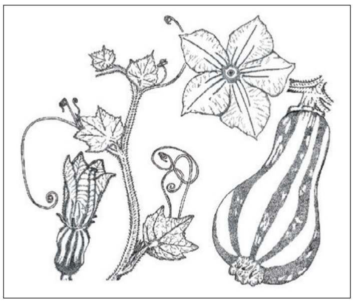
On the cover:

The first record of a naturalized alien population of *Cucurbita moschata* Duchesne in Spain is reported in the paper of Juan et al. Habit, flowers and fruit are shown in the drawing by Joaquín Moreno.



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Taxonomic importance of leaf anatomical characters for the genus *Alopecurus* L. (Poaceae)

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Abstract – Tiller leaf anatomical features of nine *Alopecurus* species collected from high mountainous areas in Turkey were evaluated in the present study. Detailed leaf anatomical descriptions of the studied taxa and an identification key generated using the anatomical features are given. Clustering and principal coordinate analysis (PCA) methods based on a total of 14 leaf anatomical characters were applied. Anatomical differences among species were detected and anatomical characters of taxonomic interest were identified in this genus. With clustering analyses, a unweighted pair group method with arithmetic mean (UPGMA) tree was obtained to show the relationship between the species studied. Based on the results of PCA, the arrangement of adaxial sclerenchyma cells, the number of adaxial ribs, the number of abaxial sclerenchyma strands, the size of epidermal cells and the number of vascular bundles are designated as the most reliable characters to separate the species.

Keywords: *Alopecurus*, leaf anatomy, numerical taxonomy, systematics

Introduction

The Poaceae Barnhart is one of the most diverse plant families, represented by about 12,000 species and 780 genera worldwide (Clayton and Renvoize 1986, Kellogg 2015, Christenhusz and Byng 2016, Soreng et al. 2017). In Turkey, Poaceae consists 658 species and infraspecific taxa within 146 genera (Cabi and Doğan 2012). *Alopecurus* L. (the foxtail genus) is a genus of the subtribe Alopecurinae with about 50 species all around the world, including many alpine species (Doğan 1988), the centre of diversity of the genus being in southwest Asia (Boudko 2014). According to Cabi and Doğan (2012) and Cabi et al. (2017), the genus *Alopecurus* is represented by 27 taxa, seven of which are endemic to Turkey. Turkish *Alopecurus* species occupy a wide variety of habitats ranging in elevation from sea level to high mountain steppes (Doğan 1985).

The grass family has highly specialized and reduced flowers and fine morphological distinctions are often essential to define differences among taxa (Ellis 1976, 1986). Therefore, Ellis (1976) indicated that anatomical data are regarded as being of undoubted importance in the jigsaw of complete systematic evidence. Leaf anatomical characters in cross-section have been the main supplementary tools to add to the morphological features for characterizing some genera of difficult taxonomy within the grass family (e.g., Ellis 1976, 1986, López and Devesa 1991, Martínez-Sagarra et al. 2017, Aykurt et al. 2022) such as *Festuca* L. (e.g., Martínez-Sagarra et al. 2017).

According to Doğan (1985), the *Alopecurus* taxa found in Turkey were classified under four sections: Sect. *Alopecurus*, Sect. *Colobachne* P.Beauv., Sect. *Pseudophalaris* Tzvelev., and Sect. *Tozzettia* (Savi) Endl. Then, as a result of the numerical taxonomy of the genus, *Alopecurus* was divided into three different sections: *Alopecurus*, *Alopecurium* Dumort. and *Colobachne* (Doğan 1997). According to Doğan (1985, 1997) the most diverse section in Turkey is *Colobachne*, which contains a total of 10 species if the recently described *A. goekyigitiana* Cabi & Soreng is counted (Cabi et al. 2017). All of the species in this section are mountainous caespitose perennials that grow in the subalpine or alpine zone (Doğan 1985, 1999, Cabi et al. 2017) with ovoid panicles and branches bearing 1–6 spikelets (Doğan 1985, Boudko 2014). The glumes gradually attenuate into long points (Doğan 1985) and are longer than the lemma. The palea of taxa in the Sect. *Colobachne* is generally present and rarely absent (Boudko 2014). The Turkish species included in this section, except for three species, which are *A. anatolicus* Doğan, *A. glacialis* K.Koch and *A. laguroides* Balansa, were

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evaluated within the scope of our study. In addition, *A. aequalis* Sobol. and *A. arundinaceus* Poir. were included in the study, because they also occur in high mountain areas. They are included in the sections *Alopecurium* and *Alopecurus*, respectively. The sections *Alopecurium* and *Alopecurus* consist of both annual and perennial species and the palea of the species is absent (Doğan 1985, Boudko 2014). The panicles are cylindrical to oblong and with 1–5 spikelets in the section *Alopecurium*. The glumes are acute to obtuse and are longer than or equal to the lemma. The section *Alopecurus* has ovoid to cylindrical panicles with each branch bearing 1–10 spikelets. Glumes are acute, equal to or longer than the lemma and connate in the lower half (Boudko 2014). Although numerous studies conducted on the morphology of the genus *Alopecurus* can be found (e.g., Doğan 1997, 1999, Soreng et al. 2007, Boudko 2014), there are limited studies conducted on the importance of leaf anatomical characters for *Alopecurus* species in the taxonomy of this genus.

In recent years, studies evaluating the phylogenetic relationships between the members of this genus have been conducted, and infrageneric and interspecific relationships have been interpreted with the use of both nuclear (ITS and ETS) and plastid markers (*trn*TLF, *rpo*B-*trn*C and *mat*K) (e.g., Soreng et al. 2007, Boudko 2014, Cabi et al. 2017). As a result of the plastid (TF+*rpo*B+*mat*K) and combined (ETS+ITS+TF+*rpo*B+*mat*K) maximum parsimony analyses made by Boudko (2014), it was shown that the infrageneric classification of the genus accepted by Doğan (1999) (sect. *Alopecurus* incl. *A. myosuroides*, sect. *Alopecurium* and sect. *Colobachne*) is not monophyletic. Phylogenetic studies involving more species are very important in order to resolve the phylogenetic relationships in both infrageneric and interspecific classification of the genus *Alopecurus* (Boudko 2014).

A large number of specimens belonging to nine *Alopecurus* species were collected in the high mountainous zone of the Western Taurus (Antalya, Turkey) during our project on the diversity of the Poaceae, and their leaf anatomy (in cross-section view) was evaluated. There are some difficulties during the identification and separation of some *Alopecurus* species with the use of morphological characters. This study aimed to (i) determine the leaf anatomical features in the *Alopecurus* species studied; (ii) determine the anatomical characters useful in identification of the species of the genus; (iii) evaluate the relationship between the taxa using the anatomical data.

Materials and methods

The tiller leaf anatomical features of mountain *Alopecurus* species that occur in subalpine and alpine areas in Turkey were evaluated in the present study. We investigated a total of nine *Alopecurus* species, seven of which are included in the Sect. *Colobachne*, which are *A. aucheri* Boiss., *A. davisii* Bor., *A. gerardii* (All.) Vill., *A. goekyigitiana, A. lanatus* Sm., *A. textilis* Boiss. and *A. vaginatus* (Willd.) Pall. ex Kunth. *Alopecurus goekyigitiana* and *A. lanatus*, endemic to Turkey. The other two species investigated, *A. aequalis* and *A. arundinaceus*, are common foxtails in different habitats including high mountainous regions. Most of the plant specimens examined were collected from natural populations during field trips between the years 2018 and 2020. We used herbarium materials deposited in AKDU and the collection data of the taxa used for our analyses are presented in Tab. 1. A total of 45 individuals, three individuals from

Tab. 1. Studied *Alopecurus* taxa and collection information of the specimens. OTU – operational taxonomic unit

OTU	Species	Locality data of specimens studied
OTU1	*A. aequalis*	Isparta, Gölcük Nature Park, 1390 m, 10.07.2013, *C. Aykurt 3397.*
OTU2	*A. arundinaceus*	Antalya, Kaş, Gömbe, Ak Mountain, around Yeşilgöl, 1825 m, 29.07.2018, *C. Aykurt 4779.*
		Antalya, Gündoğmuş, between Eğrigöl and Söbüçimen Plateau, 2087 m, 20.06.2019, *C. Aykurt 5345.*
OTU3	*A. vaginatus*	Antalya, Serik, Bozburun Mountain, 1855 m, 24.05.2019, *C. Aykurt 4940a.*
		Antalya, Alanya, Ak Mountain, 1985 m, 18.06.2019, *C. Aykurt 5245.*
OTU4	*A. textilis* subsp. textilis	Antalya, Akseki, Gidengelmez Mountains, 1950 m, 13.06.2012, *C. Aykurt 3224.*
		Antalya, Kumluca, Sarıkaya Wildlife Development Area, Bey Mountains, 2754 m, 28.06.2021, *SWDA 97-1-12.*
OTU5	*A. gerardii* var. *gerardii*	Antalya, Alanya, Başyayla environs, 1579 m, 17.06.2019, *C. Aykurt 5153.*
		Antalya, Alanya, Ak Mountain, 2044 m, 16.07.2019, *C. Aykurt 5514.*
OTU6	*A. goekyigitiana*	Antalya, Gündoğmuş, Eğrigöl to Hadim, 2205 m, 20.06.2019, *C. Aykurt 5327.*
		Antalya, Gündoğmuş, between Eğrigöl and Söbüçimen Plateau, 2087 m, 20.06.2019, *C. Aykurt 5344.*
OTU7	*A. aucheri*	Bitlis, Tatvan, Nemrut Mountain, 2480 m, 28.05.2019, *L.Y. Konuralp*.
OTU8	*A. davisii*	Izmir: Kemalpaşa, Mahmut Mountain, 1250-1368 m, 28.04.1992, *A. Aksoy 744.*
OTU9	*A. lanatus*	Antalya, Kumluca, Sarıkaya Wildlife Development Area, Bey Mountains, 2754 m, 28.06.2021, *SWDA 97-1-11.*
		Antalya, İbradı, Toka Plateau, 1514 m, 24.06.2020, *C. Aykurt 5624.*

each location, belonging to nine species were used for the anatomical measurements carried out within the scope of the study. At least three tiller leaves from each individual were analyzed.

Short pieces of tiller leaves taken from herbarium materials were kept in distilled water for approx. five minutes. Then, cross-sections ± 0.05 mm thick were cut by free hand from pieces of leaf fixed in styrofoam. This was done under a stereomicroscope using reflected light and the sections were stained in a drop of water with toluidine blue. After 1–3 minutes, depending on stainability, the sections were washed in distilled water and studied under a light microscope at a magnification of 10–40 × (general anatomical pattern) or 100 × (detailed shape of the epidermal and bundle sheath cells).

The leaf preparations were analyzed in detail and anatomical features were determined for each taxon. As a result, 14 anatomical characters that can be used for the genus *Alopecurus* were scored for each taxon and included in the numerical analysis. In this study, two different numerical analysis methods were used: clustering (UPGMA) and principal coordinate analysis (PCA) with the use of PAST Version 4.03 computer software. The anatomical characters determined for this study and evaluation of these characters for each taxon are presented in Tab. 2. In addition, the scatter biplot diagram of the studied *Alopecurus* species as operational taxonomic units (OTUs) based on the leaf anatomical characters determined was created. Ellis (1976, 1979) was used as the main source for choice of leaf anatomical characters.

Results

The results of our study are presented in two parts, in which the general leaf anatomical features and anatomical characters of *Alopecurus* taxa are evaluated taxonomically with numerical analyses.

The tiller leaf anatomical characters determined within the scope of the study are generally related to the epidermis, sclerenchyma strands and girders, both abaxial and adaxial ribs, and vascular bundles. The cross-sections taken from the tiller leaf blades of all species studied are shown in Fig. 1. The first anatomical character was determined as the shape of the leaves in cross-section. Almost all species of Sect. *Colobachne* included in the study have a U-shaped tiller leaf cross-section, except for *A. vaginatus*, which has a U- to O-shaped cross-section. In contrast, leaf cross-sections of *A. aequalis* (sect. *Alopecurium*) and *A. arundinaceus* (sect. *Alopecurus*) are flat. Characteristics determined in relation to the epidermis are the size of epidermal cells (C13), the size of bulliform cells (C14), papillae on the epidermal cells (C3) and density of macro-hairs (C7). The epidermal cells of all *Alopecurus* species studied are single-layered and generally polygonal in shape. The size of epidermal cells was categorized under three different groups according to their cell length. Accordingly, the epidermal cells of the species included in sect. *Colobachne* are quite different in size. In addition, bulliform cells are prominent in all species studied except *A. aequalis*.

Tab. 2. Tiller leaf anatomical characters for the genus *Alopecurus* and their scorings determined within the scope of the study and used in the numerical analyses.

Character	Description
C1	The shape of the tiller leaves in cross-section: flat (0) / U- to O-shaped (1)
C2	The midrib larger than the laterals (1) / not larger (0)
C3	Papillae on the epidermal cells: Outer walls of epidermal cells arched but not papillose (0) / Entire or major part of epidermis composed of thin-walled wide papillae (1) / Thin-walled wide papillae scattered throughout the epidermis (2)
C4	Arrangement of adaxial sclerenchyma strands as: scattered few groups (0) / regular groups at the level of the vascular bundles (1) / extended along the lobes (2) / T-shaped (3)
C5	The number of abaxial sclerenchyma strands: 5–14 (0) / 17–21 (1) / 24–28 (2)
C6	Depth of adaxial furrows in comparison to the leaf thickness: Medium furrows; quarter to one half the leaf thickness (0) / Deep furrows; more than one half the leaf thickness (1)
C7	Density of macro hairs: few and scattered (0) / dense (1)
C8	Abaxial ribs: absent (0) / slightly lobed (1) / distinct 14–21 (2)
C9	The number of adaxial ribs: 6–9 (0) / 12–16 (1) / 17–20 (2)
C10	Abaxial sclerenchyma strands as: very small or small strands (0) / big strands like a cap (1) / with well-developed girder (2)
C11	The number of vascular bundles: 6–10 (0) / 11–18(1)
C12	The connection of midrib with epidermal layer: midrib not connected (0) / midrib connected with abaxial side (1) / midrib connected to both sides (2)
C13	The size of epidermal cells: up to 12.5 μm long (0) / up to 17.99 μm long (1) / up to 24 μm long (2)
C14	The size of bulliform cells: not distinct (0) / 16–26 μm (1) / 35–45 μm (2)

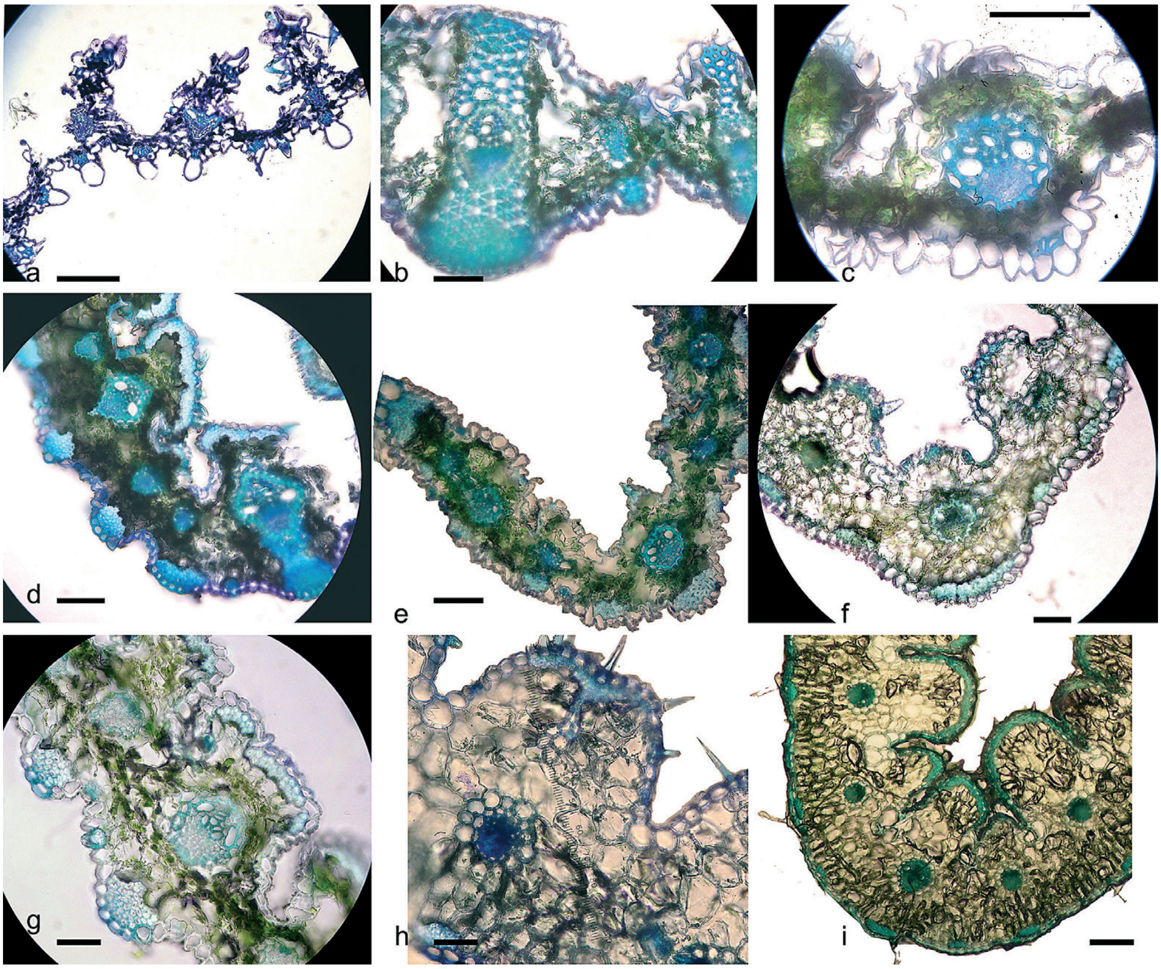

Fig. 1. Tiller leaf cross-sections of *Alopecurus* taxa studied. a – *A. aequalis* (from C. Aykurt 3397), b – *A. arundinaceus* (from C. Aykurt 5345), c – *A. vaginatus* (from C. Aykurt 4940a)*;* d – *A. textilis* subsp. *textilis* (from C. Aykurt 3224), e – *A. gerardii* var. *gerardii* (from C. Aykurt 5514), f – *A. goekyigitiana* (from C. Aykurt 5327), g – *A. aucheri* (from L.Y. Konuralp)*;* h – *A. davisii* (from A. Aksoy 744), i – *A. lanatus* (from C. Aykurt 5624). Scale bars: 50 µm.

In the species studied, the arrangement of adaxial sclerenchyma strands (C4) can be scattered as few groups, regular groups at the level of the vascular bundles, extended along the lobes or T-shaped. The adaxial sclerenchyma strands are: regular groups at the level of the vascular bundles in *A. aequalis* and *A. arundinaceus*; a few scattered groups in *A. vaginatus*, *A. gerardii* and *A. goekyigitana*; and extended along the lobes in *A. aucheri* and *A. textilis*. Only in *A. davisii* are the adaxial sclerenchyma strands T-shaped. The abaxial sclerenchyma strands (C 10) are: very small or small strands; big strands like a cap; or with well-developed girder. Abaxial sclerenchyma strands with well-developed girders appear only in *A. arundinaceous*. In *A. textilis* and *A. aucheri,* the abaxial strands are big and resemble a cap. The rest of the species studied have very small or small abaxial sclerenchyma strands.

Based on the PCA results, the variance value of the first two components is 59.58%, and the variance value of the first four components is 91.06% (Fig. 2.). The eigenvalue and the percentage of eigenvalue of the components, and the eigen vector value of the components are given in the Appendices, respectively. According to the results of the numerical analysis PC1, the first five most reliable characters are C4 (arrangement of adaxial sclerenchyma strands), C9 (the number of adaxial ribs), C5 (The number of abaxial sclerenchyma strands), C13 (the size of epidermal cells) and C11 (the number of vascular bundles), respectively; based on PC2, it is seen that they are C12 (the connection of midrib with epidermal layer), C10 (the number adaxial ribs), C14 (the size of bulliform cells), C3 (Papillae on the epidermal cells) and C2 (the ratio of midrib size to the laterals).

The obtained UPGMA dendrogram shows that *A. aequalis* (Sect. *Alopecurium*) is separated from all other species studied (Fig. 3). *Alopecurus arundinaceus* is located close to the cluster of the Sect. *Colobachne* species. *Alopecurus* Sect. *Colobachne* species are grouped together into two main branches. *Alopecurus vaginatus*, *A. gerardii* and *A. goekyigitiana* are grouped together and close to *A. textilis* (first group), whereas *A. lanatus* and *A. davisii* are grouped in a different branch close to *A. aucheri* (second group). The anatomical characters most useful for separating these two species groups are C9 (the number of adaxial ribs) and C11 (the number of vascular bundles). The number of adaxial ribs is between 6–9 in the first group, where-

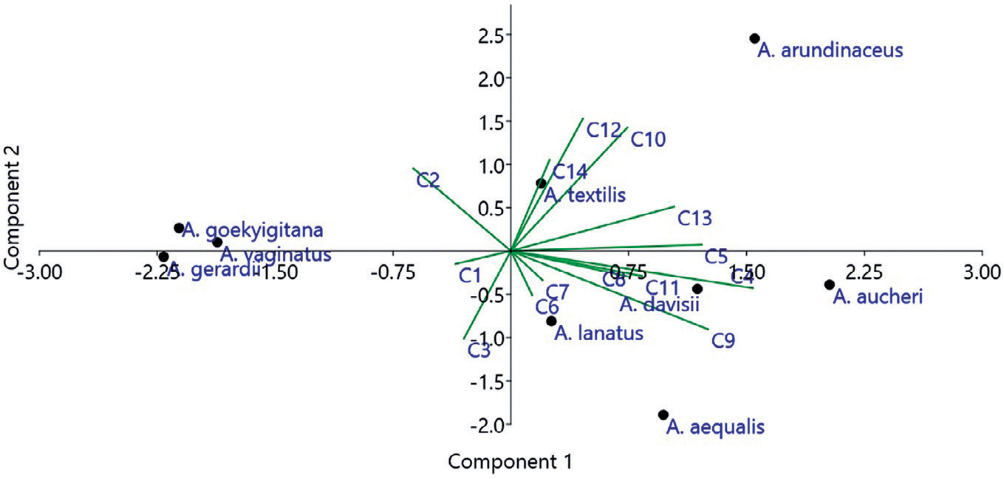

Fig. 2. Principal component analysis (PCA) biplot scatter diagram of studied *Alopecurus* species as operational taxonomic unit (OTUs) along PC1 and PC2 axes based on 14 leaf anatomical characters described in Tab 2.

as they are between 12–20 in the second group. The adaxial ribs are between 17–20 in *Alopecurus aucheri* and are between 12–16 in *A. davisii* and A. *lanatus*. While the number of vascular bundles varies between 6–10 in the first group; in the second group, it varies between 11–18. Detailed evaluations and measurements of the species studied are also given in Tab. 3.

The abaxial ribs (C8) are very prominent in only two of the species studied, which are *A. textilis* and *A. aucheri*. The abaxial ribs are slightly lobed in *A. aequalis* and are absent in the other species studied. The number of vascular bundles was evaluated under two categories, and this character is very useful to separate the species groups in the sect. *Colobachne*.

