Michael 2016). In addition to the biosynthetic pathway, a number of polyamine family transporters also contribute to the regulation of intracellular polyamine concentration. Spermine and spermidine family transporters are transmembrane proteins that can be found in organelle or plasma membranes, driving polyamine influx or efflux. The efflux of polyamines was shown to be induced with decreased growth rate and inhibited upon increased growth rate (Wallace & Keir 1981, Wallace & Mackarel 1998). This fact signifies the role of polyamine transporters in the cells along with the study that showed yeast polyamine transporter Tpo1 extends the cell cycle arrest induced upon environmental osmotic stress (Krüger *et al.* 2013).

The present study was performed in order to identify and functionally characterize spermine family transporter *caf5*$^+$ in *S. pombe*. For this purpose, the deletion mutant of *caf5*$^+$ gene was used. The mutant was scanned for normal growth rate, stress response, cell division defects and spore formation to determine any process that was defective in the absence of this gene, as an indicator of its involvement in that particular process.

Materials and Methods

Schizosaccharomyces pombe Strains and the Media

Schizosaccharomyces pombe 972 *(h- ade)* strain was used to form *caf5*$^+$ deletion mutants and as wild-type control. The strains were handled as explained in Moreno *et al.* (1991). The wild type and mutant strains were incubated in yeast extract agar (YEA) plain media (5 g/l difco yeast extract, 30 g/l glucose, 75 mg/l adenine, pH adjusted to 5.6 with HCl – supplemented with 2% (w/v) agar for agar media). The YEA agar was supplied with 1 M KCl, 120 mM $CaCl_2$ or 2 M Sorbitol for the osmotic stress response experiment. For hydroxyurea treatment, the media was supplied with 4 M hydroxyurea. A series of different concentrations of the stress agents (both salts and hydroxyurea) was initially used for stress response experiments. Specifically, the concentrations started with 0.5 M KCl, 60 mM $CaCl_2$, 1 M Sorbitol and 2 mM hydroxyurea and systematically increased up till 2M KCl, 240 mM $CaCl_2$, 4 M Sorbitol and 10 mM hydroxyurea in which even the wild type cells couldn't grow properly (so not informative to detect any difference in growth rate). Thus, the maximum salt and hydroxyurea concentrations that allowed proper wild type cell growth were shown in this study. To induce sporulation, the cells were streaked onto sporulation agar (SPA) medium [10 g/l glucose, 1 g/l KH_2PO_4, 1 ml/l 1000 X vitamin stock (1 g/l panthothenate, 10 g/l nicotinic acid, 10 g/l inositol, 10 mg/l biotin)]. The SPA medium causes nitrogen starvation, which consequently induces G1 arrest, mating (in the presence of the opposite mating type) and finally meiosis to form spores.

Forming Single Gene Mutations in S. pombe

caf5$^+$ gene deletion is performed by a PCR-based method explained in Bähler *et al.* (1998). pFA6a-kanMX6 gene deletion cassette containing kanamycin resistance gene was amplified by specific primers with complementary sequences to the immediate upstream and downstream regions of *caf5*$^+$ gene. Kanamycin resistance gene provides resistance to G418 in *S. pombe*. Upon transformation of the deletion cassette by LioAc method of transformation, the cells were initially plated onto YEA agar and after 24 hours at 30°C, they were replica-plated

onto YEA plate supplemented with 200 mg/l G418 to select for the kanamycin resistance cassette. The colonies which were resistant to G418 showed up in 3-5 days incubation at 30°C, which were then tested by colony PCR for the genomic location of the deletion cassette.

Colony PCR

The cells surviving in the G418 containing YEA agar are known to have deletion cassette in the genome. To make sure that the deletion cassette was inserted at the right location in the genome, hence the right portion of the genome was deleted, colony PCR was used. The procedure started with boiling the colonies with dNTP mix, PCR buffer and the primers, at 98°C for 10 min. After being cooled down on ice, Taq polymerase was added and the following PCR program was run: 30 cycles of 94°C for 20 s, 50°C for 40 s, and 72°C for 1 min/kb, followed by 72°C for 5 min. The expected size of the product was 305 kb for *caf5*$^+$ deletion. The forward primer was designed according to the upstream region of *caf5*$^+$ gene and the reverse primer was designed according to the deletion cassette.

Forming Double Mutants in S. pombe

caf5Δ SPBC409.05Δ double mutants were formed by crossing *h*$^-$ *caf5Δ* and *h*$^+$ *SPBC409.05Δ* cells, which were resistant to G418 and hygromycin, respectively. The single mutants were initially streaked together on SPA agar sporulation media and incubated for 3 days for G1 arrest, mating and spore formation. The cells were then replica-plated onto YEA + 200 mg/l G418 and incubated at 30°C for 3 days. Then the colonies were replica-plated onto YEA + 300 µg/ml hygromycin. The colonies that could grow in both of the agar plates were expected to have both of the deletion cassettes, and hence neither of the *caf5*$^+$ and *SPBC409.05* genes.

Growth Rate and Cell Size Analysis

For the growth rate analysis, the cells were inoculated into YEA broth and incubated overnight (O/N) at 30°C. When they reached the fast growing phase, the cells were diluted to 10^6 cells/ml and t = 0 timepoint samples were collected. Additional samples were collected at t = 2 hr, t = 4 hr, t = 6 hr, t = 8 hr and cells were count to determine how fast they multiply. The cell cultures reached saturation between 8 hr-9 hr so t = 8 hr was decided to be the last timepoint to follow. For the cell size analysis, the cells from the O/N cultures were collected at the fast growing phase, fixated with gluteraldehyde (as explained in DAPI and calcofluor staining below) and observed under the microscope. Independent samples t-test was performed using the SPSS version 22 package to compare wild type cells and mutants for statistically significant differences in growth rate at different timepoints and cell size. Shapiro-Wilk test was also performed for each sample to prove normal distribution of cell number, as a prerequisite for t-test.

Stress Protocol

Different types of stressors were scanned to understand the role of *caf5*$^+$ in the cells. Osmotic stress was induced by adding the indicated amount of KCl, $CaCl_2$ or Sorbitol into the YEA agar. The cells were initially grown O/N at 30°C in YEA broth until they reached 0.5–1 x 10^7 cells/ml. They were then diluted to a total of five serial dilutions, containing 5, 50, 5 × 10^2, 5 x 10^3 and 5 x 10^4 cells (8 µl each) and spotted onto YEA agar media (plain or with KCl, $CaCl_2$ or Sorbitol). In case of DNA damaging agents, hydroxyurea plates were prepared and the cells were spotted onto agar plates similar to the osmotic stress conditions, as explained above. For UV damage, however, the cells were spotted onto YEA plain media and exposed to different strengths of UV light immediately. The growth was observed after 3-5 days incubation at 30°C.

DAPI and Calcofluor Staining

Schizosaccharomyces pombe cells were initially grown O/N at 30°C in YEA broth until they reached 0.5x10^7 cells/ml. 1 ml of the cell culture was mixed with gluteraldehyde (2.5% final concentration) and incubated at 4°C for 30 min. After being centrifuged at 3000 rpm for 2 min at 4°C, the cells were re-suspended in 500 µl ice-cold phosphate buffer saline (PBS). They were washed with PBS three times and re-suspended in 20–30 µl ice-cold PBS. 1ml of the sample was either mixed with 1 µl of 100 µg/ml DAPI or calcofluor on the microscope slide to be examined.

Results

The Role of caf5+ in Normal Growth Rate

To understand the significance of *caf5*$^+$ gene, deletion mutants were formed and scanned in terms of a series of cellular processes. *caf5*$^+$ deletion was performed as explained in Bähler *et al.* (1998). In this procedure, pFA6a-kanMX6 gene deletion cassette containing kanamycin resistance gene was amplified by specific primers (with complementary sequences to the immediate upstream and downstream regions of *caf5*$^+$ gene). The product was transformed into the wild type cells to replace *caf5*$^+$ gene, which is driven by intrinsic homologous recombination mechanisms (Fig. 1A). The mutants were checked by antibiotic resistance for the presence of deletion cassette in the genome and by colony PCR to prove the correct genomic location (the forward and reverse primers are shown as F and R, respectively in Fig. 1A). For *caf5*$^+$ deletion, the expected PCR product is 305 bp long. Six different colonies that could grow on YEA + G418 were selected for colony PCR. The results showed that the colonies gave positive results for the presence of deletion cassette in the correct location of the genome (Fig. 1B). The fifth colony was chosen for further characterization analysis.

The first experiment was performed to check if *caf5*$^+$ gene was involved in normal growth at the optimum conditions. *caf5Δ* cells and wild type cells grown at optimum conditions were compared in a time course analysis, where the cell numbers were followed for a total of 8 hours. Samples were collected at every 2 hours and the cell numbers were count. There was no significant

difference between the wild type and *caf5Δ* cells at t = 0 (p = 0.434), t = 2 hr (p = 0.294), t = 4 hr (p = 0.364), t = 6 hr (p = 0.281) time points. However, at t = 8 hr, *caf5Δ* cells were significantly more than wild type cells (p = 0.043). At t = 8 hr, cells approached saturation, so no more time points were collected from this point on (Fig. 1C). The results showed a slightly faster growth in *caf5Δ* cells, which could only be observed in a time course experiment but not on agar plates (Fig. 1C vs. Fig. 2A YEA plate). This result indicates a small but significant role of *caf5*$^+$ in cell division under optimum conditions, in the absence of cellular stress. Mean ± standard deviation for the wild type cell numbers at different time points were 1.00 ± 0.63 for t = 0 hr, 1.88 ± 0.85 for t = 2 hr, 4.50 ± 2.14 for t = 4 hr, 6.46 ± 1.47 for t = 6 hr, 13.65 ± 6.93 for t = 8 hr. Mean ± standard deviation for the *caf5Δ* cell numbers at different time points were 1.30 ± 0.57 for t = 0 hr, 2.42 ± 0.85 for t = 2 hr, 5.75 ± 2.40 for t = 4 hr, 7.79 ± 2.46 for t = 6 hr, 20.83 ± 3.76 for t = 8 hr. The results showed normal distribution for all the samples (the significant values in Shapiro-Wilk test for the wild type and *caf5Δ* cells at different time points were as follows: wild type (0.053 for t = 0 hr, 0.062 for t = 2 hr, 0.368 for t = 4 hr, 0.258 for t = 6 hr, 0.356 for t = 8 hr); *caf5Δ* (0.228 for t = 0 hr, 0.382 for t = 2 hr, 0.201 for t = 4 hr, 0.579 for t = 6 hr, 0.437 for t = 8 hr).

The Effect of caf5+ Deletion in Cell Size

One very important aspect of life cycle in *S. pombe* is the cell size. In normal growth, *S. pombe* cells are rod shaped and when they reach a critical size, they are expected to divide. Any delay in the cell cycle results in longer cell length and premature progression of cell division is expected to result in shorter cell length. In parallel with this, many cell cycle regulator gene mutations were associated with abnormal cell size. The most extreme examples are mutants of *cdk* regulators: *cdc25* and *wee1* gene deletions in *S. pombe* are well known to have elongated and shorter phenotype, respectively. Therefore, the cell size in *caf5Δ* cells was also checked to see any such cell size defect. The results showed that *caf5Δ* cells were significantly shorter than the wild type cells (p = 0.006) (Fig.. 1D). As discussed above, this result is an indication of involvement of *caf5*$^+$ gene in cell division control. *Schizosaccharomyces pombe* spermine family transporter *SPBC409.08* gene deletion was previously identified to have a shorter cell size (Güngör & Örs Gevrekci 2016). Double mutants *caf5Δ SPBC409.08Δ* cells were formed and checked for the cell size in the second part. However, no significant difference was found between *caf5Δ* and *caf5Δ SPBC409.08Δ* in terms of cell size (p = 0.160), so the phenotype was not exacerbated in double mutation (Fig. 1D). Mean ± standard deviation of cell sizes (in µm) were 13.39 ± 1.37 for the wild type cells, 12.00 ± 1.65 for *caf5Δ,* and *12.86* ± 2.13 for *caf5Δ SPBC409.08Δ.* The results showed normal distribution for all the samples (0.677 significance in Shapiro-Wilk test for the wild type cells, 0.742 for *caf5Δ,* 0.873 for *caf5Δ SPBC409.08Δ*).

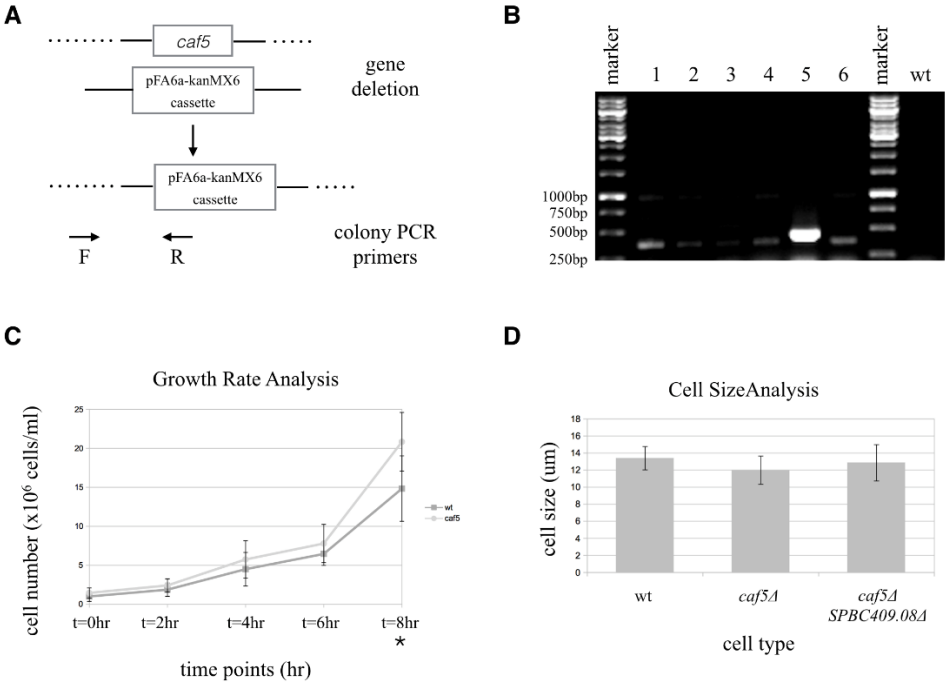

Fig. 1. Schematic representation of gene deletion (A), colony PCR (B), growth rate analysis (C) and cell size analysis of *caf5Δ* (D). (A) A summary of gene deletion and consequent confirmation of deletion by colony PCR, including the forward (F) and reverse (R) primer locations. (B) Both of the colonies (1-6) had the deletion cassette in the correct location of the genome, successfully replacing the *caf5*$^+$ gene. The expected product size was 305 kb. The colony PCR result for wild type (wt) cells with intact *caf5*$^+$ is shown as a negative control. (C) Time course experiment graph shows the cell numbers at different time points from t = 0 hr to t = 8 hr. The only timepoint with a significant difference between wild type and *caf5Δ* is indicated with an asterix (t = 8 hr). (D) Cell size comparison of wild type and *caf5Δ* cells. The bars represent standard error.

The role of caf5+ in stress response

One of the most studied and best characterized function of the polyamines is in stress response. At the next step, we checked whether *caf5⁺* was involved in the response against different environmental stressors. Wild type and *caf5Δ* cells were exposed to different kinds of environmental stress such as osmotic stress induced by salts in the media, UV irradiation and hydroxyurea. Hydroxyurea is known to inhibit ribonucleotide reductase (RNR) enzyme and deplete dNTPs, which starves DNA Polymerase for dNTPs. UV irradiation, on the other hand, induces pyrimidine dimers in the DNA. *caf5Δ* cells were plated onto YEA agar plates with high amounts of KCl, $CaCl_2$ and sorbitol, along with wild type cells to understand any osmotic stress sensitivity. It is noteworthy that different concentrations of KCl, $CaCl_2$ and sorbitol were tried to see osmotic stress response, increasing gradually starting from lower concentrations up until wild type cells stoped growing. The highest concentrations that allowed normal growth of wild type cells were shown in Fig. 2A. The results showed that *caf5Δ* cells were not different from wild type cells in osmotic stress sensitivity.

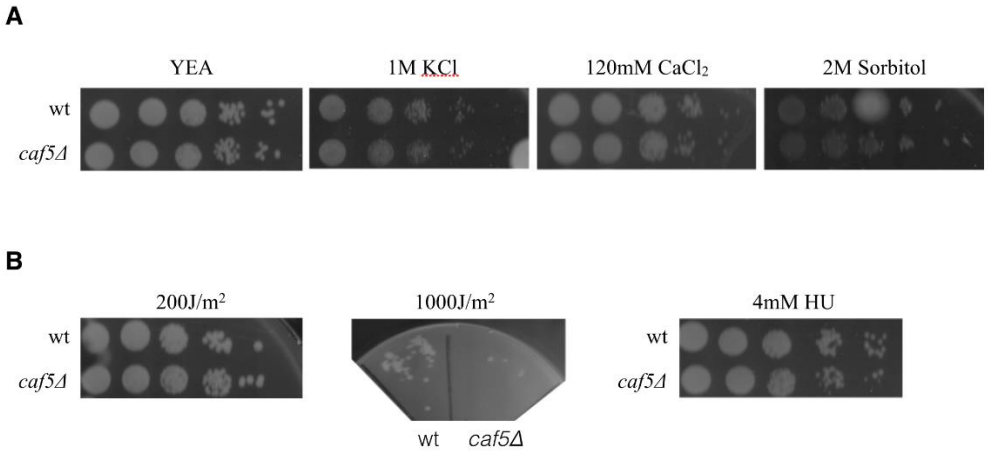

Fig. 2. Environmental stress response of the *caf5Δ.* Spots on the plates represent 10-fold serial dilutions starting with 5 x 10^4 cells, 5 x 10^3 cells, 5 x 10^2 cells, 50 cells and 5 cells, from left to right. (A) KCl, $CaCl_2$ and sorbitol were applied as osmotic stress causers. *caf5*Δ cells showed no sensitivity against osmotic stress. (B) *caf5*Δ and wild type cells were exposed to different strengths of UV irradiation and hydroxyurea. *caf5Δ* cells had reduced viability upon 1000 J/m^2 UV irradiation but not against hydroxyurea.

Another group of environmental stress factor is DNA damaging agents such as UV irradiation or hydroxyurea. c*af5Δ* cells were exposed to direct UV irradiation and plated onto YEA media to be incubated at the optimum conditions. Similar to osmotic stress, different strengths of UV irradiation was applied. It was shown that *caf5Δ* cells showed no UV irradiation sensitivity between 50-500 J/m^2 (200 J/m^2 was shown as a representative in Fig. 2B). However, *caf5Δ* cells grew slightly slower at 1000 J/m^2 compared to the wild type cells (Fig. 2B). When 1000 J/m^2 UV light was applied, even the wild type cell growth could be observed well on spot tests, so streak onto plates was preferred to be able to place more cells on the plate and hence for better observation. The next DNA damaging agent checked was hydroxyurea. Exposure to different concentrations of hydroxyurea revealed no sensitivity of *caf5Δ* cells, they grew in a similar pattern with wild type cells (Fig. 2B). The results altogether indicate involvement of *caf5⁺* gene in stress response specifically induced by UV, but not hydroxyurea, KCl, $CaCl_2$ and sorbitol.

The Mitotic and Meiotic Progression in caf5Δ Cells

Previous research has shown that polyamines were involved in cell cycle regulation and polyamine biosynthesis and transporter gene mutations were associated with defects in cell cycle progression, as explained in the introduction. In *S. pombe,* polyamine deprivation was shown to cause disintegration of the nucleus and absence of septum (Chattopadhyay *et al.* 2002).

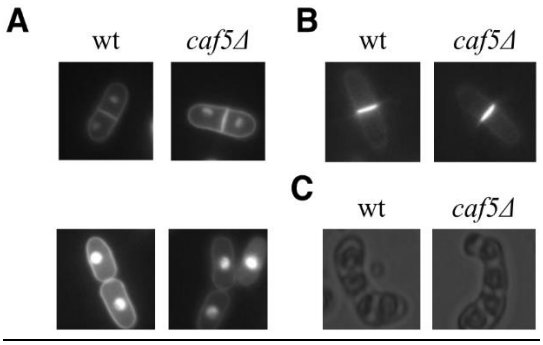

Fig. 3. Cell morphology analysis of *caf5Δ.* (A) Wild type and *caf5Δ* cells were observed under the microscope after DAPI staining to visualize the DNA. (B) Wild type and *caf5Δ* cells were observed under the microscope after calcofluor staining to visualize the septum. (C) Wild type and *caf5Δ* spores formed on the SPA medium

In an attempt to see any cell cycle defects and associated phenotypes, *caf5Δ* and wild type cells growing at optimum conditions were fixated and stained with DAPI and calcofluor to visualize DNA and septum formation. No defective phenotypes in *caf5Δ* cells at

different stages of the cell cycle were seen and the DNA seemed to segregate well in cell division (Fig. 3A). In addition, calcofluor staining showed proper septum formation (Fig. 3B).

Meiotic division was also checked in *caf5Δ* cells by observing spore formation. *caf5Δ* cells were incubated with the cells of the opposite mating type on SPA medium, upon which successful spore formation was observed (Fig. 3C).

Discussion

It is now well established that polyamines are involved in proper cell cycle progression in the cells. Consistent with the role of polyamines in both of these processes, polyamine biosynthesis enzyme ODC level is shown to oscillate during the cell cycle in *S. cerevisiae* (Kay *et al.* 1980), and polyamine depletion from the environment was shown to cause a number of cell cycle dependent phenotypes in *S. pombe* as mentioned before (Chattopadhyay *et al.* 2002). Cell cycle control is also known to be interconnected with stress response pathways. A very important checkpoint in the cell cycle is at the G1 stage, at which cells monitor the extracellular and intracellular environment for nutrients, growth factors, DNA damage etc. In the presence of stressors, cells are programmed to respond to these stress conditions and regulate the initiation of cell division accordingly. Similar to the cell cycle control, stress response is another process that includes polyamine function (Gevrekci, 2017).

Since polyamines contribute to many different functions in the cells including normal cell growth, polyamine levels in the cells are well regulated by redundant mechanisms such as *de novo* biosynthesis, interconversion, cellular uptake and release as well as degradation (Cohen 1998). Polyamine transporters are transmembrane proteins that drive influx and efflux of polyamines. They can be found in the plasma membrane as well as organelle membranes. *Schizosaccharomyces pombe* cells have a number of spermine and spermidine transporter genes with high sequence similarity. They share a major facilitator superfamily (MFS) domain in their protein sequence. Among these genes, *caf5*$^+$ codes for a spermine family transporter, which differs from the other spermine and spermidine transporters by not having a sugar transporter domain. This gene previously came up in a scan that showed its overexpression caused resistance to high doses of caffeine (Benko *et al.* 2004). In this study, we aimed to better understand the significance of *caf5*$^+$ in different cellular processes in *S. pombe*. Fission yeast *S. pombe* is a haploid organism in its normal cell cycle whose cell cycle defects can be easily observed. *caf5*$^+$ deletion mutants were created and scanned for different processes to reveal its involvement in normal growth, cell cycle and stress response, which are the conserved functions of polyamines in different organisms.

caf5Δ cells were very similar to wild type cells in many aspects such as their normal growth at optimum conditions. The viability and growth rate was not reduced in the absence of *caf5*$^+$, but a slightly faster growth could be observed at 8 hr timepoint, just before saturation in *caf5Δ* cell culture. This pattern of growth could be the result of a slightly faster cell division in *caf5Δ* cells. To better focus on the premature/faster cell cycle progression phenotype, the next experiment was designed to compare the cell size of *caf5Δ* and wild type cells. The cell size in *S. pombe* is a good indicator of premature or delayed cell division, as in the case of *wee1Δ* and *cdc25Δ* cells. The results showed that *caf5Δ* cells were shorter than the wild type cells. When slightly faster growth rate and shorter cell size phenotypes were considered together, it can be interpreted that *caf5Δ* caused earlier cell cycle initiation compared to wild type cells. This might be related to the function of polyamines in stress response and their potential to slow down the cell cycle initiation according to the environmental stress (Krüger *et al.* 2013, Gevrekci 2017).

Despite the potential effect of *caf5*$^+$ on cell cycle progression, *caf5Δ* cells showed no extreme abnormal phenotypes such as cut or nuc phenotypes or lack of septum formation. cut and nuc phenotypes are mostly induced upon anaphase defect in *S. pombe* that are seen in APC/C conditional mutants (Hirano *et al.* 1986). It is also noteworthy that in case of these phenotypes the cells could not go any further in the cell cycle and the cells are not viable. In case of *caf5Δ* cells, DAPI and calcofluor staining not only ruled out this possibility but also helped to visualize successful progression of different stages in the total population. In addition to the mitotic division, meiotic progression is also scanned by observing spore formation. *Schizosaccharomyces pombe* cells, which are haploid in normal life cycle, are known to arrest at G1 upon nutrient starvation, then mate with the opposite mating type to form diploid cells and finally form spores by meiotic division. Previous researches showed that a very low number of gene mutations (1% of the *S. pombe* genes) cause sporulation defects in *S. pombe*, unlike *S. cerevisiae* (Ucisik-Akkaya *et al.* 2014). For instance, *spo4* and *spo6* were the two genes whose mutations caused failure in the formation of four spores in sporulation media (Nakamura *et al.* 2000, 2002). *caf5Δ* cells were also checked for the presence of sporulation defect but they could successfully form four spores in SPA sporulation media, indicating proper meiosis.

Considering the significance of polyamines in resistance to environmental stressors, polyamine transporters are expected to contribute to stress response at least indirectly. Wild type and *caf5Δ* cells were exposed to different kinds of osmotic stress and DNA damaging agents to understand if loss of *caf5*$^+$ causes any sensitivity to stress. Osmotic stress was induced by KCl, $CaCl_2$ and sorbitol and *caf5Δ* cells could grow as well as wild type cells in the presence of osmotic stress. In case of DNA damaging agents, hydroxyurea and UV irradiation was used. The results showed that at high doses of UV irradiation, the viability of the *caf5Δ* cells was reduced,

but hydroxyurea did not cause a similar effect. The DNA damage caused by the UV irradiation is by the formation of dimers in the DNA, which halts the cell cycle at G1 or G2/M transition. In case of hydroxyurea, however, dNTPs are depleted, DNA replication slows down and cells cannot pass through G2/M transition. The results indicate the significance of *caf5*⁺ particularly in the UV-induced DNA damage response. The results of the stress response, in combination with cell size and growth rate analysis, might show the role of *caf5*⁺ gene in the regulation of the cell cycle regulation upon environmental stress. It can be speculated that *caf5*⁺ gene activity at G1 delays the initiation of cell division to give cells enough time for DNA repair and deletion of *caf5*⁺ could to some extend accelerate cell cycle. It is crucial to note that further research is needed to understand the cell cycle dependent activity of the transporters and their specific roles.

One of the biggest challenges of working with genes involved in polyamine transport is the fact that polyamine levels are kept under strict control by redundant mechanisms. Thus, lack of one transporter is compensated to some extent by other mechanisms. This is the reason for that deletion of polyamine transporter genes are viable at optimum conditions and might have weaker phenotypes. We have previously observed that *S. pombe* cells with mutations in spermine family transporter *SPBC409.08* only had a deviation in cell size with normal cell growth (Güngör & Örs Gevrekci 2016). A similar result was observed for *caf5*⁺, which showed small but consistent variation in cell size and cell growth. A previous study also showed that spermine family transporter SPBC36.01c was shorter in length compared to the wild type cells, as observed in *caf5*⁺*Δ* cells (Örs Gevrekci 2017). When the present and previous studies are considered together, another common phenotype between spermine family transporters and *caf5*⁺ turns out to be the fact that both deletion mutants showed sensitivity to UV. It is, however, notable that unlike spermine family transporters, *caf5*⁺ deletion did not induce any sensitivity to hydroxyurea (Örs Gevrekci 2017). Altogether, it can be concluded that polyamine transporters are involved in cell size control and DNA damage responses as redundant mechanisms. It is crucial to keep in mind that some of the phenotypes are shared between certain spermine and spermidine transporters, while each of the polyamine transporters can have unique contributions as shown by differing phenotypes at certain cellular processes.

In addition to the transporters, biosynthetic and degradation enzymes contribute to the polyamine homeostasis. Previous studies also revealed the interplay between synthesis, degradation and transport by showing that increase in polyamine levels negatively regulate polyamine synthesis and uptake in mammalian cells (Gesteland *et al.* 1999, Rom & Kahana 1994). A previous study on *S. pombe* polyamines showed that prolonged polyamine depletion from the environment was shown to induce a number of cell cycle dependent phenotypes (Chattopadhyay *et al.* 2002). This proves the significance of polyamines for the cells. The same study also showed that even a small amount of polyamine could restore normal cell growth and division. So, in summary, although they are involved in multiple crucial processes, cells have evolved different mechanisms for polyamine homeostasis, which in turn makes it harder to identify and characterize transporters/biosynthetic/degradation genes hard. As a future extension, combination of different mutants could reveal activatory and inhibitory pathways in polyamine homeostasis.

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Research Article

GENOTOXICITY AND ANTIOXIDANT ENZYME ACTIVITIES INDUCED BY THE CAPTAN FUNGICIDE IN THE ROOT OF BELL PEPPER (*Capsicum annuum* L. var. *grossum* L. cv. Kandil)

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Abstract: In this study, we investigated the toxic effects of the captan fungicide by using morphological, physiological and cytological parameters in bell pepper (*Capsicum annuum* L. var. *grossum* L. cv. Kandil) root tissue. The seeds of bell pepper were germinated in Petri dishes including different concentrations (0, 25 µM, 50 µM, 100 µM, 150 µM) of captan fungicide for 7 days. The germination rates and root lengths were significantly reduced in captan-treated seeds. All concentrations caused a significant decrease in mitotic index and increase in different types of chromosomal abnormalities such as c-mitosis and chromosome stickness in meristematic cells of bell pepper root. Captan treatment also induced oxidative stress by leading to membrane damage with an increase in root electrolyte leakage in 7 days-old bell pepper root. Catalase, glutathione reductase and total peroxidase activities increased under different concentrations as a response to oxidative stress. Our results showed that captan fungicide had negative effects on germination and growth in bell pepper seed.

Key words: Chromosomal abnormality, antioxidant enzyme activity, pesticide toxicity.

Özet: Bu çalışmada, dolmalık biberde (*Capsicum annuum* L. var. *grossum* L. cv. Kandil) kök dokusunda morfolojik, fizyolojik ve sitolojik parametreler kullanarak captan fungisitinin toksik etkisi araştırıldı. Dolmalık biber tohumları 7 gün boyunca farklı konsantrasyonlarda (0,25 µM, 50 µM, 100 µM, 150 µM) captan fungisit içeren petri kabında çimlenmiştir. Elde edilen sonuçlar, captanla muamele edilmiş tohumlarda çimlenme oranının ve kök uzunluğunun düştüğünü göstermiştir. Ayrıca, captan fungisitinin tüm konsantrasyonları, mitotik indekste önemli bir azalmaya ve biber kökünün meristematik hücrelerinde c-mitoz ve kromozom yapışkanlığı gibi farklı tipte kromozomal anormalliklerin artmasına neden olmuştur. Ayrıca, captan muamelesi, 7 günlük dolmalık biber kökündeki kök elektrolit sızıntısında bir artış ile membran hasarına yol açarak oksidatif stresi tetiklemiştir. Oksidatif stres ile başa çıkmak için katalaz, glutatyon redüktaz ve toplam peroksidaz aktivitelerinin dolmalık biber köklerinde farklı captan fungisit konsantrasyonu altında arttığı belirlenmiştir. Elde ettiğimiz sonuçlar captan fungisitinin dolmalık biber tohumundaki çimlenme ve büyümeyi olumsuz yönde etkilediğini göstermiştir.

Introduction

Pepper (*Capsicum annum* L.), a member of the Solanaceae family, is cultivated in different parts of the World and is an important vegetable with various fruit types known as bell-shaped, charleston, conic and long-green all which are consumed either as fresh, processed, pickled or powder (Dağistan *et al.* 2015). According to the production ratio, pepper is ranked the sixth among commonly grown vegetables in Turkey (Aytop *et al.* 2014). However, yield and quality of pepper is decreased by many diseases that cause great losses in its production (Abou-Zeid *et al.* 2016). Many soilborne fungal root rot and wilt pathogens such as *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tassi) Goid, *Fusarium oxysporum* Schlecht. *and F. solani* (Mart.) Sacc. have been reported to be widespread and attack pepper roots and stems causing severe losses in seed germination, plant growth and yield (Güney & Güldür 2018).

Fungicides are used to protect agricultural products against fungal infections in seed, root, shoot and leaves of plants. Members of the dicarboximide from the oldest groups of fungicides have been frequently used in these treatments since 1949s (Thomson 1997). Captan is a dicarboximide and phthalimide member non-systemic fungicide. It is one of the most commonly used fungicides in seed treatment and used to protect crops, vegetable and fruit from fungal diseases caused by pathogens such as *Phytophthora infestans* (Mont.) de Bary and *Botrytis cinerea* Pers.

Excessive use of pesticides not only poses risk for soil, water and air but also deleteriously affects non-target

organism including humans, animals and plants (Parween *et al.* 2016). Many studies showed that indiscriminate use of pesticides results in undesirable consequences one of which is induction of genetic damage on plant cells (Aktar *et al.* 2009). The most important effect of pesticides is their genotoxic, mutagenic or cytotoxic roles in non-target organism (Çavuşoğlu *et al.* 2011; Verma & Srivastava 2018). The alkylating abilities of pesticides break DNA and cause damages in DNA replication (Kaur *et al.* 2011). Pesticides also lead to mitotic disorders such as abnormal chromosomes, micronucleus formation, chromosomal bridges and polyploidy (Iqbal *et al.* 2019). Studies revealed that pesticides caused a wide range of genotoxic effects in *Allium cepa* L. (Türkoğlu 2012, Karaismailoğlu 2017), *Vicia faba* L. (Singh *et al.* 2013) *Helianthus annuus* L. (Karaismailoğlu 2014) and *Lycopersicon esculentum* Mill. (Akpınar 2014).

Pesticide toxicity can deleteriously affect various metabolic processes by inhibition of germination, retardation of growth, reduction in photosynthesis and alteration of nitrogen/carbon metabolism (Dias 2012). At cellular level, high pesticide concentrations induce oxidative damage which result in accumulation of reactive oxygen species, injury and increase ion leakage of the cell membrane (Parween *et al.* 2012). Plants are able to develope multiple complex enzymatic antioxidant system including catalase (CAT), peroxidase (POX) and glutathione reductase (GR) to protect themselves against harmful effects of oxidative stress.

In the present study, we investigated the potential genotoxic effect of the captan fungicide on bell pepper *Capsicum annuum* L. var. *grossum* L. cv. Kandil by determining mitotic index and chromosomal and mitotic aberrations in root meristems. The toxic effects of captan were also evaluated by considering the alterations in some growth parameters (root length, germination rate and protein content), root electrolyte leakage and antioxidant enzyme activities.

Materials and Methods

Experimental Design

The test substance Captan (N-triklorometilmercapto-4-siklohekzen-1)(IUPAC Name: $C_9H_8Cl_3NO_2S$, CAS No: 133-06-2, molecular weight: 300, 59 g/mol, purity of 99,6 %) was purchased from Pestanal (32054) (Steinheim, Germany) and prepared by ultrapure water. 500 µM stock solution was stored in the dark and under +4°C. The seeds of *Capsicum annuum* (2n = 24) were purchased from a seed company (Agrogen) operating in Tekirdağ, Turkey. All chemicals were obtained from Sigma Aldrich (St. Louis, MO).

The seeds were sterilized in 1% sodium hypochlorite and imbibed in deionized water for 6 hours. The imbibed seeds were kept on Petri dishes (9 cm diameter) containing different concentrations (25 µM, 50 µM, 100 µM, 150 µM) of captan solutions for 7 days at 25°C under dark conditions in an incubator. The seeds of the control group were kept in deionized water.

EC50 Determination

Bell pepper seeds were primarily treated with different concentrations of captan ranging from 12.5 µM to 200 µM for EC50 determination. Test concentrations which caused 50% reduction in root length in comparison with the control group were designated as EC50. EC50 values for captan was 25 µM and 150 µM. After EC50 determination, four different concentrations (25 µM, 50 µM, 100 µM, 150 µM) were selected for the test applications. In the selection of the experimental concentrations, the doses used by local farmers in agricultural fields were considered.

Germination Rates and Root Growth

The number of germinating seeds and the root length was determined at the end of the 7^{th} days following applications.

Mitotic Index and Assays

The root tips were randomly collected from each Petri plate in triplicate for cytological studies. Primary roots were fixed in freshly prepared Carnoy's solution (acetic-ethanol: 1:3, v/v) in separate vials. Squash preparations were made using 2% aceto-orcein. For each replicate, about 1000 cells were examined in roots and analyzed with respect to mitosis and chromosomal aberrations. For each treatment, mitotic index was calculated as a percentage by the ratio of dividing cell number to total cell number. Chromosomal abnormalities were counted in prophase, metaphase, anaphase and telophase stages and expressed as a percentage of the total number of abnormalities in the dividing cells.

Electrolyte Leakage

Electrolyte leakage from fine roots (REL) was determined using the relative conductivity method of Wilner (1955). Roots were washed three times with deionized water to remove surface ions. Each root sample was put into 28 mL glass bottles containing 16 mL deionized water of a known conductivity. The sealed bottles were left at room temperature for 24 h after shaking. The conductivity of the solution in the bottle which was shaken again was measured using a conductivity probe with in-built temperature compensation. After samples were autoclaved at 110°C for 10 mins, it was cooled to room temperature and the total conductivity was measured for each sample. The 24 h conductivity was expressed as a percentage (%).

Analysis of Antioxidant Enzyme Activities

Root samples were extracted in 0.05 mM potassium phosphate buffer (pH: 7.8) containing 1 mM EDTA and 2% PVPP (Polyvinylpyrrolidone). The homogenate was centrifuged at 14.000×*g* for 30 min at +4°C, and the obtained supernatant was used in determination of protein and enzyme activity. All spectrophotometric analyses were conducted on Epoch 2 Microplate Spectrophotometer (United States). The soluble protein content was determined by Bradford (1976) method using bovine serum albumin as a standard. Total peroxidase activity was

assayed by following the increase in absorbance by oxidation of 3,3-diaminobenzidine tetrahydrochloride (DAB) at 465 nm, according to the method of Herzog & Fahimi (1973). Catalase (CAT, EC 1.11.1.6) activity was analyzed by measuring the rate of decomposition of H_2O_2 at 240 nm, as described by Bergmeyer (1970). Glutathione reductase (GR, EC 1.8.1.7) activity was measured by following the change in 340 nm as oxidised glutathione (GSSG)-dependent oxidation of NADPH, according to the method of Foyer & Halliwell (1976).

Statistical Analysis

All experimental data were analyzed using the mean ± standard deviation values of at least 5-10 replicates. The data is suitable for normal distribution according to Shapiro-Wilk test. The significance of differences between the mean values were determined by a one-way ANOVA followed by Tukey Post Hoc Test analysis. All analyzes were performed on GraphPad Prism version 5.2 for Windows (GraphPadSoftware, San Diego, CA).

Results

The Effect of Captan Fungicide on Seed Germination and Root Growth

The effect of captan fungicide on germination percentage and root length were given in Table 1. 25, 100 and 150 µM concentrations caused approximately 32, 55 and 66 % reduction in seed germination, respectively. On the other hand, 50 µM captan treatment significantly increased germination rate when compared to the control. The root length values significantly decreased by approximately 40, 59, 72 and 74 % with 25, 50, 100 and 150 µM/L captan treatments, respectively. 150 µM/L treatment caused the highest inhibition on germination and root length in bell pepper (Table 1).

The Effect of Captan Fungicide on Genotoxicity

The genotoxic effects of the captan fungicide were evaluated by the mitotic index and the percentage of chromosomal abnormalities in root meristems. The results of the mitotic index (%) and chromosome abnormality under fungicide treatments were shown in Table 2. Mitotic activity in root meristem cells gradually decreased with increasing captan concentrations. The highest inhibition of mitotic activity (38%) was observed in 150µM captan treatment. The microscopic investigations revealed that captan treatments induced various chromosomal aberrations in mitosis phase in root meristem cells (Fig. 1). The fungicide treatments reduced cell division frequency in comparison with the control and the maximum inhibition was determined in 150 µM treatment. The treatments also significantly affected the anaphase among other phases. The total abnormality frequency increased by 35, 35, 49 and 59% at 25, 50, 100 and 150 µM/L treatments, respectively. The types of chromosome abnormalities were indicated according to sticky chromosome, lagging chromosome, multipolarity, fragment, c-mitosis, chromosome bridge and binucleated (Table 2). When compared to the control, sticky chromosome and multipolarity were commonly observed in dividing cells following captan treatments (Table 2).

The Effect of Captan Fungicide on Membrane Damage

The root electrolyte leakage (REL) increased significantly, when compared to the control, by 37, 57, 94, 105 and 111,08 % in 25, 50, 100 and 150 µM captan treatments, respectively (Fig. 2).

The Effect of Captan Fungicide on Antioxidant Enzyme Activities

Captan fungicide negatively affected protein content in bell pepper root tissues. The increasing treatment concentrations decreased protein content (Fig. 3) and the reduction was significant for 100 and 150 µM/L treatments. We investigated POX, CAT and GR activities in roots treated with different fungicide concentrations (Fig. 4). POX activity increased by 26, 29, 68 and 80% at 25, 50, 100 and 150 µM/L treatments, respectively. CAT and GR activities significantly increased with only 150 µM/L treatment by 17 and 32%, respectively (Fig. 4).