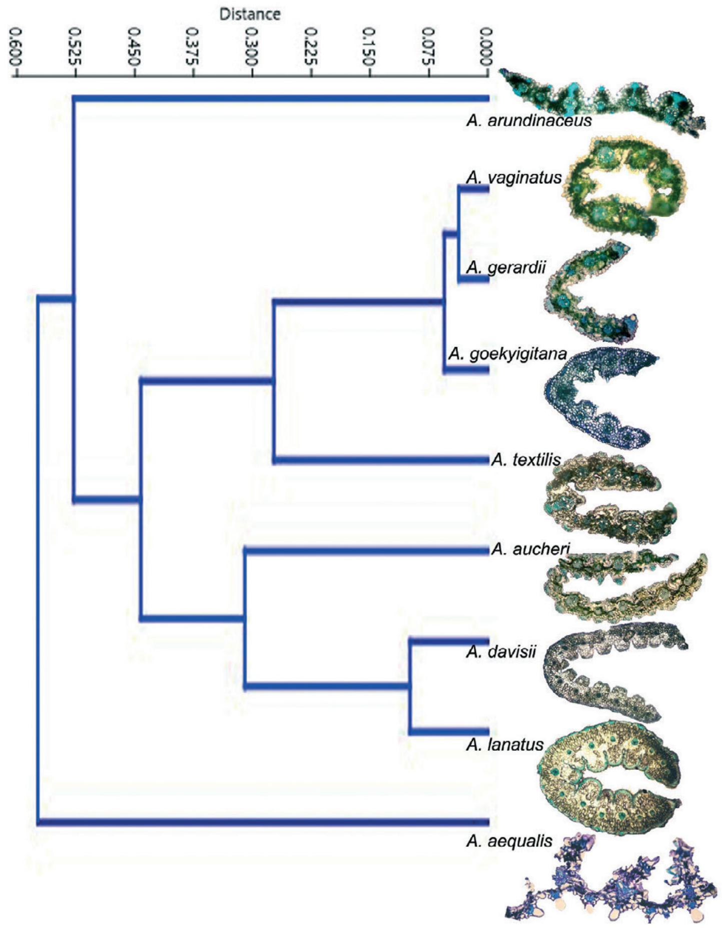

Fig. 3. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram created according to the Gower similarity index by using anatomical character matrix of *Alopecurus* species studied (see Tab. 2).

Tab. 3. Evaluation, measurements and scoring of the characters used in numerical analyses according to the tiller leaf anatomical features of *Alopecurus* species studied as described in Tab. 2.

Leaf anatomical characters	***A. aequalis***	***A. arundinaceus***	***A. vaginatus***	***A. textilis***	***A. gerardii***	***A. goekyigitiana***	***A. aucheri***	***A. davisii***	***A. lanatus***
The shape of the tiller leaves in cross-section (C1)	Flat (0)	Flat (0)	U- to O-shaped (1)	U-shaped (1)	U-shaped (1)	U-shaped (1)	U-shaped (1)	U-shaped (1)	U-shaped (1)
The midrib larger than the laterals or not (C2)	Not larger than the laterals (0)	Larger than the laterals (1)	Larger than the laterals (1)	Larger than the laterals (1)	Larger than the laterals (1)	Larger than the laterals (1)	Not larger than the laterals (0)	Not larger than the laterals (0)	Not larger than the laterals (0)
Papillae on the epidermal cells (C3)	Thin-walled wide papillae scattered throughout the epidermis (2)	Outer walls of epidermal cells arched but not papillose (0)	Entire or major part of epidermis composed of thin-walled wide papillae (1)	Outer walls of epidermal cells arched but not papillose (0)	Entire or major part of epidermis composed of thin-walled wide papillae (1)	Outer walls of epidermal cells arched but not papillose (0)	Outer walls of epidermal cells arched but not papillose (0)	Outer walls of epidermal cells arched but not papillose (0)	Outer walls of epidermal cells arched but not papillose (0)
Arrangement of adaxial sclerenchyma strands (C4)	Regular groups at the level of the vascular bundles (1)	Regular groups at the level of the vascular bundles (1)	Scattered few groups (0)	Extended along the lobes (2)	Scattered few groups (0)	Scattered few groups (0)	Extended along the lobes (2)	T-shaped (3)	Extended along the lobes (2)
The number of abaxial sclerenchyma strands (C5)	24-26 (2)	24-28 (2)	5–7 (0)	17–18 (1)	9–11 (0)	12–14 (0)	17–21 (1)	17–18 (1)	17–18 (1)
Depth of adaxial furrows in comparison to the leaf thickness (C6)	Deep furrows (1)	Medium furrows (0)	Medium furrows (0)	Medium furrows (0)	Medium furrows (0)	Medium furrows (0)	Medium furrows (0)	Medium furrows (0)	Medium furrows (0)
Density of macro-hairs (C7)	Few and scattered (0)	Few and scattered (0)	Few and scattered (0)	Few and scattered (0)	Few and scattered (0)	Few and scattered (0)	Few and scattered (0)	Dense (1)	Dense (1)
The number of abaxial ribs (C8)	Slightly lobed (1)	Absent (0)	Absent (0)	16–19 (2)	Absent (0)	Absent (0)	14-21 (2)	Absent (0)	Absent (0)
The number of adaxial ribs (C9)	15–20 (2)	15–16 (1)	6–8 (0)	7–9 (0)	6–7 (0)	6–7 (0)	11–20 (2)	13–15 (1)	12–13 (1)
Abaxial sclerenchyma strands (C10)	Very small or small strands (0)	With well-developed girder (2)	Very small or small strands (0)	Big strands like a cap (1)	Very small or small strands (0)	Very small or small strands (0)	Big strands like a cap (1)	Very small or small strands (0)	Very small or small strands (0)
The number of vascular bundles (C11)	12–20 (1)	17–20(1)	7–8 (0)	7–10 (0)	6–7 (0)	7–8 (0)	12–14 (1)	15–17 (1)	14–18 (1)
The connection of midrib with epidermal layers (C12)	Midrib not connected (0)	Midrib connected to both sides (2)	Midrib not connected (0)	Midrib connected with abaxial side (1)	Midrib not connected (0)	Midrib not connected (0)	Midrib not connected (0)	Midrib not connected (0)	Midrib not connected (0)
The size of epidermal cells (µm × µm) (C13)	11.24–14.79 × 8.57–8.87 (1)	15.31–20.33 × 8.67–10.11 (2)	9.36–17.57 × 7.57–12.42 (1)	10.11–11.90× 5.65–8.33 (0)	11.51–17.87 × 7.87–9.39 (0)	10.62–16.32 × 3.88–6.99 (0)	17.51–24.20 × 8.91–10.50 (2)	13.22–17.35 × 6.33–9.09 (1)	10.08–12.35 × 7.05–8.23 (0)
The size of bulliform cells (µm × µm) (C14)	Not distinct (0)	40.46–44.79 × 15.02–26.58 (2)	19.09–20.90 × 8.48–10.28 (1)	16.07–22.6× 9.31–16.45 (1)	16.36–19.39 × 12.12–15.75 (1)	16.48–23.67 × 12.39–17.28 (1)	20.26–26.36 × 11.51–13.93 (1)	32.50–35.26 × 17.90– 24.51 (2)	22.94–25.88 × 9.41–17.64 (1)

The depth of adaxial furrows is more than one half of the leaf thickness (C6), and thin-walled wide papillae are easily visible as scattered throughout the epidermis (C3) in *A. aequalis.* With these characters, *A. aequalis* can be easily distinguished from other species. *Alopecurus arundinaceus* is the only species having a midrib connected to both sides (C12). The sclerenchyma bands around the mature vascular bundles of this species are elongated to both abaxial and adaxial surfaces.

Results from the PCA analysis showed that the least reliable characters are C6 (Depth of adaxial furrows in comparison to the leaf thickness) and C7 (Density of macrohairs) according to PC1.

Identification key for *Alopecurus* taxa studied

1. Leaves flat in cross-section 2
1. Leaves U- or O-shaped in cross-section 3
2. Abaxial and adaxial surfaces connected with sclerenchyma cells; depth of adaxial furrows quarter to one half the leaf thickness; outer walls of epidermal cells arched but ot papillose .... *A. arundinaceus*
2. Abaxial and adaxial surfaces not connected with sclerenchyma cells; depth of adaxial furrows more than one half the leaf thickness; thin-walled wide papillae scattered throughout the epidermis .... *A. aequalis*
3. The number of vascular bundles 6–10 4
3. The number of vascular bundles 11–18 7
4. Adaxial sclerenchyma strands scattered in several groups 5
4. Adaxial sclerenchyma strands extended along the lobes .... *A. textilis*
5. Outer walls of epidermal cells arched but not papillose .... *A. goekyigitiana*
5. Entire or major part of epidermis composed of thin-walled wide papillae 6
6. Epidermal cells up to 12.5 μm long .... *A. gerardii*
6. Epidermal cells up to 17.99 μm long ...... *A. vaginatus*
7. Abaxial ribs distinct .... *A. aucheri*
7. Abaxial ribs absent 8
8. Adaxial sclerenchyma bands T-shaped; epidermal cells up to 17.99 μm long .... *A. davisii*
8. Adaxial sclerenchyma bands not T-shaped; epidermal cells up to 12.5 μm long .... *A. lanatus*

Discussion

We focused on the tiller leaf anatomical characters of the high mountain *Alopecurus* species in Turkey in the present study. All *Alopecurus* species adapted to subalpine and alpine mountainous areas in Turkey are caespitose perennials classified in the Sect. *Colobachne* (Doğan 1985, 1997, 1999, Cabi et. al. 2017). The tiller leaves of these morphologically similar species are filiform, narrowly lanceolate and generally convolute. The mountain *Alopecurus* species studied exhibit leaf anatomical characters similar to those of high mountain *Festuca* species. Abaxial sclerenchyma patterns, number of vascular bundles and number of ribs are among the most distinctive morphological characters for *Festuca* (Martínez-Sagarra et al. 2017). Considering the results obtained from our study, it is seen that these characters are similar to the most important characters in PC1 in the high mountain species of the genus *Alopecurus* (C4, C9, C5 and C8).

The anatomical characters analyzed within the scope of our study can be easily observed in leaf cross-sections that can be taken from herbarium materials. Since dry samples were used, some distinguishing anatomical characters specified by Ellis (1976, 1979) could not be used, such as vascular bundle, vascular bundle sheath, and leaf mesophyll (chlorenchyma and colourless parenchyma cells) in the present study. The results obtained showed that especially the number of both adaxial and abaxial ribs, and features of sclerenchyma strands such as number, arrangement and girders are very important and useful anatomical characters. Metcalfe (1960) stated that the ribs are generally characteristic of and more fully developed on the adaxial than the abaxial surface. Adaxial ribs are distinct in all of the species evaluated within the scope of the study, while abaxial ribs are distinct only in *A. textilis* included in the first group and *A. aucheri* included in the second group. According to Ellis (1976), ribs and furrows may vary in depth, transverse shape, spacing and location in different species. In *A. aequalis* the adaxial furrows are distinct and much deeper than in the other species studied, which have medium furrows. In the PCA results obtained, the length of epidermal cells is one of the most significant anatomical characters. *Alopecurus textilis*, *A. gerardii* and *A. goekyigitiana* have the smallest epidermal cells, in contrast to *A. arundinaceus* and *A. aucheri*, which have the biggest epidermal cells.

The least significant leaf anatomical characters according to the PC1 results are depth of adaxial furrows (C6), density of macro hairs (C7), size of bulliform cells (C14) and papillae on the epidermal cells (C3), respectively. We evaluated C7 as scattered and dense; accordingly, *A. davisii* and *A. lanatus*, which have densely hairy leaves, were scored differently from the rest of the species studied. In the dendrogram obtained, the relationships among the taxa are similar to the results obtained previously by different researchers, from both morphological and phylogenetic studies (e.g., Doğan 1997, 1999, Cabi et al. 2017). This is an indication that anatomical characters are useful for the taxonomy of the genus *Alopecurus*. *Alopecurus aequalis* was separated into a different branch from all other species. Among the studied species, *A. aequalis*, which is an annual and grows in damp places and marshy habitats, has distinct anatomical differences from the others in terms of the characters C3, C6, C8 and C14. Although *A. arundinaceus* is close to other species studied, it is separated into a different branch. It differs markedly from the other species studied in that the midrib is connected to both the abaxial and adaxial surfaces via sclerenchyma cells, and its leaves are flat in cross-section. Doğan (1985) stated that *A. arundinaceus* is rare in South Anatolia. However, we observed during the field studies that this species was widespread in the high mountainous areas of southern Anatolia.

The section *Colobachne* is divided into two groups in the dendrogram according to the number of adaxial ribs (C9), the number of vascular bundles (C11) and the size of midrib in comparison to the laterals (C2). In the first group composed of *A. vaginatus, A. gerardii, A. goekyigitiana* and *A. textilis*, the number of vascular bundles is between 6–10, the number of adaxial ribs is between 6–9 and the midvein is larger than the laterals. In the second group, which includes *A. aucheri, A. davisii* and *A. lanatus*, the number of vascular bundles is between 11–18, the number of adaxial ribs is between 12–20 and the midvein is not larger than the laterals.

Alopecurus goekyigitiana as described by Cabi et al. (2017) was evaluated as closely related to *A. gerardii* and *A. vaginatus*. According to our results, it is similar to these species in terms of its leaf anatomical features. Unlike these species, however, the outer walls of the epidermal cells in *A. goekyigitiana* are arched but not papillose (C3). The entire or major part of the epidermis in *A. gerardii* and *A. vaginatus* is composed of thin-walled wide papillae. It was also noted by Codignola et al. (1987) that *A. gerardii* has a bulbous epidermis. When evaluated in terms of this character (C3), *A. goekyigitiana* resembles *A. lanatus* and *A. davisii*. *Alopecurus davisii* and *A. lanatus*, indicated as closely related species by Doğan (1985), have some differences in terms of their leaf anatomical characters such as C4, C13 and C14 (Tab. 3). The most distinctive anatomical feature that can be used to distinguish between these two species is the arrangement of the adaxial sclerenchyma strands (C4); the adaxial sclerenchyma bands are T-shaped in *A. davisii*, whereas the sclerenchyma strands are extended along the lobes in *A. lanatus*.

Among the studied species, only *A. aucheri* and *A. textilis* have prominent abaxial ribs. Although these two species are similar in many anatomical characters, they differ in the most reliable characters, C9 and C13. This has caused these species to be included in separate groups.

The results obtained show that the leaf anatomical characters we determined within the scope of the study are very useful in grouping close species and separating species from each other for the genus *Alopecurus*. The tiller leaf anatomical features of the species groups and of the species should be clarified by further analysis of the anatomical features by using fresh materials for this genus. With this study, it has been shown that tiller leaf anatomical characters will contribute greatly to the systematics of the genus.

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The epiphytic bryophyte succession of *Buxus sempervirens* forests in the Fırtına Valley, Rize (North Türkiye)

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Abstract – In this study, the epiphytic bryophyte succession of the *Buxus sempervirens* L. forests in Fırtına Valley (Çamlıhemşin-Rize, North Türkiye), one of the nine biodiversity hotspots in Türkiye, was investigated. For this purpose, a total of 60 sampling plots were taken from the live trunks of *B. sempervirens* trees of different ages. Twenty-nine epiphytic bryophyte species were determined (24 mosses and 5 liverworts) within the sample plots. Also, six different life form types and four different habitat affinity categories were determined. Among them, the mat type life form is in first place with 34.4% and the cortico-saxicolous species are the most common with 51.7%. Two-way indicator species analysis (TWINSPAN) classified the epiphytic bryophyte communities on *B. sempervirens* trunks at the second level into two main clusters (A and B) and three sub-clusters (A1, B1 and, B2). Detrended correspondence analysis (DCA) axis 1 was interpreted as gradient along the height of the epiphytic habitat (from the lower base to the upper zone) on trunks and the DCA axis 2 was interpreted as gradient of moisture (from mesic to xeric). *Exsertotheca crispa* (Hedw.) S. Olsson, Enroth & D. Quandt was the species with the highest index of ecological significance (IES) value on the lower bases of the aged trees. Species diversity and epiphytic cover in the upper zones were lower than in the basal and middle zones in the study area. While *Metzgeria furcata,* (L.) Corda*, Oxyrrhynchium hians* (Hedw.) Loeske, *Plagiothecium nemorale* (Mitt.) A.Jaeger, and *Radula lindenbergiana* Gottsche ex C.Hartm were only found on old trees, *Ctenidium molluscum* (Hedw.) Mitt. and *Pseudoleskeella nervosa* (Brid.) Nyholm were only found on middle-aged trees.

Keywords: community, index of ecological significance, liverworts, mosses, ordination analyses

Introduction

Bryophytes, the pioneer plants of different substrate types, are one of the most important component of forest ecosystems (Longton 1992, Baldwin and Bradfield 2005, Ezer 2017, Mellado-Mansilla et al. 2017). Bryophytes are poikilohydric organisms whose their moisture content rapidly equilibrating with environmental conditions (Green and Lange 1994). Therefore, they are highly sensitive to environmental factors (Schofield 2001). In particular, abiotic ecological factors such as humidity directly or indirectly affect the colonization and the distribution of bryophytes in the epiphytic habitat (Mazimpaka and Lara 1995, Schofield 2001, Mishler 2003, Mazimpaka et al. 2009). In addition to environmental drought, phorophyte-type, physical and chemical properties of bark characteristics such as rugosity, water retention capacity, bark pH, and dust deposition are also important for the spatial distribution of bryophytes on epiphytic habitats (Lara and Mazimpaka 1998, Mazimpaka et al. 2010, Ezer 2017).

Some studies on the succession of epiphytic bryophyte communities have revealed that the succession gradient of epiphytes is highly complex due to changes in positive and negative interactions among species within epiphytic communities as trees age (Mazimpaka et al. 2010, Ódor et al. 2013, Bargali et al. 2014, Ezer 2017, Ezer et al. 2019).

Although phytosociological studies on epiphytic bryophytes in Türkiye have made progress in the last decade

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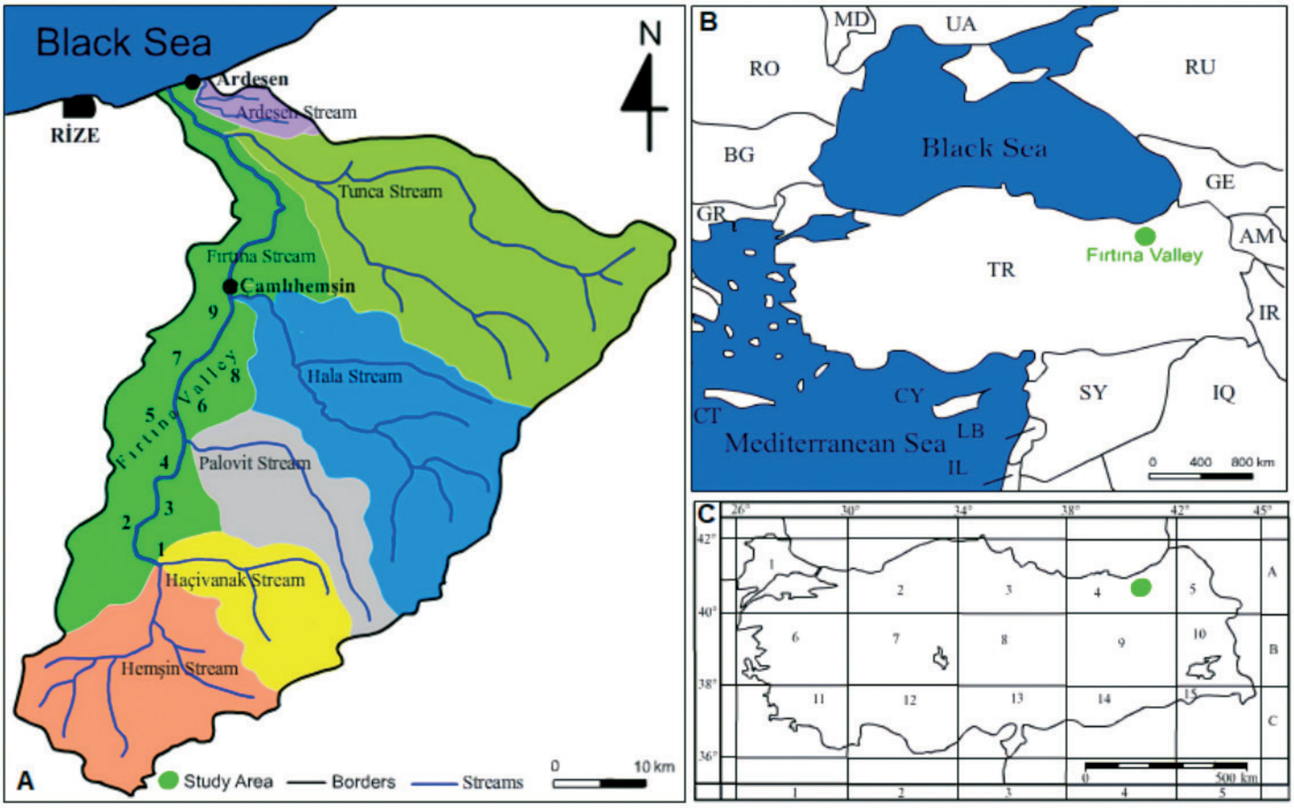

Fig. 1. The Fırtına Valley where epiphytic bryophyte species were investigated (A), its location in Türkiye (B) and in the Henderson's grid system (1961) (C).

(Alataş et al. 2017, 2021, Alataş 2018, Can Gözcü et al. 2018), few studies have been done on the spatial distributions and community composition of epiphytic bryophytes in successional stages (Ezer and Kara 2013, Ezer 2017, Ezer et al. 2019).

The present study focuses on the vertical distribution patterns of epiphytic bryophytes and community composition in the successional stages on trunks of *Buxus sempervirens* trees in Fırtına Valley. This study aimed to reveal the successional trends of bryophyte communities on the epiphytic habitats of boxwood trees and to contribute to bryo-ecological studies in Türkiye.

The study area

The Çamlıhemşin (Rize, Türkiye) District, in which the study area is located, is surrounded by Pazar and Ardeşen to the north, Çayeli, Hemşin and İkizdere districts to the west, and Artvin Province Yusufeli district to the east. The distance of the district to Rize city center is 62 km. The *B. sempervirens* forests, located in the Fırtına Valley and within the boundaries of the Çamlıhemşin District, are located within the A4 square according to the Henderson (1961) grid-square system and are in the colchic zone of the Euro-Siberian phytogeographic region (Anşin 1983, Fig. 1).

The Fırtına Valley, which exhibits a unique phytological diversity, hosts many rare species due to the presence of all the main habitats of the region. *Buxus sempervirens*, which has a very wide distribution in the Euro-Siberian Phytogeographic Region, forms remarkable communities in the valley (Kurdoğlu et al. 2004). Moreover, in 1999, the WWW (World Wildlife Fund) identified Europe's 100 forest areas valuable in terms of biodiversity and in urgent need of protection (Myers et al. 2000). Nine of these areas, which are called "Hot Spots of European Forests", are located in Türkiye (Satar and Güneş 2014). One of these nine hot spots is the Fırtına Valley. Only one study on bryophytes has been conducted in the valley so far (Abay et al. 2006).

Fırtına Valley, like the whole of the Eastern Black Sea Region, is an area very open to natural disasters such as landslides, rockfalls and avalanches due to the very steep slopes, rainy climatic conditions and, soil cover (Tunçel 1990).

Lithologically, there are units from almost all geological times in the valley (Bayrakdar 2006). In Türkiye, not rich in current glaciers, Kaçkar Mountains (3932 m) located to the south of the Fırtına Valley are one of the regions where current glaciers are found (Çiner 2003). The Kaçkar Mountains, the Verçenik (Üçdoruk) Mountain, the Bulut Mountains and, the Altınparmak Mountains are mountain ranges that limit the study area in the SW-NE direction. In the valley, which is also very rich in terms of rivers, Fırtına Stream is divided into branches at various degrees and forms the smaller Tunca, Hala, Palovit, Haçivanak and Hemşin creeks. These creeks also forked among themselves and surround the valley like a net system.

Tab. 1. Sampling localities from where epiphytic bryophyte specimens were collected and their characteristics.

Localities		Altitude (m)	Date	GPS coordinates
1	Çat Valley, Doğa Village	1275	17.05.2020	N 40°51’48.95” E 40°55’58.08”
2	Between Çatköy and Meydanköy	1231	17.05.2020	N 40°51’59.44” E 40°55’46.26”
3	Meydanköy Exit	1176	17.05.2020	N 40°52’13.82” E 40°55’37.52”
4	Meydanköy	1108	26.08.2020	N 40°52’48.29” E 40°55’43.37”
5	Meydanköy Entrance	1061	26.08.2020	N 40°53’20.49” E 40°55’51.11”
6	Meydanköy, Pul Place	1026	26.08.2020	N 40°53’40.83” E 40°56’31.71”
7	Gito Plateau- Southeast slopes	1018	09.10.2020	N 40°54’18.08” E 40°56’52.30”
8	Between Zilkale and Meydanköy	1004	09.10.2020	N 40°54’27.30” E 40°56’53.71”
9	Zilkale Place	956	09.10.2020	N 40°54’47.61” E 40°56’52.38”

The forest formation, which starts at 200 m in the north of the study area, is very rich in terms of under-forest flora, depending on the oceanic and temperate climatic conditions in all seasons. This forest formation, which is dominated by broadleaf trees, loses its colchic feature with the increase in altitude and gives way to mixed forests and then to coniferous forests (Özçağlar et al. 2006). The vegetation in the Fırtına Valley shows a distinct difference from other valleys, especially with its particular forest formations and flora richness. There are basically three types of forest formation such as alluvial forests, hardwood forests found in river floodplains and regularly flooded for a portion of the growing season; boxwood forests; and old growth forest formations in the Fırtına Valley (Kurdoğlu et al. 2004).