Table 1. The effect of different concentrations of captan fungicide on germination rate (%) and root length (cm) in bell pepper. Data are means (±) standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control as revealed by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

Treatment	Germination (%)	Root Length (cm)
Control	15.66±0.54	1.62±0.34
25 µM/L	10.63*±0.55	0.98*±0.28
50 µM/L	58.1*±0.47	0.67*±0.11
100 µM/L	7.10*±0.51	0.46*±0.13
150 µM/L	5.33*±0.49	0.42*±0.12

Table 2. Mitotic index and the frequency of chromosome abnormalities in the root meristematic cells in bell pepper treated with different concentrations of captan (S=Sticky chromosome; L=Lagging chromosome; M=Multipolarity; F=Fragment; C=C-mitosis; B=Bridge; BN=Binucleated). Data are means (±) standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

Concentration	MI (%)	Dividing Cell	Abnormality							
			S	L	M	F	C	B	BN	Total Abnormality Frequency (%)
Control	12.55 ± 0.74	127	2	-	-	-	-	-	-	1.64 ±1.17
25 µM	9.6* ± 1.09	107	20	-	9	1	4	-	3	34.58* ± 2.21
50 µM	9.53* ± 1.62	102	14	6	12	1	-	2	1	35.29* ± 3.12
100 µM	7.82* ± 0.97	81	19	2	5	2	3	1	8	49.38* ± 2.42
150 µM	7.73* ± 1.99	78	21	3	11	1	5	-	5	58.97* ± 3.53

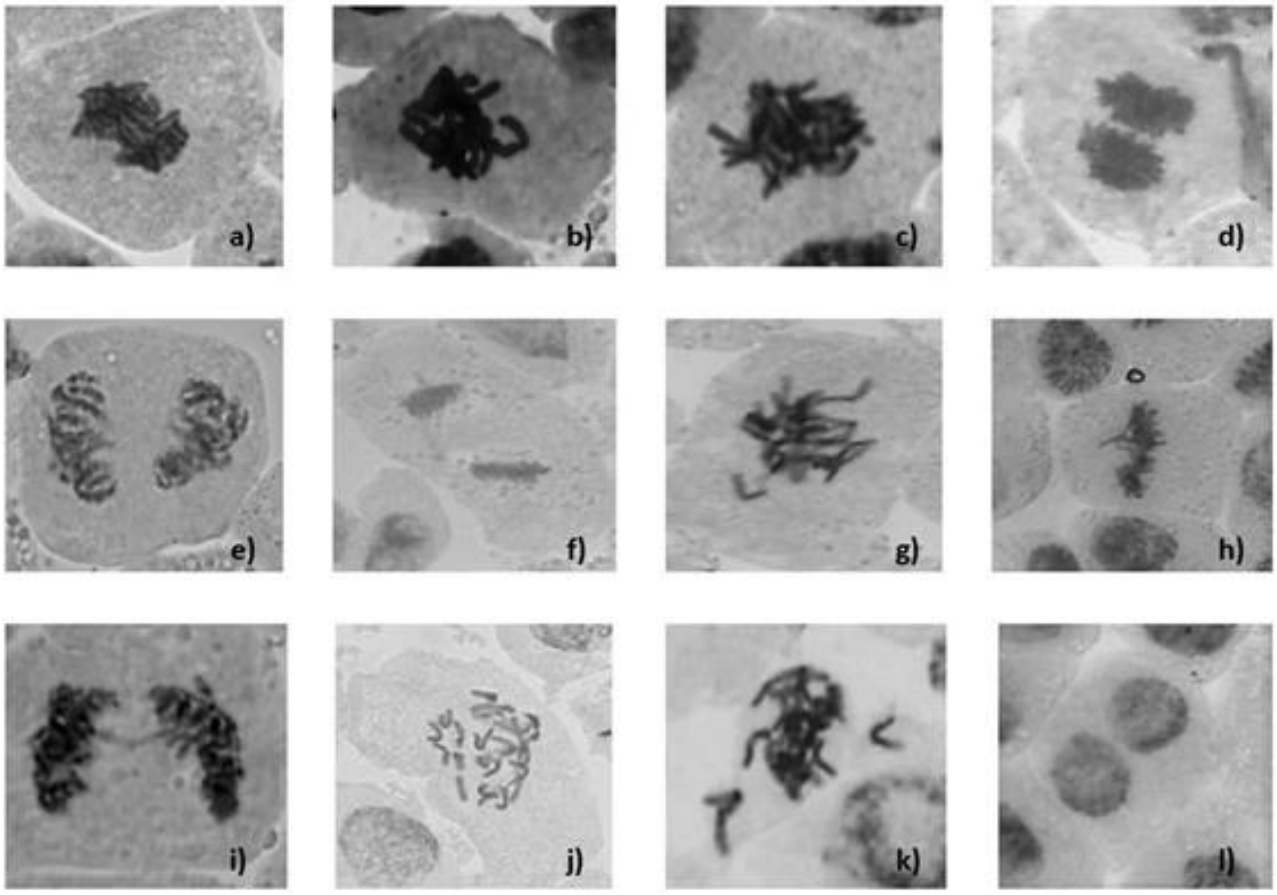

Fig. 1. Some chromosomal abnormalities which were seen in the root meristem cells in bell pepper following captan treatments of different concentrations. a-d) Stickiness; e-f) Multipolarity; g-h) Lagging Chromosomes; i) Bridge; j) C-mitosis; k) Fragmentation; l) Binucleated cell.

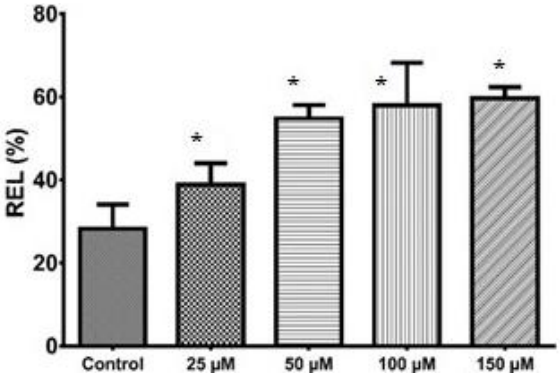

Fig. 2. The effects of different concentrations of captan fungicide on REL (%). Bars represent standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control as revealed by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

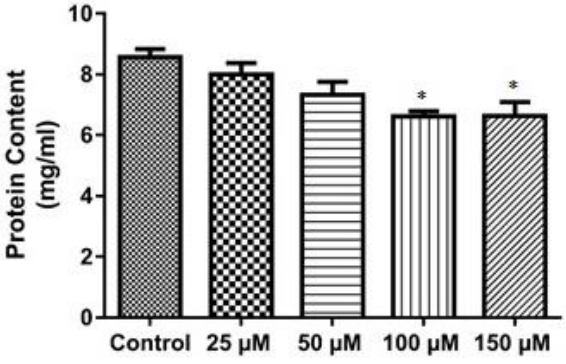

Fig. 3. The effects of different concentrations of captan fungicide on soluble protein content in bell pepper root. Bars represent standard deviations (SD). (*) represent adjusted p values ($p<0.0001$) for statistically significant differences from the control as revealed by one-way ANOVA analysis followed by Tukey Multiple Comparison Test.

Discussion

Random, excessive and unconscious use of pesticides have recently been an important pollutant for the environment (Parween *et al.* 2016). The soil is the first compartment affected by the pesticide toxicity, which in turn leads plant root sytems to be directly or indirectly influenced from pesticide related pollution. Thus, it is important to understand effects of pesticides on root systems in plant development. In the first step, pesticide toxicity causes an inhibition on seed germination and reduction on growth and development. In this study, we showed that the captan fungicide negatively influenced seed germination and root growth in bell pepper. This finding revealed that the fungicide had a toxic effect for bell pepper germination and root growth when used at concentrations from 25 to 150 µM. The reduction in

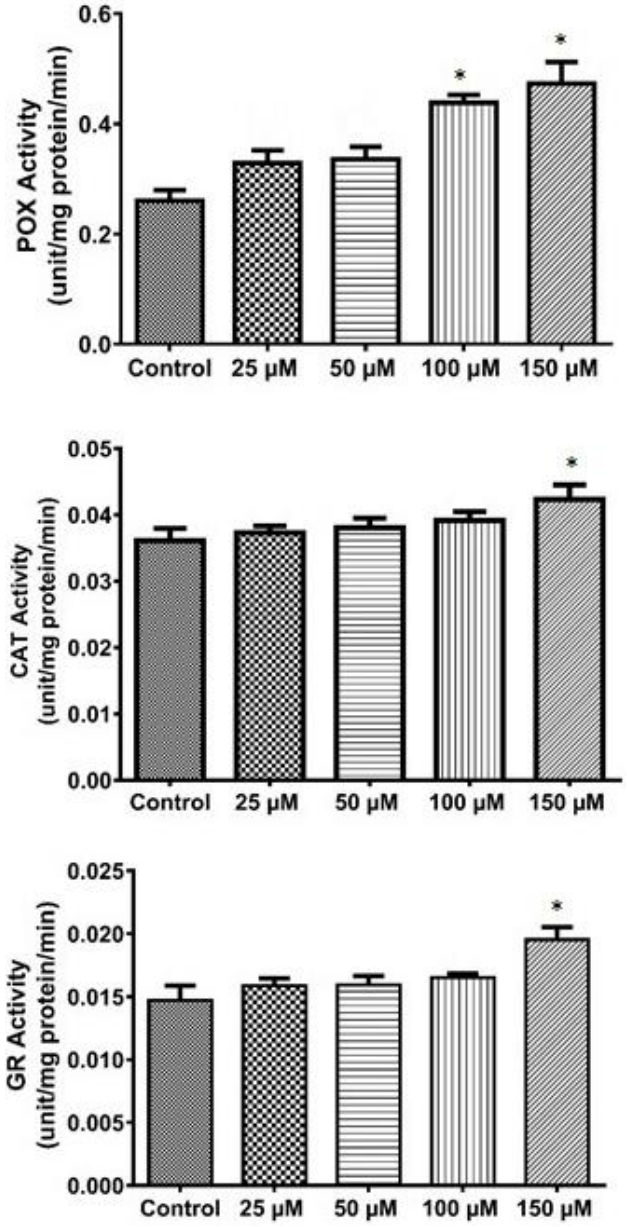

Fig. 4. The effects of different concentrations of captan fungicide on POX, CAT and GR activities in bell pepper root. Bars represent standard deviations (SD). (*) represent adjusted p values for statistically significant differences from the control as determined by one-way analysis of ANOVA followed by Tukey Multiple Comparison Test. POX activity (*): $p < 0.001$, CAT activity (*): $p < 0.01$, GR activity (*): $p < 0.006$.

germination may be explained by inhibition of important enzymes like amylase and protease, inhibition of imbibition of water and inhibition of mobilization of sugar (Gange *et al.* 1992). You & Barker (1997) showed that root fresh weight of tomato plants were decreased by 47% after 6 days of glufosinate herbicide treatment. Karaismailoğlu & İnceer (2017) determined that the insecticide deltamethrin decreased root growth in sunflowers (*Helianthus annuus*). Another study revealed that increasing concentration of tricyclazole and thiabendazole fungicides caused inhibition of germination in the tropical crop plant *Trigonella foenum - graecum* L (Mahapatra *et al.* 2019).

Mitotic index is commonly known as an indicator for determination of cytogenetic damage under stressful conditions. In this study, different concentration (25, 50, 100 and 150 µM) of captan fungicide significantly reduced the mitotic index in the root meristem of bell pepper as compared to their controls (Table 2). Pesticides may cause to inactivation of the cell cycle specific proteins and inhibition of DNA synthesis enzymes such as DNA polymerase and hindering in the G2-phase of the cell cycle, preventing the cell from entering mitosis (Mahapatra *et al.* 2019). Singh *et al.* (2013) determined that mitotic index and induction of chromosomal abnormalities increased under application of different concentration of alphamethrin and endosulfan insecticides in the meristematic cells of *V. faba* roots. Gill & Shaukat (2000) observed that mitotic index reduced by application of 5, 10, 20 and 40 ppm captan fungicide in meristematic cells of *A. cepa*.

Agrochemical toxicity directly leads to genotoxicity in many plant species. In recent studies, *A. cepa* and *V. faba* have commonly been used for indicating the genotoxic effect of pesticide usage. These studies showed a decrease in mitotic index and an increase in chromosomal abnormalities (Bonciu *et al.* 2018). The structural changes in the chromosomes are explained by alteration in the organization of histone and other proteins. These negative effect of protein organization cause changes on structure and adhesiveness of nuclear chromatin (Kurás 2004). Besides, stickiness can be formed by reaction of pesticides with DNA or proteins (Aksoy & Deveci 2012). Our result indicated that the captan fungicide had a harmful effect on chromosome structure by enhancement of sticky and multipolarity in meristematic cells of bell pepper root. The stickiness of chromosomes may cause incomplete separation of sister chromosomes as a result of cross-linkage chromoproteins (Aksoy & Deveci 2012). Gill & Shaukat (2000) indicated that captan fungicide led to chromosomal aberrations including chromosome stickiness, anaphasic bridges, and distribution of prophase in *A. cepa* cells. Aksoy & Deveci (2012) reported that Pomarsol Forte WP 80 fungicide triggered an increase of chromosomal abnormalities in soybean (*Glycine max* L.).

Excessive use of pesticides has triggered the induction of oxidative stress due to the production of reactive oxygen species (ROS) in many plant species (Yüzbaşıoğlu & Dalyan 2019). ROS accumulation damage cell membrane structure via changing the composition of the lipid bilayer and its result in leakage of potassium so electrolyte leakage is associated with membrane damage and potassium efflux in the cells (Demidchik *et al.* 2014). The present study revealed that different concentrations of captan induced oxidative stress by enhanced REL in bell pepper root. Similarly, different concentrations of monocrotophos insecticide causedan increase in electrolyte leakage in *Azolla microphylla* Kaulfuss (Raja *et al.* 2012).

Plants can response to oxidative stress via induction of antioxidant enzyme systems including POX, CAT and GR. These enzymes play a role for scavenging of the member of highly toxic ROS such as H_2O_2 and superoxide radical (Yüzbaşıoğlu *et al.* 2017). Our result showed that CAT, POX and GR were induced in 150 µM/L captan-treated root of bell pepper. In addition, all concentrations of captan enhanced the activity of POX enzyme in bell pepper root. POX enzyme is highly sensitive to pesticide application in plants because it plays a role in response to pesticides by detoxifying the pesticides and eliminating H_2O_2 in plants (Yüzbaşıoğlu & Dalyan 2019). Our previous study determined that thiram fungicide treatment caused oxidative stress. Further, it increased the antioxidant enzyme activities including CAT, POX and GR in tomato seedlings (Yüzbaşıoğlu & Dalyan 2019). Another study has indicated that mancozeb and chlorpyrifos increased CAT and POD activities in *A. cepa* seedling in a time and concentration-dependent manner (Fatma *et al.* 2018).

Conclusion

Despite the fact that use of a wide range of fungicides have risen productivity of crops, fungicides have also harmful effects on plant growth and development. As revealed by our results, the captan fungicide caused genotoxic effects in meristematic cells of bell pepper. Also, its toxicity has affected the membrane permeability and antioxidant enzyme activities in bell pepper root. These findings can contribute to increasing knowledge about the side effects of pesticides and it may help to develop a new perspective to minimize the destructive effects of pesticides in plant growth, development, and yield.

Acknowledgement

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Research Article

COMPARATIVE MORPHOLOGICAL AND ANATOMICAL STUDIES ON *Iris peshmeniana* Güner & T. Hall. AND *Iris aucheri* (Baker) Sealy (IRIDACEAE)

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Abstract: In this study, morphological and anatomical features of two morphologically similar species (*Iris peshmeniana* Güner & T. Hall. and *Iris aucheri* (Baker) Sealy) within the *Scorpiris* Spach subgenus (Juno iris) of the genus *Iris* L. were determined and similarities and differences between the two species were discussed. *Iris peshmeniana* is endemic to Turkey and its spreading area is protected because of exctinction threat. *Iris aucheri* is not endemic to Turkey and it has a limited distribution in the country. The cross-sections from root, scape and leaves and surface-sections from leaves of species were taken. Anatomical and morphological evaluations of the collected and obtained samples revealed differences in plant size, leaf number, width and length, periant tube length, fall length, standard structure, capsule length and width, bract and bracteole structure, length of style branches and testa structure of seeds and in the number of exoderma and cortex layers, margin structure of cortex paranchyma cells, structure of the root center cylinder, xylem strand number, status micropapillae in lower epiderma, layer number of palisade and spongy parenchyma and status of sclerenchyma cap. Although there were some differences in morphological and anatomical features, based on similar anatomical and morphological features revealed in the present study, it has been suggested that *I. peshmeniana* may be a subspecies of *I. aucheri*.

Key words: *Scorpiris* taxa, morphology, anatomy.

Özet: Bu çalışmada, *Iris* L. cinsinin *Scorpiris* Spach altcinsinde (Juno iris) morfolojik olarak benzer iki türün (*Iris peshmeniana* Güner & T. Hall. ve *Iris aucheri* (Baker) Sealy) morfolojik ve anatomik özellikleri belirlendi ve iki tür arasındaki benzerlikler ve farklılıklar tartışıldı. *Iris peshmeniana* Türkiye'ye endemiktir ve tükenme tehlikesi altında olduğundan onun yayılış alanı koruma altındadır. *Iris aucheri* Türkiye'ye endemik değildir ve ülkede sınırlı bir yayılışa sahiptir. Türlerin kök, skap ve yapraklarından enine kesitler, yapraklardan yüzeysel kesitler alındı. Toplanan ve elde edilen örneklerin anatomik ve morfolojik değerlendirmeleri, bitki boyu, yaprak sayısı, eni ve boyu, periant tüp boyu, dış tepal boyu, iç tepal yapısı, kapsül boyu ve eni, brakte ve brakteol boyu, stilus parçalarının boyu ve tohumların testa yapısı ve eksoderma ve korteks tabakalarının sayısı, korteks parankima hücrelerinin kenar yapısı, kök merkezi silindirin yapısı, ksilem kol sayısı, alt epidermadaki mikropapillaların durumu, palizat ve sünger parankimanın tabaka sayısı ve sklerenkima kümesinin durumundaki farklılıklarla ortaya çıkarıldı. Morfolojik ve anatomik özelliklerdeki bazı farklılıklara rağmen, bu çalışmada ortaya konan benzer morfolojik ve anatomik özelliklere dayanarak *I. peshmeniana*'nın *I. aucheri*'nin alttürü olabileceği öne sürülmüştür.

Introduction

The genus *Iris* L. is a different and crowded group of *Iridaceae* family in Turkey (Mathew 1984). Some species of the genus are used as ornamental plant because of their showy and fragrant flowers and some species which have various secondary metabolities in some organs, especially in rhizomes and bulbs, are used to treat many diseases (Baytop 1999, Fang *et al.* 2008, Sabrin *et al.* 2012, Kukula-Koch *et al.* 2015). Therefore, representatives of the genus are among significant geophytes both inTurkey and over the world.

The two taxa, *I. peshmeniana* Güner & T. Hall and *I. aucheri* (Baker) Sealy studied in the present study are placed in the subgenus *Scorpiris* Spach (Juno irises) of the genus. *Scorpiris* is represented in Turkey with 10 naturally growing taxa of which 5 are endemic to the country (Güner 2012). The subgenus is a different subgenus among other subgenera of *Iris* with its rootstock, bulbs, fleshy persistent roots, floral morphology, falcate leaves, bifacial leaf anatomy, reduced standards, well developed falls, polen morphology, and arils on the seeds (Rudall & Mathew 1993, Mathew 2001, Kandemir & Yakupoğlu 2016). The distributions of most *Scorpiris* members in Turkey are rather limited due to various reasons (ecological factors, fires, road and dam construction, human pressures, grazing, expansion of agriculture areas). For instance, *I. aucheri* is distributed only in the vicinity of Gaziantep and Diyarbakır. It is an endangered species because of dense dam and road constructions in this region and therefore needs an urgent protection. *Iris peshmeniana*, on the other hand, is distributed only around Malatya-Şakşak Mountains. Although *I. peshmeniana* is morphologically very similar to *I. aucheri*,

former differs from the latter with its creamy yellow flowers and less leaves. *Iris aucheri* has whitish to dark blue flowers and many leaves. *Iris peshmeniana* was first collected from Kubbe Passage of Şakşak Mountains and published as a new species in List of Turkey Plants (Vascular Plants) in 2012 (Güner 2012). Although there are irregular grazing and road widening activites in distributional area of *I. peshmeniana*, it has very strong growth potential. Even so, its distribution area is protected for future destruction. Since it has showy and nice flowers, it can potentially be used as an ornamental plant in future. As *Iris aucheri* has large bulbs and powerful plant structure, high young bulb growing ability, grows and reproduces fast in dry and wet areas and has beautiful colourful flowers and to be used in winter and in rock gardens and balconies (Usta 2002, Güner 2012).

The purpose of this study is to determine the similar and different morphological and anatomical features of the two species and to solve problems about them.

Materials and Methods

Iris aucheri was collected in April 2014 from natural populations in Şanlıurfa (Karaca Mountain) and Gaziantep (Sof Mountain) provinces at an altitude of 1100-1200 m. asl. *Iris peshmeniana* was obtained from Nezahat Gökyiğit Botanical Garden and was collected from Malatya-Pötürge, Kubbe Passage of Şakşak Mountains at 1855 m. asl. The localities where the specimens were collected are shown in Fig. 1.

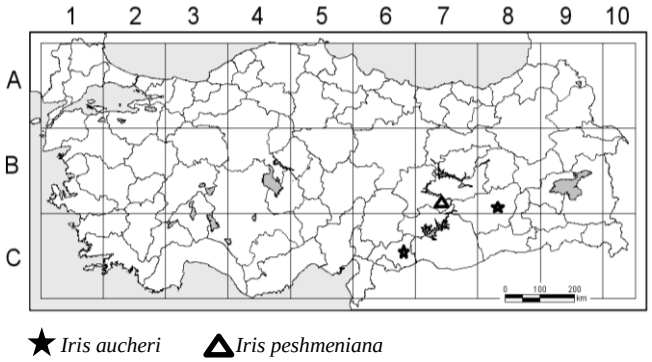

Fig. 1. Map of the region where the plant samples are collected

Taxonomic description of the taxa were made according to Mathew (1984) and Güner (2012). Fresh plant samples were fixed in 70 % ethyl alcohol solution and anatomical investigations were carried out using these samples. The cross sections of root, scape and leaves and surface section of leaves were taken and photographed by research microscope (Leica ICC50 HD). The sartur reagent was used for the cross and surface-sections of above and below-ground parts (Çelebioğlu & Baytop 1949). Anatomical and morphological measurements were made with a micrometric ocular and a ruler, respectively. The features considered for anatomical measurements are given in Table 1. Mean epidermal and stoma cells numbers per 1 mm^2 of surface section were determined (Table 2). Cell counts were obtained by using a research microscope. The stomata index was calculated according to the descriptions of Mesdner & Mansfield (1968).

Table 1. Anatomical features of studied *Iris* species (all dimensions are in µm).

Anatomical features	*I. aucheri*	*I. peshmeniana*
Root		
Width of epidermal cells	20-22	18-22
Layer number of exodermis	3-5	2-4
Layer number of cortex	18-22	13-15
Diameter of cortex cells	60-85	65-85
Margin structure of cortex parenchyma cells	apparent undulated	light undulated
Diameter of endoderma	20-25	22-26
Tracheae number of pith region	1	3
Xylem strand number	8-10	9-11
Trachea diameter	50-60	50-70
Dark contents	present	present
Scape		
Diameter of epidermal cells	20-22	22-26
Status of vascular bundles	scattered	scattered
Leaf		
Diameter of upper epidermal cells	54-56	48-53
Side wall structure of upper and lower epidermal cells	straight	straight
Cuticle thickness	9-13	10-15
Length of parenchyma cells	30-37	27-31
Width of parenchyma cells	25-31	23-26
Tracheae diameter	16-18	14-15
Diameter of lower epidermal cells	30-36	30-34
Micropapillae	light	dense
Keels	not apparent	extremely apparent
Structure and layer number of mesophyll	bifacial, 7-8	bifacial, 7

Table 2. Stomata measurements and stomata index for the studied *Iris* species. The lower surfaces of the leaves were considered for both species.

	Number of epidermal cells	Number of stoma cells	Stomata length (µm)	Stomata width (µm)	Stomata index (%)
I. aucheri	102	138	36-40	30-32	57.5 %
I. peshmeniana	108	133	38-42	33-35	55.2 %

Results

The results of anatomical and morphological measurements and the obtained measurement and index data related to stomata are given in Tables 1-3.

Morphological features

Iris aucheri

Plant 15-40 cm in length; bulb 2.5-3.5 cm in diameter, covered with papery tunics; storage roots fleshy; stem concealed by leaves at anthesis; leaves 5-12 well developed at anthesis, 25 × 2.4-4.3 cm, lanceolate, usually falcate, canaliculate, glabrous or minutely papillose, glossy green above, upper shorter and bract-like and pale at base (Fig. 2A); bract and bracteole long-acuminate, 5.1-6.3 × 1.6-2.4 cm and 5.8-6.3 × 1.5-2.5 cm, respectively; perianth tube 5-6.6 cm in length; flowers 3-6 deep blue to nearly white; falls 4-5.5 cm in length; claw winged, 2.5-3.5 × 2-2.4 cm, lamina orbicular or eliptic, with band of yellow hair along side of middle line, 1.5-2 × 1.4-2.3 cm undulate; crest prominent, erose-crenulate, yellow or cream, 2.3-2.5 mm; standards patent to deflexed, obovate, 2.1-3.4 × 0.5-1.3 cm; filament 1.2-1.6 cm in length; anther 1.0-1.2 cm in length; style branches 3.5-5 cm in length, pale blue; ovary narrow, 10-12 × 2-4 mm; capsule narrowly cylindrical, 6-7 × 1.2-1.4 cm, without beak; seeds 3 mm, rugose, dark brown.

Iris peshmeniana

Plant 9-16 cm in length; bulb 2.2-3.6 cm in diameter, long ovoid, tunic papery and dark brown; storage roots fleshy; stem hidden by leaves, unbranched, with 1-4 flowers (Fig. 2B); leaves 5-8, strongly falcate, canaliculate, linear lanceolate, lowermost leaves 7.5-15 ×1.7-3.7 cm, slightly undulate, regulary veined, pale green, glossy green above, margins white and smooth; bract and bracteoles equal, lower straw coloured and rounded, upper green and carinate; bracts 5.3-6.1 × 1.8-2.6 cm, lanceolate scabridulous at margin and very narrow transparent stripe; bracteole 5.7-6.5 × 1.7-2.3 cm, lanceolate, acuminate in flower; perianth tube 1.8-4 cm in length; flowers yellow with a yellow central patch and crest on falls; falls 4.3-4.6 × 2.3-2.8 cm, panduriform, creamy yellow with a large yellow patch; claw 2.7-3 × 2.3-2.8, winged with a 3 mm wide band of yellow hair; blade 1.5 × 1.8-2.2 cm, oblong, narrower than claw; crest 2.4 mm, crinkly and yellow; standards reflexed, 2.3-3.1 × 1.1-1.5 cm, spathulate to obovate, creamy yellow, rarely with a yellow line; filaments 1.5-1.8 cm in length; anthers 1.2-1.3 cm in length; style branches 4.1-4.5 × 1-1.6 cm, creamy yellow with a central yellow line; crests vertical to ascending, outer margins irregularly crenate; stigma bilobed, 0.3 × 0.6 cm; ovary 10-13 × 3-4 mm; capsule oblong triangular with rounded margins, 2.5-3.5 × 0.8 cm without beak, 30-42 with seed; seeds pear shaped, acute, dark brown, 0.3 × 0.2 cm, slightly rugose.

The root anatomical features

Iris aucheri

Epiderma is made of a single layer of small and rectangular shaped cells. Exoderma is 3-5 layered and large celled. Cortex parenchyma is multilayered (18-22 layered). Parenchyma cells are large, oval shaped and have dense dark contents. Margins of parenchyma cells are apparently undulated. Endoderma and pericycle are single layered and parenchymatic. The thickness in endodermal cells are three sided and oriented to pericycle. There is no thickness towards cortex. Xylem has 8-10 strands and phloem elements are obvious. In the pith region, there is one large trachea (Fig. 3A).

Iris peshmeniana

Epiderma is made of a single layer of large and rectangular shaped cells. Exoderma is 2-4 layered. Cortex is multilayered (13-15 layered) and parenchymatic. Parenchyma cells are large and oval or hexagonal shaped and have dense dark contents. Margins of parenchyma cells are lightly undulated. Endoderma is single layered and parenchymatic. The thickness in endoderma are three sided and oriented to pericycle. Pericycle is single layered, oval shaped and parenchymatic. Phloem is obvious, xylem has 9-11strands. There are metaxylem elements in the center of the root (Fig. 3B). The pith area is absent in the root. There are three large trachea in the pith.

The scape anatomical features

Iris aucheri

The cuticle is thick in the cross-section of scape. Epiderma is made of a single layer of small and square shaped cells. Papillae and micropapillae are seen on epiderma and cuticle, respectively. Cortex is composed of oval or circular shaped and large parenchyma cells (Fig. 4A). There are dense druses and rafita crystals and rare dark contents in the cortex parenchyma cells (Fig. 5A). Large and small vascular bundles are scattered. Xylem elements are in half-moon form. The vascular bundles in the pith are large, vascular bundles in the outer layers are small. The pith parenchyma cells are larger than the cortex parenchyma cells.

Iris peshmeniana

In the outer surface of the scape, there is a thick cuticle. Epiderma is made of a single layer of square shaped cells and with papillae. Cortex contains oval or circular shaped and large parenchyma cells. The sclerenchymatic cylinder in the cortex is not present. Vascular bundles are scattered in the cortex and central cylinder (Fig. 4B). The xylem and phloem elements are apparent in the vascular bundles. Xylem elements are in half-moon form. Bundle sheath is not obvious around vascular bundles. In the cortex parenchyma, there are rare dark contents, dense rafita and druses crystals (Fig. 5B). The pith consists of large, thin walled parenchyma cells

Fig. 2. General appearance of herbarium samples of *Iris aucheri* (A) and *Iris peshmeniana* (B). r: root, b: bulb, s: scape, l: leaf, f: flower.

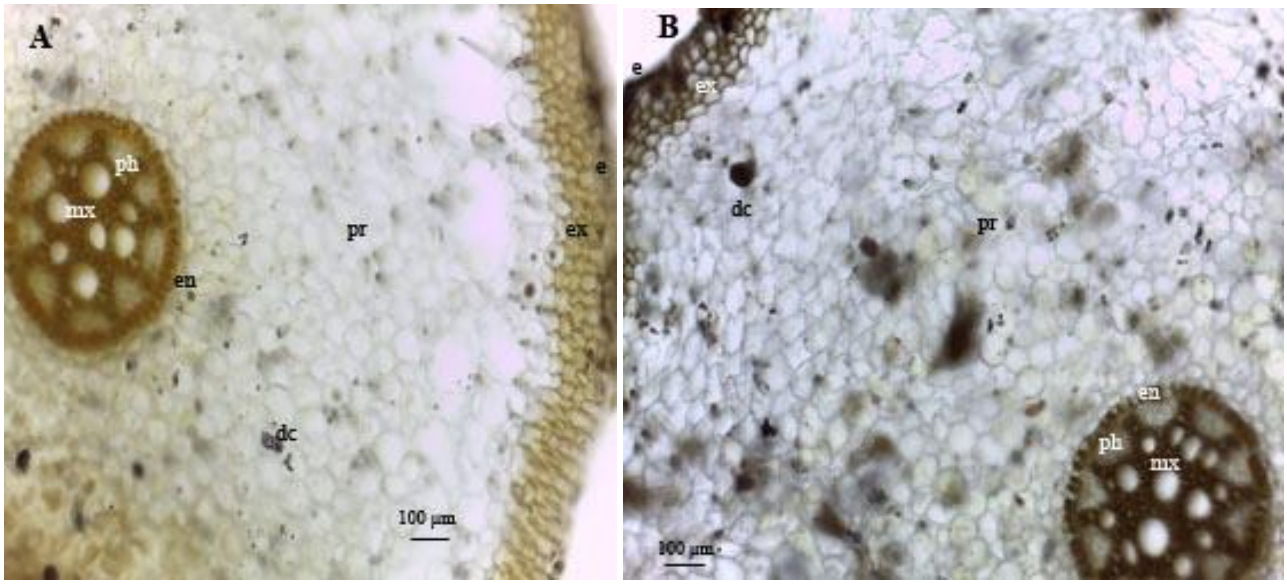

Fig. 3. Root cross sections of *Iris aucheri* (A) and *Iris peshmeniana* (B). e: epiderma, ex: exoderma, pr: parenchyma, en: endoderma, mx: metaxylem, ph: phloem, dc: dark content.

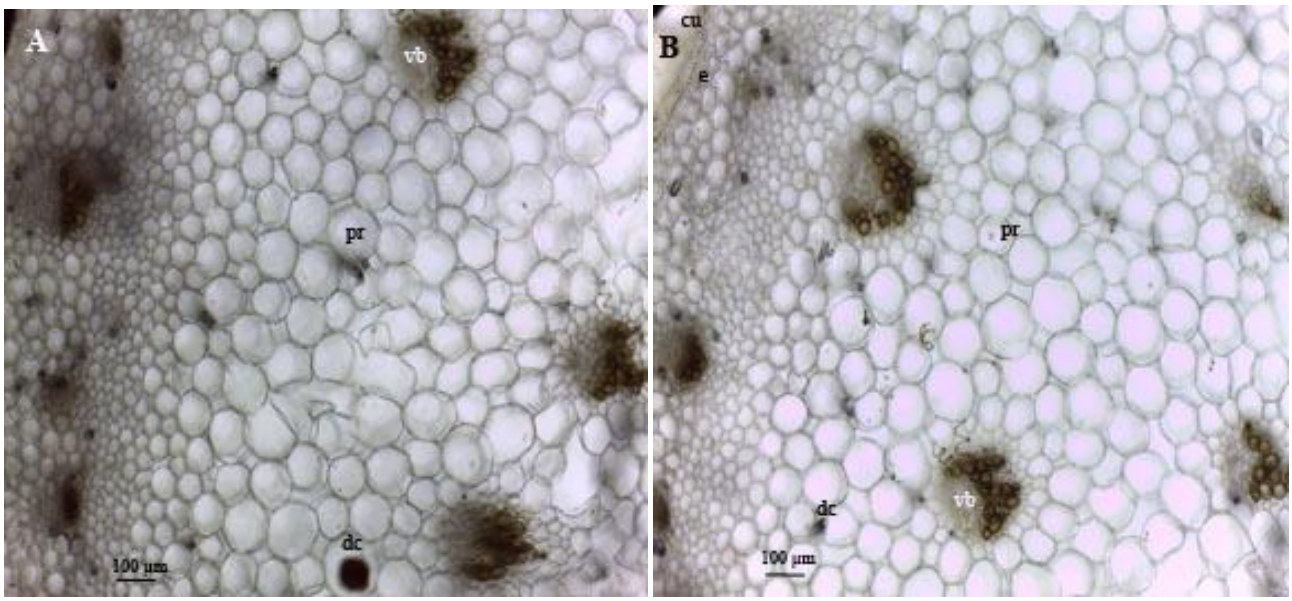

Fig. 4. Scape cross sections of *Iris aucheri* (A) and *Iris peshmeniana* (B). cu: cuticle, e: epiderma, pr: parenchyma, vb: vascular bundle, dc: dark content.

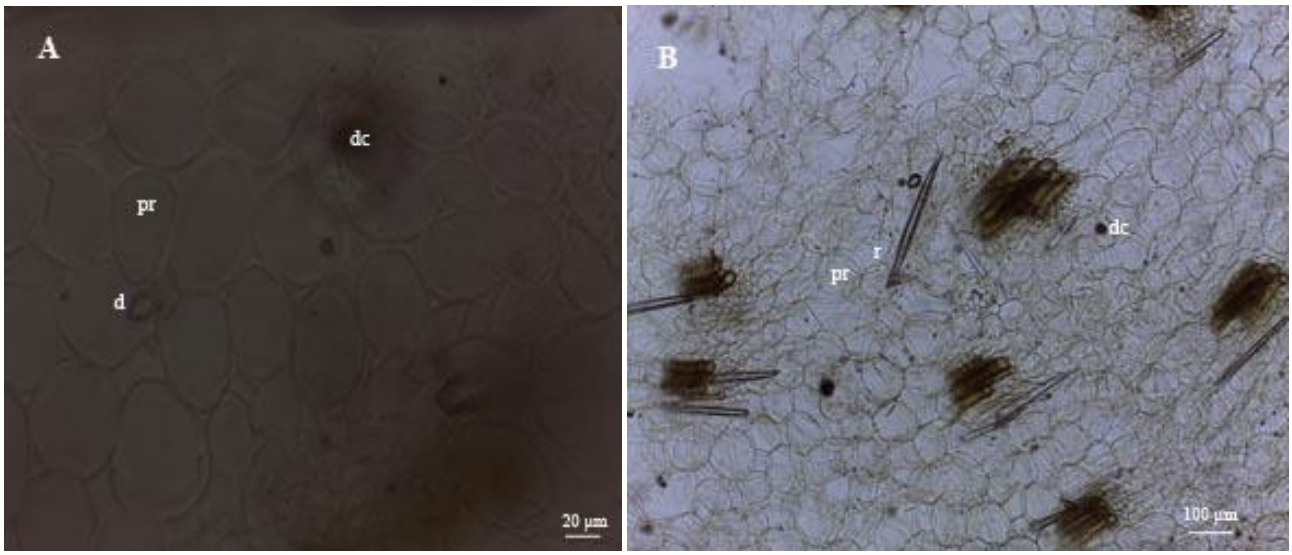

Fig. 5. Crystals in scape cross sections of *Iris aucheri* (A) and *Iris peshmeniana* (B). pr: parenchyma, d: druse crystals, r. rafita crystals, dc: dark content.

The leaf anatomical features

Iris aucheri

Leaf is in bifacial type (Fig. 6A). Upper epidermal cells are rectangular shaped, very large, single layered and have papillae. Cuticle layer is thick and it has micropapillae. Lower epidermal cells are rectangular shaped, small and have papillae. The upper epidema without stomata. Stomata are dense and large in lower epiderma (Fig. 7A). In the upper epiderma, there are dense crystal granules, rare hexagonal crystals and bulliform cells (Fig. 8A). Mesophyll is 7-8 layered with rare styloids. The palisade-like parenchyma is 3-5 layered in the upper epiderma. These cells are large and with dense chloroplast. The spongy-like parenchyma is 2-3 layered, oval shaped and with less chloroplast. Rare styloids are observed in the mesophyll cells. Vascular bundles are typically in a single row. Sclerenchyma cap is rarely seen at the phloem poles of vascular bundles, leaf margin and the keels in the lower epiderma. Keels are light round shaped and are not apparent. There are subadjacent marginal epiderma in large vascular bundles. At xylem pole of vascular bundles, sclerenchyma cap is not seen. There are oval shaped, large parenchyma cells lacking chloroplast at the xylem pole of vascular bundles.

Iris peshmeniana

Leaf is in bifacial type. Upper and lower epiderma are single layered, upper epiderma is with large square shaped cell and lower epiderma is small rectangular shaped cells. Papillae are seen on the upper and lower epiderma. Cuticle with micropapillae is thick. Dense micropapillae

present in lower epiderma (Fig. 6B). Stomata are not seen in the upper epidema. Stomata are large and frequent in the lower epiderma (Fig. 7B). There are more dense crystal granules, rare hexagonal crystals and bulliform cells in upper epiderma (Fig. 8B). Crystal granules in lower epiderma are rare. Bifacial mesophyll is 7 layered, the palisade-like parenchyma cells with dense chloroplast are 1-3 layered. The spongy-like parenchyma cells, which are large, oval shaped and with rare chloroplast, are 3-4 layered. There are rare styloids in the mesophyll. Sclerenchyma cap is found at the xylem pole of vascular bundles, leaf margin and the keels in lower epiderma. Keels are round shaped and extremely apparent. Mesophyll cells present between sclerenchyma cap and phloem of vascular bundles. Sclerenchma cap is almost adjacent to lower epiderma. In the mesophyll cells, rare styloids are found. At phloem pole of vascular bundles, sclerenchyma cap is not obtained. The subadjacent marginal epiderma is in large vascular bundles.

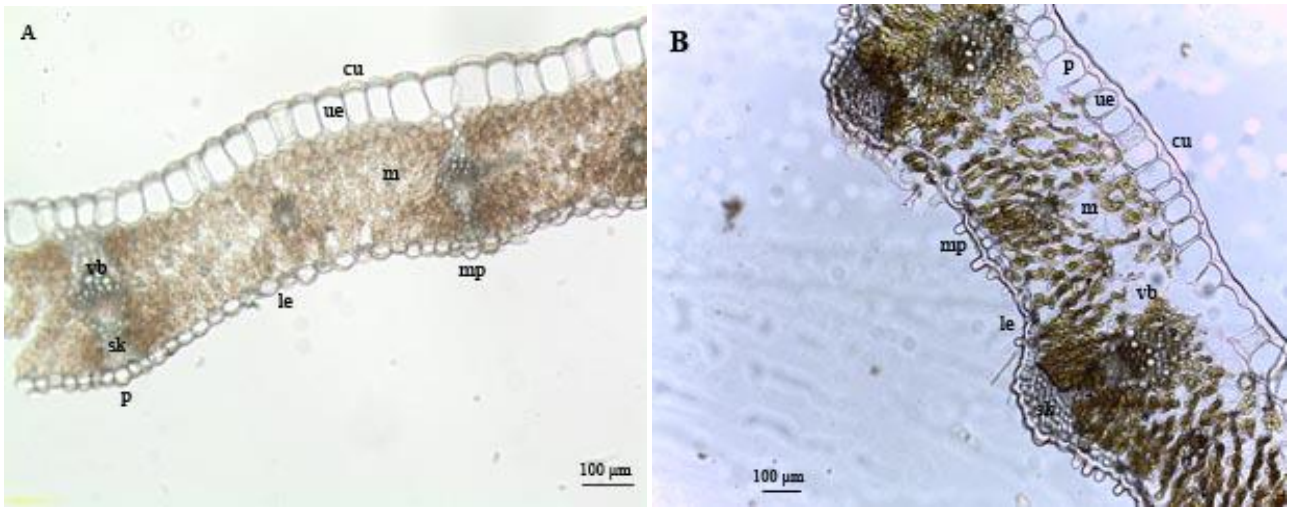

Fig. 6. Cross sections of leaves of *Iris aucheri* (A) and *Iris peshmeniana* (B). cu: cuticle, ue: upper epiderma, le: lower epiderma, m: mesophyll, vb: vascular bundle, p: papillae, mp: micropapillae, sk: sclerenchyma.

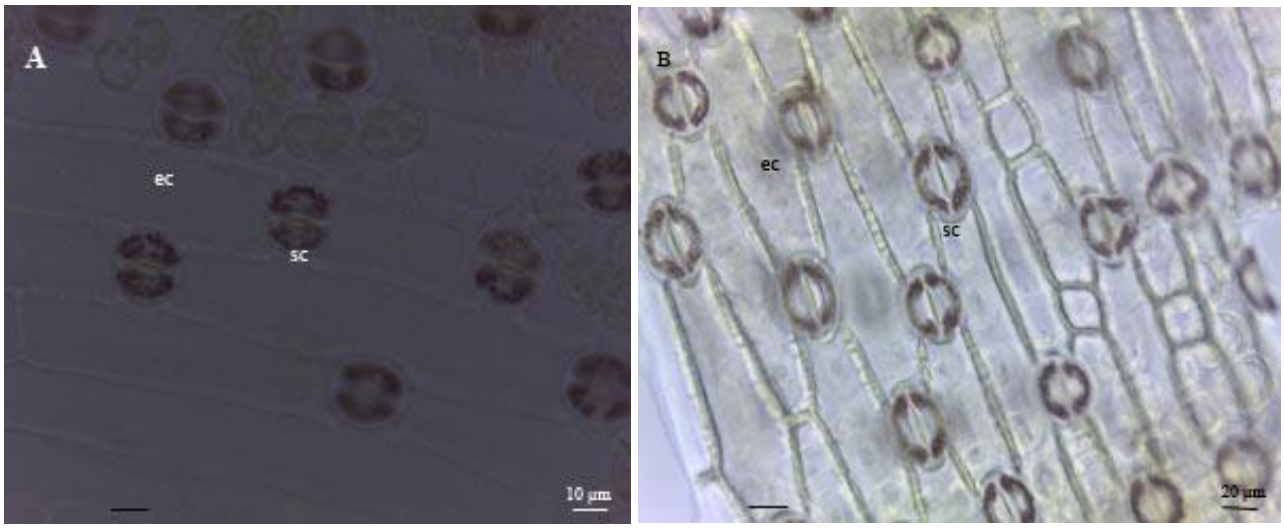

Fig. 7. Surface sections of leaves of *Iris aucheri* (A) and *Iris peshmeniana* (B). ec: epidermal cell, sc: stomata cell.