The *B. sempervirens* forests spread along the Fırtına Stream and its tributaries, between 200-1500 meters of the study area. Although these forests are seen along the streams, they are also found in large groups on the valley slopes. These forests are widely found between 900-1300 meters along the Çamlıhemşin-Meydan road, in Şimşirlik place, within the Gito Forests and Palovit Valley.

The study area generally has a temperate Oceanic climate. The annual average precipitation is 2192 mm and, the annual average temperature is 8.3 °C in Çamlıhemşin. The hottest month of the year is August and the coldest is January. The absence of a dry season indicates that the Fırtına Valley is under the influence of an Oceanic climate (Akman 2011).

Materials and methods

Field sampling procedures

The epiphytic bryophyte specimens were collected from the living trunks of *B. sempervirens* in Fırtına Valley during field studies in 2020. The locality details are given in Tab. 1. A total of 60 sampling plots were taken from the trunks of 20 boxwood trees of different ages in the valley. Spatial distributions and community structures of epiphytic bryophytes in successional stages on boxwood trees were investigated according to tree diameter at breast height (dbh) and tree age. The trees were divided into three age classes using an indirect method as young (dbh 20-35 cm, 21 plots), middle-aged (dbh 40-50 cm, 18 plots), and aged trees (dbh 60-80 cm, 21 plots). Each boxwood tree was divided into the lower base zone (0 - 40 cm from the ground), the middle zone (40 - 120 cm), and the upper zone (120 - 180 cm) as proposed by Moe and Botnen (2000).

Sampling plots from tree zones were defined by 20 × 20 cm^2, determined according to species diversity on the living trunks of *B. sempervirens.* In the present study the branches of the boxwood trees did not provide sample plots, only the trunks. The percentage cover of the species within the each sample plot was visually estimated and ecological data such as humidity, aspect and light of the epiphytic habitat were recorded.

The nomenclature of the epiphytic bryophyte species determined within the sample plots follows Ros et al. (2013) and Hodgetts et al. (2020) (Tab. 2). Habitat affinity types of bryophytes were established following Mazimpaka and Lara (1995) and Draper et al. (2003). Life forms of the taxa were determined according to Mägdefrau (1982). Voucher specimens were deposited in the herbarium of Niğde Ömer Halisdemir University.

Data analysis

The relative frequency of each taxon in the sample plots was determined by the index of ecological significance (IES) described by Lara and Mazimpaka (1998), Albertos et al. (2001) and Mazimpaka et al. (2009). The formula used to calculate the IES values for each taxon is as follows:

$$\mathrm{IES} = \mathrm{F}\ (1 + \mathrm{C})$$

$$\mathrm{C} = \Sigma\ \mathrm{ci} / \mathrm{x}$$

where F is the relative frequency (100 x / n), and C is the cover of the taxon (Σ ci / x), while x represents the number of sample plots containing the taxon, n is total number of sample plots, and ci is cover class assigned to the taxon in each sample plot.

Cover classes of taxa were established using the six-point Lara and Mazimpaka (1998) scale: [0.5 (< 1%), 1 (1-5%), 2 (6-25%), 3 (26-50%), 4 (51-75%) and, 5 (76-100%)]. In addition, the IES values were combined in the following abundance classes: very scarce (< 25), scarce (26-50), moderately abundant (51-100), abundant (101-200), and dominant (> 200).

Tab. 2. List of epiphytic bryophyte species found on the *Buxus sempervirens* trees, their families and life form types and affinity for epiphytic habitats (Mägdefrau 1982, Mazimpaka and Lara 1995, Draper et al. 2003).

Species	Family	Life form	Affinity for epiphytic habitats	Frequency (%)
MOSSES				
Alleniella besseri (Lobarz.) S.Olsson, Enroth & D.Quandt	Neckeraceae	fan	Cortico-saxicolous	65
Alleniella complanata (Hedw.) S.Olsson, Enroth & D.Quandt	Neckeraceae	fan	Cortico-saxicolous	76.6
Anomodon viticulosus (Hedw.) Hook. & Taylor	Anomodontaceae	tail	Cortico-saxicolous	3.3
Brachythecium rutabulum (Hedw.) Schimp.	Brachytheciaceae	weft	Preferantially not corticolous	5
Ctenidium molluscum (Hedw.) Mitt.	Myuriaceae	weft	Indifferent	3.3
Exsertotheca crispa (Hedw.) S.Olsson, Enroth & D.Quandt	Neckeraceae	fan	Cortico-saxicolous	93.3
Fissidens serrulatus Müll.Hal.	Fissidentaceae	fan	Indifferent	5
Homalia trichomanoides (Hedw.) Brid.	Neckeraceae	fan	Cortico-saxicolous	16.6
Hypnum cupressiforme Hedw.	Hypnaceae	weft	Indifferent	20
Isothecium alopecuroides (Lam. ex Dubois) Isov.	Lembophyllaceae	mat	Cortico-saxicolous	28.3
Leucodon sciuroides (Hedw.) Schwägr.	Leucodontaceae	tail	Cortico-saxicolous	33.3
Orthotrichum pumilum Sw. ex anon.	Orthotrichaceae	cushion	Customary epiphyte	30
Oxyrrhynchium hians (Hedw.) Loeske	Brachytheciaceae	weft	Indifferent	1.6
Palamocladium euchloron (Müll.Hal.) Wijk & Margad.	Brachytheciaceae	tail	Cortico-saxicolous	15
Plagiothecium nemorale (Mitt.) A. Jaeger	Plagiotheciaceae	mat	Indifferent	1.6
Pseudanomodon attenuatus (Hedw.) Ignatov & Fedosov	Neckeraceae	mat	Cortico-saxicolous	31.6
Pseudoamblystegium subtile (Hedw.) Vanderp. & Hedenäs	Amblystegiaceae	weft	Cortico-saxicolous	6.6
Pseudoleskeella nervosa (Brid.) Nyholm	Pseudoleskeellaceae	mat	Cortico-saxicolous	1.6
Pterigynandrum filiforme Hedw.	Pterigynandraceae	tail	Cortico-saxicolous	6.6
Sciuro-hypnum flotowianum (Sendtn.) Ignatov & Huttunen	Brachytheciaceae	mat	Cortico-saxicolous	35
Sciuro-hypnum populeum (Hedw.) Ignatov & Huttunen	Brachytheciaceae	weft	Indifferent	3.3
Thamnobryum alopecurum (Hedw.) Gangulee	Neckeraceae	dendroid	Indifferent	5
Thuidium delicatulum (Hedw.) Schimp.	Thuidiaceae	weft	Indifferent	6.6
Ulota crispa (Hedw.) Brid.	Orthotrichaceae	cushion	Customary epiphyte	31.6
LIVERWORTS				
Frullania dilatata (L.) Dumort	Frullaniaceae	mat	Cortico-saxicolous	10
Frullania tamarisci (L.) Dumort.	Frullaniaceae	mat	Cortico-saxicolous	8.3
Metzgeria furcata (L.) Dumort.	Metzgeriaceae	mat	Indifferent	5
Radula complanata (L.) Dumort	Radulaceae	mat	Customary epiphyte	51.6
Radula lindenbergiana Gottsche ex C. Hartm.	Radulaceae	mat	Customary epiphyte	1.6

Here, TWINSPAN and DECORANA (Hill 1979, Seaby and Henderson 2007) were used to explore the community composition and spatial patterns of epiphytic bryophyte communities and their relationship with the associated environmental factors of the epiphytic habitat. In this context, TWINSPAN and DECORANA were applied to the matrix of cover in 60 sample plots according to the computer program CAP (Community Analysis Package-5) of Seaby and Henderson (2007).

Results

Floristical results

Twenty-nine species belonging to 17 families and 25 genera were determined as a result of the identification of 362 specimens. Among them 24 are mosses (21 pleurocarpous and 3 acrocarpous), and 5 are liverworts. Neckeraceae (6 species, 20.6%) and Brachytheciaceae (5 species, 17.2%), both pleurocarpous moss families, are the most species-rich families found in epiphytic habitats on boxwood trees in the study area (Tab. 2). *Exsertotheca crispa* (Hedw.) S.Olsson, Enroth & D.Quandt is the most common species in the *B. sempervirens* forests of Fırtına Valley.

Ecological results

Six different life forms were determined. Among them, the mat life form prevailed (34.4%), followed by the weft life form (24.1%). The dendroid life form was negligible (3.4%) (Tab. 2). In addition, four different habitat affinity categories as cortico-saxicolous, indifferent, customary epiphyte and preferentially not corticolous were determined belonging to the species. While cortico-saxicolous species were the most common with 51.7% within the sample plots, the preferentially not corticolous type habitat affinity is least common with one species (Tab. 2).

Lower base zone

The spatial distributions and community structures analyses showed that a total of 11 species, all of them are mosses, were found on the base zone of young boxwood trees (dbh 20-35 cm). Among of the mosses *E. crispa* was the most frequent and the most dominantm with the highest IES value (271). *Isothecium alopecuroides* was co-dominant with 214 IES values. *Homalia trichomanoides* and *Sciuro-hypnum flotowianum* were abundant species with 171 and 143 IES values on the lower base of trunks of *B. sempervirens*. *Brachythecium rutabulum*, *S. populeum* and *Thamnobryum alopecurum* which have the lowest IES values (43) were scarce on the lower base (Tab. 3). Whereas the weft life form was the most dominant (36.3%) in the base zone, the life forms mat and fan were co-dominant (27.2%). Also, the cortico-saxicolous type habitat affinity of the species was the most common with 54.5% on the base zones of young boxwood trees.

Fifteen mosses were collected from the lower base of the middle-aged *B. sempervirens* (dbh 40-50 cm). Among them, *E. crispa* (266 IES value) and *H. trichomanoides* (216 IES value) were the two most dominant species. *Sciuro-hypnum flotowianum* was the most abundant on the base zones of the middle-aged trees with the 183 IES value. *Anomodon viticulosus*, *Pseudoleskeella nervosa* and *S. populeum* were scarce with the lowest IES values (33). Moreover, *Ctenidium molluscum* and *P. nervosa* were only found on the lower bases of middle-aged trees (Tab. 3). While the fan life form is the most dominant (33.3%), the life forms mat and weft were co-dominant (26.6%) on the lower base of middle-aged boxwood trees. Cortico-saxicolous type affinity of epiphytic habitats (60%) were the most common on the basal zone of middle-aged trees.

Twelve species (11 mosses, one liverwort) were determined on the lower bases of aged boxwood trees (dbh 60-80 cm). *Exsertotheca crispa* was still the most frequent and the most dominant with the highest IES value (371). This value is also the highest among all tree-size groups (Tab. 3). While, *Hypnum cupressiforme, I. alopecuroides, Pseudanomodon attenuates* and, *S. flotowianum* were abundant, *Fissidens serrulatus, Oxyrrhynchium hians* and, *Plagiothecium*

Tab. 3. Index of ecological significance (IES) values in each tree-size groups according to tree diameter at breast height (dbh) and tree age at lower base zone, middle zone and upper zone.

Species	Young trees (dbh 20–35 cm)			Middle-aged trees (dbh 40–50 cm)			Aged trees (dbh 60–80 cm)		
	Lower base	Middle zone	Upper zone	Lower base	Middle zone	Upper zone	Lower base	Middle zone	Upper zone
MOSSES									
Alleniella besseri	–	328	271	50	316	233	–	314	300
Alleniella complanata	100	328	285	115	266	250	71	342	342
Anomodon viticulosus	–	43	–	33	–	–	–	–	–
Brachythecium rutabulum	43	–	–	50	–	–	57	–	–
Ctenidium molluscum	–	–	–	67	–	–	–	–	–
Exsertotheca crispa	271	314	357	266	216	366	371	314	328
Fissidens serrulatus	–	–	–	67	–	–	28	–	–
Homalia trichomanoides	171	–	–	216	50	–	57	–	–
Hypnum cupressiforme	71	71	–	–	–	–	128	114	28
Isothecium alopecuroides	214	43	–	130	33	33	171	85	–
Leucodon sciuroides	–	–	257	–	50	300	–	–	300
Orthotrichum pumilum	–	–	200	–	–	100	–	28	200
Oxyrrhynchium hians	–	–	–	–	–	–	28	–	–
Palamacladium euchloron	–	128	–	–	150	–	–	85	28
Plagiothecium nemorale	–	–	–	–	–	–	28	–	–
Pseudanomodon attenuatus	85	128	71	100	150	33	114	57	–
Pseudoamblystegium subtile	–	28	–	–	33	–	–	43	28
Pseudoleskeella nervosa	–	–	–	33	–	–	–	–	–
Pterygynandrum filiforme	–	71	–	–	–	–	–	57	–
Sciuro-hypnum flotowianum	143	86	28	183	116	33	128	28	–
Sciuro-hypnum populeum	43	–	–	33	–	–	–	–	
Thamnobryum alopecurum	43	–	–	83	–	–	–	–	–
Thuidium delicatulum	85	–	–	100	–	–	–	–	–
Ulota crispa	–	–	200	–	–	167	–	–	200
LIVERWORTS									
Frullania dilatata	–	–	57	–	–	33	–	28	57
Frullania tamarisci	–	–	–	–	33	–	–	86	43
Metzgeria furcata	–	–	–	–	–	–	–	57	28
Radula complanata	–	200	114	–	133	100	71	171	143
Radula lindenbergiana	–	–	–	–	–	–	–	28	–

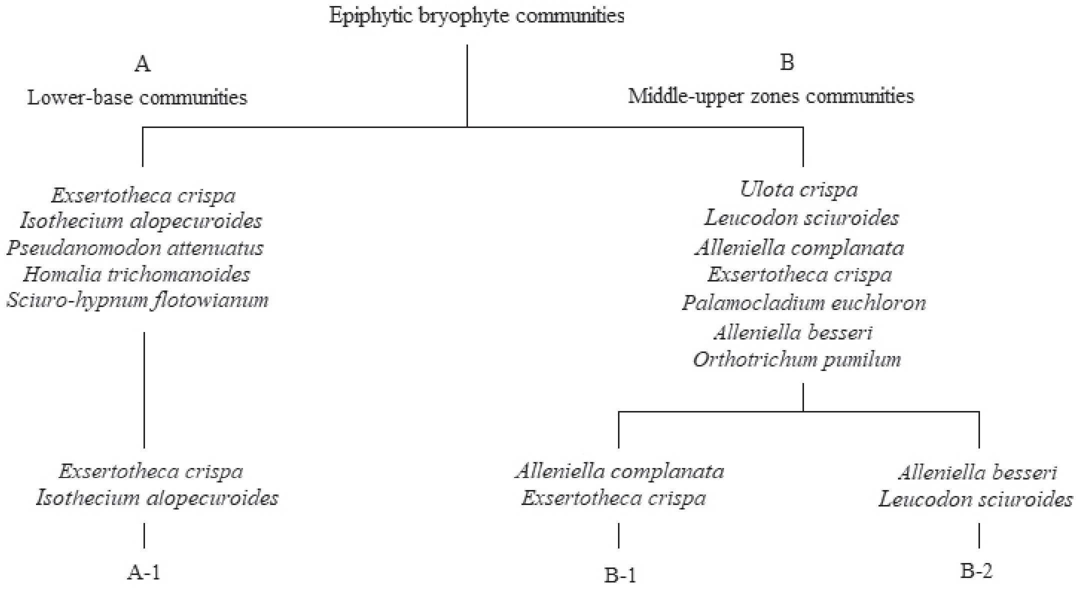

Fig. 2. Classification of TWINSPAN based on the matrix of 29 epiphytic bryophyte species over 60 sample plots.

nemorale usually not epiphytic, were scarce with the lowest IES values (28). The mat life form was the most dominant with 41.6% and cortico-saxicolous species (50%) were still the most common on base zones of aged *B. sempervirens.*

Middle zone

Twelve species (11 mosses, one liverwort) were determined on the middle zones of the young boxwood trees. *Alleniella besseri* and *A. complanata* were the most frequent and the most dominant with the highest IES values (328). While *E. crispa* was co-dominant with 314 IES values, *Radula complanata* (200), *P. attenuatus* (128) and, *Palamacladium euchloron* (128) were abundant on the middle parts of the trunks of the young *B. sempervirens* (Tab. 3). The mat life form with 33.3% and the cortico-saxicolous type habitat affinity with 83.3% were still most dominant on the middle parts of trunks of young boxwood trees.

Twelve species were collected from the middle zones of the middle-aged boxwood trees. *Alleniella besseri* was the most dominant with the highest IES values (316). *Alleniella complanata* and *E. crispa* were co-dominant, both with 266 IES values. *Frullania tamarisci, I. alopecuroides* and, *Pseudoamblystegium subtile* were scarce with the same IES values (33) on the middle zones of middle-aged trees (Tab. 3). The life form mat was the most dominant with 41.6% and cortico-saxicolous species were conspicuously the most common with the rate of 91.6% on middle parts of the middle-aged *B. sempervirens.*

A total of sixteen species, five of which were liverworts, were determined in the middle zones of the aged boxwood trees. All of the liverworts, which were determined in the epiphytic habitats of the boxwood forests, were found on the middle parts of the old trees. While *A. complanata* was the most frequent and the most dominant, with the highest IES value (342), *E. crispa* and *A. besseri* were co-dominant with the same IES values (314) (Tab. 3). The mat life form (50%) and the cortico-saxicolous type affinity (68.75%) were the most dominant on the middle zones of aged trees.

Upper zone

Ten species were found on the upper zones of young trees. Among them, eight were mosses and, two were liverworts. *Exsertotheca crispa* was still the most frequent and the most dominant with the highest IES value (357). *Alleniella complanata, A. besseri* and, *Leucodon sciuroides* were co-dominant with the higher IES values (>200) on the upper zones of the young *B. sempervirens.* While *Orthotrichum pumilum* (IES value 200), *Ulota crispa* (200) and, *R. complanata* (114) were abundant on these zones, *S. flotowianum* (28) was scarce with the least IES value (Tab. 3). The mat life form (40%) and the habitat affinity type cortico-saxicolous (70%) were the most common on the upper parts of young trees.

Eleven species (nine mosses, two liverworts) were collected from upper zones of the middle-aged boxwood trees. While *E. crispa* was still the most frequent and the most dominant with the highest IES value (366), *A. besseri*, *A. complanata* and, *L. sciuroides* were co-dominant (> 200). And also, *U. crispa* (167) was abundant on the upper zones of the middle-aged *B. sempervirens* (Tab. 3). The mats (45.45%) and cortico-saxicolous species (72.72%) were the most dominant.

A total of thirteen species were determined on the upper zones of the old *B. sempervirens*. Among them, four were liverworts and nine were mosses. *Alleniella complanata* was the most frequent and the most dominant with the highest IES value (342). While *A. besseri* (IES value 300), *E. crispa* (328) and, *L. sciuroides* (300) were co-dominant with the higher IES values, *O. pumilum* (200), *R. complanata* (143) and, *U. crispa* (200) were abundant on the upper parts of aged boxwood trees (Tab. 3). The life form mat (30.7%) and the habitat affinity type cortico-saxicolous (53.8%) were the most common again on the upper parts of old trees.

TWINSPAN classification

TWINSPAN classified the epiphytic bryophyte communities on the trunks of *B. sempervirens* in the Fırtına Valley at the second level into two main clusters (A and B) and three sub-clusters (A1, B1 and, B2) (Fig. 2). These main and sub-clusters were named according to the dominant, co-dominant and abundant species which were the distinctive species within the communities. The main cluster, A, occurred in lower-base communities and it was characterized by dominant species *E. crispa* and *H. trichomanoides*, co-dominant *I. alopecuroides* and abundant *P. attenuatus* and *S. flotowianum*. The second main cluster, B, occurred in middle and upper zone communities. Cluster B was characterised by dominant species *A. besseri* and *A. complanata*, co-dominant *E. crispa* and *L. sciuroides,* abundant species *O. pumilum, P. euchloron*, *R. complanata*, and *U. crispa*.

Epiphytic bryophyte communities

The A1 community was named *Exsertotheca crispa-Isothecium alopecuroides* due to the frequency, constancy and, dominancy of these species within the lower-base community. Both *E. crispa* and *I. alopecuroides* had the highest IES value on the lower bases of all tree-size groups (young, middle-aged and old boxwood trees) (Tab. 3). The community was represented with 19 moss species in a total of 20 sample plots. Moderately abundant *R. complanata* was the only liverwort in the lower-base community. *Exsertotheca crispa-Isothecium alopecuroides* community was co-dominated by *H. trichomanoides*. In this community, *P. attenuatus* and *S. flotowianum* were also abundant. While the dominant life forms within the community were weft and mat (31.5%), cortico-saxicolous species (47.3%) were dominant and indifferent type affinity was also co-dominant (42.1%).

The B1 community was named *Alleniella complanata-Exsertotheca crispa* according to its dominant and co-dominant species. It was represented by 19 species (15 mosses, 4 liverworts) in a total of 17 sample plots from the middle parts of the young, middle-aged and old boxwood trees. The liverwort *R. complanata* was still present in the middle parts of the trunks with relatively high IES values. Also, *P. euchloron* was abundant in the community B1 on the middle zones, particularly of young and middle-aged trees particularly (Tab. 3). While mats were the most dominant with the rate of 42.1% within the middle parts of community B1, cortico-saxicolous species were the most common with 63.1%.

The B2 community was named *Alleniella besseri-Leucodon sciuroides* according to its co-dominant species. This community, consisting of 17 species (13 mosses and four liverworts), was found on the upper zones of boxwood trees. The community B2 was represented by a total of 23 sample plots. *Orthotrichum pumilum*, *R. complanata*, and *U. crispa* were abundant species in the upper zones community. While mats were the most dominant with the rate of 41.1% within the middle parts of community B2, the cortico-saxicolous type habitat affinity was the most common with 70.5%.

DECORANA ordination

DECORANA grouped the sample plots on axis 1 and axis 2 according to the similarity and the environmental gradients (Fig. 3). While the DCA axis 1 was interpreted as

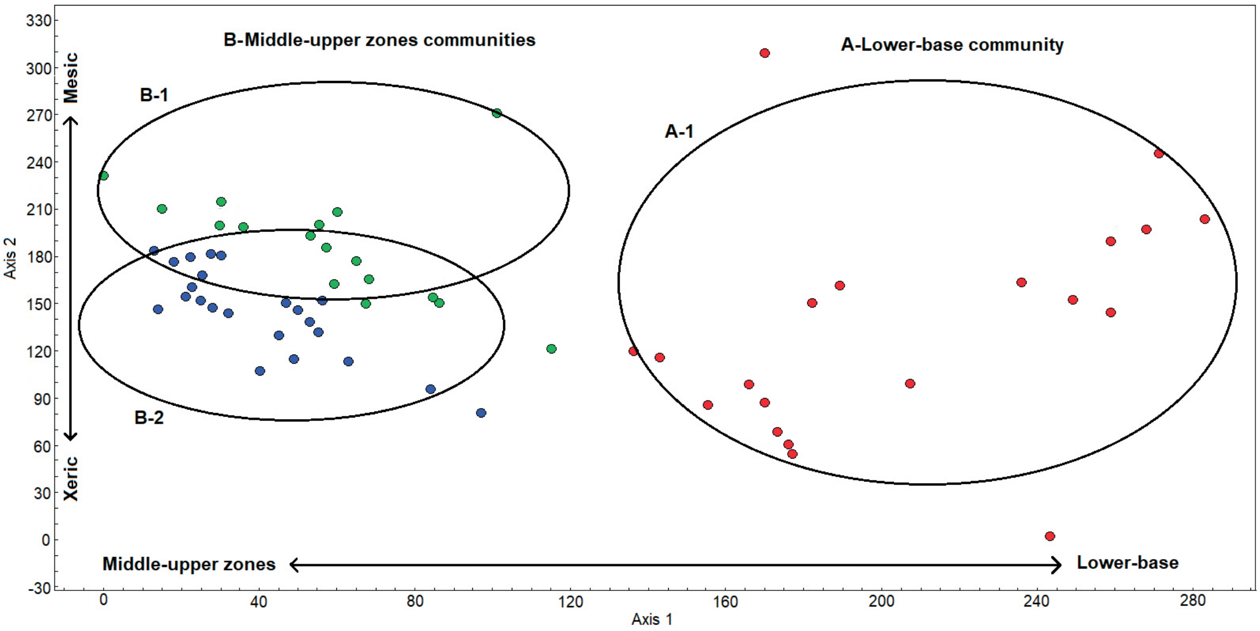

Fig. 3. The relationship between the three epiphytic bryophyte groups generated after the application of TWINSPAN classification technique on 60 sample plots and the distribution of the groups along the environmental gradient on the first and second axes of DECORANA. A–1: *Exsertotheca crispa-Isothecium alopecuroides*, B–1: *Alleniella complanata-Exsertotheca crispa*, B–2: *Alleniella besseri-Leucodon sciuroides*, axis 1: the gradient of height of the epiphytic habitat, axis 2: the gradient of moisture.

gradient along the height of the epiphytic habitat (from the lower base to the upper zone) on trunks, the DCA axis 2 was interpreted as gradient of moisture (from mesic to xeric) (Fig. 3).

Discussion

When the epiphytic habitats on the boxwood trees in the Fırtına Valley were examined in terms of species diversity and species composition, the lower bases and the middle-parts were the richest zones with equal numbers of species (19 species), while the upper zones contain 17 epiphytic species. Large pleurocarp mosses such as *E. crispa*, *H. trichomanoides,* and *I. alopecuroides* were common on the basal parts of the trunks of *B. sempervirens*. These strong competitor members of pleurocarpous type mosses are more sensitive to drought, have a faster growth habit than acrocarpous mosses and spread horizontally in a carpet-like appearance on the substratum (Schofield 2001, Ezer 2017). The basal parts of the trunks are usually more humid and more nutrient rich than the other parts (middle and upper) due to soil proximity. Therefore, basal zones allow early establishment and rapid colonization of bryophytes due to higher water retention capacity, higher soil humidity, low evapotranspiration rate and low insolation (Lara and Mazimpaka 1998, Mazimpaka et al. 2009, Ezer and Kara 2013, Ezer 2017). In this respect, the lower base parts of the trees can be considered an extension of the forest floor (groundlayer) environment. For this reason, species such as *B. rutabulum, C. molluscum*, *O. hians, P. nemorale, P. nervosa, S. populeum, T. alpecurum*, *Thuidium delicatulum,* and *F. serrulatus*, which usually grows on the soil, were encountered only in this part. This caused the weft life form and indifferent type habitat affinity to co-dominate with the mats and cortico-saxicolous species were the strong competitor of robust pleurocarpous in basal parts of boxwood trees.

Alleniella besseri, *A. complanata* and *E. crispa* were most dominant on the middle zones of the boxwood trees. Also, the mesophytic species *P. euchloron*, which was not present in the base zone, was abundant in the middle zone. Therefore, *P. euchloron* can be considered a characteristic and distinctive species of the communities in the middle zones. The customary epiphyte xerophytic species *O. pumilum*, which in the present study was generally abundant in the upper zones, in the present study was found for the first time only in the middle zones of old trees. As the middle and upper parts of the trunks move away from the soil effect, they are periodically exposed to higher insolation and desiccation that makes colonization of species on epiphytic habitats difficult (Moe and Botnen 2000). Therefore, as in the present study, small cushion-type mosses which have xerophytic characters such as Orthotrichaceae members and photophilous or heliophilous species such as *A. besseri*, *A. complanata,* and *L. sciuroides* are most common species within the middle and upper zone communities. Mat type life forms were predominant in the middle parts of old trees, due to the presence of cortico-saxicolous liverworts.

Although mesoscale climatic factors such as seasonal climatic variables are among the most important determinants of bryophyte species richness and species diversity, forest structure and habitat characteristics are also important for community compositions and spatial distributions of epiphytic bryophytes on epiphytic habitats (Medina et al. 2014, Ezer 2017). Species diversity and epiphytic cover on the upper zones were found to be lower than in the base and middle zones. The xerophytic robust pleurocarpic species *L. sciuroides* was dominant on the upper parts of all tree-size groups. Besides *A. besseri*, *A. complanata* and *E. crispa* there were other dominant pleurocarpic species in the upper zones of boxwood trees in the Fırtına Valley. Therefore, weak competitor species such as liverworts *Frullania dilatata* and *Metzgeria furcata* and small cushion-type mosses (such as *O. pumilum* and *U. crispa*) remain under these large pleurocarpous species and decreased or disappeared from the epiphytic habitats in the valley.

Some studies on the succession of epiphytic bryophyte communities have demonstrated that variables of the epiphytic bryophyte composition in the successional stages are closely related to tree age, trunk height (basal, middle and upper zones) and bark characteristics (Lara and Mazimpaka 1998, Mazimpaka et al. 2010, Ódor et al 2013, Bargali et al. 2014, Ezer 2017). However, in the present study, trunk height rather than tree age and bark characteristics were effective in the variability of epiphytic bryophyte composition in the per successional stage. Hygrophytic pleurocarpous species that usually grow on soil, such as *B. rutabulum*, *C. molluscum*, *F. serrulatus, O. hians*, *P. nemorale P. nervosa*, *S. populeum*, *T. alpecurum*, and *T. delicatulum* were particularly the pioneer colonizers in the early successional stages on the basal parts particularly of middle aged *B. sempervirens*. While the xerophytic small cushions *O. pumilum* and *U. crispa* were the pioneer colonizers in the early successional stages, the large pleurocarpous *L. sciuroides* was a secondary colonizer in the advanced stages on the upper parts of all tree-size groups. In addition, cortico-saxicolous species *P. euchloron*, a characteristic and distinctive species on the middle zones, was pioneer colonizer in the early successional stages on the middle parts of all tree-size groups. In this study, *A. besseri, A. complanata*, and *E. crispa* were other colonizers in the advanced successional stages on the middle zones. Considering the morphological physiognomy of the bryophyte communities on trunks of *B. sempervirens* in the Fırtına Valley; all communities were dominated by large pleurocarpous mosses. *E. crispa* was in particular the most constant and the most dominant within all communities in the *B. sempervirens* forests. In sum, the succession of the epiphytic bryophyte communities of the boxwood forests in the Fırtına Valley has reached the climax.

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The accelerated spread of a neophyte introduced to Europe long ago – First occurrence of *Sporobolus indicus* (Poaceae) in Hungary

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Abstract – The first occurrence of *Sporobolus indicus* in Hungary is reported. The neotropical *S. indicus* is one of the oldest introduced neophytes to Europe. From the middle of the 19th century until the last decade of the 20th century, apart from a few occasional occurrences, it expanded only in the Mediterranean area. However, the number of observations has dramatically increased in the past two decades, even outside the Mediterranean region. Its recent rapid spread is evident along roads, on lawns and in tourist places subject to trampling (e.g., campsites). Tourism certainly contributes to the very successful recent diffusion of the species. Still, global warming, including the increasingly mild winters in continental Europe, can certainly enhance the establishment and further dispersal of this cold sensitive species.

Keywords: alien species, anthropochory, global warming, plant invasion, Poaceae

Introduction

The genus *Sporobolus* (Clayton et al. 2006, Peterson et al. 2014, Govaerts et al. 2021), is very rich in species (~ 200 species / 160–222 species, depending on genus delimitation), the majority of them being of tropical or subtropical origin. In the temperate zone of North America, it numbers approximately 30 indigenous species, while in Europe only *S. pungens* (Schreb.) Kunth, occurring in the Mediterranean, is considered native, excluding the species of *Crypsis* and *Spartina* that, according to some authors (e.g., Peterson et al. 2014), should also be classified in the genus *Sporobolus*. Most *Sporobolus* species found in Europe are thus alien taxa. They include both ephemerals and dangerous habitat-modifying invasive species (Hansen 1980, Verloove 2006, Celesti-Grapow et al. 2010). It is mainly the species of temperate America that spread successfully (*Sporobolus cryptandrus* (Torr.) A. Gray, *S. neglectus* Nash, *S. vaginiflorus* (Gray) Alph. Wood.) with the exception of *S. indicus* (L.) R.Br. with a tropical or subtropical origin. It was described based on specimens collected in tropical Eastern Australia (Brown 1810), but presumably it is a neotropical species (Clayton et al. 2006, POWO 2021). According to Thellung (1912), it was introduced to Europe from South America (Argentina or Brazil), more than a century and a half ago.

Currently, the occurrence of *S. vaginiflorus* (Király and Hohla 2015), *S. neglectus* (Király 2016), and *S. cryptandrus* (Polgár 1933, Török and Aradi 2017, Török et al. 2021) are known from Hungary. Out of these, *S. cryptandrus* has become a dangerous invasive species within a short time (Török et al. 2021). Although *S. indicus* is the oldest introduced *Sporobolus* species in Europe, the proliferating number of new observations (Niketić 1998, Glasnović and Jogan 2009, Celesti-Grapow et al. 2010, Lauber et al. 2018, Perić et al. 2013, Eichberger et al. 2015, Pachschwöll et al. 2016, Amarell and Himpel 2020) testifies its accelerating spread.

Sporobolus indicus arrived to Europe as a vagrant with various commodities (e.g. crops), but primarily with shipments of wool (Thellung 1912, p. 654). Based on collecting data from botanists and herbalists of that time, Thellung deduced that in and near port cities in the south-west of France, in industrial areas (harbours, loading bays, wool-driers), the species was observed as early as the mid-19th century (in Bayonne and Biarritz: 1847; in Montplaisir: 1869). It emerged as a weed in the botanical garden of Montpellier in 1905 and by that time it was already naturalized in the valley of the river Orb (southern France) as well (Thellung 1912). Almost at the same time as the first observations in France, *S. indicus* was also detected by Bubani in the north-east of Spain, in the port town of Roses: Sept. 1853 (Bubani 1901). Casasayas

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(1990) erroneously thought this was the first observation of the species in Europe. Gómez-Lama et al. (2006) cited several herbarium specimens from the Iberian Peninsula. The oldest specimens in Spain were collected very close to the town of Roses, along the road and rail line connecting Montpellier and Barcelona (Figueres in 1904, Girona in 1916, Barcelona in 1910). Since then, it has been detected at several locations in the Iberian Peninsula, but its rate of spread has slowed down. It occurred in southern Spain in the past few decades (Devesa et al. 1990, Zarco 2004, Gómez-Lama et al. 2006). From Portugal, it has been known since 1944 (Pinto da Silva 1946) and it was soon further dispersed over much of the country (Almeida 1999). According to Lauber et al. (2018) and Info Flora (2021) it occurred in some places in the southern, climatologically favourable, part of Switzerland as early as in 1930, and since the late 1990s there has been a growing number of observations; nowadays it is spreading along roads and highways. In Italy, it was detected in several places in the 1930s (Fiori 1933, Montelucci 1935), but it is particularly interesting that it was discovered in Sicily only a few years ago (Galasso et al. 2015). Occasional introductions of the species were reported around wool processing plants in Germany, France, and the Czech Republic (Issler 1934, Probst 1949, Dvořák and Kühn 1966), and once after an oil exploration drilling (Rastetter 1966). In Slovenia, it was found in 2005 (Glasnović and Jogan 2009). In the Balkans, Hansen (1980) reported the species from Bulgaria. In the late 1990s it was found in Montenegro (Niketić 1998) and in Greece (Krigas et al. 1999). The species is listed in the grass-checklist of Istria, Croatia (Starmühler 2003). More recently, it has been reported from Serbia (Perić et al. 2013), Austria (Eichberger et al. 2015, Pachschwöll et al. 2016) and Germany (Amarell and Himpel 2020).

In this paper, we report the first observation of *S. indicus* in Hungary, and evaluate the prospects of its spread and the expected role of the species in European vegetation.

Materials and methods

The fieldwork was carried out in the autumn of 2021 as a part of a systematic flora survey of the Külső-Somogy region and the southern shore of Lake Balaton, Hungary. We used a MobileMapper60 satellite navigation device to record geographic coordinates. The grid number of the site is provided in accordance with the Central European Flora Mapping System (Niklfeld 1971). The herbarium acronyms mentioned in the text follow Thiers (2021).

The plant material was identified using, among others, Clayton (1965), Baaijens and Veldkamp (1991), Simon and Jacobs (1999) as well with reference to herbarium specimens found in the collections at BR and BP. The specimens collected at the new Hungarian site were deposited in the BP and BR herbaria. The nomenclature of the taxa follows the Euro+Med Plantbase (EuroMed 2006); for taxa non-native in Europe the Plants of the World Online (POWO 2021) database.

For the meaningful presentation of occurrence data and distribution of *S. indicus* in Europe, we assigned the geographic coordinates of the settlements (or larger geographic units) and the year of the observation to the published data. QGIS 2.18 software (QGIS 2022) was used to develop the cartographic representation.

Results

Sporobolus indicus was found on October 24, 2021 in the residential area of Zamárdi, characterised by detached houses with gardens (46.89088217 N, 17.979621 E, CEU-quadrat: 9173.2). About 15 specimens of *S. indicus* emerged from a mown lawn. The last mowing may have taken place in late summer. Shoot formation, flowering, and ripening must have taken place in September–October. The specimens of *S. indicus* were in the best phenophase in terms of identification, i.e. in the fruiting stage (Fig. 1). The immediate vicinity of the occurrence of the species in the recreation area of Lake Balaton, as well as in similar habitats of neighbouring settlements, were surveyed and checked systematically in the next few weeks, but no further specimens of *S. indicus* were found. Based on the 15 specimens found at only one site so far, we assume that it has been present in the lawn patch for a few years, and late autumn ripening appears to be a particularly good strategy to develop a presumably self-sustaining small population in the lawn.

The local habitat of *S. indicus* is a mown dry lawn (Fig. 2) between the sidewalk and the asphalt road, dominated by disturbance- and trampling-tolerant species of natural sandy grasslands (*Cynodon dactylon* (L.) Pers., *Bothriochloa ischaemum* (L.) Keng), with very few accompanying species

Fig. 1. *Sporobulus indicus* in fruiting stage in Zamárdi, Hungary. October 30, 2021. Photo: N. Bauer.

Fig. 2. Habitat of *Sporobolus indicus* in Zamárdi, Hungary. October 30, 2021. Photo: N. Bauer.

(*Berteroa incana* (L.) DC., *Chrysopogon gryllus* (L.) Trin., *Euphorbia seguieriana* Neck., *Petrorhagia saxifraga* (L.) Link). It is assumed that the habitat has not been overseeded, since in such semi-natural mown lawns, many species of the former sand grassland vegetation of Külső-Somogy (see Soó 1930) can still be found. These lawns are maintained by mowing until the end of the summer holiday season, but are not treated thereafter, which is beneficial for species blooming in autumn. This is evidenced by the presence of *Spiranthes spiralis* (L.) Chevall. stocks that are found in the same type of urban habitat-islands near the occurrence of *S. indicus* (and in several settlements along the shore of Lake Balaton). Further, weed species typically introduced from large cities are also present on the streets of Zamárdi (e.g., *Cenchrus spinifex* Cav., *Eleusine indica* (L.) Gaertn., *Euphorbia maculata* L., *E. prostrata* Aiton), which is not surprising, taking into consideration the Siófok region's being one of the most popular destinations for tourists by Lake Balaton, as well as for the visitors of several pop-music festivals, resulting in about one million guest nights per year (based on the data of the Hungarian Central Statistical Office, https://www.ksh.hu).

Discussion

As with many adventive species in Hungary, the first observations of *Sporobolus* species can be attributed to Sándor Polgár around the railway loading area of the former Meller vegetable oil factory in Győr (Polgár 1918, 1933), but none of these records was, unfortunately, substantiated by a herbarium specimen. Some uncertainty therefore surrounds these claims and most of them also require nomenclatural updating: "*S. subinclusus* Phil." (= *S. cryptandrus*), "*S. Argutus* (Nees) Kunth" (=*S. pyramidatus* (Lam.) Hitchc.), "*S. elongatus* (Lam.) R. Br." (=*S. elongatus* R. Br.). The latter taxon is a member of the *S. indicus* complex (Clayton 1965, Baaijens and Veldkamp 1991, Simon and Jacobs 1999) and very similar to *S. indicus* s.str. Until recently, these taxa had not been observed anywhere else in Hungary. *Sporobolus cryptandrus* was rediscovered in 2016 (Török and Aradi 2017, Török et al. 2021), while the discovery of *S. indicus* in 2021 is reported in this paper, but these are certainly new introductions, independent of any earlier occurrences.

While, as mentioned in the introduction, the species was initially limited to the Mediterranean region, in recent years more and more observations have been published from areas further north, outside the Mediterranean: from Serbia (Perić et al. 2013), Austria (Eichberger et al. 2015, Pachschwöll et al. 2016), France (Tinguy 2016, Amblard et al. 2018), and Germany (Amarell and Himpel 2020) although in some cases it is uncertain whether genuinely naturalized populations are involved. However, even in areas where *S. indicus* used to be an ephemeral alien, it is obviously in the process of initial naturalization. In Belgium, for instance, where it has been known since 1886 as a repeatedly introduced but strictly casual wool and grain alien (Verloove 2006), it naturalized locally in recent years, especially – as in Hungary – in lawns. The same applies, even further north, to the Netherlands where it was found, among other places, in campsites, resulting from the unintentional introduction of seeds by tourists returning from southern Europe (Verloove et al. 2020), an introduction vector that is potentially similar to the one observed in Hungary.

Pre-World War I data are almost exclusively from the vicinity of ports or along roads and railways and each documented the rapid spread of the species. Unsurprisingly, *S. indicus* is one of the first neophytes to be recognized as a species spreading along roads and railways (Codina 1908, Cadevall 1911). We believe that earlier conditions for the transportation of crops may have facilitated the spread of many introduced species. Thellung (1912) mentioned one of the oldest European data (Montplaisir 1869) related to a

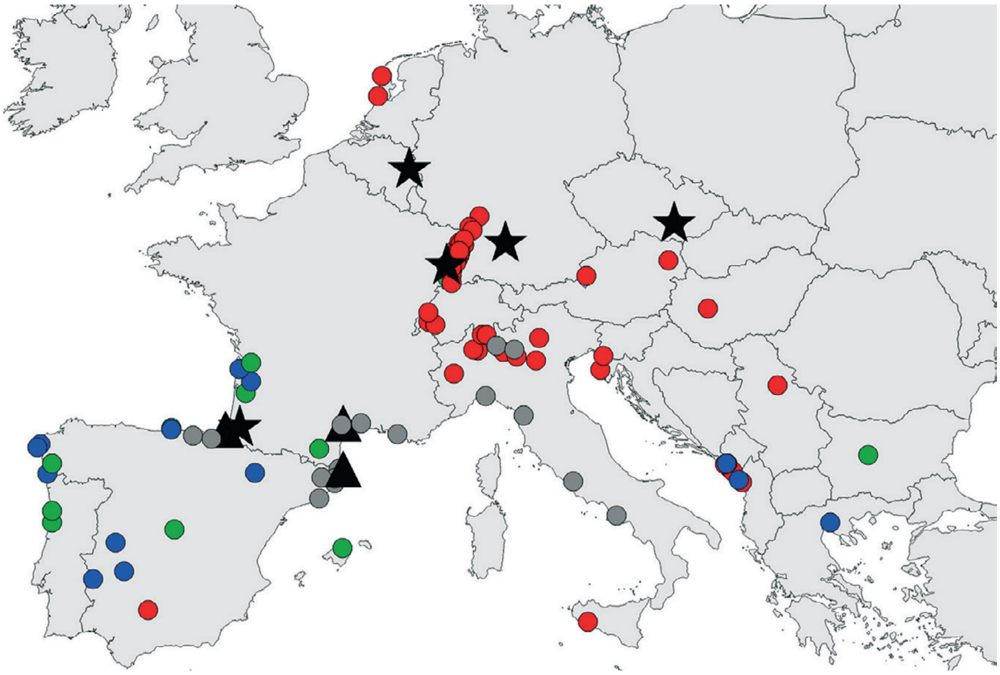

Fig. 3. Distribution of *Sporobolus indicus* in Europe based on published data. Legends are indicated on the map (black triangle: 1850–1900; grey circle: 1901–1950; green circle: 1951–1980; blue circle: 1981–2000; red circle: 2000–; black star: casual introductions to industrial areas).

wool drying plant, and it is noteworthy that it was also discovered much later at some sites of wool processing plants in Western and Central Europe (Issler 1934, Probst 1949, Dvořák and Kühn 1966), but these were casual occurrences in all cases. In the Mediterranean and Atlantic areas of Europe, it gradually spread in the 20th century, but by now it has colonised large parts of southern France, Italy, and Spain (Kerguélen 1975, Celesti-Grapow et al. 2010, Tison et al. 2014). Further north, it has reached northwestern France (Tinguy 2016, Amblard et al. 2018), Belgium, and even the Netherlands (Verloove 2006, Verloove et al. 2020), although this can only partially be explained by changing climate: its distribution area towards the north becomes fragmented, there is no gradual expansion of its entire secondary distribution area. On the contrary, in Western Europe, while plants of *S. indicus* introduced a long time ago were not able to survive and were thus mere ephemerals, this species has adapted to the local climate. In the climatologically less favourable areas (mostly with a more continental climate) it is still rare or was only detected long ago, as a casual alien (e.g., Pyšek et al. 2012).

Based on published and confirmed data, it is obvious that *S. indicus* crossed the Mediterranean and Atlantic subareas of Europe only in the last 2–3 decades (On-line Suppl. Tab. 1, Fig. 3). Although several older data are missing and some data may be questionable or inaccurate, the visualization of the GBIF data retrieved from herbaria and citizen science (Fig. 4) clearly shows that *S. indicus* has spread widely in France, Belgium, and in northwestern Italy (GBIF.org. 2022). The acceleration of its spread over the past few decades is observable, also in the Mediterranean region (Veldkamp 1990, Krigas et al. 1999, Lakušić et al. 2004, Galasso et al. 2015). Sporadic occurrences of the kind observed in the internal, continental areas of Europe over the past decade are also typically reported from areas with intense human pressure, mostly in cities or along roads (Eichberger et al. 2015, Meneguzzo et al. 2016, Amarell and Himpel 2020, etc.). Its accelerated spread clearly evidences anthropogenic spread associated with transportation and successively increasing vehicle traffic, as has been observed for other adventitious *Sporobolus* species. As it is a tropical and subtropical species, however, it also seems likely that increasingly mild winters and hot summers have also contributed to its successful spread, especially in urbanised areas with a more continental climate, where expected temperature increase may considerably exceed the global warming rate (Bartholy et al. 2009). Based on the experience with *S. indicus* elsewhere in Europe, it can be expected to spread further rapidly – but it will probably appear in habitats of strong anthropogenic impact, in settlements, along roads, and in trampled weed associations.