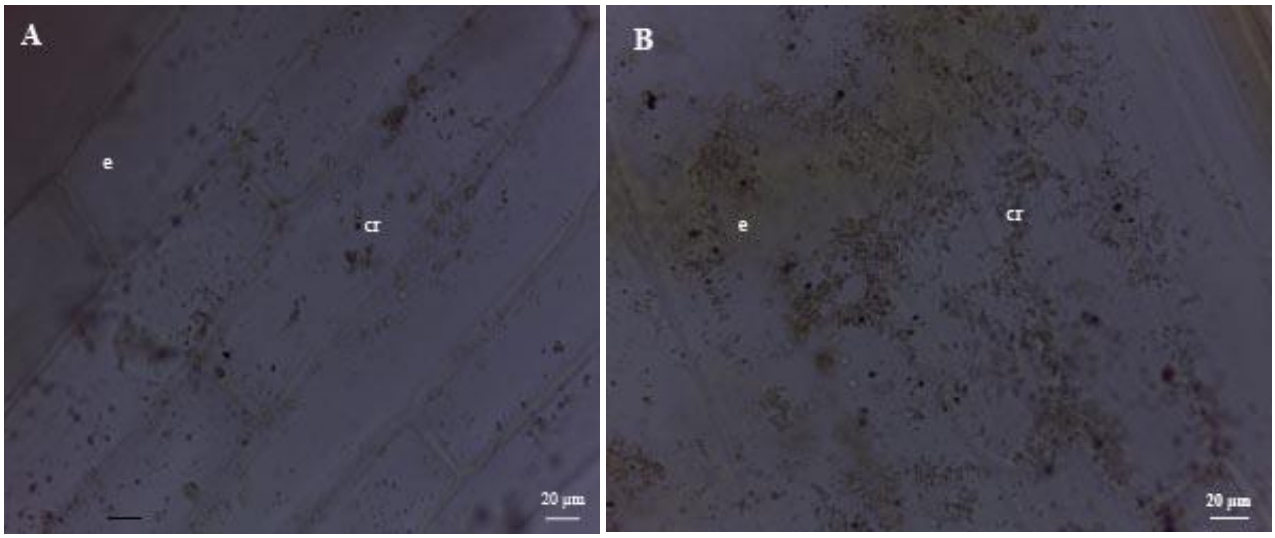

Fig. 8. Crystals in leaf cross sections of upper epiderma of *Iris aucheri* (A) and *Iris peshmeniana* (B). e: epiderma, cr: crystal granules.

Discussion

In this study, morphological and anatomical features of two endangered species of the subgenus *Scorpiris* in Turkey, *Iris aucheri* and *I. peshmeniana* were compared. The distinguishing anatomical features of taxonomic value were determined and given in Tables 1-2.Variations were seen in flower colours of the studied species and according to these variations, these taxa were reported as different species (Güner 2012). Variations were also found in some other flower features of other *Scorpiris* taxa in Turkey and the world (Mathew 2001, Guo 2015, Kandemir & Yakupoğlu 2016). The evolutionary significance of these different features in flower characters is been known yet. Moreover, the pollination biology of *Scorpiris* is little known. Due to the above situations, taxonomic problems of taxa placed within *Scorpiris* are still waiting to be solved. The present results showed that differences were also present between the studied species in plant size, leaf number, width and length, periant tube, fall length, standard structure, capsule length and width, bract and bracteole structure, length of style branches and testa structure of seeds. These differences may be an outcome of the effects of the differing distributions of the species. On the other hand, if there are also intraspecific differences in morphological features of the samples collected in different localities, it reveales that ecological conditions affect morphological features of the plants.

The problems in taxonomy of *Scorpiris* group are caused by changes in flower colours, shape and size of perigon segments of this subgenus species (Boltenkov 2016). *Scorpiris* is also a very different group within the genus *Iris* with its members having fleshy storage roots, different bulbs, well developed falls, reduced standards, petaloid style branches, different testa structure and bifacial leaf anatomy (Rudall & Mathew 1993, Mathew 2001, Dönmez & Pınar 2001, Hall *et al*. 2001, Kandemir &Yakupoğlu 2016). Despite these differences, this subgenus was not considered as separate genus by Tillie *et al*. (2000) who evaluated *Scorpiris* as a monophyletic group. On the contrary, Crespo *et al.* (2015) evaluated the Juno irises as a separate genus, according to recent taxonomic studies. On the other hand, there have been a lot of phylogenetic studies on *Iris* genus. However, data in phylogenetic studies are not enough to solve the relationships and problems in subgenera of the genus (Makarevitch *et al*. 2003, Wilson 2009, 2011, Guo & Wilson 2013).

In the anatomical study, differences were obtained in the number of exoderma and cortex layers, margin structure of cortex paranchyma cells and the root center cylinder. In the roots of *I. peshmeniana*, xylem strands, number of exoderma and cortex layers, margin structure of cortex paranchyma cells were 9-11, 2-4, 13-15 and light undulated, respectively. Xylem strands, number of exsoderma and cortex layers, margin structure of cortex paranchyma cells were 8-10, 3-5, 18-22 and apparent undulated in the root of *I. aucheri*, respectively. In the root pith region of *I. peshmeniana*, there is three large trachea. However, in the root pith region of *I. aucheri*, one large trachea is seen. The above mentioned root anatomical features may be used as distinguishing characters between the two taxa. The similar situation was found in the roots of some other *Iris* taxa by researchers (Nicolic & Mitic 1991, Mitic *et al*. 2000, Gontova & Zatylnikova 2013, Kandemir & Yakupoğlu 2016, Kandemir & Çelik 2017). No significant distinguishing features were found in the scape of the studied species. Meaning that, the two species are very similar to each other in terms of scape anatomical features.

Although the leaves of the studied species have bifacial type from anatomical point of view, some differences and simililarities were seen between the two species. The upper and lower epidermal cells of *I. aucheri* are larger than *I. peshmeniana*. In *I. aucheri*, the upper and lower epidermal cells are rectangular or square and rectangular shaped, respectively. However, the upper and lower epidermal cells of *I. peshmeniana* are square and

rectangular shaped, respectively. Micropapillae and papillae on cuticle and the upper epiderma of the two species are rare. In lower epiderma of *I. peshmeniana*, micropapillae are more conspicuous and denser than *I. aucheri*. Also, cuticle layer on both epiderma of *I. aucheri* is thinner than *I. peshmeniana*. The cuticles on both epiderma layers of *I. peshmeniana* are too thick. In the upper epiderma of *I. aucheri* and *I. peshmeniana*, dense crystal granules, rare hexagonal crystals and bulliform cells were found. However, dense crystal granules, hexagonal crystals and bulliform cells were not found in the lower epiderma of two species. Also, stomata were not seen in the upper epidermis, while stomata were seen dense and large in the lower epidermis of the two species. Stomata of *I. peshmeniana* are larger and rarer than *I. aucheri*. Mesophyll is 7-8 layered in *I. aucheri* and 7 layered in *I. peshmeniana*. The palisade and spongy-like parenchymatous cells are 3-5 and 2-3 layered in *I. aucheri*, respectively. But, the palisade and spongy-like parenchymatous cells are 3-4 and 2-4 layered in *I. peshmeniana*, respectively. In *I. peshmeniana*, sclerenchyma cap in the large vascular bundles is at the xylem poles of vascular bundles, leaf margin and the keels in the lower epidermis. Keels are round shaped and extremely apparent. There are mesophyll cells between sclerenchyma cap and phloem of vascular bundles. Sclerenchyma cap in large vascular bundles of *I. aucheri* is rare at the phloem poles of vascular bundles, leaf margin and the keels in the lower epiderma. Rudall (1991) in *Tigridieae* species, Kandemir (2015) in *Hermodactyloides* species, Kandemir & Çelik (2017) in three *Iris* species, Kandemir *et al*. (2019) in some *Scorpiris* species reported sclerenchyma cap at the phloem poles in leaf anatomy. Keels in lower epiderma are light round shaped and are not apparent and they are not quite frequent. The subadjacent marginal epidermis is seen in large vascular bundles of both taxa. The similar phenomenon was seen in leaf anatomy of *Tigridieae* and *Scorpiris* taxa (Rudall 1991, Celep 2011, Kandemir &Yakupoğlu 2016, Kandemir *et al*. 2019). Rare styloids are obtained in the mesophyll cells of the studied species. The bifacial mesophyll structure is evidently conspicuous in leaf anatomy of *I. aucheri*.

Although there are different morphological and anatomical features with some taxonomic value between the two species, they are very close to each other. Although no consensus has been made about the taxonomic status of *I. aucheri* and *I. peshmeniana*, we suggest that *I. peshmeniana* and *I. aucheri* are dependent species on the base of the morphological and anatomical features. In other words, *I. peshmeniana* should be a subspecies of *I. aucheri*.

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Research Article

ON NEWLY DISCOVERED DEUTONYMPHAL STAGE OF *Stigmaeus kumalariensis* Akyol & Koç (ACARI: STIGMAEIDAE) FROM TURKEY, WITH NUMERICAL AND STRUCTURAL VARIATIONS IN ADULTS

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Abstract: Deutonymph of *Stigmaeus kumalariensis* Akyol & Koç (Acari: Stigmaeidae) is herein described and illustrated based on specimens collected from litter and soil from Ekşisu marsh, Erzincan (Turkey). This is the first record of deutonymphal stage of *S. kumalariensis.* In addition, variations in the number of aggenital setae and structure of the median zonal shields in some adult specimens of the species are reported.

Key words: Ekşisu, immature, mite, *Stigmaeus*, variation, Turkey.

Özet: *Stigmaeus kumalariensis* Akyol & Koç (Acari: Stigmaeidae)'in deutonimf evresi, Erzincan'ın Ekşisu sazlığından toplanan döküntü ve toprak örnekleri içindeki bireyler üzerinden tanımlandı ve şekilleri çizildi. Bu, *S. kumalariensis*'in deutonimf evresinin ilk kaydıdır. Buna ilaveten, türün bazı ergin bireylerinde gözlenen, median zonal plağın yapısı ve aggenital kılların sayısındaki varyasyonlara da değinildi.

Introduction

Stigmaeidae is one of the most diverse mite families in Raphignathoidea with 33 genera one of which is *Stigmaeus* Koch (Fan *et al*. 2019, Khaustov 2019). The genus *Stigmaeus* is a large group with 147 described species (Fan *et al*. 2016, Bingül *et al*. 2017a, Khaustov *et al*. 2017, Doğan 2019a,b) of which 46 species have been recorded so far from Turkey (Erman *et al*. 2007, Doğan 2007, 2019a,b, Doğan *et al*. 2015a, 2016, 2017, Dilkaraoğlu *et al*. 2016a, Bingül *et al*. 2017a, Akyol 2019) and 24 of these species, including *Stigmaeus kumalariensis* Akyol & Koç, are known only from Turkey (Doğan & Ayyıldız 2003, Koç 2005, Akyol & Koç 2007, Dönel & Doğan 2011, Özçelik & Doğan 2011, Dönel *et al*. 2012, Doğan *et al*. 2015a, 2017, Uluçay 2015a-c, Bingül *et al*. 2017a, Doğan 2019a,b).

It is important to know morphological features of immature stages of mites as in many organisms for understanding history of developmental morphology. Most mite species are known only with adults, although immature instars show a diversity of characters and possess remarkable features potentially useful for understanding mite taxonomy, phylogeny and biology (Zhang 2018), making descriptions of immature stages important to know morphological diversity in all life stages. There are five different life stages - egg, larva, protonymph, deutonymph and adult - in members of Stigmaeidae (Fan & Zhang 2005, Fan & Flechtmann 2015). Recently, Doğan *et al*. (2019) reported presence of the third nymphal stage in Stigmaeidae. The deutonymphal stage can be distinguished from adults by absence of genital setae and fewer setae on some leg segments. *Stigmaeus kumalariensis* has been known so far with only females and males and no data have been published on its immature stages. In the present study, the deutonymphal stage of *S. kumalariensis* specimens collected from litter and soil has been revealed for the first time. We also reported variations in *S. kumalariensis* for the first time. Variations in the genus *Stigmaeus* have been observed so far in *S. elongatus* Berlese, *S. longiclipeatus* Doğan, Doğan & Erman, *S. longipilis* (Canestrini), *S. erzincanus* Doğan, Bingül, Dilkaraoğlu & Fan, *S. solidus* Kuznetsov, *S. bifurcus* Bingül, Doğan & Dilkaraoğlu and *S. miandoabiensis* Bagheri & Zarei (Dilkaraoğlu *et al*. 2016b, Doğan *et al*. 2016, 2017, 2019, Bingül *et al*. 2017a,b). Our results also contributed to the knowledge about variations in the genus *Stigmaeus*.

Materials and Methods

Specimens of *S. kumalariensis* were collected from litter and soil in Ekşisu, Erzincan located 11 km to the east of Erzincan Province. The specimens were extracted by using Berlese-Tullgren funnels, cleared in 60% lactic acid and mounted on microscopic slides in Hoyer's medium. The specimens were examined by using a Leica DM 4000B phase-contrast microscope. The photographs of the specimens were taken by an Olympus BX63-CBH DIC microscope. Measurements of the deutonymphs (four specimens) identified within the sampled material were taken in micrometers (µm) with the aid of the Leica Application Suite (LAS) Software Version 3.8. The mean values followed by the range values in paranthesis were given. Dorsal idiosomal and leg setal designations follow Kethley (1990) and Grandjean (1944), respectively. Specimens examined are deposited in Acarology Laboratory of Erzincan Binali Yıldırım University, Erzincan, Turkey.

Results

Superfamily: Raphignathoidea

Family: Stigmaeidae

Genus: *Stigmaeus* Koch

Stigmaeus kumalariensis Akyol & Koç

Description

Deutonymph (Figs 1-5)

Length of body 269 (231-292), width 191 (179-199).

Dorsum (Fig. 1). Dorsal integument striated except for the shields. Dorsal shields reticulated. Propodosomal shield bearing a pair of eyes and setae *vi*, *ve* and *sci*. Eyes 9 (8-9) in diameter. A pair of auxiliary shields bearing setae *sce*. Setae c_1 and d_1 on central hysterosomal shield. Setae d_2 located on marginal shields. Setae e_2 located on lateral zonal shields. Median zonal and intercalary shields paired, bearing setae e_1 and f_1. Suranal shield entire, bearing two pairs of setae (h_1 and h_2). All dorsal setae long and faintly barbed. Lengths and distances of dorsal setae as follows: *vi* 30 (28-31), *ve* 48 (46-50), *sci* 18 (17-19), *sce* 40 (37-43), c_1 38 (35-41), c_2 37 (35-41), d_1 38 (37-40), d_2 40 (37-44), e_1 40 (38-42), e_2 44 (42-46), f_1 49 (48-50), h_1 48 (47-49), h_2 45 (45-46), *vi*-*vi* 17 (17-18), *ve*-*ve* 44 (42-46), *vi*-*ve* 28 (25-30), *sci*-*sci* 78 (74-82), *ve*-*sci* 20 (19-21), *sce*-*sce* 125 (117-136), *sci*-*sce* 25 (22-28), c_1-c_1 55 (52-58), c_2-c_2 182 (175-194), d_2-d_2 157 (145-170), c_1-d_1 53 (52-54), c_1-d_2 57 (54-63), d_1-d_1 53 (49-57), d_2-d_1 53 (50-56), e_2-e_2 125 (111-147), d_2-e_2 60 (58-62), d_1-e_1 49 (43-54), d_1-e_2 53 (49-57), e_1-e_1 44 (41-48), e_2-e_1 42 (32-48), f_1-f_1 68 (62-74), e_1-f_1 37 (35-40), f_1-h_1 39 (35-44), f_1-h_2 30 (28-34), h_1-h_1 22 (20-23), h_2-h_2 49 (45-53), h_1-h_2 12 (11-13).

Venter (Fig. 2). Humeral shields situated ventro-laterally between coxae II and III, bearing setae c_2. Setae c_2 faintly barbed as those of dorsal setae. Coxisternal shields divided in midline, bearing three pairs of intercoxal setae (1*a*, 3*a* and 4*a*). Lengths and distance of these setae: 1*a* 10 (9-11), 3*a* 12 (11-12), 4*a* 11 (10-11), 1*a*-1*a* 20 (17-23), 3*a*-3*a* 32 (30-33), 4*a*-4*a* 20 (18-21). Three pairs of aggenital setae (ag_{1-3}) presenton the aggenital shield. Genital shield and setae (*g*) absent. Anal shields subterminal, bearing three pairs of pseudanal setae (ps_{1-3}). Lengths of aggenital and pseudanal setae:ag_1 9 (8-9), ag_2 8 (7-8), ag_3 10 (10-11), ps_1 25 (24-26), ps_2 23 (22-24), ps_3 11 (11-12).

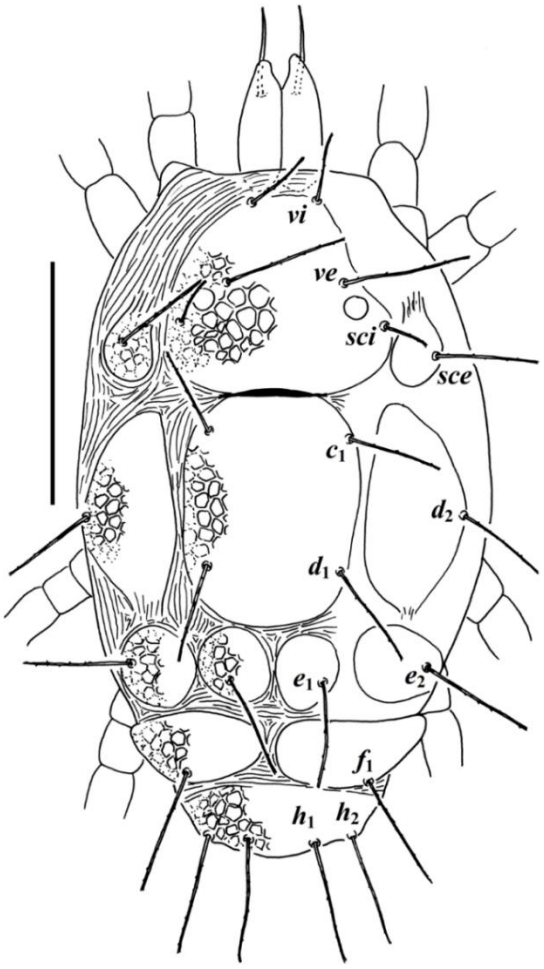

Fig. 1. Dorsal view of idiosoma of *Stigmaeus kumalariensis* deutonymph. Scale 100 µm. (*vi* = internal pair of vertical setae; *ve* = external pair of vertical setae; *sci* = internal pair of scapular setae; *sce* = external pair of scapular setae; c_1 = internal pair of humeral setae; d_1 = internal pair of dorsal setae; d_2 = external pair of dorsal setae; e_1 = internal pair of lumbral setae, e_2 = external pair of lumbral setae; f_1 = internal pair of sacral setae; h_1 = 1st pair of clunal setae; h_2 = 2nd pair of suranal setae)

Legs (Figs 3, 4). Leg I 138 (133-141), leg II 118 (115-121), leg III 120 (116-124), leg IV 132 (128-135) long. Numbers of setae on legs I-IV: coxae 2–2–2–2, trochanters 1–1–2–0, femora 6–4–3–2, genua 2(+1κ)–2(+1κ)–0–0, tibiae 5(+1φρ+1φ)–5(+1φρ)–5(+1φρ)–5(+1φρ), tarsi 13(+1ω)–9(+1ω)–7(+1ω)–7(+1ω).

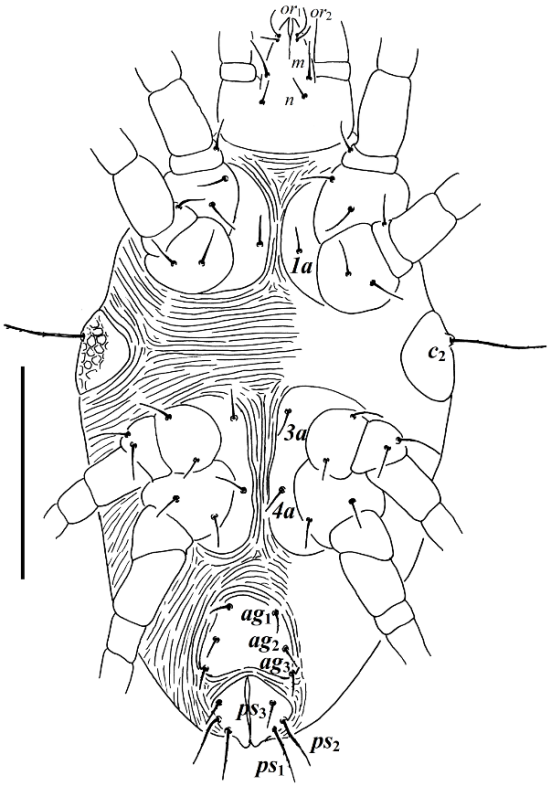

Fig. 2. Ventral view of idiosoma of *S. kumalariensis* deutonymph. Scale 100 µm. (or_1 = 1st pair of adoral setae; or_2 = 2nd pair of adoral setae; *m* = anterior pair of subcapitular setae; *n* = posterior pair of subcapitular setae; c_2 = external pair of humeral setae; *1a* = 1st pair of intercoxal setae; *3a* = 2nd pair of intercoxal setae; *4a* = 3rd pair of intercoxal setae; ag_1 = 1st pair of aggenital setae; ag_2 = 2nd pair of aggenital setae; ag_3 = 3rd pair of aggenital setae; ps_1 = 1st pair of pseudanal setae; ps_2 = 2nd pair of pseudanal setae; ps_3 = 3rd pair of pseudanal setae).

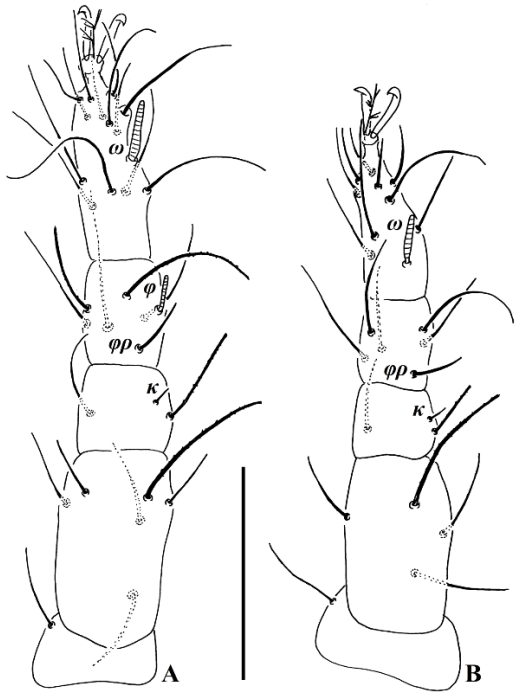

Fig. 3. Legs in *S. kumalariensis* deutonymph. A) Leg I, B) Leg II. Scale 40 µm. (ω = solenidion on tarsi; φ = solenidion on tibia I; $\varphi\rho$ = proximal solenidion on tibiae; κ = famulus on genua).