According to Casasayas (1990), *S. indicus* is a highly invasive species; its local invasion has already been observed in Austria (Englmaier and Wilhalm 2018). As it has so far appeared only in ruderal habitats of Central Europe, it does not seem to be as dangerous as *S. cryptandrus*, which has become a vegetation-transformer invasive species in the sandy areas of the Pannonian Basin within a few years (Török et al. 2021). It should also be noted that the behaviour of *S. indicus* in natural habitats has remained largely unknown. However, the massive spread of closely related species of the *S. indicus* complex (e.g., *S. africanus* Poir., *S. fertilis* (Steud.) Clayton) poses a serious ecological challenge in several areas (USA, Australia, New Zealand, and South Africa), and a number of methods have been developed to control them and curb their spread (e.g., Palmer 2004). The

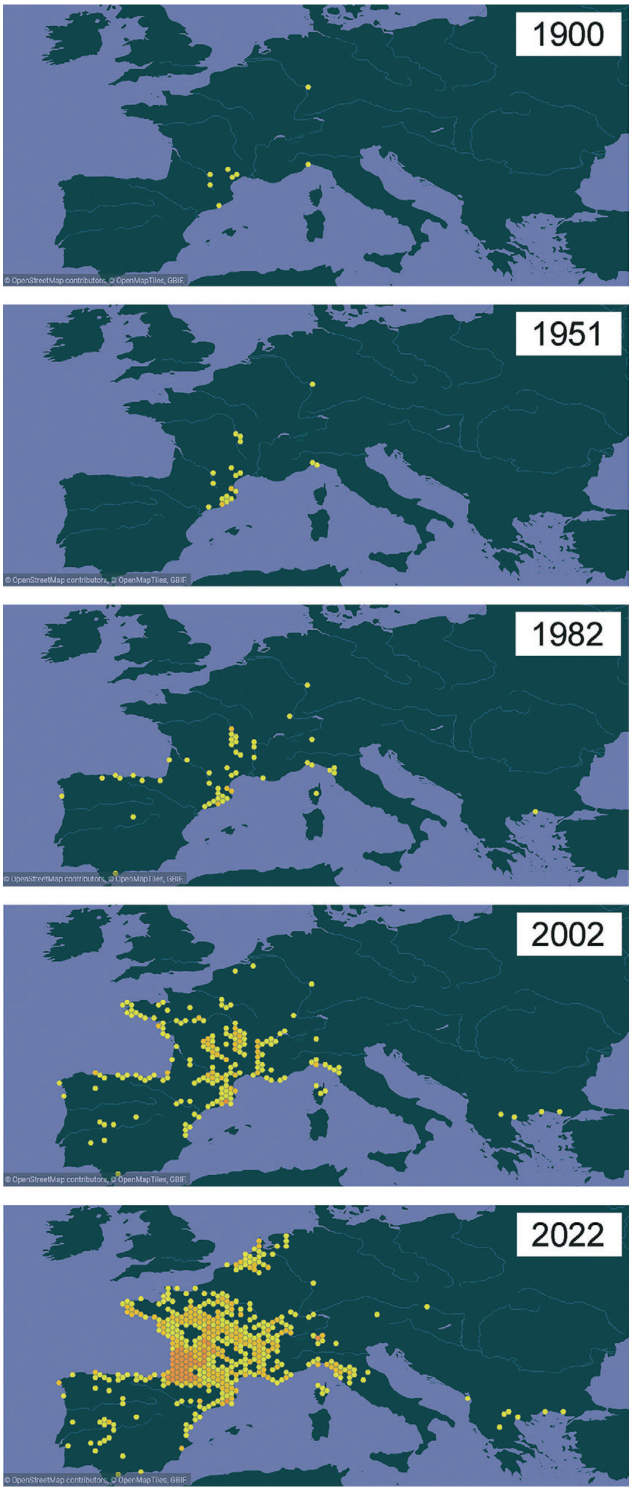

Fig. 4. Distribution and spread of *Sporobolus indicus* in Europe from 1900 to 2022 based on GBIF data (GBIF.org. 2022).

monitoring and control of the occurrence of *S. indicus* in Europe is of great importance for the conservation of grasslands close to nature.

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First record of alien naturalized populations of the crop *Cucurbita moschata* (Cucurbitaceae) in Spain, with remarks on typification status

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Abstract – As a result of a floristic survey carried out in riparian ecosystems of the south-eastern part of the Iberian Peninsula (Spain), the first report of well-established populations of the alien cultivated plant species *Cucurbita moschata* Duchesne for the Iberian Peninsula is provided here. Data about the morphological description (compared to other *Cucurbita* species), certain clarification aspects about the typification status of this name and related synonyms, and the ecological and climatic conditions of the riparian area are given together with an identification key of the *Cucurbita* species to facilitate further identification. The alien status and distribution of *C. moschata* together with its relatives *C. ficifolia*, *C. pepo* and *C. maxima* are reviewed for the Spanish references. This study outlines the first record of a naturalized population of *C. moschata* in Spain, well supported by the stability of the population over the years and in the ecological conditions. Finally, detailed ecological data indicate that agricultural activities together with riparian habitatsare starting points and corridors, respectively, for seed dispersal for the process of the invasion of alien plants in the south-eastern Iberian Peninsula.

Keywords: alien plants, *Cucurbita*, Mediterranean, riparian habitats, Spain, typification, xenophyte

Introduction

Cucurbita L. (Cucurbitaceae) comprises about 12-13 species widely distributed on the American continents, of which five species are cultivated (Paris 2016). In Europe, the most important cultivated species are *C. pepo* L., *C. maxima* Duchesne, *C. moschata* Duchesne and *C. ficifolia* Bouché (Teppner 2004, Henning et al. 2017). Tardío et al. (2018) recently included these four species in the Spanish list of the traditional crops for agricultural biodiversity, with *C. pepo* as the main crop species of the genus due to its great commercial importance. In the framework of the *Flora iberica* project, Fernandes (2005) reported *C. pepo*, *C. maxima* and *C. ficifolia* as crop species, without any mention of the possible findings of spontaneous specimens of them, in any Spanish or Portuguese geographical area. In addition, Fernandes (2005) did not include the existence, not even as a crop, of *C. moschata* for the Iberian Peninsula and Balearic Islands. According to The Euro+Med Plant Base (Henning et al. 2017), crops of *C. moschata* are mostly reported from eastern European countries, though the cultivation of this species is also mentioned for Mediterranean countries including Spain (Tardío et al. 2018) and Italy (Lust and Paris 2016). However, as previously stated by Quintero (1981), *C. pepo* and *C. maxima* were the most widely cultivated species along the south-eastern Iberian territories. Probably due to the extensive agricultural use of *C. pepo* and *C. maxima*, there are some reports of the existence of non-cultivated subspontaneous individuals of these two species, but always close to their original field cultures (e.g., Serra 2007, Herrera and Campos 2010). There are a couple of mentions of non-cultivated individuals of *C. moschata*, reported to be ephemerals, for Portugal and Spain (Verloove and Alves 2016, Gómez-Bellver et al. 2019), otherwise no specific geographic reference to the presence of this species outside of cultivation (Mateo et al. 2015). Although species of the genus *Cucurbita* have been considered as invasive alien flora (Sanz Elorza et al. 2004), any specific geographical indications of

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their presence, degree of naturalization, abundance and habitat as alien species are remarkably lacking.

On the basis of the ongoing study of the alien flora of the River Vinalopó (Alicante province, south-eastern Spain), the fieldwork conducted on this river has recently revealed the presence of a high number of casual and even naturalized alien plants (Juan et al. 2019, Terrones et al. 2021). The aims of this study are (i) to report the presence of well-established populations of *C. moschata* outside cultivation, which would correspond to the first record of the species as a naturalized alien population, (ii) to update the presence and alien status of the species of *Cucurbita* in Spain, and (iii) to identify ecological conditions that would favour the fruit dispersal and seed germination of these species, especially focused on *C. moschata*. In addition, the typification of the name *C. moschata* and related synonyms is reviewed.

Materials and methods

The present study was based on fieldwork carried out during the period 2016-2020 on the River Vinalopó (south-eastern Iberian Peninsula, Spain). The ecological features of this river dramatically change from its source, in a mountainous area characterized by a high-water quality, to its mouth, located in saline wetlands close to the Mediterranean Sea. Most of the river flows throughout semiarid territories, which causes the salinization of the waters (José Ramón Coves, pers. comm.) and even a seasonal total lack of flow in several non-continuous areas. The quality and physical-chemical properties of the waters of the river were described by José Ramón Coves (pers. comm.). Climate features of the closest meteorological station to the studied area (Elda) were obtained from the Climate-data online database (https://www.es.climate-data.org), which compiles data from the last 30 years. Based on these data, the bioclimatic characteristics of the area followed the classification of Rivas-Martínez et al. (2001).

Information on species distribution was based on herbarium specimens from GBIF (Global Biodiversity Information Facility – www.gbif.org) and literature. Morphological features were based on the specialized literature (Merrick and Bates 1989, Nee 1990, Lira and Rodríguez Arévalo 1999, Teppner 2004, Paris 2016, OECD 2016), together with the observations based on the population found during this research. The collected plant material of *C. moschata* is preserved in the ABH Herbarium (Thiers 2020), which was used to draw up detailed morphological pictures of this species. In this contribution, an identification key is reported to facilitate further identification. The typification status of *C. moschata* and related names was updated based on original descriptions and the inspection of available material from various herbaria (F, L, MO, NL, U, WAG; Thiers 2020). Ecological data of the alien *Cucurbita* populations are described, including remarks about the origin, degree of naturalization, habitat and the importance of the plant dispersal processes.

Results

Description and typification notes

Cucurbita moschata Duchesne, Essai Hist. Nat. Courges: 7 (1786)

Gymnopetalum calyculatum Miq. in Fl. Ned. Ind., Eerste Bijv. (2): 332 (1861)

Lectotypus (designated here): Label 1: "Gymnopetalum ? calyculatum Mq, Banka, (J. Amand)"; Label 2: A. Cogniaux Monogr. Cucurb. H "Cucurbita moschata Duch." (probably Cogniaux's handwriting); U (U0001457!).

An annual species characterized by softly hairy, non- to shallowly 3-5 lobed leaves, 15-30 × 20-40 cm, without or with whitish blotches; tendrils bifids or trifids; sepals free, mostly linear, but sometimes lanceolate together with flowers with broadened apices (Fig. 1); corolla yellow-orange, 8-12 cm long; fruiting peduncle 5-ribbed thickened and dilated or cylindrical, 5-10 cm long, widely flat at the fruit attachment; fruits covered by a wipeable waxy layer, dumbbell and solid green or pyriform and cream-coloured with light green longitudinal reticulate mottled stripes (usually in bands), up to 35 cm length; seeds elliptical, 8-15 × 4-7 mm, with a rounded marginal bulge, margin mostly with a colour shade slightly different from the surface, marginal wings developed very strongly. Most of these morphological features correspond to the main features used to distinguish it from the other domesticated species of this genus, *C. ficifolia*, *C. maxima* and *C. pepo* (Tab. 1).

Regarding the protologue of *C. moschata*, Paris (2000) already stated that this name was validly published by Duchesne (1786) in the Essai sur l'Historie Naturelle des Courges, and hence, Duchesne was solely responsibility for the authority. On the GBIF database (www.gbif.org), three different entries were found related to the type specimens of the name *C. moschata*. Firstly, the mention of the type specimen of this name held at the Missouri Botanical Garden (MO1722018) should be considered a mistake, since no voucher is apparently held at MO only at F, LPB and NY (Teisher, pers. comm.). The voucher information corresponds to the collection of T.J. Killien (nº 1267) made in Bolivia during 1989, but no publication has been found about this likely typification and, at least, the voucher at F (barcode F2013050!; Bolivia, Santa Cruz, Cordillera, Tatarenda, 30 km S of Rio Grande on road to Camiri, 19º08'S 63º15'W, 700 m, first range of Andes with semi-deciduous forest and slash and burn agriculture, soils generally sandy, Tim Killeen 1267, 16 Oct 1985) does not bear any identification of type material. Therefore, the typification of the name *C. moschata* is still an uncompleted task, as with many other cultivated species, which were initially described without any direct reference to a herbarium voucher or collection. The second mention concerns the accepted synonym *Gymnopetalum calyculatum* Miq., the description of which (Miquel 1860) was based on material collected from Bangka by J. Amann (pseudonym of W.S. Kurz, see Van Steenis-

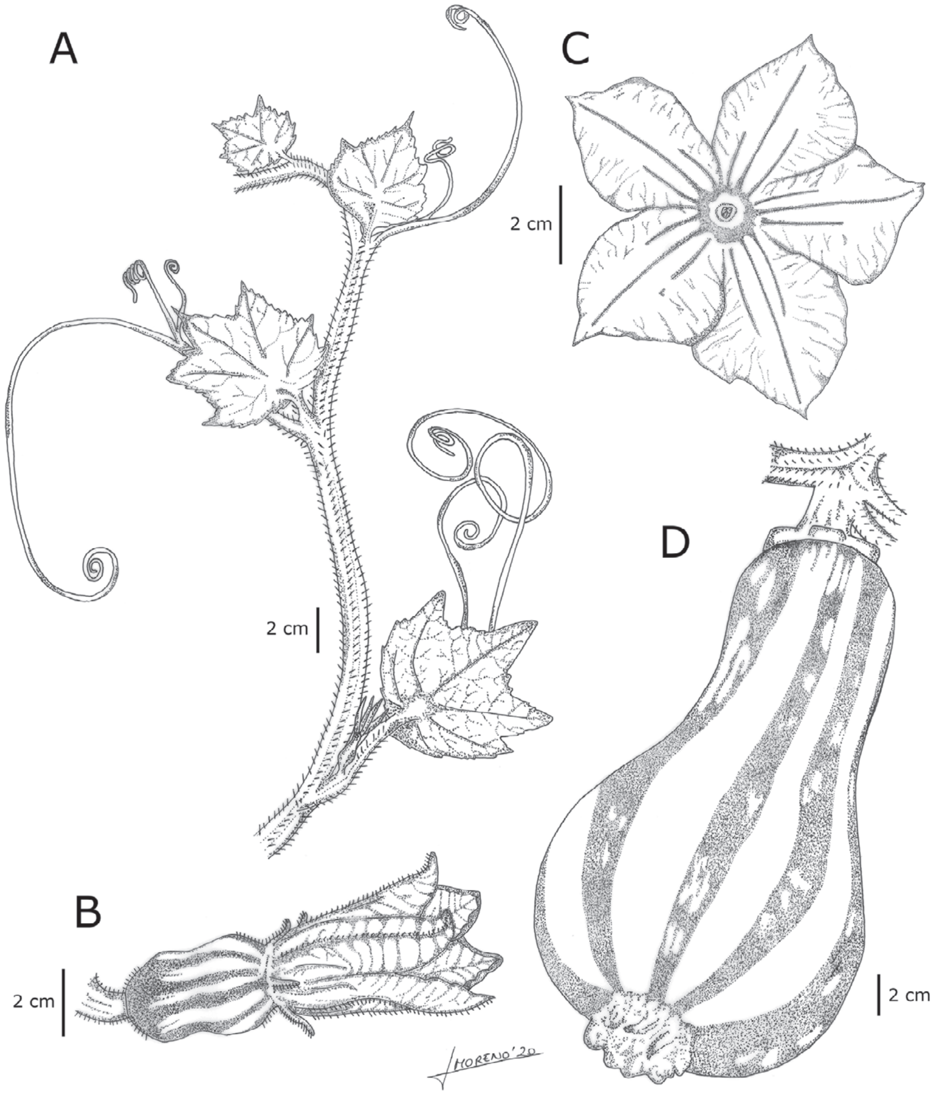

Fig. 1. *Cucurbita moschata* Duchesne. A – habit, B – flower (side view), C – flower (front view), D – mature fruit (drawing by Joaquín Moreno).

Kruseman and Van Steenis 1950). De Wilde and Duyfjes (2010) designated a voucher kept at the herbarium U (barcode U0001457!) as the holotype. However, the use of the term holotype by these authors cannot be adequate, as Miquel (1860) only referred to Amann's gathering activity but not to a specific sheet. Hence, there is no certainty that

Tab. 1. Main morphological features differentiating the four species of *Cucurbita* reported in Spain (based on Lira 1995, Lira and Rodríguez Arévalo 1999, Teppner 2004).

	C. ficifolia	*C. maxima*	*C. moschata*	*C. pepo*
Habit	Perennial	Annual	Annual	Annual
Leaves	Lobed	Not lobed/ shallowly lobed	Not lobed/ shallowly lobed	Shallowly to deeply lobed
Segment leaves	Rounded	Rounded	Acute	Acute
Indument	With short glandular hairs	Stiff-haired/ hispid	Soft-haired	Spiculate
Sepals	Linear, apex non-broadened	Linear, apex non-broadened	Linear or with apex often broadened like leaves	Linear, apex non-broadened
Seed color	Dark brown to black	Orange or white	Tan to brown	Pale tan
Fruit peduncle	Ribbed, moderately broadened at attachment	Cylindrical, non-broadened at attachment	Ribbed, at attachment widely broadened (flat)	Ribbed, slightly, broadened at attachment

other duplicate material was not used to prepare the description of this name by the original author (ICN, Art. 9.1), though no additional specimens were found at NL, L, U and WAG (M. Scherrenberg, pers. comm.). Therefore, the voucher U0001457 should be considered as the lectotype of the name *G. calyculatum*. Finally, the third reference corresponds to a specimen of the name *C. sulcata* Blanco kept at L (barcode L0585284!; *Cucurbita sulcata*, Merrill, Species Blancoanae nº 152, labelled as neotype by J.K. Veldkamp 2/03, labelled as *C. moschata* by W.J.J.O de Wilde & Duyfjes, 2011), which was selected as neotype by Veldkamp on the specimen; however, Veldkamp never published his designation, which is thus ineffective. The name *C. sulcata* is currently considered a synonym of the species *C. maxima*, but the identification of this particular voucher was corrected to *C. moschata* on the specimen by De Wilde and Duyfjes. To avoid the effect of destabilizing the nomenclature of the name *C. sulcata*, an effective typification should be based on a specimen whose identification corresponds to the original description (Blanco 1837).

Habitat and populations

The populations of *C. moschata* were found growing in the central part of the course of the River Vinalopó (named the Middle Vinalopó district), which runs across the inner central part of Alicante Province (Spain), crossing semiarid and arid areas. At this geographical zone of the river, the watercourse is not continuous, and it can remain totally dry for long periods of time, even in excess of one year. This ecological peculiarity, added to the climatic characteristics of the area, contributes to soil salinity.

Four scattered populations, with up to 10 specimens each, of *C. moschata* have been discovered along five kilometres of this river basin, most of them growing clearly away from the direct influence of the main course of the water. The largest population, about 10 individuals, appeared on sandy soils on the upper part of the terrace of the river. At a distance of approximately two kilometres downstream, another well-established population with 5 individuals grew close to secondary dry channels of this river, characterized by gravel and sandy soils. The other two populations, formed by 2–3 individuals, and some isolated individuals of *C. moschata* were placed on the shallow area of meander scars and along the edges of the riverbed, both upstream and downstream in reference to the largest populations.

The first observations of them were noted in 2016, when only the two above-mentioned largest populations were initially identified with five and two reproductive individuals, respectively. Over the years these populations of *C. moschata* have developed autonomously, with increasing numbers of individuals, while new subpopulations or isolated individuals have been found far from them, being fully reproductive. Although the size of the population may be different from year to year, the annual appearance of new specimens seems to be entirely independent, based on the development of functional seeds, which seem to germinate under the ecological conditions of this area. In addition, no crops were found close to the observed populations.

The populations of *C. moschata*, in general, appear intermixed with wild shrubby and grassland vegetation (e.g., *Atriplex halimus* L., *Cynodon dactylon* (L.) Pers., *Tamarix gallica* L., together with other naturalized alien species along the river, such as *Physalis peruviana* L., *Stenotaphrum secundatum* (Walt.) Kuntze, *Solanum lycopersicum* L. and *S. sisymbriifolium* Lam. Independently of the size and location of the population, the observed individuals were fully reproductive, and well-developed mature fruits were easily found.

Climate and watercourse features

On the basis of the recent studies of José Ramón Coves (pers. comm.), the main features of the water of the River Vinalopó along the studied area are an average water temperature of about 20 °C, with an electric conductivity ranging from 2.8 to 5.9 mS cm^{-1}, pH 7.66 (7.3–7.8) and an average nitrate concentration of 75 mg L^{-1} (25–180 mg L^{-1}). The weather conditions of the studied area are characterized by typically Mediterranean climate, with remarkably dry summers. The annual average rainfall is 345 mm, with maximum values in September (43 mm) and minimum in July (6 mm). The average monthly values of temperatures are always above 0 °C, January being the coldest month with minimum average temperatures of 7.7 °C whereas the maximum values were registered in both July and August (mean values of 25.1 °C and 24.9 °C, respectively). According to these rainfall and temperature data, the studied area corresponds truly to a semiarid area belonging to an inferior Mesomediterranean belt.

Naturalized specimens observed of *Cucurbita moschata*

Hs, Alicante: Elda, River Vinalopó, 38°27'09''N 0°48'12''W, 337 m, 12 Nov 2017, A. Juan AJ117 (ABH 82475). Elda, River Vinalopó, left side of the river, upper part of the terrace of the river, 38°27'06''N 0°48'13''W, 335 m, 20 Nov 2017, A. Juan & I. Juan AJ118 (ABH 82476). Elda, River Vinalopó, upper part of the terrace of the river, 38°27'04''N 0°48'12''W, 335 m, 14 Sept 2018, A. Juan, J. Moreno & A. Terrones (ABH 82477). Elda, River Vinalopó, secondary dry channel, 38°26'51''N 0°48'13''W, 330 m, 27 October 2018, A. Juan, J. Moreno & A. Terrones (v.v.). Elda, River Vinalopó, terrace of the river, 38°28'16''N 0°48'15''W, 370 m, 24 Nov 2019, A. Juan, J. Moreno & A. Terrones (v.v.).

Distribution of the *Cucurbita moschata* in Spain

Specific geographic references to *Cucurbita moschata* as crop have been reported only from the northeast of the Iberian Peninsula (Barcelona province), where it also behaves as an occasional alien growing on the margin of the river (Gómez-Bellver et al. 2019). The newly found *C. moschata* populations in Alicante are very distant. The populations of *C. moschata* found in Alicante Province have been documented since 2016 and most recently have been observed to be composed of at least 25 reproductive individuals.

Discussion

Morphological aspects

The collected specimens of *Cucurbita moschata* show morphological features that fully fit the typical diagnostic characteristics of the species (Teppner 2004, De Wilde and Duyfjes 2010), though certain variabilities related to the leaves, sepals and fruits were detected among the studied specimens. The leaves were shallowly lobed, and either without or with whitish blotches. Most of the samples were characterized by the unique presence of linear sepals, but some specimens showed flowers with lanceolate sepals together with flowers with broadened sepals. Two types of fruits were observed: (i) dumbbell and solid green with a fruiting pedicel widely flat at the fruit attachment, and (ii) more rarely, pyriform and cream-coloured with light green longitudinally reticulated mottled stripes (usually in bands) with 5-ribbed thickened and dilated peduncles at the fruit attachment. The observed minor morphological variations are likely derived from the existence of numerous cultivars of this species, which vary greatly in fruit shape and colour (Lira 1995, Teppner 2004, De Wilde and Duyfjes 2006).

The main morphological characters distinguishing *C. moschata* plants or fruits from the closely related species, *C. pepo* and *C. maxima*, are basically related to the indument, leaves and fruit stalk (Lira and Rodríguez Arévalo 1999, Teppner 2004, Tab. 1). While *C. moschata* is a soft-haired plant with non- to shallowly lobed leaves, smoothly grooved stems and a hard, smoothly angled fruit stalk widened at the apex, *C. maxima* is characterized by hispid, unlobed (or slightly lobed) leaves and rounded stems, with the fruit stalk soft and rounded, not enlarged at the apex. Finally, *C. pepo* is typically spiculate with grooved stems and palmately lobed, often deeply cut and prickly leaves, with a hard and markedly angular fruit stalk sometimes slightly widened at the apex.

The following dichotomous key is based on Fernandes (2005) to which *C. moschata* was added to facilitate the plant identification:

1. Plants with short glandular hairs, generally climbing; leaves 3-5-lobed, with rounded or obtuse segments; fruit with white flesh, seeds black . . . . *C. ficifolia*
– Plants hispid with short, stiff hairs, prostrate or climbing; leaves from entire to clearly lobed, with triangular segments; fruit with pale or yellow or yellow-orange flesh, seeds white or orange 2
2. Leaves entire or slightly lobed; rounded stems; fruit stalk subcylindrical, rounded, soft . . . . *C. maxima*
– Leaves shallowly to deeply lobed; grooved stems; fruit stalk different 3
3. Plant prickly, spiculate; leaves palmate, shallowly to deeply lobed; fruit stalk notably angled, slightly broadened at attachment on the fruit apex; fruit with light-coloured yellow to orange flesh; seed margin with similar colour and texture as the surface . . . . *C. pepo*
– Plant softly hairy; leaves shallowly lobed; fruit stalk slightly angled, mostly abruptly broadened at attachment on the fruit apex; fruit with orange flesh; seed margin with a different colour and texture than the surface . . *C. moschata*

Distribution and alien status of *C. moschata* and related species

The presence of *C. moschata* out of cultivation is quite scarce not only around the Mediterranean basin but also around the European continent. Ardenghi and Mossini (2015) have reported the presence of two non-cultivated populations in Italy, located along an irrigation canal among rice fields, together with scattered vegetable refuse. According to these authors, the populations almost certainly grow from seeds rejected as food waste, since the observed seedlings likely derived from fruits produced in previous years. In addition, occasional reports of *C. moschata* from northern European territories have been also indicated (Jonsell and Karlsson 2010, Verloove 2018). Jonsell and Karlsson (2010) stated the doubtful mention of this taxon in Sweden during the 1950s and no new mention is available, and, Verloove (2022) reported it on a dump in Belgium. In Spain, Gómez-Bellver et al. (2019) recently reported alien plants of *C. moschata* growing on the margin of the river Llobregat. Based on Richardson et al. (2000), these mentions might be considered as ephemeral casual alien plants. Contrary to these previous reports, our direct field observations yielded the lack of any vegetable crops close to this stretch of the river, including cultivated plants of *C. moschata*. Therefore, the existence of these non-cultivated populations of *C. moschata* might have originated from seeds of old discarded agricultural waste in some remote area upstream of the river, as neither farming litter nor current cultivation has been observed close to the studied area. The existence of a large number of individuals cut off from the riverbed together with their repetitive presence during several years would denote a certain population stability. Consequently, the presence of *C. moschata* populations along the basin of the River Vinalopó, and their subsequent propagation over the years, would be nowadays considered as autonomous and well established, and not directly bound to any current agricultural activity. Although most free-living plants of the cultivated species of *Cucurbita* are reported as casual offspring from nearby fields, on the basis on our observations we consider the described Alicante populations of *C. moschata* as the first ones to be naturalized in Spain.

Regarding closely related species (*C. ficifolia*, *C. maxima* and *C. pepo*), only two mentions (Barcelona and Valencia, based on herbarium specimens) were reported for *C. ficifolia*, growing in fields of carob trees. Conversely, the species *C. pepo* and *C. maxima* both have been largely reported for different Spanish provinces (e.g., Serra 2007, Sanz Elorza et al. 2009, and more than 250 occurrences on GBIF), including the Balearic and the Canary Islands. Most of the observations of these three species based on the labels of vouchers and existing literature reported the presence of scarce specimens out of the crops (e.g., Serra 2007, Gómez-Mercado 2009, Herrera and Campos 2010, among others). They were considered as casual alien plants as they typically grew in disturbed habitats near crop fields.

Ecological aspects

The existence of numerous small parcels of farmland on which are grown tomatoes, broad beans, peas, onions, and melons, among others things, basically for subsistence, is fairly extensive along the Vinalopó Valley, though they were not the main agricultural use for this territory (Juárez 2010). Nowadays, the discovered naturalized populations of *C. moschata* did not grow close to any pumpkin crops, and therefore, the river would be the main method of seed dispersal along the Vinalopó Valley. In fact, riparian habitats can act as 'conveyor belts' for propagules (Richardson et al. 2007), and they might be important corridors for seed dispersal, both for native and alien species (Pyšek and Prach 1995, Stohlgren et al. 1998). Many alien species spread along watercourses (Richardson et al. 2000), and their invasion success largely depends on their dispersal ability (Pyšek and Prach 1995). Ecological conditions also play an important role on the entrance into and stabilization of alien plant populations along watercourses (Iamonico 2021), especially for annual *Cucurbita* species (Lira 1995, OECD 2016). *Cucurbita* fruits can be buoyant in the watercourse (OECD 2016), and hence, the river would represent a potential means of long seed dispersal. Under the appropriate environmental conditions, including no severe frost, seasonably warm temperatures and well-drained soils (OECD 2016), the germination of seeds and development of offspring of *C. moschata* would be favoured, even in the existence of relative drought. The observed climatic conditions of the studied area coincided with the ecological requirements, although the water quality of this river is slightly alkaline and saline (José Ramón Coves, pers. comm.). In fact, a high number of non-cultivated individuals of *Solanum lycopersicum* (Solanaceae) belonging to different cultivars, including cherry and plum tomatoes, appear clearly naturalized along the Vinalopó river channel and its terrace, comprising well-established populations without direct intervention by humans (pers. obs.). The number of individuals of *S. lycopersicum* is quite high and their presence is so frequent that this species already coexists with the riparian natural vegetation of the river, and the long-time permanence of these individuals would be autonomous and even might be considered as invasive. Similarly, other alien non-agricultural species, as *Drosanthemum hispidum* (L.) Schwanthes, *Ulmus pumila* L., *Rumex cristatus* DC., *Cotula coronopifolia* L. or *Datura inoxia* Mill., have also been observed and their populations are easily found along the River Vinalopó (Serra 2016, Juan et al. 2019). Among them, the species *D. hispidum* clearly shows an invasive behaviour, being the dominant species along the studied upper fluvial terraces of the River Vinalopó and close slopes (pers. obs.).

Conclusions

Agricultural activities are confirmed again as a starting point for the process of naturalization of alien plants, which together with the presence of a river greatly favours the dispersion of these alien plants. Conversely to the casual alien populations of *Cucurbita pepo*, *C. ficifolia* and *C. maxima*, the herein identified populations of *C. moschata* are able to reproduce regularly without any human activities and they are well-established along the central area of the River Vinalopó (southern Iberian Peninsula, Spain), where no regular crops of this species have been located. Therefore, the non-cultivated Spanish populations of *C. moschata* are catalogued as naturalized alien populations, which would be the first reference for Western Mediterranean countries. Nevertheless, further investigations are needed to identify new possible localities of this taxon, both upstream and downstream of the sites discussed.

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Pollination patterns of flora and vegetation in northern Croatia with reference to *Apis mellifera*

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Abstract – Pollination patterns i.e. the proportions of entomophilous, anemophilous, autogamous and hydrophilous plant species and those useful for the European honey bee (*Apis mellifera* L.) in the flora and vegetation of northern Croatia have been determined. The survey included 507 plant taxa, belonging to 95 plant families. The results show that most plant species employ insect pollination (73.6%), followed by self-pollination (30%), wind (25%) and water pollination (0.6%). For some plant species there are one, two or more modes of pollination; the largest group consists of pure insect pollination (43%), followed by both insect and self-pollination (27%), pure wind pollination (22%), insect and wind pollination (2.6%), and so on. Overall, 54% of plant species useful to European honey bees were found, 51% of which provide pollen and 47% nectar. These results suggest that *A. mellifera* could be a potential pollinator for about half of the flora. Analysis shows significant differences in pollination patterns among habitat types and that most entomophilous plant taxa are found in grassland, forest and ruderal sites, indicating that these habitats are most important for pollinators. Other characteristics of plant species, such as flowering time, plant family, life form and origin, were also analysed to determine a possible relationship with pollination.

Keywords: European honey bee, insect pollinators, life forms, plant families

Introduction

Pollination is one of the key ecosystem services, enabling the reproduction of wild and cultivated plant species, i.e. the production of seeds and fruits. In Europe, in the area of temperate continental climate, various insects are pollinators. Most numerous are the hymenopterans (*Hymenoptera*), butterflies (*Lepidoptera*), flies (*Diptera*) and beetles (*Coleoptera*) (Kevan and Baker 1983, Ollerton 2021). In addition to wild insects, European honey bees (*Apis mellifera* L.) play a very important role in pollination. Beekeeping is also used for the production of honey, pollen, propolis, royal jelly, bee venom, wax, queens and bee communities, as well as in apitherapy and apitourism.

Scientific studies have shown a declining trend in pollinator numbers (Potts et al. 2010, Goulson et al. 2015, Sánchez-Bayo and Wyckhuys 2019), mostly relating to habitat degradation and loss, urbanisation, agricultural intensification, pesticide and fertiliser use, pollution, pathogens, climate change, alien species and synergistic action of several factors. The most common declines involve specialists or species closely associated with a particular plant species or habitat, while a small number of generalists are increasing in number (Klein et al. 2007, Sánchez-Bayo and Wyckhuys 2019). However, some generalists are also declining, including the European honey bee. There are also other problems, e.g. competition between European honey bees and wild pollinators for forage (Goulson et al. 2015), a large knowledge gap about wild pollinators, etc. Along with the decline in pollinators, a decline in wild plant species pollinated by insects has been observed in some parts of the world (e.g., the UK) (Biesmeijer et al. 2006, Potts et al. 2010).

Ollerton et al. (2011) indicate that, in temperate regions of the world, about 78% of wild plant species are pollinated by animals, while Klein et al. (2007) have found that, of 107 leading crops worldwide, 91 species (85%) depend to varying degrees on animal pollination. According to Potts et al. (2010) pollination by insects, primarily bees, is necessary for 75% of all crops. However, there is relatively little literature on this topic. In Croatia, there are studies that deal with pollination from different aspects. One study refers to different taxonomic groups and species of insect pollinators in different habitats in north-eastern Croatia (Kovacic et al. 2016). A few papers present the results of melissopalynological analysis of honey samples from different areas of continental Croatia (Sabo et al. 2011, Štefanić et al. 2012,

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Špoljarić Maronić et al. 2017, Rašić et al. 2018), where the botanical origin (plant species used by European honey bees as nectar and pollen sources) was determined on the basis of pollen grains. Nevertheless, due to the economic importance of beekeeping in Croatia, several books and lists of plant species useful for *A. mellifera* have been published (e.g., Umeljić 2004, 2018, Bačić and Sabo 2007, Zima 2007, Bučar 2008, 2018, Zima and Štefanić 2018). There are several botanical studies that include an analysis of plant species useful for pollinators, especially European honey bees, according to specific habitat types (Martinis and Lovašen-Eberhart 1986, Dujmović Purgar and Hulina 2007, Britvec et al. 2013, Dujmović Purgar et al. 2015, Ljubičić et al. 2017, Štefanić et al. 2020). Franić (2019) provides an overview of the interaction between forestry and beekeeping in Croatia. However, none of the above papers includes an analysis of the proportion of insect-pollinated plant species and those useful to *A. mellifera* in the entire flora and all habitat types.

Given the lack of data on the proportion of plant species pollinated by insects in the total flora and in all habitat types, at both regional and global level, this paper presents such an analysis in Croatia for the first time. Given the aforementioned decline in pollinators and insect-pollinated plant species, such scientific research data is of the utmost importance, as it can help in determining best practices for ecosystem management.

The objectives of this study were therefore (i) to determine the pollination patterns of the flora and vegetation in the continental part of Croatia, (ii) to determine the proportions of plant species useful to *A. mellifera* in the flora and by habitat type, and (iii) to analyse how pollination is related to by various characteristics of plant species, including flowering time, plant family, origin and life form.

Materials and methods

Study area

The study of flora and habitats was carried out in the area of the settlement Bedekovčina, with about 3400 inhabitants, in northern Croatia (On-line Suppl. Fig. 1). The study area is located partly in the valley of the River Krapina and partly in a hilly area at an altitude of 148 to 237 m a.s.l., over an area of about 30 km^2. The landscape consists of a built-up area, arable land with annual crops, traditional gardens, vineyards, orchards, forest, a small number of mown meadows, abandoned arable land and meadows in various stages of succession. Aquatic ecosystems include the River Krapina, numerous streams and canals, and five artificial lakes that have an area of about 11.2 ha. The area is characterized by a temperate continental climate, belonging to the C_{fwbx} type according to the Köppen classification, and to the humid climate according to the Thornthwaite classification, with an average annual air temperature between 10 and 11 °C and an average annual precipitation from 900 to 1000 mm (Zaninović et al. 2008).

Data collection

The field research into the flora and habitats was carried out in the period from 1992 to 2021. Plant species were identified using the Flora Europaea (Tutin et al. 1964-1980, 1993) and Exkursionsflora von Österreich (Adler et al. 1994). The nomenclature of the plant taxa and their taxonomic positions follows Euro+Med PlantBase (2006-2021). For some taxa only, Flora Croatica Database (hereafter: FCD) (Nikolić, 2021) and Pladias (2021) were used, because these taxa could not be found in the Euro+Med PlantBase (2006-2021). These include aggregate species, subspecies of the genus *Leontodon*, genus *Corydalis* and *Medicago x varia* Martyn.

Species were classified into 11 habitat groups according to their affiliation to plant communities: (i) forest unaffected by flooding (ii) scrubland unaffected by flooding, (iii) floodplain forest and scrubland, (iv) forest-edge vegetation, (v) wet and mesic grassland, (vi) dry grassland, (vii) aquatic freshwater vegetation, (viii) marsh vegetation, (ix) ruderal vegetation, (x) weed vegetation and (xi) vegetation of walls. For each habitat group, the corresponding habitat types according to the National Habitat Classification of the Republic of Croatia (Anonymous, 2018) and vegetation classes according to the Classification System for European Vegetation (EuroVeg CheckList, Mucina et al. 2016) were added (see On-line Suppl. Tab. 1).

Data on the mode of pollination (autogamy, entomophily, anemophily, hydrophily), flowering time, origin of taxa and life forms were taken from FCD (Nikolić 2021) and Pladias (2021).

Plant species useful to *A. mellifera* have been divided into the following categories depending on the food source they offer: nectar, pollen, honeydew and propolis. The data were taken from Maurizio and Grafl (1969), Bačić and Sabo (2007), and Bučar (2008, 2018).

All collected data are presented in On-line Suppl. Mat.

Data analysis

The data were treated statistically using Excel and Statistica v7. Contingency tables, displaying the multivariate frequency distribution of the variables, were constructed using Excel, while Pearson Chi-squares (χ^2) were calculated using Statistica v7 software.

Results

Flora

In the Bedekovčina area, a total of 507 plant taxa (On-line Suppl. Mat.) were identified, belonging to 95 plant families (On-line Suppl. Tab. 2), of which *Compositae* are the most numerous (54 taxa), followed by *Poaceae* (51), *Fabaceae* (28), *Lamiaceae* (26), *Cyperaceae* (23), etc. According to the affiliation to higher taxonomic groups, the class *Magnoliopsida* prevailed (496 taxa), followed by *Polypodiopsida* (10) and *Pinopsida* (1).

Relatively few threatened species were found: one endangered (EN), seven vulnerable (VU) and five near-threatened species (NT) (On-line Suppl. Mat.).

Habitat types

Regarding habitat types, most plant taxa were recorded in ruderal vegetation (30%), followed by wet and mesic grassland (28%), forest unaffected by flooding (28%), weed vegetation (12%), marsh vegetation (9%), floodplain forest and scrubland (5%), scrubland unaffected by flooding (5%), forest-edge vegetation (4%), dry grassland (2%), freshwater aquatic vegetation (2%) and vegetation of walls (0.2%). Some plant species occur in two or more habitat types.

Pollination patterns

Among the pollination modes, expressed in absolute percentages in relation to the total number of plant species, insect pollination (entomophily) is the most widespread, with 73.6%, followed by self-pollination (autogamy) with 30%, wind pollination (anemophily) with 25%, and water pollination (hydrophily) with 0.6% (Fig. 1a). There are also ferns whose fertilisation requires water (2%). The sum of the percentages exceeds 100% because some plant species have more than one mode of pollination.

Pollination in the largest proportion of species is done exclusively by insects (43%) (Fig. 1b). Both insect and self-pollination occur in 27% of plant species, followed by wind pollination (22%), insect and wind pollination (2.6%), etc. (Fig. 1b). The values are expressed in relative percentages.

Certain modes of pollination are associated with specific plant families. Among the families with the largest number of species, *Compositae*, *Fabaceae*, *Lamiaceae*, *Apiaceae*, *Rosaceae*, *Caryophyllaceae* and *Plantaginaceae* are predominantly insect-pollinated and to a lesser extent self-pollinated, while *Poaceae* and *Cyperaceae* are wind-pollinated (On-line Suppl. Fig. 2).

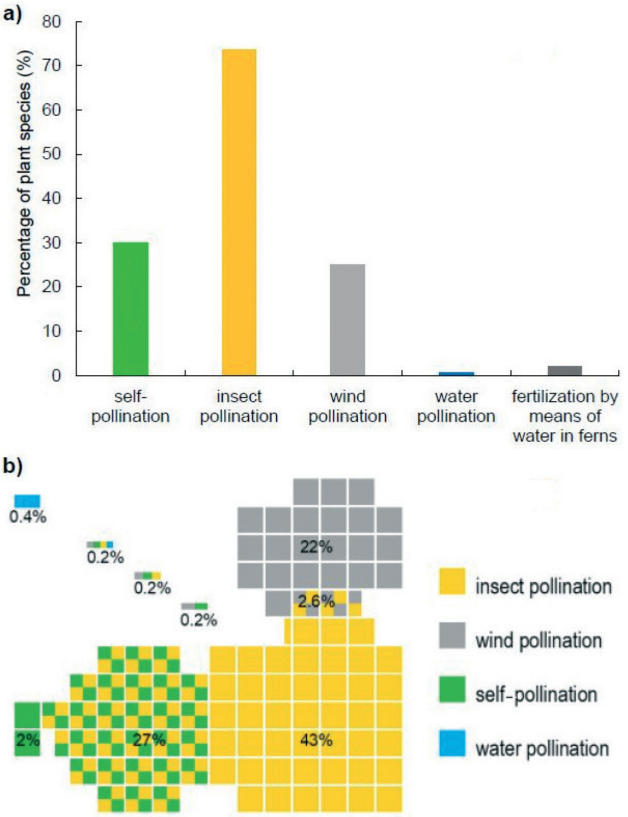

Fig. 1. Contributions of the different modes of pollination in the flora studied in the northern Croatia: a) representation of individual modes of pollination in absolute percentages (where the sum exceeds 100% because some plant species have more than one mode of pollination), b) contribution and overlap of specific modes of pollination in relative percentages.

Insect pollination is prevalent in all habitat types, and is shown in absolute percentages (Fig. 2a), with the highest proportion in ruderal (24%), forest (22%) and grassland habitats (20%). As can be seen from Tab. 1, for the grassland, forest and ruderal habitats, the calculated Chi-square ($\chi^2 = 14.5$, $P < 0.05$) indicates their statistically significant difference, with insect pollination as the dominant mode. The proportion of wind- and self-pollinated plant species varies by habitat group (Fig. 2a). The largest proportion of wind-pollinated plant species (9%) is found in open habitats, such as grassland. No wind-pollinated species were found in forest-edge vegetation, probably because these habitats are sheltered from the wind. Self-pollinated plant taxa make up a significant proportion in ruderal (11%) and weed habitats (6%), because there are many annual species with a short life cycle, thus ensuring survival. Pollination by water is represented only in aquatic vegetation. Representation of pollination modes by habitat type in relative percentages and with an overlap of pollination modes (Fig. 2b) shows that pollination patterns vary considerably among habitat types ($\chi^2 = 39.8$, $P < 0.001$). Obtained variability of pollination modes (Fig. 2b): insect pollination in the range of 26–60%, both insect and self-pollination ranging between 6 and 45%, wind pollination ranging from 0 to 38%, self-pollination ranging from 0 to 9%, and both insect and wind pollination

Tab. 1. Contingency table showing number of plant species in certain habitat type in relation to pollination modes. * for denoted habitats, there is a statistically significant difference (χ^2: 14.5; $P < 0.05$).

Habitat type	Insect pollination	Insect and self-pollination	Wind pollination	Other modes of pollination	Total
Grass veg.*	48	22	37	3	110
Forest veg.*	62	36	23	11	132
Ruderal veg.*	54	37	22	8	121
Other habitats types	55	44	29	16	144
Total	**219**	**139**	**111**	**38**	**507**

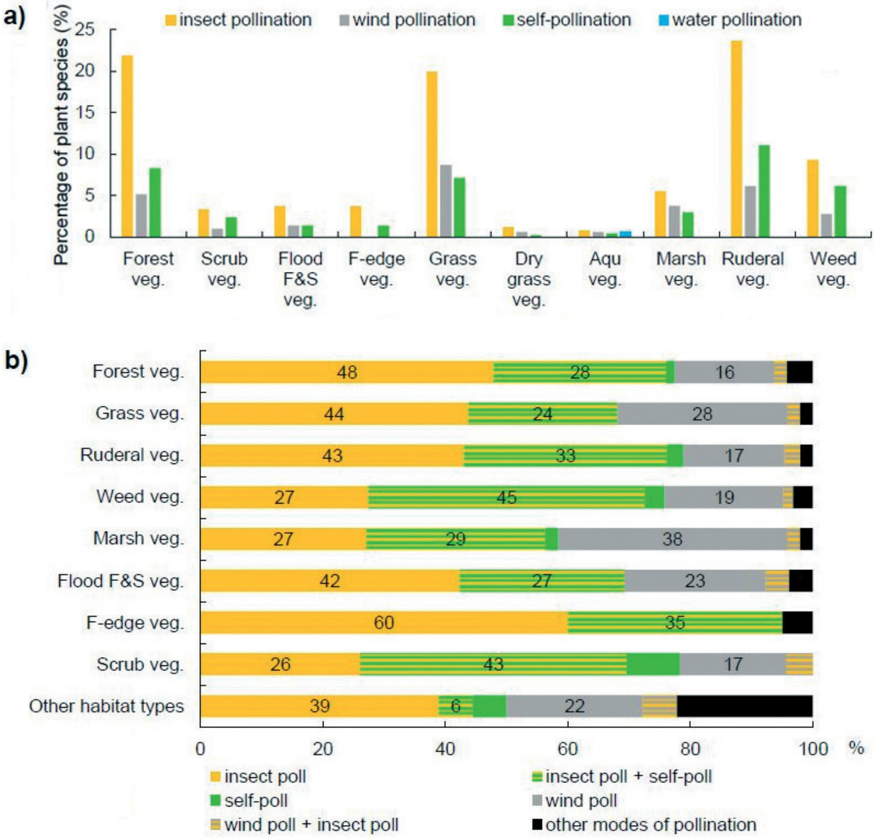

Fig. 2. Percentages of different pollination modes in different habitat groups: a) representation of individual modes of pollination by habitat in absolute percentages (sum exceeding 100% because some plant species have more than one mode of pollination), b) representation of the proportion and overlap of specific modes of pollination by habitat type in relative percentages (where the habitat groups differ significantly with respect to pollination mode: $\chi^2 = 39.8$, $P < 0.001$). Forest veg. – forest vegetation unaffected by flooding, scrub veg. – scrub vegetation unaffected by flooding, flood F&S veg. – floodplain forest and scrub vegetation, F-edge veg. – forest-edge vegetation, grass veg. – wet and mesic grassland vegetation, dry grass veg. – dry grassland vegetation, aqu. veg. – aquatic freshwater vegetation, marsh veg. – marsh vegetation, ruderal veg. – ruderal vegetation, weed veg. – weed vegetation, wall veg. – wall vegetation.

ranging between 0 and 6%. Pure insect pollination is most prevalent in forest-edge vegetation, followed by forest, grassland and ruderal vegetation. Both insect- and self-pollination are best repesented in weed, scrub, forest-edge and ruderal vegetation. Pure wind pollination is most prevalent in marsh and grassland vegetation.

Plant species useful for *Apis mellifera*

The European honey bee plays a very important role in the pollination of plant species. In this study, a total of 54% of plant taxa useful to *A. mellifera* were identified: 47% as a nectar source, 51% as a pollen source, 4% as a honeydew source, and 1% as a propolis source (On-line Suppl. Tab. 3). Of the plant species that depend only on insect pollination (43% of total species), 67% (29% of total species) can be used by European honey bees as a nectar source and 63% (27% of total species) as a pollen source (Fig. 3). Of the plant species with both insect and self-pollination (27% of total species), European honey bees can potentially use 63% (17% of total species) each as a nectar and/or pollen source. Of the wind-pollinated plant species (22% of total species), European honey bees can use 18% (4% of total species) as a pollen source.

The distribution of plant species useful to *A. mellifera* per habitat type is shown in Fig. 4. As can be seen from the figure, most plant species providing nectar to *A. mellifera* were found in ruderal (16%), grassland (15%) and forest habitats (14%), while there were fewer in other habitat types. A similar trend was observed for plant species serving as a source of pollen: the highest numbers were found in ruderal (17%), forest (16%) and grassland habitats (16%). Relatively few species are known to be a source of honeydew (up to 2%) and propolis (< 1%), and they grow in forest and scrub vegetation.

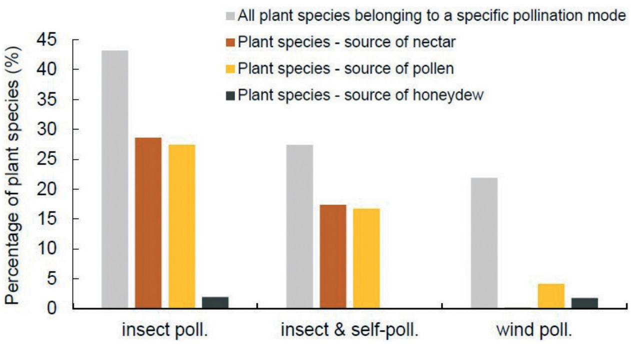

Fig. 3. Percentages of plant species useful for *Apis mellifera* (as a source of nectar, pollen and honeydew) by pollination mode.