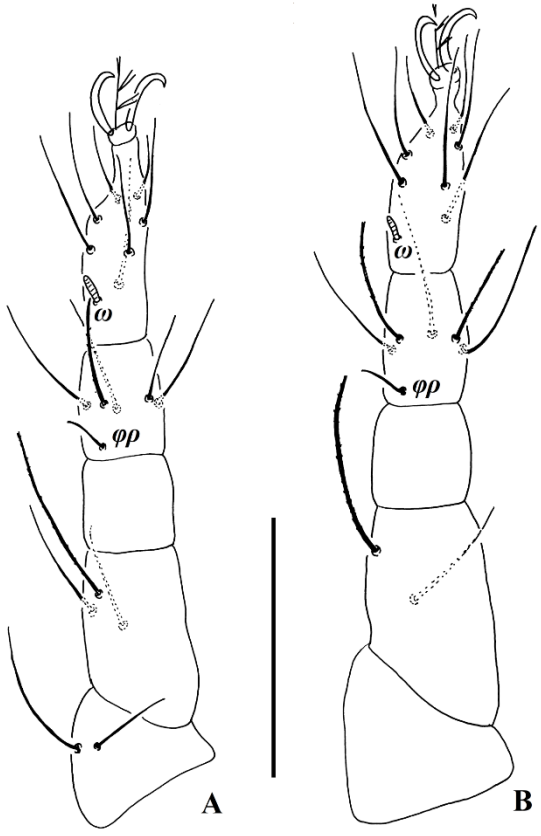

Fig. 4. Legs in *S. kumalariensis* deutonymph. A) Leg III, B) Leg IV. Scale 40 µm.

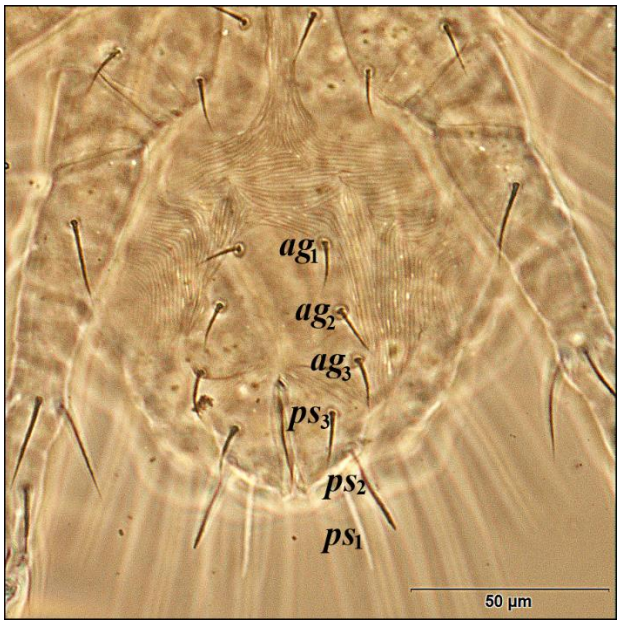

Fig. 5. Anogenital region and setae in *S. kumalariensis* deutonymph.

Gnathosoma. 53 (51-55) long. Subcapitulum with two pairs of setae. Dimensions and distances between subcapitular setae, *m* 14 (13-14), *n* 9 (8-9), *m–m* 17 (16-18), *n–n* 14 (13-15), *m–n* 7 (5-8). Chelicerae 71 (69-72) long. Palp 69 (68-71).

Female (n = 5) (Figs 6-9)

Length of body 320 (309-338), width 206 (191-226). Integument striated except for the shields. Dorsal shields reticulated. Propodosomal shield bearing a pair of eyes and setae *vi*, *ve* and *sci*. A pair of auxiliary shields bearing setae *sce*. Setae c_1 and d_1 on central shield. Setae d_2 located on marginal shields. Setae e_2 located on lateral zonal shields. Median zonal and intercalary shields paired, bearing setae e_1 and f_1. Suranal shield entire, bearing two pairs of setae (h_1 and h_2). All dorsal setae faintly barbed (Fig. 8). Humeral shields reticulated, bearing setae c_2. Coxisternal shields divided in midline, bearing three pairs of intercoxal setae (1*a*,3*a* and 4*a*). Three pairs of aggenital setae ($ag_{1\text{-}3}$) on the aggenital shield. Genital shield bearing a pair of genital setae (g_1). Anal shields bearing three pairs of pseudanal setae ($ps_{1\text{-}3}$) (Fig. 6). Numbers of setae on legs I-IV: coxae 2–2–2–2, trochanters 1–1–2–1, femora 6–5–3–2, genua 2(+1κ)–2(+1κ)–0–0, tibiae 5(+1φρ+1φ)–5(+1φρ)–5(+1φρ)–5(+1φρ), tarsi 13(+1ω)–9(+1ω)–7(+1ω)–7(+1ω).

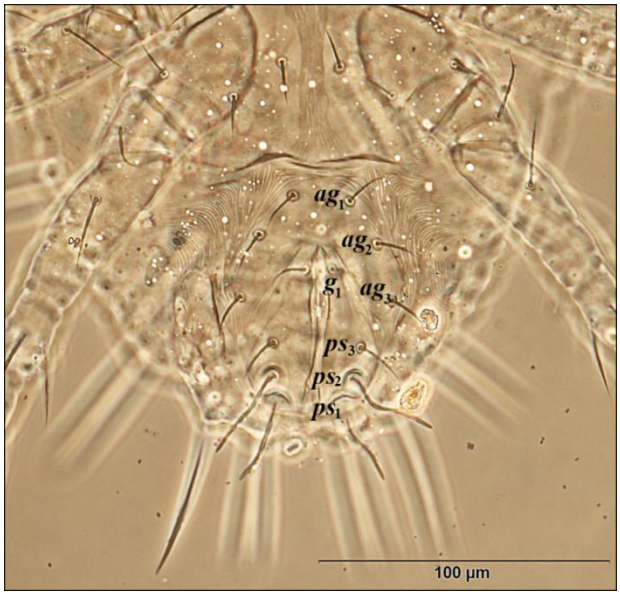

Fig. 6. Anogenital region and the setae in *S. kumalariensis* female. (g_1 = 1st pair of genital setae).

Male (n = 5) (Fig. 10)

Length of body 294 (288-305), width 173 (167-178). Resembles female in general appearance, but posterior of body narrower, genital setae absent, tarsus I-IV bearing two solenidia (ω and ω♂).

Other immature stages: Unknown.

Material examined: 5 females from litter in *Juncus heldreichianus* T. Marssoon ex Parl. (Juncaceae), 39°43'44"N 39°37'28"E, 31 January 2014; 31 females, 6 males and 4 deutonymphs from litter and soil, 39°42'37.11"N 39°37'43.52"E, 1139 m. a.s.l., 13 March 2018, Ekşisu marsh, Erzincan, Turkey.

Distribution: Turkey (Afyonkarahisar, Hakkari and Erzincan) (Akyol & Koç 2007, Doğan *et al*. 2015b, Uluçay 2015a, Doğan 2019b).

Variation

In the present study, a total of 46 specimens (36 females, 6 males and 4 deutonymphs) of *S. kumalariensis* were examined and variations in some adult specimens were found. The variations were determined in the structure of median zonal shields of one adult female (Fig. 9) and one adult male (Fig. 10), and in the number of aggenital setae (*ag*) of two adult females (Fig. 7). The median zonal shield in *S. kumalariensis* is normally divided (Fig. 8), and symmetrically three pairs of aggenital setae are present (Fig. 6). In the abnormal specimens examined, the median zonal shield is undivided (Figs 9, 10) and one aggenital seta on the left side of aggenital shield is absent (Fig. 7). This unilateral absence of the seta is a form of asymmetry.

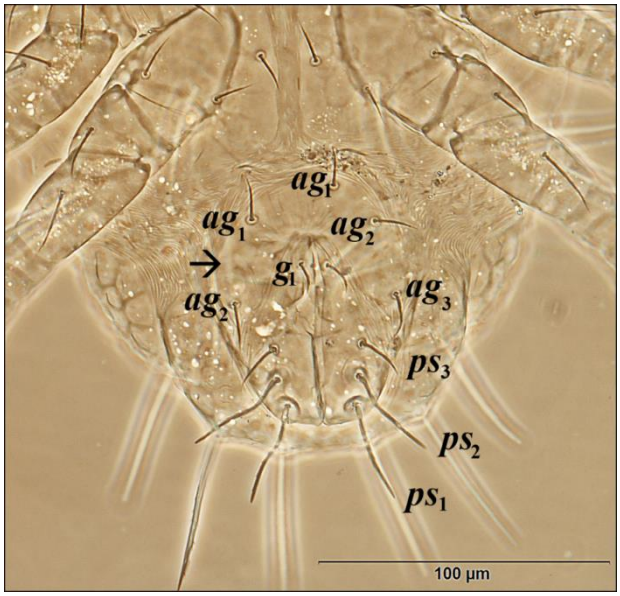

Fig. 7. Variation in the number of aggenital setae in *S. kumalariensis* female.

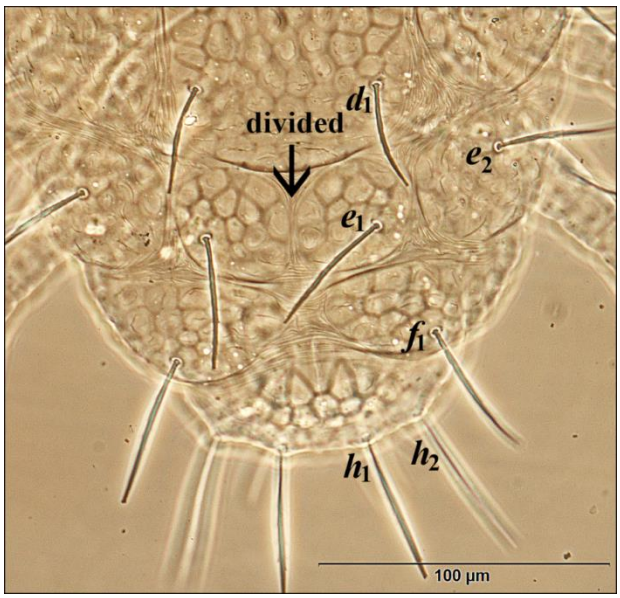

Fig. 8. Divided median zonal shields in *S. kumalariensis* female.

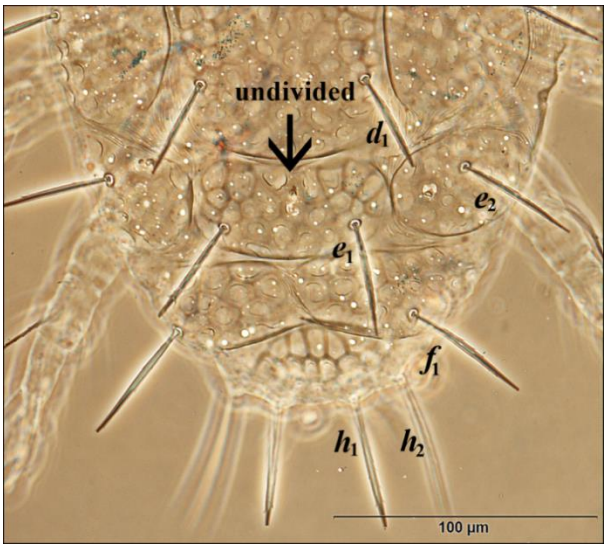

Fig. 9. Undivided median zonal shield in *S. kumalariensis* female.

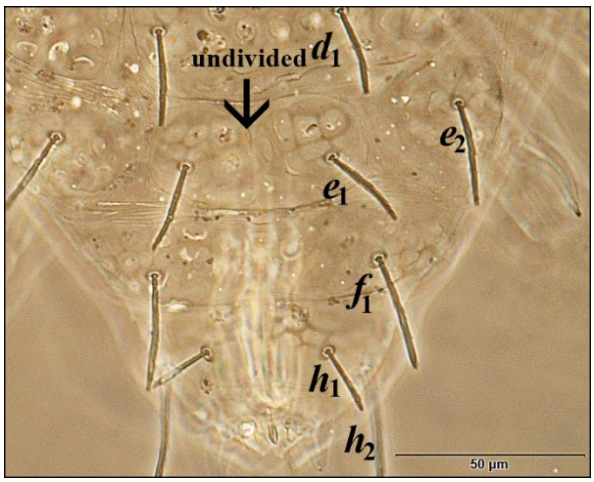

Fig. 10. Undivided median zonal shield in *S. kumalariensis* male.

Remarks

Stigmaeus kumalariensis was first discovered in Afyonkarahisar (type locality), and later reported from Hakkari and Erzincan in Turkey. Until now, female and male of this species have been known. The deutonymphal stage of the species is described for the first time in this study. Deutonymph specimens resemble adult females. Dorsal idiosoma and setae as in adult female. Ventral idiosoma similar to adult female, but genital setae absent (Figs 2, 5), with fewer setae on segments of the legs (trochanter IV and femur II) than those of adult female. Also, famulus κ is present on genu I and II in both adult female and male specimens as in Hakkari male specimens of the species (Uluçay 2015a), but in Afyonkarahisar specimens genu II lacks famulus κ (Akyol & Koç 2007).

No variation was reported so far for *S. kumalariensis* (Akyol & Koç 2007, Uluçay 2015a). In the present study, variations in the structure of median zonal shields and in the number of aggenital setae were observed. In some studies, variations in the genus *Stigmaeus* have been reported. Variations in *S. elongatus* Berlese were recorded as lack of unilateral intercalary shield and numerical variations in form of presence or absence of intercoxal, suranal, dorsal, genital and aggenital setae (Doğan *et al*. 2019). Variations in location of aggenital setae in *S. longiclipeatus* Doğan, Doğan & Erman was mentioned by Doğan *et al*. (2017). Variations on location of central and aggenital setae and numerical variations in form of absence of intercoxal and aggenital setae have been reported in *S. longipilis* (Canestrini) by Dilkaraoğlu *et al*. (2016b). Variations in *S. erzincanus* Doğan, Bingül, Dilkaraoğlu & Fan were observed as lack of unilateral intercalary shield and as presence or absence of suranal, dorsal, genital and aggenital setae by Bingül *et al*. (2017b). Doğan *et al*. (2016) mentioned variations in structure of suranal setae of *S. solidus* Kuznetsov. Variations in the shape of some dorsal setae were reported in both *S. bifurcus* Bingül, Doğan & Dilkaraoğlu and *S. miandoabiensis* Bagheri & Zarei, as well as asymmetric variations in the number and structure of some setae in *S. bifurcus* (Bingül *et al*. 2017a). In conclusion, it is clear that most of the variations in *Stigmaeus* are in the form of unilateral or bilateral presence or absence of some body setae.

Unilateral absence of aggenital setae observed in *S. kumalariensis* is a common variation in *Stigmaeus*, but variation in structure of the median zonal shields is reported for the first time with this study. Entire or divided median zonal shields for identification of the species are considered to be an important character in the genus *Stigmaeus.* This variation was observed in only 2 adult specimens of all examined specimens. We consider that the fusion of median zonal shields in this species is a rare, but an important variation. Such variations among individuals of the same species can lead to misidentifications.

There is no certain explanation about what factors causes the variation. Observed asymmetric and structural variations may be the result of the interactions of genetic and environmental factors (Bingül *et al.* 2017b, 2018).

Acknowledgement

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Research Article

EVALUATION OF THE ANTIVIRAL ACTIVITY OF *Ballota glandulosissima* Hub.-Mor. & Patzak EXTRACTS AGAINST RESPIRATORY SYNCYTIAL VIRUS (RSV)

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Abstract: In order to find new and effective antiviral agents from natural sources, the antiviral properties of methanol and aqueous extracts of *Ballota glandulosissima* Hub.-Mor. & Patzak were evaluated by Colorimetric XTT test against Respiratory syncytial virus (RSV). The concentration required to provide 50% protection against cytopathic effects caused by the virus was defined as EC_{50}, the selectivity index (SI) was determined from the ratio of CC_{50} (50 % Cytotoxic concentration) to EC_{50}. The results showed that both the methanol (EC_{50} = 12.45 µg/mL; SI = 24.84) and aqueous extracts (EC_{50} = 19.12 µg/mL; SI = 24.59) of *B. glandulosissima* had almost the same strong anti-RSV activity as well as ribavirin, used as a positive control against RSV (EC_{50} = 3.25 µg/mL, SI = 34.89). In conclusion, we can say that *B. glandulosissima* extracts are worthy of further studies in order to develop an alternative to the drugs used in clinical practice against RSV. This is the first report on the anti-RSV activity of *B. glandulosissima*.

Key words: *Ballota glandulosissima*, methanol and aqueous extracts, antiviral activity, respiratory syncytial virus.

Özet: Doğal kaynaklardan yeni ve etkili antiviral etkenler bulmak amacıyla yapılan bu çalışmada, *Ballota glandulosissima* Hub.-Mor. & Patzak'dan elde edilen metanol ve su ekstraktlarının antiviral özellikleri Respiratuvar Sinsityal Virus (RSV)'una karşı kolorimetrik XTT testi ile değerlendirilmiştir. Virüsün neden olduğu sitopatik etkilere karşı %50 koruma sağlaması için gerekli konsantrasyon EC_{50} olarak tanımlanmış, CC_{50} (%50 Sitotoksik Konsantrasyon)'nin EC_{50}'ye oranından da seçicilik indeksi (SI) belirlenmiştir. Araştırma sonucunda, *B. glandulosissima*'nın hem metanol (EC_{50} = 12.45 µg/mL; SI = 24.84) hem de su ekstraktının (EC_{50} = 19.12 µg/mL; SI = 24.59) RSV'ye karşı; pozitif kontrol olarak kullanılan ribavirin (EC_{50} = 3.25 µg/mL, SI = 34.89)'e göre önemli sayılabilecek oranda anti-RSV aktiviteye sahip olduğu tespit edilmiştir. Sonuç olarak, *B. glandulosissima* ekstraktlarının, RSV'ye karşı klinikte kullanılan ilaçlara karşı bir alternatif olarak geliştirilebilmesi için; daha ileri çalışmalara layık olduğunu söyleyebiliriz. Bu çalışma, *B. glandulosissima*'nın anti-RSV aktivitesine yönelik ilk rapordur.

Introduction

Acute respiratory infections caused by viruses are a major reason of morbidity and mortality in children worldwide. Human Respiratory Syncytial Virus (HRSV) is the most important cause of pneumonia and bronchiolitis in infants, young children and adults (Hruska *et al.* 1982, Treanor & Falsey 1999) and can be destructive in immunosuppressed populations (Wyde *et al.* 1998). In addition, recurrent infections are a common phenomenon showing that naturally acquired immunity does not provide long-term protection (Dubovi *et al.* 1981). Although many vaccine development studies have been conducted on RSV, efforts to develop effective vaccines against RSV have been unsuccessful (Chin *et al.* 1969, Kim *et al.* 1969, Wyde *et al.* 1998). Moreover, even if the use of one of the vaccines developed is accepted, this may not be appropriate in some RSV-sensitive populations, e.g., in very young infants and in immunocompromised individuals (Wyde *et al.* 1998). Ribavirin (RBV) and immunoglobulins containing high titer RSV-specific neutralizing antibodies are currently recognized as antivirals for the treatment and prevention of RSV infections (Kneyber *et al.* 2000) although they are expensive and not easy to apply. For instance, RBV has been reported to be myelocytotoxic when administered intravenously, and therefore it is only allowed to be used as small aerosol particles (Smith *et al.* 1991, Lewinsohn *et al.* 1996).

Aerosol administration of drugs to patients, especially babies and children, is difficult to control and use at home, and therefore patients should go to hospitals for chemotherapy. The absence of a vaccine available to

prevent RSV and the presence of RBV as the single antiviral agent used only in severe infections are still a problem in pediatric medicine. Therefore, it is necessary to develop specific anti-RSV drugs that can be administered orally or parentally (Ma *et al.* 2002).

Ballota L. species are perennial herbaceous plants belonging to the Lamiaceae family. The genus *Ballota* is represented by 35 species and 14 subspecies in the world of which 12 species and 8 subspecies are present in Turkey. The rate of endemism in high with 8 species in Turkey (Davis 1982, Greuter & Raus 1998, Patzak 1958, Patzak 1960). The genus is well known in Europe due to its spasmolytic and sedative effects (Garnier *et al.* 1961), and its members are called in different regions of Turkey with the local names şalba, çalba, balotu, ballık otu, nemnem otu, ısırgan, gezgez otu, köpek otu, karayer pırasası, elkurtaran, pat pat otu, leylim kara, somruk and karınca somurcağı. Some species are used by people in the treatment of cough, asthma, headache, nausea, haemorrhoid and wound (Baytop 1984, Eryaşar & Tuzlacı 1998, Meriçli *et al.* 1988, Tolon Fenercioğlu & Tuzlacı 1998, Tuzlacı & Tolon 2000, Yeşilada *et al.* 1993, Yeşilada *et al.* 1995). The main components of *Ballota* species are flavonoids, labdan diterpenoids and phenyl propanoids. Diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) and 14 flavonoids (kumatakenin (jaranol), pachypodol, 5-hydroxy-7,3',4' trimethoxy flavone, velutin, velutol, salvigenol, korimbosol, retusol, corymbosine, 5-hydroxy 3,7,4'trimethoxyflavone, retusin, 5-hydroxy 7, 4'dimethoxy flavone, flindulatine, ladanein) were isolated from different *Ballota* species and chemically characterized and analyzed by HPLC (Çitoğlu *et al.* 1998, Çitoğlu *et al.* 1999, Saltan Çitoğlu *et al.* 2003a, Saltan Çitoğlu *et al.* 2003b). Pachypodol (Ro 09-0179) was first isolated from *Plectranthus cylindraceus*, *Pogostemon cablin*, *Heterotheca grandiflora*, *Aglaia andamanica*, *Euodia glabra* and *Larrea tridentata* (Valesi *et al.* 1972, Fraser & Lewis 1973, Sakakibara *et al.* 1976, Wollenweber *et al.* 1985, Miyazawa *et al.* 2000, Orabi *et al.* 2000) has antiviral effect against RNA virus such as poliovirus and rhinovirus. This antiviral effect was shown by inhibiting RNA polymerase in viral RNA synthesis and inhibiting viral replication (Arita *et al.* 2015, Ishitsuka *et al.* 1986, Ninomiya *et al.* 1985, Pérez & Carrasco 1992). Some of these flavonoids isolated from *B. glandulosissima*, a species naturally growing in southern Anatolia, has also been reported to have antimicrobial activity (Saltan Çitoğlu *et al.* 2003a, Saltan Çitoğlu *et al.* 2003b).