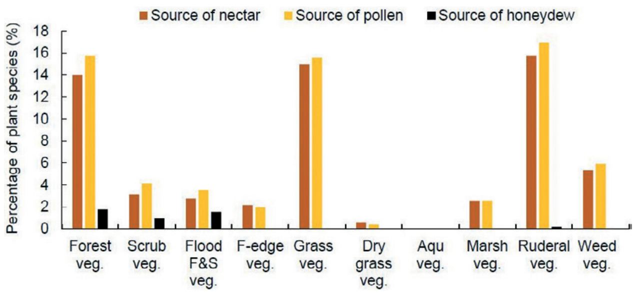

Fig. 4. Percentage contribution of plant species that are a source of nectar, pollen and honeydew for *Apis mellifera* by habitat group. (For habitat abbreviations see caption of Fig. 2).

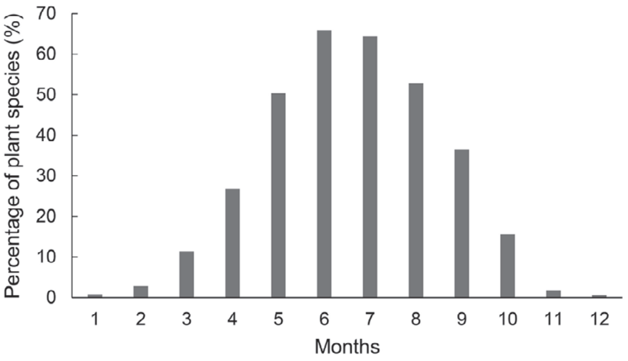

Fig. 5. Percentage contribution of plant species in Bedekovčina flora according to flowering time.

Flowering time

Most plant species flower in June (66%), and fewest in December (0.6%) and January (0.8%). During the ten month flowering period, from February to November, pollinators and *A. mellifera* can use nectar and pollen (Fig. 5).

Life forms

With regard to life forms in the flora, herbaceous perennials or hemicryptophytes predominate (53%), followed by annual plant species or therophytes (21%), geophytes (17%), woody plants or phanerophytes (11%), hydrophytes and chamaephytes (4% each), with some species associated with two life forms.

By habitat type, hemicryptophytes predominate in grassland, ruderal and forest habitats; therophytes have a high proportion in ruderal and weed habitats; geophytes are most numerous in forest habitats; phanerophytes in forest and scrub vegetation; chamaephytes in forest, and hydrophytes in marsh and aquatic vegetation (On-line Suppl. Fig. 3).

Insect pollination prevails in all life forms (Tab. 2, On-line Suppl. Fig. 4), while wind and self-pollination are less well represented. Theorophytes also have a considerable amount of self-pollination, whereas aquatic pollination occurs only in hydrophytes (On-line Suppl. Fig. 4).

Origin of plant species

By origin, indigenous or native plant species are most abundant (79.1%), followed by archaeophytes (11.8%), neophytes (8.5%) and three taxa (0.6%) of uncertain origin. Indigenous plant species dominate in all habitat types except weed vegetation, where archaeophytes have a higher proportion (On-line Suppl. Fig. 5). Furthermore, ruderal and weed vegetation contains a considerable proportion of archaeophytes and neophytes.

Analysis of pollination modes by origin of plant species shows that, in all three groups (indigenous plant species, archaeophytes and neophytes), plant species pollinated by insects dominate, while wind pollination and self-pollination are less well represented (On-line Suppl. Fig. 6). Only among the archaeophytes are there slightly more plant species with self-pollination than with wind pollination. The importance of insect pollination for plants of different origins can also be seen in Tab. 3, which shows that this mode of pollination is particularly prevalent in native plant species and neophytes ($\chi^2 = 19.6$, $P < 0.01$).

Discussion

The flora studied depends mostly on insect pollination (73.6%). Our results are in agreement with Ollerton et al. (2011) and Potts et al. (2010), who state that about 78–80% of wild plant species in temperate zones are pollinated by insects. A similar percentage was obtained in a study by

Tab. 2. Contingency table showing number of plant species by life form in relation to pollination modes. Life form abbreviations: H – hemicryptophytes, T – therophytes, G – geophytes, Ch – chamaephytes, P – phanerophytes, Hy – hydrophytes.

Life forms	Insect pollination	Insect and self-pollination	Wind pollination	Other forms of pollination	Total
H	112	53	48	9	222
T	27	38	17	9	91
G	28	11	16	10	65
P	25	12	14	2	53
Ch	6	7		1	14
Hy	5		2	6	13
Combinations	16	18	14	1	49
Total	**219**	**139**	**111**	**38**	**507**

Tab. 3. Contingency table showing number of plant species by origin in relation to pollination modes. A – archaeophytes; I – indigenous; N – neophytes. All types of analysed plant species were found to be different with respect to existing pollination modes (χ^2: 19.6; $P < 0.01$).

Origin of plant species	Insect pollination	Insect and self pollination	Wind pollination	Other modes of pollination	Total
I	172	104	91	34	401
A	19	28	10	3	60
N	27	7	8	1	43
Total	**218**	**139**	**109**	**38**	**504**

Štefanić et al. (2020) in NE Croatia, with the finding that 72.6% of plant species on field margins are beneficial to pollinators, although not all habitat types were included. For the flora of the Czech Republic, Chytrý et al. (2021) show only maps with the proportions of pollination modes influenced by relief and climate. Melendo et al. (2003) indicate, for the endemic flora in the south of the Iberian Peninsula with a Mediterranean climate, that 91% of the plant species are biotically pollinated, mainly by insects.

According to the data collected, about two thirds of plant species depend on only one mode of pollination, while about one third of plant species have two or, less frequently, several pollination modes. Durka (2002) determined exactly the same proportion of insect pollination (43%) for the flora of Germany as in N Croatia, slightly less for both insect and self-pollination (21%), much more for self-pollination (22%), less for wind pollination (18.5%), and almost the same for water pollination (0.5%). The data are not fully comparable, as Durka (2002) used, for plant species with several pollination modes, only the dominant one. Somewhat later, Kühn et al. (2006) mapped the distribution of pollination modes across the whole of Germany, with the help of modelling. Altitude and wind speed were strongly correlated with the proportions of pollination modes. Remarkable spatial differences were obtained: insect pollination in the range of 41.9–63.1%, wind pollination in the range between 15.5–32.7%, and self-pollination in the range of 16.1–29.9%. A coarse spatial resolution was used with a cell size of about 130 km^2 and a different method for calculating the proportion of pollination modes than in this paper.

To our knowledge, an approach combining multiple pollination modes of the whole flora and all habitat types, as used in this study, is not to be found in the available literature, so further comparison is not possible.

The proportion of certain pollination modes in a given area is influenced by ecology and evolution. The dominance of insect-pollinated plant species on the global level is explained by the high rate of diversification during evolution (Givnish 2010). Wind pollination of angiosperms probably evolved from insect pollination in response to unfavourable weather conditions in some areas (strong wind, heavy rain and low temperatures) and the associated lack of insect pollinators (Culley et al. 2002, Friedman and Barrett 2008). In some plant species, a transitional stage between wind and insect pollination i.e. ambophily is still present (Culley et al. 2002). In the flora studied, plant species that use both wind and insect pollination are relatively rare. Self-pollination is a typical feature of annual species (Lloyd 1992) or therophytes. Such plant species are not dependent on the availability of pollinators, weather conditions and pollen transmitters (animals, wind and water), which is particularly important when a species is rare in its habitat (Lloyd 1992). According to Pyšek et al. (2011), self-pollination is a crucial feature for the alien plant species invasion process. In the flora studied, there are very few plant species that are only self-pollinated, but a considerable proportion that are both insect- and self-pollinated. To ensure their survival, some plant species exhibit multiple pollination modes.

On a broad spatial scale, according to Givnish (2010), 202 out of 379 plant families are animal-pollinated, and only 39 are wind- or water-pollinated. The same trend, with the largest number of insect-pollinated plant families, has been found in N Croatia, and a small number are wind pollinated. Most wind-pollinated species belong to herbaceous families of open habitats such as marsh and grassland vegetation (*Poaceae, Cyperaceae, Juncaceae*) and woody species (*Betulaceae, Corylaceae, Fagaceae, Moraceae*) which are tall and exposed to the wind and flower before they form leaves.

The results of this study revealed that insect pollination is the predominant mode of pollination for most life forms as well as for plant species of different origins. However, the analyses showed that the distribution pattern of life forms and plant species by origin is more influenced by habitat types rather than pollination modes. In fact, it has been found that habitat types, and then affiliation to plant families, have the greatest influence on the distribution of pollination modes.

Different plant species have different flowering times, thus occupying different temporal niches and providing food for different species of pollinators during the vegetation season (Fenster et al. 2004). Depending on the species, the duration of the flowering period varies. There are also rare species that bloom all year, and even in December and January, but due to low temperatures, short daylight and lack of dormant insects, it is hard to speak of pollination. From February, the number of flowering species and active pollinators increases until June, and then the number decreases until November.

Recently, the phenology of plant species has been significantly affected by climate change (Tylianakis et al. 2008, Gordo and Sanz 2009). That is, climate change is causing plant species to begin flowering much earlier than usual, which can affect the temporal matching of pollinators and plant species (Tylianakis et al. 2008).

Among pollinators, *A. mellifera* could be a potential pollinator for about half of the flora, according to the research results of this study. The actual number is probably even higher, because there are no data for each wild plant species on whether it is visited by European honey bees. As already mentioned, for bees the most important group is that of insect-pollinated plant species, and somewhat less the group of insect- and self-pollinated plant species. In these groups, about two thirds of the plant species can be used by *A. mellifera* as a source of nectar and pollen. In addition, bees use less than one fifth of wind-pollinated plant species as a pollen source. Comparison with the literature is not possible, as no comparable data are available, which underlines the need for further studies in this field.

Potts et al. (2010) also highlight the fact that the contribution of European honey bees to the pollination of wild plant species is not well supported by empirical data. For example, regarding *A. mellifera*, the entomophilous plant species are relatively well known. They all produce pollen in greater or lesser amounts, and most nectar, but not all (nectarless species: *Chelidonium majus* L., *Clematis vitalba* L., *Papaver rhoeas* L., *Rosa canina* L., and others) (Maurizio and Grafl 1969). Anemophilous plant species produce large amounts of pollen through wind pollination, which is a very important food for many insect pollinators and the European honey bee. These include many widespread tree species (e.g., *Alnus glutinosa* (L.) Gaertn., *Betula pendula* Roth, *Corylus avellana* L., *Fagus sylvatica* L., *Populus tremula* L., *Quercus petraea* (Matt.) Liebl., *Q. robur* L., etc.), and also common herbaceous plant species (e.g. *Plantago lanceolata* L., *P. major* L., *Rumex* spp., etc.) (Maurizio and Grafl 1969). Of the other anemophilous plant species, *A. mellifera* is known to use plant taxa from *Poaceae* (total annual pollen yield may be as high as 1–10%), *Cyperaceae* (Maurizio and Grafl 1969), and probably many others. However, it is not completely known which species are involved. Thus, the number of anemophilous species used by *A. mellifera* is probably much higher than presented in this paper.

It is known that bees use the most suitable species among those available (Maurizio and Grafl 1969). Which plant species are used by European honey bees can be determined by melissopalynological analysis. Several such studies have been published for the continental part of Croatia (Sabo et al. 2011, Štefanić et al. 2012, Špoljarić Maronić et al. 2017, Rašić et al. 2018). In the papers cited, pollen grains from 4 to 33 plant taxes were found in honey samples. However, the final number of plant species visited by the bees is certainly much higher, since in the cited works not all honey samples were analysed during the vegetation season, and pollen samples collected separately by the bees were not analysed at all.

As *A. mellifera* is the best-studied insect pollinator, many findings from this study can be applied to wild pollinators, especially from the *Hymenoptera* group, which have similar foraging behaviour.

Which pollinators are associated with particular plant species can be found, in part, in the CrypTra database (Ellis and Ellis-Adam 1993), whose analysis shows that relationships are not characterised by specialisation. In the plant pollination system, Johnson and Steiner (2000) point out that, in Europe, generalists among pollinators prevail over specialists.

The study area is characterised by a diverse relief and a mosaic landscape. The great diversity of habitats is enhanced by the very small areas of land individually owned characteristic of this part of N Croatia. As some plant species only grow in certain habitats, habitat diversity is a prerequisite for flora biodiversity. The results show that habitat types differ significantly in terms of pollination patterns. In this study, three groups of habitats were identified where most insect-pollinated plant species occur, and which are also useful for *A. mellifera*. These habitats include grassland, forest and ruderal sites.

Grassland habitats belong mostly to the wet and mesic meadows of the class *Molinio-Arrhenatheretea* Tx. 1937. These are still very species-rich habitats, although much of the former meadows have been abandoned and are in various stages of succession. The reason for this is the change in the way of life of the local residents in the last 30 years. People have abandoned traditional agriculture and livestock breeding (mainly cows). Significantly reduced grassland areas result in a reduced food source for pollinators. The importance of such habitats for *A. mellifera* in the continental part of Croatia is highlighted by Ljubičić et al. (2017), and in the Mediterranean part of Croatia by Britvec et al. (2013). Comprehensive research in several European countries has also shown that semi-natural habitats (grassland) are very rich in bee pollinators (*Hymenoptera: Apiformes*) (Westphal et al. 2008). Restoration of grassland habitats is possible and involves the reintroduction of traditional extensive management, e.g. mowing two to three times a year.

Forest habitats belong mainly to beech (*Fagus sylvatica* L.) communities of the class *Carpino-Fagetea sylvaticae* Jakucs ex Passarge 1968. They are located in the hills, outside the influence of flood waters. Other types of woody vegetation (scrubland unaffected by flooding, floodplain forest and scrubland) cover relatively small areas. Compared to other habitat types, forest is the least changed. However, it is highly fragmented which negatively affects insect pollination (Kolb and Diekmann 2005), mostly privately owned, and affected by frequent and unplanned logging. Wind-pollinated plant species predominate among woody species. Herbaceous plant species develop in the ground layer and usually flower in the spring before tree leaves form.

In ruderal habitats there is a very heterogeneous group of plant communities in phytosociological terms (Mucina et al. 2016). In the study area, these are places alongside buildings, roads, railway lines and ditches, on construction sites, yards, landfills, composting sites, and filled and trampled areas. In general, these are habitats where humans prevent the development of natural vegetation through various disturbances. In addition to typical ruderal species, those of weed, grassland and, to a lesser extent, other habitat types grow in these stands. A large part of these habitats is mown and forms replacement habitat for grassland species, namely those that are resistant to frequent mowing. For pollinating insects, such habitats can be a food source, but only if mowing is not too frequent and if the plants have enough time to form flowers. The results of other studies (Dujmović Purgar and Hulina 2007, Dujmović Purgar et al. 2015) in the continental part of Croatia show the importance of ruderal habitats for *A. mellifera*. Studies in urban areas in the UK have also confirmed the importance of such habitats for flower-visiting insects (Baldock et al. 2015).

The entire study area in N Croatia is under significant anthropogenic influence. This is evident not only from the large areas covered with ruderal and weed vegetation, but also from a significant proportion of archaeophytes and neophytes in the composition of the flora, as well as from a small number of threatened species. Although neophytes pose a threat to native plant species and habitat diversity, some neophytes (*Robinia pseudoacacia* L., *Amorpha fruticosa* L., *Solidago gigantea* Aiton, etc.) can also serve as an additional nectar and pollen source for *A. mellifera* (Zima and Štefanić 2018). Even a common invasive alien species that is allergenic to humans, *Ambrosia artemisiifolia* L., serves as a pollen source for European honey bees (Špoljarić Maronić et al. 2017). Similarly, entomophilous neophytes serve as a food source for many wild pollinators (Suni et al. 2022). Visitation of alien plant species by entomofauna demonstrates their integration into the network of native pollinators, but there are controversial views on whether this is a positive or negative phenomenon (Potts et al. 2010). On the positive side, alien plant species, including many ornamental plants, provide food for pollinators; and, on the negative side, native plant species may be deprived of pollinators (Tylianakis et al. 2008). Suni et al. (2022) have shown that pollinators in urban areas prefer invasive alien plant species over native ones.

Various anthropogenic activities are known to cause declines in biodiversity at all levels of biological organization, including declines in insect pollinators (Potts et al. 2010, Goulson et al. 2015, Sánchez-Bayo and Wyckhuys 2019), which can lead to declines in plant species (Biesmeijer et al. 2006), and vice versa. Of all pollination modes, only insect pollination is threatened.

To preserve the biodiversity of pollinators, it is necessary to preserve the biodiversity of flora and natural and semi-natural habitats. Dennis et al. (2003, 2007), Garibaldi et al. (2014), Goulson et al. (2015) and Bretagnolle and Gaba (2015) suggest implementing various practices: providing nesting opportunities for pollinators, increasing heterogeneity of agricultural land (smaller fields), leaving or restoring areas of natural or semi-natural vegetation between or near crops, leaving weeds between crops (which can reduce crop yields but promote pollinator biodiversity), sustainable and/or organic agriculture, reducing the use of pesticides and machinery, no-tillage farming, seeding (wild) flower strips between and along crops and roads, planting hedgerows, seeding flowering crops, managing plant phenology (sowing plants that flower at different times), introducing pollinator monitoring, preventing the introduction of non-native bees, prohibiting the keeping of European honey bees in some natural areas to stimulate wild pollinators, enforcing effective quarantine measures for the movements of European honey bees to prevent the spread of pathogens and parasites, etc.

Some scientists point to the importance of cultivated plant species in maintaining wild pollinator biodiversity and providing food for *A. mellifera* (Garbuzov and Ratnieks 2014a, b, Salisbury et al. 2015). However, cultivated plant species can only be considered an additional food source when a particular crop is sown or planted and for only a certain period of year. It is unlikely that a diversity of cultivated plant species in a given area will provide food for pollinators throughout the vegetation season. From the mid-twentieth century to the present, various pesticides used in crop production have had lethal or sublethal effects on pollinators (Goulson et al. 2015), which is difficult to reconcile with pollinator stimulation. In addition, studies of insect foraging show that some commonly planted non-native ornamental species are unused or rarely used by pollinators (Garbuzov and Ratnieks 2014b, Lowenstein et al. 2019).

In Croatia, the food source for insect pollinators is still dominated by wild plant species. In wild plant and insect species, there is an evolutionary specialization of individual functional groups of insect pollinators to specific plant functional groups, which are linked in so-called pollination syndromes (Fenster et al. 2004).

Conclusions

The pollination pattern of the flora studied shows that insect pollination predominates, followed by self-, wind and water pollination. About two-thirds of the plant species depend on only one mode of pollination (mostly insect and wind pollination), while about one-third of the plant species depend on two (mostly both insect and self-pollination) and less frequently on several modes of pollination.

The distribution of pollination patterns is mainly influenced by habitat types. Detailed studies on this topic are needed in the future. Most insect pollinated plant species are found in grassland, forest, and ruderal habitats, highlighting their importance to pollinators. Among habitats, semi-natural grassland is most threatened because of the cessation of mowing.

In addition to habitat types, plant family affiliation also has a considerable influence on the distribution of pollination modes.

The European honey bee can potentially participate in the pollination of about half of the flora.

Given the predominance of wild plant species in N Croatia as a food source for pollinators in terms of the number of species, the area they cover, and their various temporal niches, it is crucial to preserve the biodiversity of wild flora and associated habitats.

The results of this work, with minor variations, can most likely be generalized to most of inland Croatia and to other temperate regions with similar relief, climatic conditions and habitats.

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Pollen morphology and flower visitors of *Leiotulus aureus* (Sm.) Pimenov & Ostr. (Apiaceae)

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Abstract – The pollen grains of *Leiotulus aureus* (syn. *Malabaila aurea* (Sm.) Boiss.) were examined by light and scanning electron microscopy in order to contribute to the taxonomical and melissopalynological studies of the species. Flower visitors have also been observed and analyzed aiming at the clarification of some pollination aspects including the species contribution to bee pasture. The pollen grains of *L. aureus* are isopolar, radially symmetrical, medium to large in size, tricolporate and perprolate. They are slightly equatorially constricted with obtuse polar caps and triangular in polar view. The sculpturing pattern is rugulate–microperforate. With regard to flower visitors, the following pollination types occurred: melittophily, myophily and sapromyophily and cantharophily. Some insects attracted by *L. aureus* cannot be considered pollinators but casual visitors. The flowers were the most frequently visited by honey bees during midday.

Keywords: *Malabaila aurea*, light microscopy – LM, palynomorphology, scanning electron microscopy – SEM, insect pollinators

Introduction

According to the latest taxonomic revision, genus *Leiotulus* Ehrenb., belonging to the Apiaceae tribe *Tordylieae* Koch., contains 10 species and subspecies, previously mainly assigned to the genera: *Malabaila* and *Pastinaca* (POWO 2022). Species *Leiotulus aureus* is known under the following homotypic synonims: *Heracleum aureum* Sm. (basionym), *Lophotaenia aurea* (Sm.) Griseb., *Malabaila aurea* (Sm.) Boiss., and *Pastinaca aurea* (Sm.) Calest., while the accepted name is proposed as a new name combination by Pimenov and Ostroumova (1994), based on carpological investigations. The genus included species characterized as intermediate between *Pastinaca* L. (incl. *Malabaila* s.str.) and *Zosima* Hoffm. (e.g. *Malabaila involucrata* Boiss. & Spruner, *M. pastinacifolia* Boiss. & Balansa etc.).

L. aureus is a herbaceous biennial, a plant of the native range from SE Europe to NW Turkey (POWO 2022), prefering warm and sunny places along with open rocky habitats. It is caracterised by a semirosette growth form, erect, hollow, striate and somewhat viscid stem up to 50 cm tall, pinnately divided leaves with ovate leaflets near the ground and linear-lanceolate in upper stem ones. Bright yellow flowers are grouped in terminal and subterminal, regularly compound umbels (Tutin et al. 1981). The mature fruit is obcordate – suborbicular, flattened, surrounded by a wide somewhat thickened margin, cordate at the apex, with persistent style (Fig. 1). Although the plant is slightly aromatic, a literature search did not reveal any reference to its local use in traditional medicine or cooking.

Investigations regarding apiaceaen pollen morphology have been reported by a number of authors, including Erdtman (1971), Van Zeist and Bottema (1977) and Cerceau-Larrival (1981). However, although pollination biology within the Apiaceae has not received much attention, some aspects were studied in a few taxa including *Chaerophyllum*, *Heracleum, Seseli*, *Thaspium*, *Zizia* and *Daucus* (Lindsey 1984, Lindsey and Bell 1985, Pimenov and Ostroumova, 1994, Lamborn and Ollerton 2000, Langenberger and Davis 2002a,b, Rovira et al. 2002, Wróblewska 1993, Mačukanović-Jocić et al. 2016). Besides phylogenetic studies (Pimenov and Ostroumova 1994, Ajani et al. 2008, Downie et al. 2010), scientific papers relating to other reproductive aspects of the *Leiotulus* species are rather rare. Although some palynological studies within the genus *Leiotulus* are modest, except on *L. secacul* (Mill.) Pimenov & Ostr., *L. kotschyi* (Boiss.) Pimenov & Ostr. (Van Zeist and Bottema 1977), the pollen features of *L. aureus* have been unexplored.

The present study aimed to provide palynomorphological features of the species that can be used for the taxon identification and clarification of higher level relationships within the family, as well as to contribute to melissopalyno-

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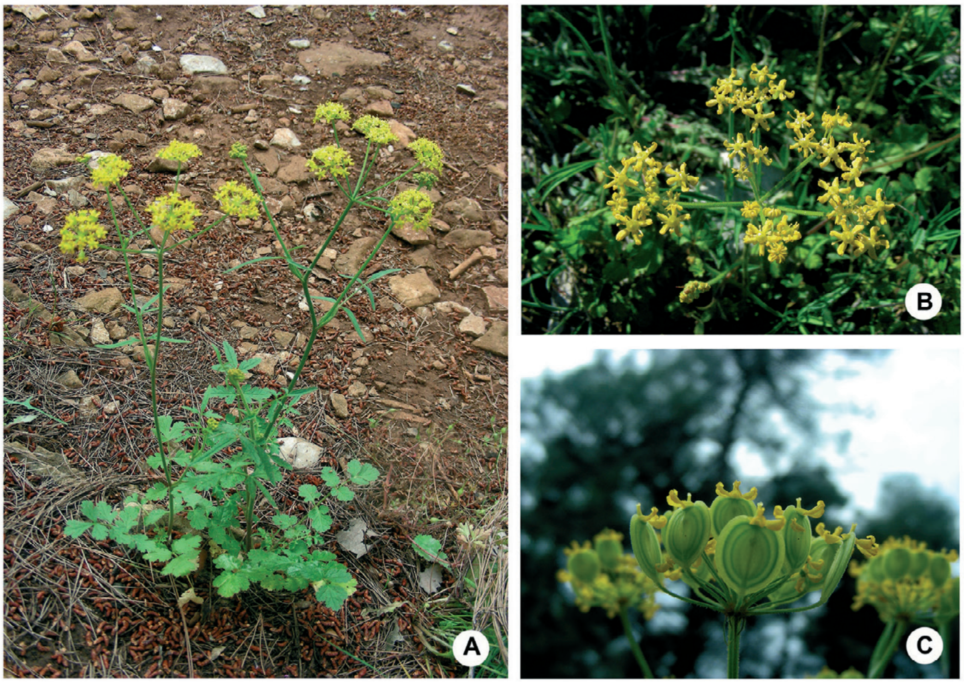

Fig. 1. Habitus of *Leiotulus aureus* (A), inflorescence close up (B), detail of immature fruits (C) (photo: Danijela Stešević).

logical studies and to the pollen atlas of the region. In addition, the study aimed to examine the attractiveness of this species to the honey bee, in terms of its contribution to honey bee pasture, by providing evidence about flower visitors.