Although it is well reported that *Ballota* species have antinociceptive, anti-inflammatory, hepatoprotective, antilisterial, antiproliferative and antioxidant activities (Çitoğlu *et al.* 1998, Çitoğlu *et al.* 1999, Saltan Çitoğlu *et al.* 2003a, Saltan Çitoğlu *et al.* 2003b, Erdoğan-Orhan *et al.* 2010, Özbek *et al.* 2004, Rigano *et al.* 2016, Saltan Çitoğlu *et al.* 2004a, Saltan Çitoğlu *et al.* 2004b, Saltan Çitoğlu *et al.* 2004c, Sever Yılmaz *et al.* 2005, Sever Yılmaz *et al.* 2006), researches on the antiviral activities of *Ballota* species with different biological activities are limited only to picornaviruses. *Ballota* species, which have different components one of which is pachypodol, have not been searched for antiviral activity against HRSV.

This study aimed to find new and reliable antiviral agents against RSV. For this purpose, crude extracts obtained from *B. glandulosissima* were used to evaluate the antiviral activity against RSV.

Materials and Methods

Plant materials

Ballota glandulosissima specimens were collected from Antalya in 2016 during flowering period (in July). The samples were identified by Prof. Dr. Osman TUGAY from Pharmacy Faculty of Selçuk University. Aerial parts of the specimens were dried in the shade, ground into a fine powder by a mill and stored in sterile black glass jars at room temperature. A voucher sample is kept at KNYA Herbarium, Selcuk University, Science Faculty, Biology Department, Konya, Turkey.

Preparation of the extracts

20 g powder samples were placed separately in 400 ml of methanol and 400 ml of sterile distilled water and extracted for 1 hour with an ultrasonicator at 37°C. The extracts were filtered through Whatman No: 1 filter paper, and the solvents used were completely evaporated at 40°C under reduced pressure in a rotary evaporator (Heidolph Laborota 4000, Germany). After evaporation, the extracts were lyophilized at -110°C under reduced pressure in a lyophilizer (Labconco, USA). Each 1000 mg of the lyophilized methanol and aqueous extracts were dissolved in 10 mL of EMEM (serum-free) and stock solutions were prepared at a concentration of 100 mg/mL. The stock solutions were sterilized with 0.22 µm Millipore filter, put in 2 mL tubes at a rate of 1 mL concentrations and stored at +4°C until use. Dilutions of the extracts for cytotoxicity and antiviral activity tests were prepared from these stocks. Ribavirin (RBV, R9644-10 mg, Sigma, USA), a drug approved for the treatment of RSV infections in humans, was purchased and used as positive control. 10 mg RBV was dissolved in 5 mL of EMEM (serum-free). This 2 mg/mL stock concentration were filtered with a 0.22 µm Millipore filter and stored at -80°C or +4°C (When stored at + 4° C, it was used within 1 week).

Cells and the virus

Human larynx epidermoid carcinoma cells [HEp-2; ATCC (the American Type Culture Collection) CCL 23] were used to culture HRSV (RSV Long strain: ATCC VR-26). Reagents and medium for cell culture were purchased from different companies. Cells were propagated at 37°C under 5% CO_2 in EMEM supplemented with 10% fetal bovine serum (FBS, ATCC-30-2020), 10000 U/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B. The virus was propagated on 90% confluent cell monolayer in

EMEM with 2% FBS and antibiotics as described above. Viral titer was determined by 50% tissue culture infectious dose ($TCID_{50}$) method and expressed as $TCID_{50}$ per 0.1 mL (Kaerber 1964). The virus was stored at -80°C until use.

Cytotoxicity assay

The method described by Eskiocak *et al.* (2008) was used to determine the cytotoxic effects of RBV and methanol and aqueous extracts *of B. glandulosissima* on HEp-2 cells.

The cytotoxicity test was carried out as follows;

First column of a 96-well microplate was used as the medium control (MC) and 150 µL EMEM (serum-free) was added in each of the 8 wells contained in this column. Except for the 3rd column, 100 µL EMEM (serum-free) was put in the remaining 10 columns (viz, 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 column). An experiment solution of 2 mg/mL (2000 µg/mL) was prepared using EMEM (serum-free) from stock solution of the extracts (100 mg/mL).

200 µL from experiment solution of the extracts were added in each of the 8 wells in the 3rd column and serial two fold dilutions according to log2 base (2000, 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 µg/mL) were prepared. The second column of the microplate was used as cell control (CC).

The same procedures were performed for RBV using another microplate. First column of the microplate was used as MC and 150 µL EMEM (serum-free) were put in each of the 8 wells. Except for the 3rd column, 100 µL EMEM (serum-free) was put in the remaining 10 columns (viz, 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 column). An experiment solution of 750 µg/mL was prepared by using serum-free EMEM from stock solution of RBV (2000 µg/mL). 200 µL RBV from 750 µg/mL experiment solution was placed in each of the 8 wells. Serial two fold dilutions were made from the 3rd to the 12th columns (750, 375, 187.5, 93.75, 46.88, 23.44, 11.72, 5.86, 2.93 and 1.46 µg/mL).

50 µL HEp-2 cell suspensions containing 1×10^5 cells per mL were added to each well in columns 2 to 12 making the final concentrations of the extracts in the wells as 1333.33, 666.67, 333.33, 166.67, 83.33, 41.67, 20.83, 10.42, 5.21 and 2.60 µg/mL, while the final concentrations of RBV in the wells were 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 µg/mL. The plates were incubated in 5% CO_2 humidified incubator at 37°C for 3 days. Then 50 µL suspensions mixed with 5 mL XTT reagent and 0.1 mL PMS activator were placed in each well. The plates were incubated for a further 3 hours to form the XTT formazan product. Optical densities (OD) were recorded in an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 490 nm and a reference wavelength of 630 nm to record OD averages from 8 wells. The test was performed in triplicate and the results were shown as the ratio of the average cytotoxicity to the cell control.

The following formula was used to calculate the percentage of cytotoxicity of the extracts on HEp-2 cells and RBV.

$$\text{Cytotoxicity (\%)} = \frac{(A - B)}{A} \times 100$$

A: The optical density of cell control

B: The optical density for the cells treated with extracts or RBV.

The cytotoxicity percentages calculated were plotted according to the corresponding concentrations of the tested samples (extracts and RBV). 50% cytotoxic concentration (CC_{50}), defined as the concentration reducing the OD values of the cells treated with extracts or RBV by up to 50% compared with CC, was determined with non-linear regression analysis run in the GraphPad Prism Version 5.03 statistical program. The maximum non-toxic concentrations (MNTCs) of the extracts and RBV were also determined by comparing OD with CC. These MNTCs were used to determine the antiviral activity of the extracts and RBV.

Antiviral activity assay

After MNTCs were determined against HEp-2 cells, 10 times more concentrated dilutions of the extracts and RBV were prepared. The dilutions were diluted according to Log_2 base and their antiviral activities against RSV (diluted at 100 $DKID_{50}$ dose) were tested by XTT method (Chiang *et al.* 2002). The method was applied as described below;

Suspensions were prepared such that the HEp-2 cells were at a concentration of 1.43×10^5 cells / mL using 2% FBS-containing EMEM (cell maintenance medium). From these cell suspensions, the wells of 96-well culture plates (except for 8 wells in the 1st column of the plate used as MC) were seeded at a volume of 70 µL per well (~ 10^4 cells / well). 100 µL aliquots of cell maintenance medium were placed in 8 wells used as MC and incubated in 5% CO_2 at 37ºC for 4 hours. Then, 20 µL of RSV suspension diluted in 100 $TCID_{50}$/0.1 mL using the maintenance medium were put in the wells (except for 8 wells in the 1st column used for MC and the 2nd column used for CC). 8 wells in the 3rd column of the microplates were used as Virus Control (VC). 20 µL aliquots of the maintenance medium were placed in 8 wells in the 2nd column used as CC and the plate was incubated for 2 hours. 10 × MNTC dilutions containing 2% FBS were prepared from stock solutions (100 mg / mL) of the extracts. Subsequently, serial two fold dilutions [(10×MNTC)/2, (10×MNTC)/4, (10×MNTC)/8, (10×MNTC)/16, (10×MNTC)/32, (10×MNTC)/64, (10×MNTC)/128, (10×MNTC)/256] using the maintenance medium were prepared from the extract solutions of 10 × MNTC concentrations. After incubation for 2 hours, 10 µL of the dilutions prepared 10×MNTC

concentration were put in 8 wells of the 4th column. In the remaining 8 columns of the microplates (viz, wells on columns 5, 6, 7, 8, 9, 10, 11 and 12), 10 µL of the extract dilutions [(10×MNTC)/2, (10×MNTC)/4, (10×MNTC)/8, (10×MNTC)/16, (10×MNTC)/32, (10×MNTC)/64, (10×MNTC)/128, (10×MNTC)/256] were added to the each well.10 µL cell maintenance medium was placed in the wells of the microplates used as CC and VC wells. The same procedures were also performed for RBV using another microplate. A dilution of 10×MNTC containing 2% FBS was prepared from the stock solution (1000 µg/mL) of RBV. Subsequently, two fold dilutions [(10×MNTC)/2, (10×MNTC)/4, (10×MNTC)/8, (10×MNTC)/16, (10×MNTC)/32, (10×MNTC)/64, (10×MNTC)/128, (10×MNTC)/256] using the maintenance medium were prepared from the RBV solution in 10 × MNTC. Next, the steps in determining the antiviral activity of the extracts were followed. The plates were incubated in 5% CO_2 at 37ºC for 3 days. Then, 5 mL of the XTT reagent was mixed with 0.1 mL of the PMS activator and 50 µL was added to each well. The plates were gently shaken to homogeneously distribute the dye into the wells. The plates were incubated for a 3 hours to form the XTT formazan product. OD values were read at 490 nm test and 630 nm reference wavelengths in an ELISA reader, and OD averages from 8 wells were recorded. The protection percentages of extracts or RBV concentrations against viruses were calculated from the following formula where A, B and C indicate the absorbance of the extracts or RBV, the virus and the cell controls, respectively (Andrighetti-Fröhner *et al.* 2003):

Protection percentage = [(A−B) / (C−B) x 100]

EC_{50} value, defined as the concentration of the extracts or RBV that provides protection in 50% of the infected cells, was determined using nonlinear regression analysis in GraphPad Prism Version 5.03 taking into account the % protection rates determined with extract or RBV concentrations. The selectivity index (SI) of the extracts and RBV was calculated from the CC_{50} / EC_{50} ratio. The experiments were done in triplicate.

Results

Virus titration

Titres of RSV in HEp-2 cells were determined as $TCID_{50} = 10^{-4}$ / 0.1 mL at the end of the 3rd day.

Cytotoxicity assay

In order to determine the MNTC and CC_{50} values of *B. glandulosissima* methanol and aqueous extracts against HEp-2 cells, the obtained cytotoxicity rates are shown in Table 1 and Fig. 1, respectively. The CC_{50} value of *B. glandulosissima* methanol extract was determined as 309.30 µg/mL and the MNTC as 10.42 µg/mL. The CC_{50} and MNTC of *B. glandulosissima* aqueous extract were determined as 470.10 47 µg/mL and 2.60 µg/mL (Table 1 and Fig. 1). The MNTC and CC_{50} values (113.40 µg/mL and 0.98 µg/mL, respectively) of RBV are shown in Table 1 and Fig. 1.

Table 1. The results of cytotoxicity and antiviral activity assays for RBV and methanol and aqueous extracts of *B. glandulosissima*.

	Extract type	Cytotoxicity		Antiviral activity	
		MNTC (µg/mL)	CC_{50} (µg/mL)	EC_{50} (µg/mL)	SI
B. glandulosissima	Methanol	10.42	309.30	12.45	24.84
	Aqueous	2.60	470.10	19.12	24.59
Ribavirin (RBV)	--	0.98	113.40	3.25	34.89

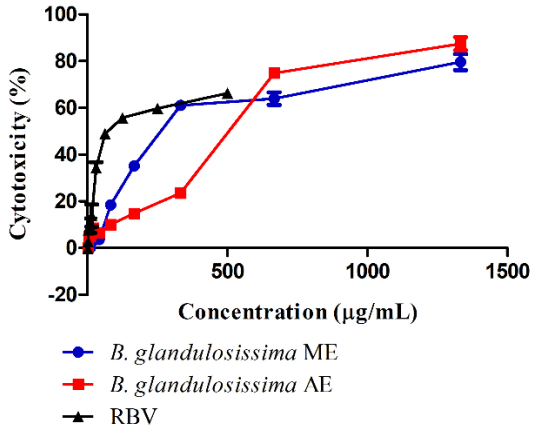

Fig. 1. Cytotoxic activities of methanol and aqueous extracts of *B. glandulosissima* and RBV.

Antiviral activity assay

Percentage protection rates of methanol and aqueous extracts starting from MNTC dilutions against RSV are shown in Table 1. The EC_{50} values of methanol and aqueous extracts were determined as 12.45 µg/mL and 19.12 µg/mL, respectively (Fig. 2). SI (CC_{50}/EC_{50}) values of methanol and aqueous extracts are 24.84 and 24.59, respectively (Table 1).

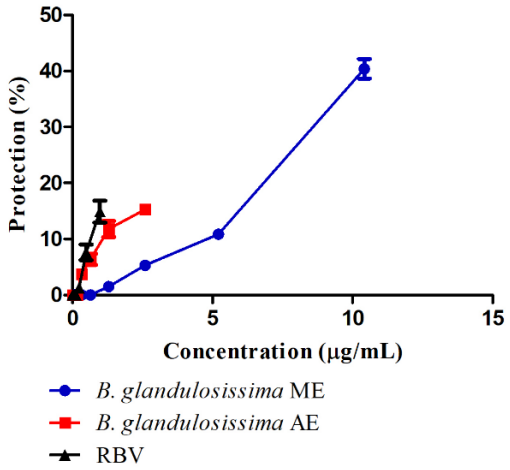

Fig. 2. Antiviral activities of methanol and aqueous extracts of *B. glandulosissima* and RBV.

The protection rates of RBV against RSV are given in Table 1. The EC_{50} value of RBV was determined as 3.25 µg/mL (Fig. 2). The selectivity index (SI) of the RBV was determined as 34.89 from the CC_{50}/EC_{50} ratio (Table 1).

Discussion

RSV is the most common cause of acute respiratory infections in infants and children. Although the mortality rate is generally low, it can also cause heart or respiratory failure up to 37-73% and bone marrow transplantation up to 36-45% in infants (Kimura *et al.* 2000, MacDonald *et al.* 1982, Englund *et al.* 1988, Harrington *et al.* 1992). Original anti-RSV compounds with better efficacy and safety than ribavirin have been the target of researches. Natural products may constitute different sources of antiviral agents. For example, pachypodol was isolated from many plant species, in addition to *B. glandulosissima*, and it has been reported to have antiviral activity against RNA viruses such as poliovirus and rhinovirus (Arita *et al.* 2015, Ishitsuka *et al.* 1986, Ninomiya *et al.* 1985, Pérez & Carrasco 1992).

Cytotoxic and antiviral activities of methanol and aqueous extracts prepared from the aerial parts of *B. glandulosissima* and RBV were evaluated by colorimetric XTT test (see Table 1. The results showed that the extracts with CC_{50} values in the range of 309.30–470.10 µg/mL were found to be non-toxic to HEp-2 cells according to the criteria of Rukunga & Simons (2006). According to the classification of Rukunga & Simons (2006), extracts with a CC_{50} value less than 2 µg/mL are cytotoxic, extracts with a CC_{50} value in the range of 2-89 µg/mL are partially (moderately) cytotoxic and extracts with a CC_{50} value greater than 90 µg/mL are non-toxic.

The antiviral activity experiments revealed that methanol and aqueous extracts of *B. glandulosissima* had a significant antiviral activity comparable to RBV. The EC_{50} values of the extracts were determined as 12.45 µg/mL and 19.12 µg/mL, respectively, while SI values were determined as 24.84 and 24.59, respectively. Vanden Berghe & (1993) suggested that the antiviral activity of crude plant extracts should be detectable in at least two consecutive maximum non-toxic dilutions to discriminate between the virus-induced CPE and the CPE resulting from the toxic effect of the extracts. In addition, Cos *et al.* (2006) defined quality standards for basic evaluation of activity screening of natural products. As a standard for the antiviral efficacy of natural products, such as plant extracts, investigators have proposed a mandatory endpoint of EC_{50} values of less than 100 µg/mL. Apart from EC_{50} values, SI values of 10 or greater are generally reported to be considered as indicators of positive antiviral activity (Chattopadhyay *et al.* 2009).

In this study, our results showed a parallelism with the studies of Vanden Berghe *et al.* (1993), Cos *et al.* (2006) and Chattopadhyay *et al.* (2009). Extracts with more than two consecutive maximum non-toxic dilution in the concentration of antiviral effect has been determined, and EC_{50} values less than 100 µg/mL and SI values greater than 10 were obtained. Therefore, it was concluded that the extracts of *B. glandulosissima* had a reliable antiviral activity. Various components may be responsible for this antiviral activity. For instance, the rich flavonoid components identified in this plant can especially be effective on the virus (Saltan Çitoğlu & Sever 2002). Saltan Çitoğlu & Sever (2002) isolated seven flavonoids (kumatakenin (jaranol), pachypodol, 5-hydroxy-7,3',4' trimethoxy flavone, velutin, salvigenin, corymbosin, retusin) from *B. glandulosissima* extracts among which pachypodol has an antiviral effect. This effect is shown by inhibiting RNA polymerase in viral RNA synthesis and inhibiting viral replication (Arita *et al.* 2015, Ishitsuka *et al.* 1986, Ninomiya *et al.* 1985, Pérez & Carrasco 1992).

This is the first study to determine the anti-RSV activities of *B. glandulosissima*. Therefore, no comparison could have been done with other studies.

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Research Article

OBTAINING CANDIDATE SALT TOLERANT WHEAT MUTANT LINES DERIVED FROM COMBINATION OF SODIUM AZIDE MUTAGENESIS AND SOMATIC EMBRYOGENESIS

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Abstract: Plant mutants are important bio-resources for crop breeding and functional gene studies. In the present study, conventional chemical mutagenesis technique was combined with somatic embryogenesis to obtain candidate salt tolerant mutant wheat lines. For this purpose, 0-5 mM Sodium Azide (NaN_3) was applied for 30 minutes to embryonic calli under *in vitro* conditions to produce genetic variations in the bread wheat (*Triticum aestivum* L. cv. Adana 99). Treated and non-treated calli were put in somatic embryo induction media, and 3 and 4 mM NaN_3 were determined as optimum mutation doses for somatic embryo induction. The obtained somatic embryos from these optimum mutagen doses were then screened for tolerance in regeneration media containing 125 mM NaCl to be used to improve tolerance to salt stress. In NaN_3 treatment, 14 mutants with moderate salt tolerance were obtained. The results suggest that the *in vitro* technique in combination with chemical mutagenesis may be a useful approach for accelerating breeding strategies to create enough genetic variation in populations and to get fourth generation putative salt tolerant wheat mutant lines in less than 1.5 years.

Key words: Sodium azide, *in vitro* mutagenesis, salt tolerance, somatic embryogenesis, wheat.

Özet: Bitki mutantları, bitki ıslahı ve fonksiyonel gen çalışmaları için önemli biyo-kaynaklardır. Mevcut çalışmada, tuza toleranslı aday mutant buğday hatları elde etmek için somatik embriyogenez ile konvansiyonel kimyasal mutajenez tekniği birleştirildi. Bu amaçla; ekmeklik buğdayda (*Triticum aestivum* L. cv. Adana 99) genetik varyasyonlar yaratmak için embriyonik kalluslara 0-5 mM Sodyum Azid (NaN_3), 30 dakika boyunca *in vitro* ortamda uygulandı. İşlem görmüş ve görmemiş kalluslar, somatik embriyo teşvik ortamına konularak somatik embriyo teşviki için 3 ve 4 mM NaN_3 uygulaması optimum mutasyon dozları olarak tespit edildi. Ardından bu mutasyon dozları ile muamele edilen kalluslardan elde edilen somatik embriyolar tuz stresine tolerans geliştirmek için kullanılacak 125 mM NaCl içeren rejenerasyon ortamlarında tolerans açısından tarandı. NaN_3 muamelesinde, orta düzeyde tuza toleransı olan 14 mutant elde edildi. Elde edilen sonuçlar; kimyasal mutagenez ile kombine halde *in vitro* teknik uygulamasının, popülasyonlarda yeterli genetik varyasyon oluşturmak ve 1.5 yıldan daha az bir sürede dördüncü jenerasyon tuz toleranslı aday buğday mutant hatlarını ıslah sürecini hızlandırarak elde etmek için kullanışlı bir yöntem olabileceğini göstermektedir.

Introduction

Wheat (*Triticum aestivum* L.), the world's most important food crop, covers a cultivated land of 219 million hectares at the global level with more that 758 million tons of annual yield production (Hububat Sektör Raporu, 2017). High level of salinity in agricultural soil is one of the brutal environmental factors for many crops and reduces plant growth and productivity due to its toxic effects (Shrivastava & Kumar 2015), leading to interruption of sustainable agricultural production. The way to minimize this interruption is to increase salt stress tolerances of the crops in question, which is generally achieved through improvement of the crops via breeding techniques. Induced mutation through mutagenic agents, combined with selective breeding strategy is highly efficient for generating crops with improved traits. These mutagenic agents alter genes or break chromosomes. Sodium azide (NaN_3) is one of the chemical mutagens and has become an important tool to enhance agronomic traits of crops with its easy handling and low cost. Chemical mutagens induce not only loss-of-function or enhanced function mutations, but also mutate genes that confer novel functions (Al-Qurainy & Khan 2009). Besides the conventional mutation breeding, *in vitro* mutagenesis is an important tool to generate rapid phenotypic and genotypic variations in plants. Compared with conventional breeding techniques, *in vitro* mutagenesis

can overcome some of the limitations, such as lack of screening of effective mutant treatment techniques and production time for mutated plants (He *et al.* 2009; Wannajindaporn *et al.* 2014). Chemical mutagen applications are based on somatic embryogenesis, one of the biotechnological techniques widely used in plant breeding for rapid and large scale propagation of mutant population and mutant selection besides disease free *in vitro* plants (Suprasanna *et al.* 2012). The present study was performed in order to (i) combine chemical mutagenesis with somatic embryogenesis to create genetic variation to obtain mutant wheat populations, and (ii) to screen the populations for salinity tolerance using combination of *in vitro* and *in vivo* techniques.

Materials and Methods

Material

The bread wheat cultivar Adana-99 (*Triticum aestivum* L. cv.) was obtained from the Eastern Mediterranean Agricultural Institute in Adana, Turkey. Adana-99 was selected as the cultivar to be studied considering the results of pre-trials which showed that the percentage of plant regeneration from somatic embryos was determined to be high.

Determination of NaCl threshold concentration for screening salt tolerance

In order to determine threshold NaCl concentration of the target bread wheat cultivar, its seeds were sterilized and were imbibed in sterile water for 2 hours at 35°C, and mature embryos were removed under aseptic conditions (Ozgen *et al.* 1998). The mature embryos were subsequently inoculated in Murashige and Skoog (MS) medium (Murashige & Skoog 1962) basal salt mixture containing 0.05 mgL^{-1} 2,4-Dichlorophenoxyacetic acid (2.4-D), 20 gL^{-1} sucrose, 0.8% (w/v) agar and different concentrations of NaCl [0 (as a control), 50, 100, 150 and 200 mM NaCl]. The pH of the screening media was adjusted to 5.8 before autoclaving. The cultures were incubated in a growth chamber at 26±2°C for 28 days under 16 hours of light: 8 hours of dark photoperiod and irradiance of 500 µmol $m^{-2}s^{-1}$ photon flux density. The regeneration ratios, plant heights and fresh weights of the 28-day-old cultures were measured and the screening concentration of NaCl was selected accordingly.

In vitro mutagenesis and screening mutants for salt tolerance

Callus induction:

The mature embryo explants from sterilized wheat seeds were removed under aseptic conditions as described above and inoculated in petri dishes with a callus induction medium consisted of mineral salts of MS, 2 mgL^{-1} 2.4-D, 20 gL^{-1} sucrose, and 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving. The cultures were incubated in a growth chamber at 28±2°C for 21 days under dark conditions.

In vitro mutagenesis, somatic embryo induction, indirect regeneration and *in vitro* selection:

Three-week-old embryonic calli derived from mature embryos were treated with NaN_3 (pH 3.5) at 0.0 (as a control), 1.0, 2.0, 3.0, 4.0, and 5.0 mM NaCl concentrations for 30 minutes (He *et al.* 2009; Wannajindaporn *et al.* 2014) and then transferred to non-selective medium. After 72 hours of recovery, the calli were transferred to somatic embryo induction medium. The medium was prepared with MS salts, 20 gL^{-1} sucrose, 2 mgL^{-1} 2.4-D, 100 mgL^{-1} myo-inositol, 500 mgL^{-1} glutamin, 300 mgL^{-1} casein hydrolysate, 1 mlL^{-1} B5 vitamin (Gamborg *et al.* 1968) complex (1000x), and 8 gL^{-1} agar. Its pH was adjusted to 5.8 before autoclaving. Embryogenic callus and somatic embryo cultures were incubated in a growth chamber at 26°C for 4 weeks in dark conditions (Zair *et al.* 2003). One month later, the ratios of somatic embryo induction were recorded and according to these records, the optimum mutagen dosages were detected to be 3 and 4 mM NaN_3. 500 calli were treated with each concentration to create mutant populations. Four weeks later, obtained somatic embryos were transferred to selective indirect regeneration media, which contained MS, 20 gL^{-1} sucrose, 0.5 mgL^{-1} indole-3-acetic acid (IAA), 1 mlL^{-1} B5 vitamin complex (1000x), 0 (as a control) and 125 mM NaCl, and 8 gL^{-1} agar, and cultured at 26±2°C under 16 hours of light/ 8 hours of dark photoperiod for two months.

Transferring into soil, acclimating process and segregating the mutant lines up to fourth generation (M_1- M_4):

The rooted seedlings of 60-day-old mutant lines and parental line were transferred to pots for acclimation in a greenhouse at a temperature of 25°C. After acclimation, NaCl was gradually added to the 1/10 Hoagland Solution (Hoagland & Arnon 1950) in increasing increments of 25 mM every week until the salt concentration reached the final treatment level of 125 mM. Plantlets were irrigated with two days intervals until anthesis. After anthesis, 1/10 Hoagland Solution was used for irrigation. Once the first generation mutants (M_1) became 90 days old, their immature seeds were collected and embryos were isolated under aseptic conditions. The immature embryos were transferred to ½ MS media with 20 gL^{-1} sucrose, 125 mM NaCl and 8 gL^{-1} agar. The pH was adjusted to 5.8 before autoclaving. These cultures were incubated in a growth chamber at 26°C for 14 days under 16 hours of light/ 8 hours of dark photoperiod. Two-week-old seedlings were transferred to pots. When the second generation mutant lines (M_2) became 90 days old, the same procedure was applied to generate third and subsequently fourth generation mutant lines (M_3 and M_4). Fig. 1 shows the basic steps of obtaining candidate salt tolerant mutant lines.

Data analyses

One-way analysis of variance (ANOVA) followed by Student-Newman Keuls post-hoc test was used to make statistical analysis by running SPSS computer program (IBM Inc., Chicago, IL, USA), based on fresh weight and plant height. All values were expressed as mean ± standard error mean (Zar 1984).

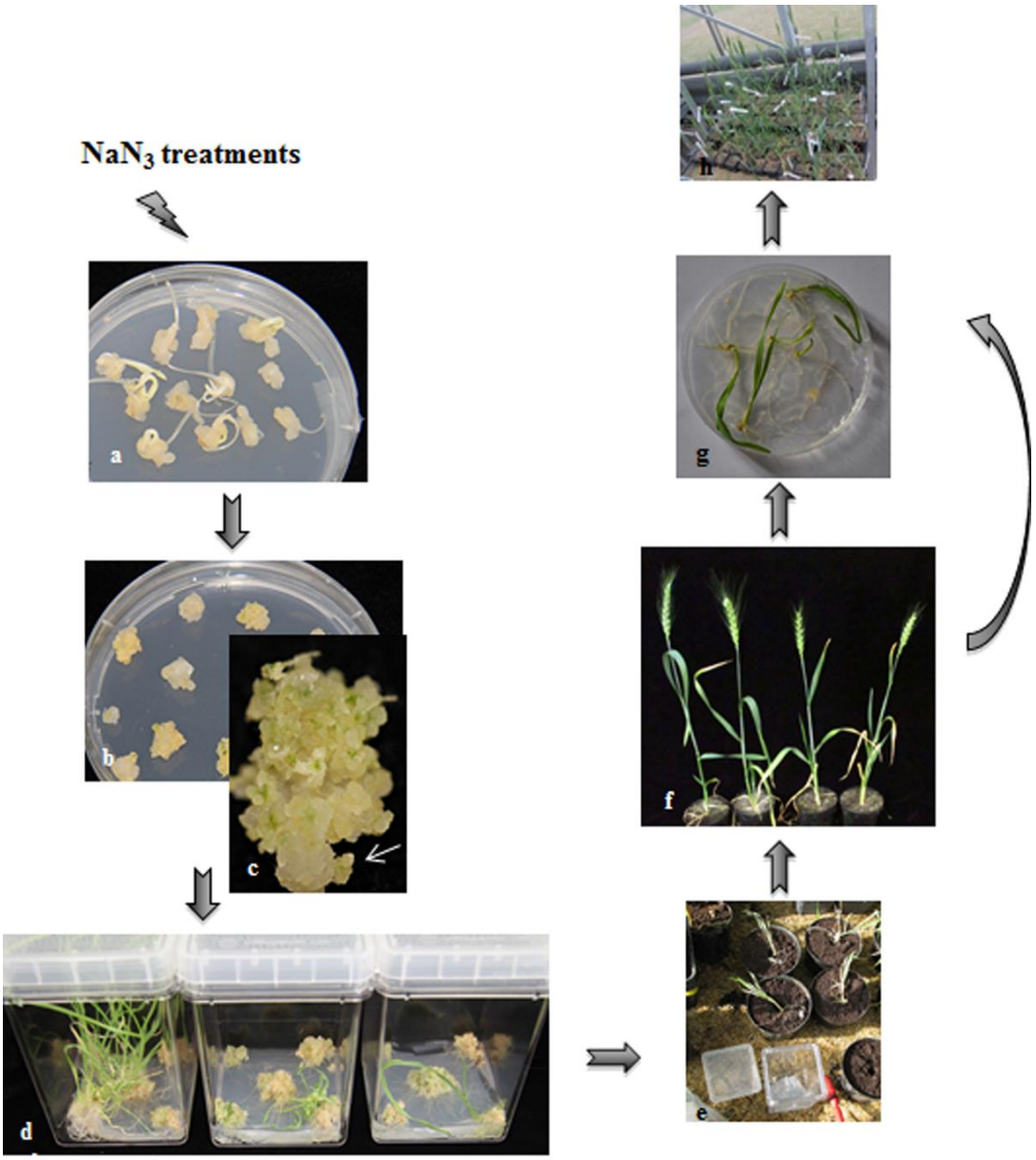

Fig. 1. The basic steps for obtaining candidate salt tolerant wheat mutant lines. Inducing embryonic calli in induction medium and the treating embryonic calli with NaN_3 (a), NaN_3 treated embryonic calli were to transferred somatic embryo induction medium (b and c), obtained somatic embryos were transferred to indirect regeneration media containing NaCl (d), the rooted candidate salt-tolerant mutant lines were transferred to pots (e), mutant lines were growth up to setting seeds (f), once the first generation of mutant lines seeds set, their immature embryos were isolated under aseptic conditions and transferred to ½ MS media with 20 gL^{-1} sucrose, 125 mM NaCl and 8 gL^{-1} agar for germinations (g). Then, these were two-week-old mutant lines were transferred to pots and grown until plantlets set seeds (h). The steps from f to h were repeated four times for fixing genome.

Results

Determination of threshold NaCl concentrations

Table 1 shows that increased salt concentration significantly decreased the regeneration ratio (16.67-90%), average plant fresh weight (17.07-81.38%), and average plant height (11.95-67.92%). 100 and 150 mM NaCl concentrations reduced the average plant fresh weight and length approximately by 50%-60% compared to the control. We therefore chose the 100 mM as the threshold concentration and growth media with 125 mM NaCl, which was slightly above the threshold concentration, was used to select the candidate salt tolerant plants.

Determination of mutagen concentration(s) and selection of mutant individuals under in vitro conditions, transferring plantlets into soil and segregating them up to fourth generation

In order to determine optimum mutagen concentration(s), firstly 50 embryonic calli were treated with NaN_3 concentrations from 0 to 5 mM. Then, these calli were inoculated into somatic embryo induction medium. Somatic embryo induction rates were calculated

to be 80.00%, 85.50%, 87.75%, 92.31%, 93.25% and 77.30%, under 0 (without NaN_3 application), 1, 2, 3, 4 and 5 mM NaN_3 treatments, respectively. According to these results, 3 and 4 mM NaN_3 concentrations were selected to be optimum mutagen concentrations for creating mutant populations. In the next step, 500 embryonic calli from each concentration were treated with 3 and 4 mM NaN_3 and inoculated into somatic embryo induction medium. Then, the generated somatic embryos were put into selective regeneration medium including 0 (as a control) and 125 mM NaCl. These cultures were monitored during two months. In the control group (without NaN_3 treatment), 25 plantlets regenerated in the culture of 125 mM NaCl. Eight of them set seeds under NaCl treatments. A total of 334 plantlets were obtained under NaN_3 treatments. 180 of them showed growth and morphological abnormalities (i.e., sterility, dwarf, awn, and abnormal head morphology) due to either NaN_3 or NaCl treatments. The fertile plantlets were calculated as 154 (91 for 3 mM and 63 for 4 mM NaN_3) with 125 mM NaCl treatment.

In order to obtain M_2 mutant lines, the first-generation immature embryos from 162, 90-day-old fertile plantlets derived from either somatic variation or NaN_3 induction were collected and transferred to ½ MS media with 125 mM NaCl under aseptic conditions to grow the second generation. After two weeks, 101 M_2 mutant lines were transplanted into greenhouse. The mutant lines were irrigated with 125 mM NaCl twice a week until full grain filling. Nearly 85 M_2 mutant lines set seeds. The M_2 generation immature embryos from mutant lines were collected and transplanted into pots. Of the 85 mutant lines, 55 were regenerated of which 33 set seeds. When the same experimental procedures were repeated to obtain further generation mutant lines, the number of seed setting plants were recorded as 25 in M_3 and 14 in M_4 mutants. The distribution of obtained candidate salt tolerant M_4 mutant lines are given in Table 2.

In the second generation, no fertile mutant plantlet from somatic variation derived at the first generation mutant plantlets (without NaN_3 treatment control plantlets) under NaCl treatments was recorded. This result creates the impression that 125 mM NaCl application reduced fertility. Additionally, due to genetic segregations, decreasing in fertility rate was observed in each mutant population under salt application.

Table 1. Regeneration percentages, average fresh plant weights and plant heights of 28-day-old Adana 99 cultivar exposed to different concentrations of NaCl.

Experimental Groups	Explant Number	Regeneration %	Average Plant Fresh Weight (mg)	Average Plant Height (cm)
Control	30	90.00	709 ± 13^{a}	14.65 ± 1.98^{a}
50 mM NaCl	30	80.00	$588 \pm 18^{b*}$	12.9 ± 1.3^{a}
100 mM NaCl	30	66.67	$385 \pm 21^{c*}$	$8.5 \pm 0.81^{b*}$
150 mM NaCl	30	50.00	$264 \pm 26^{d**}$	$6.8 \pm 0.87^{c*}$
200 mM NaCl	30	16.67	$132 \pm 16^{e***}$	$4.7 \pm 0.64^{d**}$

Means followed by a different letter are significantly different *p<0.05, ** p<0.01, ***p<0.001, (*) comparison with the control.

Table 2. NaN_3 and NaCl concentrations (mM) and morphological characteristics of 14 M_4 wheat mutants.

Mutant Lines	NaN_3 (mM)	NaCl (mM)	Morphological Characteristics
Adn99-1	3	125	Normal appearance
Adn99-2	3	125	Normal appearance
Adn99-3	3	125	Normal appearance
Adn99-4	3	125	Earliness
Adn99-5	3	125	Normal appearance
Adn99-6	3	125	Normal appearance
Adn99-7	4	125	Normal appearance
Adn99-8	4	125	Normal appearance
Adn99-9	4	125	Normal appearance
Adn99-10	4	125	Normal appearance
Adn99-11	4	125	Normal appearance
Adn99-12	4	125	Normal appearance
Adn99-13	4	125	Without awn
Adn99-14	4	125	Different spike morphology

Discussion

Salinity constrains crop growth and agricultural productivity in many regions in the world. It has been estimated that 20% of the world's irrigated land is affected by salinity (Wang *et al.* 2003). In Turkey, 3.6 million ha of land are not suitable for farming due to salinity and high subsoil water. More than 1.5 million ha of these lands making almost 2% of Turkey's total area are saline soils (Kanber *et al.* 2005). The improvement of salt-tolerant crops would be a practical solution to this problem and different strategies are being developed to serve this solution. In the presented study, we modified *in vitro* somatic embryogenesis technique combined with NaN_3 applications to improve salt tolerance in the Turkish bread wheat cultivar Adana 99. We determined the genetic tolerance capacity of Adana 99 against salinity and our results showed that, in agreement with previous studies (Zair *et al.* 2003; Yumurtaci & Uncuoglu 2012), increased NaCl concentration negatively affected plant growth and 125 mM NaCl concentration was determined as a selection concentration.

Mutants generated via conventional mutation technology usually take a substantial amount of time and work to screen, especially for large quantities. However, through the combination of *in vitro* mutation and tissue culture, researchers can rapidly isolate variants with desired agronomic traits (Serrat *et al.* 2014). In our study, we used NaN_3 as a chemical mutagen to induce variations in the embryonic cultures. NaN_3, in contrast to natural mutations, generally induces mutation bias on AT→ GC base pair substitutions resulting in amino acid changes, which change the function of proteins and alter phenotypes (Olsen *et al.* 1993) and is frequently used to create mutant crop genotypes (Al-Qurainy & Khan 2009). In a study on embryonic calli from immature maize embryos, the treatment of 20 Gy of gamma ray and 1 $mmolL^{-1}$ of NaN_3 was identified as the most effective for inducing mutation (He *et al.* 2009). Ganesan *et al.* (2005) showed that somatic embryo germination in cotton increased from 44.6% to 50.9% with a 10 mM NaN_3 treatment. Ikram-ul-Haq *et al.* (2011) used 0.5% NaN_3 at pH 4.6–4.7 to induce mutation in sugarcane calli to obtain salt-tolerant mutants. Ahmad *et al.* (2010) used 0.0–0.5 mM NaN_3 to induce mutation in potato calli. In our study, 3 and 4 mM NaN_3 concentrations were selected as the optimum mutagen concentrations for creating mutant populations because these concentrations increased the frequency of somatic embryo formation.

Plant breeding for determination of tolerance to salt stress requires reliable and rapid screening techniques. Screening in field conditions is difficult due to various factors such as stress heterogeneity, the presence of other soil-related stresses, temperature, relative humidity and solar radiation. However, a laboratory-based *in vitro* strategy can increase genetic variation and is the easiest way to screen large cell populations in a short time and on a year-round basis. Somatic embryogenesis is one of the regeneration methods by which somatic embryos arise from single cells and an efficient method of plant regeneration allowing rapid production of large number of "true to type " plants. Each mutated single cell can develop into a somatic embryo and regenerate a mutant plant. The combination of *in vitro* mutation with conventional breeding as well as *in vitro* selection increased the variation of genetic diversity that produced a superior variety in a recent genetic diversification program (Jain 2010). *In vitro* mutagenesis strategies have been used for different plants. Wannajindaporn *et al.* (2014) reported that 28 Dendroium'Earsakul'mutants were obtained with protocorm-like bodies using 0-5 mM NaN_3 under i*n vitro* conditions. Abiotic and biotic stress-tolerant mutants have been obtained by applying chemical mutagens coupled with *in vitro* systems in chrysanthemum (salt), sugarcane (salt), banana (drought) and banana (viruses) (Hossain *et al.* 2006; Ikram-ul-Haq *et al.* 2011; Bidabadi *et al.* 2011; El-Sayed *et al.* 2012). The general strategy in breeding is that segregating the offspring, whose genomes have been modified such a kind of techniques mutagen applications or hybridizations, at least six generations are needed to fix their modified genomes. In this study, 14 fourth-generation candidate salt tolerant mutant lines were obtained with combination of somatic embryogenesis approach with 125 mM NaCl application. The study is still continuing to stabilize the mutated genome gained by the new character.

In conclusion, the results suggest that the *in vitro* technique in combination with chemical mutagenesis may be a useful approach for accelerating breeding strategies to create enough genetic variation in populations and to get fourth generation candidate salt tolerant wheat mutant lines in less than 1.5 years.

Acknowledgement

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Yazı hazırlama ilkeleri

Yazılar, Yayın Komisyonu'na **MS Word** kelime işlemcisiyle **12 punto** büyüklüğündeki **Times New Roman** tipi yazı karakteriyle ve 1,5 aralıklı yazılmış olarak gönderilmelidir. İletişim bilgileri yazının ilk sayfasında tek başına yazılmalı, daha sonraki sayfada yazar isimleri ve iletişim bilgileri bulunmamalıdır. Tüm yazı her sayfası kendi arasında **satır numaraları** içerecek şekilde numaralandırılmalıdır. Yazar adları yazılırken herhangi bir akademik unvan belirtilmemelidir. Çalışma herhangi bir kurumun desteği ile yapılmış ise, teşekkür kısmında kurumun; kişilerin desteğini almış ise kişilerin bu çalışmayı desteklediği yazılmalıdır.

Yazı aşağıdaki sıraya göre düzenlenmelidir:

Yazarlar: Yazının ilk sayfasında sadece yazar isimleri ve adresleri bulunmalıdır. Adlar kısaltmasız, soyadlar büyük harfle ve ortalanarak yazılmalıdır. Adres(ler) tam yazılmalı, kısaltma kullanılmamalıdır. Birden fazla yazarlı çalışmalarda, yazışmaların hangi yazarla yapılacağı yazar ismi altı çizilerek belirtilmeli (sorumlu yazar) ve **yazışma yapılacak yazarın adres ve e-posta adresi yazar isimlerinin hemen altına yazılmalıdır. Bu sayfaya yazı ile ilgili başka bir bilgi yazılmamalıdır. Yazı, takip eden sayfada bulunmalı ve yazar-iletişim bilgisi içermemelidir.**

Başlık: İngilizce olarak Kısa ve açıklayıcı olmalı, büyük harfle ve ortalanarak yazılmalıdır.

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.

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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.

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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.

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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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