Materials and methods

Study site

The research was focused on *L. aureus* plant population in the region of Gorica hill (Podgorica), Montenegro (N 42° 26′ 57′′ E 19° 16′ 2′′, elevation 114 m). The population inhabits xerophilous rocky pastures dominated by *Salvia officinalis* L. and *Stipa eriocaulis* Borbás, classified within NATURA 2000 as habitat type 62A0 – Eastern sub-mediterranean dry grasslands (*Scorzoneratalia villosae*). Using D.A.F.O.R. scale for species abundance presence (%): **D** = Dominant (51–100%), **A** = Abundant (31–50%), **F** = Frequent (16–30%), **O** = Occasional (6–15%), **R** = Rare (1–5%), *L. aureus* is observed as "Abundant" in a given area. Pollen collection and insect observations were conducted in 2017 during the blooming period in May. Three mounted and labeled plant voucher specimens (1500402, 1500403, 1500404) were processed and deposited in the herbarium collection of the Faculty of Natural Sciences and Mathematics, University of Montenegro (TGU). Digital photographs of each completed specimen and the accompanying data have also been provided.

Sampling and analysis of pollen

For scanning electron microscopy (SEM) and light microscopy (LM) analysis, the umbels (flowers) at full flowering stage were collected from 10 plants of wild populations.

For SEM study, the pollen grains from fully open flowers were mounted directly on the stub. Aiming to avoid any deformation of the pollen grains or any swelling attributed to solvents, preparation was carried out without the previous acetolysis method (Dustmann and von Der Ohe 1993). Samples were coated with gold (in BAL-TEC SCD 005 Sputter Coater, 100 seconds in 30 mA) and observed using a JEOL JSM – 6390 LV electron microscope at an acceleration voltage of 20 kV. Pollen grains were photographed in polar and equatorial view, and measurements were done on a sample of 50 or more grains for each morphological character. The following features describing pollen grains were examined: size, shape, ornamentation, apertures, polarity, symmetry, length of polar (P) and equatorial axis (E) (in SEM) and exine thickness (in LM). Description of pollen morphology was performed according to Punt et al. (2007) and Erdtman (1971).

For LM, the pollen grains were mounted on slides in a drop of saturated solution of fructose in water, observed without additional staining with a Leica DM2000 microscope equipped with a digital camera (Leica DFC320) and Leica IM1000 software.

Field monitoring and identification of insect visitors

Field observations were carried out during the peak flowering period of *L. aureus*, from 7 a.m. to 7 p.m., due to the absence of insect activity outside during the rest of the day. Furthermore, nocturnal insects or those active in the evening (such as sphingid moths) were not recorded. In addition to recording honey bee visits, imaging and sampling of all other flower visitors were performed. The insects were photographed with a Nikon Colorpix P500 digital camera. For the purpose of insect sampling, some insects were identified on sight, but for most species an accurate identification was made only after a specimen had been captured.

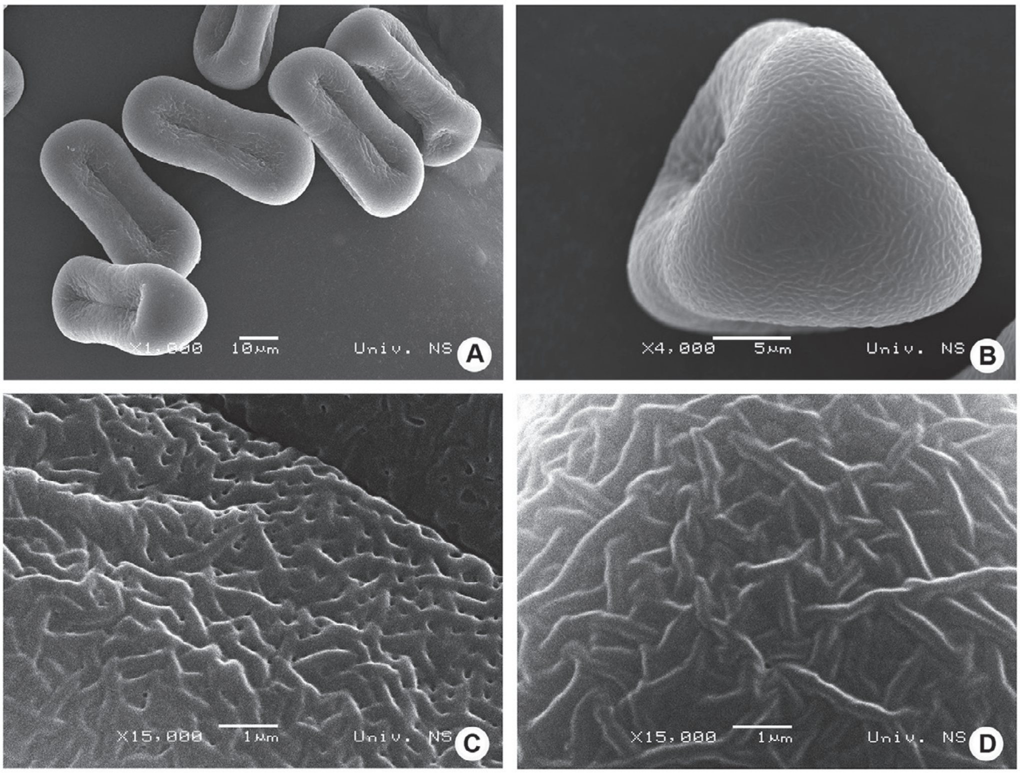

Fig. 2. Scanning electron microscopy of *Leiotulus aureus* (*Malabaila*–type) pollen: the perprolate tricolporate pollen grains are bone-shaped in equatorial (A) and triangular in polar view (B). A detail of exine surface showing rugulate – microperforate ornamentation (C, D).

Specimens were collected by entomological net and exhauster and stored for further determination in the laboratory by experts according to the relevant literature (Bouchard et al. 2011, Cassis and Schuh 2012, Lupoli 2017, Oosterbroek 2006, Sivell 2021, Van Veen 2004, Vazquez 2002).

In order to estimate the frequency of honey bee visits, three plants were randomly chosen and marked at the locality. For diurnal dynamics of frequency of visits, honey bees were monitored five times a day at three-hour intervals. Percentage of visited flowers per plant was calculated as total number of honey bee visits to each marked plant multiplied by the number of umbels visited by one honey bee, divided by the total number of open umbels per plant. The ratio of visited umbels per plant were calculated according to modified formula by Dafni et al. (1988). The following parameters were monitored in the field: A: The total number of bees visiting per marked plant in a time interval of 5 min, B: The number of umbels that each bee visited on the marked plant, C: The total number of umbels per plant. From these data, the percentage of umbels visited by honey bees was calculated according to the formula as follows: (AxB/C)x100. Also, the time each bee spent on the plant was measured.

Results

Pollen morphology

The pollen grains of *L. aureus* are isopolar, radially symmetrical and at the interface between medium-sized and large, according to Punt et al. (2007). The ratio between the

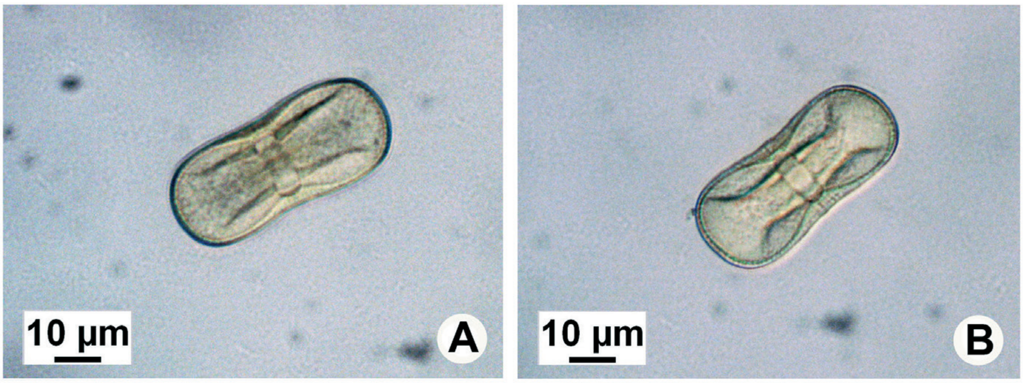

Fig. 3. Light microscopy micrographs of mesocolpial side of *Leiotulus aureus* (*Malabaila*–type) pollen grains showing clearly visible pores (A) and thicker exine (B) in the area of equatorial constriction.

polar axis length (P = 49.4 ± 2.1 μm) and the equatorial diameter (E = 19.5 ± 1.1 μm) amounts to 2.5 ± 0.2 indicating perprolate shape. The grains are slightly equatorially constricted with blunt polar ends (Figs. 2A, 3A, 2B), and in polar view they are triangular with interangular furrows (Fig. 2B). The grains are tricolporate with three straight sunken ectocolpi arranged regularly meridionally, of mean length 28.5 ± 2.2 μm, each with one endopore positioned in the indentations between the mesocolpial lobes (Figs. 2A, 3A, 2B). Mesocolpial width averaged 9.9 ± 1.6 μm. The sculpturing pattern, clearly visible in SEM is rugulate - microperforate (Fig. 2C). Exine is found to be, as observed in LM, 1.22 ± 0.25 μm thick at the poles and twice as thick in the equatorial region (2.59 ± 0.49 μm) (Fig. 3B).

Flower visitors

The golden yellow flowers of this species, arranged in terminally compound umbels, were visited by varyingly efficient pollinator insects. The results of the current study suggest that some floral features are attractive for different insect visitors belonging to four orders: Hymenoptera (*Apis mellifera* – Fig. 4A), Diptera (*Episyrphus balteatus* – Fig. 4B, *Scaeva pyrastri* – Fig. 4C, *Sphaerophoria scripta* – Fig. 4D, *Lucilia* sp. – Fig. 4E, *Sarcophaga* sp. – Fig. 4F, and *Bibio* sp. – Fig. 4G), Coleoptera (*Oedemera lurida* – Fig. 4H, *Mordella aculeata* – Fig. 4I, *Malachius bipustulatus* – Fig. 4J) and Hemiptera (*Closterotomus* sp. – Fig. 4K, *Graphosoma lineatum* – Fig. 4L). These insects are classified as primary and secondary pollinators and or accidental visitors.

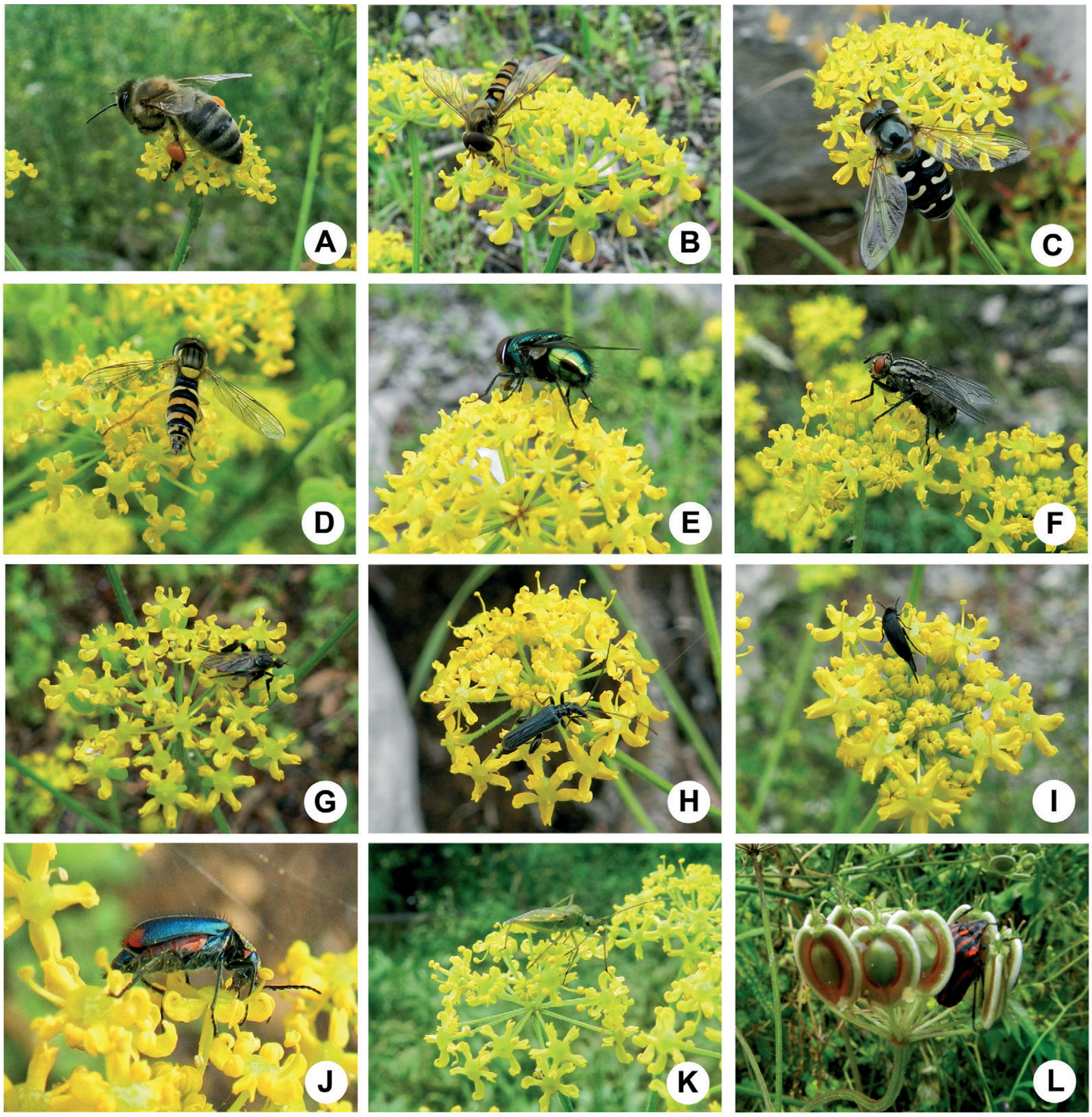

Fig. 4. The flower visitors of *Leiotulus aureus* during the observation period: fam. Apidae (*Apis mellifera* – A), fam. Syrphidae (*Episyrphus balteatus* – B, *Scaeva pyrastri* – C, *Sphaerophoria scripta* – D), fam. Calliphoridae (*Lucilia* sp. – E), fam. Sarcophagidae (*Sarcophaga* sp. – F), fam. Bibionidae (*Bibio* sp. – G), fam. Oedemeridae (*Oedemera lurida* – H), fam. Mordellidae (*Mordella aculeata* – I), fam. Melyridae (*Malachius bipustulatus* – J), fam. Miridae (*Closterotomus* sp. – K), fam. Pentatomidae (*Graphosoma lineatum* – L) among maturing fruits.

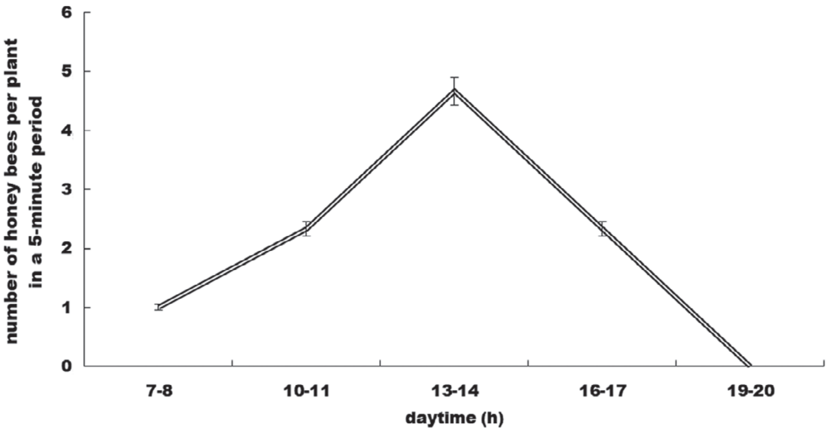

Fig. 5. Diurnal dynamics of honey bee visit frequency to *Leiotulus aureus.*

Regarding the observed flower visitors, pollination types are as follows: melittophily including pollination by honey bees, myophily and sapromyophily including specialized and non-specialized flies, and cantharophily (beetle pollination). Nevertheless, field observations have shown that the plant could be considered melliferous, as the umbels were gladly and frequently visited by honey bees.

During daytime, the average number of honey bees that visited each plant within a 5 minute interval was 2.0 ± 1.7. From 7 a.m. the frequency of honey bee visits increased reaching a maximum between 1–2 p.m., attaining 4.7 ± 1.5 bees per plant, followed by a decreasing tendency toward the evening and no visitors were recorded during the final observing period (7–8 p.m.) (Fig. 5). The average time a bee spent on the inflorescence amounted 6.06 ± 2.64 seconds (ranging from 3.57 ± 0.60 seconds at 7–8 a.m., to 9.47 ± 1.15 seconds at 1–2 p.m.).

The total number of umbels per visited plant averaged 6.3 ± 0.5 and average flower number per umbel amounted 34.4 ± 5.7. The percentage of visited umbels per plant during daytime had a pattern similar to that shown by the diurnal dynamics of honey-bee visiting frequency (Fig. 6), reaching a peak between 1 and 2 p.m., with decreasing tendency towards evening.

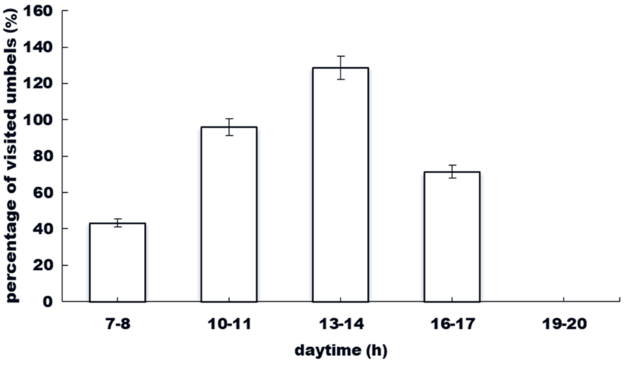

Fig. 6. Percentage of visited umbels per plant during daytime (refers to honey bees). In some periods of the day these values exceed 100%, since it can happen that the same bee during foraging will visit some umbels more than once.

Discussion

Pollen grains within the Apiaceae are usually stenopalynous, radially symmetrical, isopolar and prolate to perprolate in shape (Erdtman 1971). Grains are commonly tricolporate with slit-like ectocolpi and very distinctive and broadband-like costae (Punt 1984, Perveen and Qaiser 2006). The tectum is commonly striate-rugulate or simple striate (Punt 1984), psilate to granulate (Perveen and Qaiser 2006). There are a few different criteria accepted for classification of the pollen grains of the Apiaceae. Various characters have been used, such as polar/equatorial ratio (P/E), exine pattern, etc. For example, Cerceau-Larrival (1962) proposed 5 pollen types occurring within the family based on shape index: sub-rhomboidal (type 1, P/E:1-1.5), sub-circular (type 2, P/E:1-1.5), oval (type 3, P/E:1.5-2), sub-rectangular (type 4, P/E:2), and equatorially constricted (type 5, P/E:over 2). According to this classification, pollen grains of *L. aureus* in the current study should fit into type 5. In addition, according to Van Zeist and Bottema (1977) the pollen grains of the Apiaceae could be divided into 9 pollen types: *Anisosciadium, Bunium, Bupleurum, Eryngium, Ferula, Malabaila, Pimpinella, Sium erectum* and *Turgenia* types. The pollen features characterizing the *Malabaila* type are as follows: subrectangular to slightly oval shape with rounded poles, P/E is 2, fairly long colpi, transversal furrow oval-rectangular, distinct columellae 0.5 to 0.8 µm in diameter, slight variations in the wall thickness (exine up to 2 µm thick), grain size ranging from 35 to 40 µm. This type comprises *Malabaila secacul, M. kotschyi, Orlaya grandiflora, Heracleum maximum, H. lasiopetalum, H. persicum, Ormosciadium aucheri, Scandix iberica, Stenotaenia nudicaulis* and *Turgeniopsis foeniculacea.* Recently, *Orlaya* has already been described as having its own pollen type (Beug 2015), while *Malabaila secacul* and *M. kotschyi* were transferred to genus *Leiotulus* (as *L. secacul* (Mill.) Pimenov & Ostr and *L. kotschyi* (Boiss.) Pimenov & Ostr.)) (Pimenov and Ostroumova 1994, POWO 2022). The results of palynomorphplogical research of *L. aureus* (which previously also belonged to *Malabaila* genus) are not completely in accordance with characters described for the

Malabaila pollen type, such as P/E ratio and grain size, provided by Van Zeist and Bottema (1977). The pollen grains, described in the current paper, are slightly larger. It is known that the size may vary depending on the liquid medium used to mount samples on microslides for light microscopy (Faegri and Iversen 1989, Pospiech et al. 2021). However, the measurements of pollen size in the present paper are based on micrographs obtained by SEM, without using any mounting media which may cause enlargement of pollen grains. Extensive palynological research provided by Punt (1984), who recognized 50 types of pollen, as well as by Perveen and Qaiser (2006) who distinguished three pollen types based on tectum features within 27 Apiaceae genera, did not cover *Malabaila*/*Leiotulus* species.

The Apiaceae species have a uniform umbel and flower structure, however within the family diverse pollination systems occur varying from completely self-pollination to obligate cross-pollination (Koul et al. 1993). Flowers of umbellifers attract numerous insect taxa from several taxonomic orders supplying them with pollen and nectar (Niemirski and Zych 2011). Hence, umbellifers are often connected with generalized pollination system, indicating that their floral nectar is easily accessible to flower-visiting insects of different pollination efficiency due to the various degree of mouthpart modification for feeding on nectar, pollen or petals (Proctor et al. 1996, Olesen et al. 2007). These include mostly flies, but also hymenopterans and coleopterans (Proctor et al. 1996, Lamborn and Ollerton 2000, Zych 2007, Davila and Wardle 2008, Carvalheiro et al. 2008, Mačukanović-Jocić et al. 2016). In the current study, flowers of *L. aureus*, representing an open dish-shaped blossom type, were visited by twelve insect species from four taxonomic orders. With regard to the observed flower visitors, the following pollination types occurred: melittophily, myophily, sapromyophily and cantharophily. Dipterans were the most frequent group of visitors. Most of the recorded dipterans were of the "hoverfly type" (i.e. members of the family Syrphidae) and the "muscoid type" (i.e. members of the families Sarcophagidae (flesh flies) and Calliphoridae (blow flies)). Although not highly specialized in pollination, flower-pollinating species have been reported in the Bibionidae in previous research (Goldblatt et al. 2005). Adults of march flies (Bibionidae) are known as pollen-collecting and nectar feeding flower visitors pollinating fruit trees and some other crops (Freeman and Lane 1985, Woodcock et al. 2014)**.** In the present study carrion flies were found to be less important from the pollination point of view, since *Leiotulus* is an aromatic plant more attractive to Apidae and Syrphidae. Despite a few Coleopteran species observed in the current and similar studies on umbellifers (Zych 2006), they are of minor importance as they are not considered to be efficient pollinators (Mačukanović-Jocić 2010). Hemipterans observed in this study could not be considered as pollinators, since their pollination activity is negligible, except in rare cases (Ishida et al. 2009). Unlike previous research on some other Apiaceae species (Zych 2006, Mačukanović-Jocić et al. 2016), in the present study neither moths nor butterflies were observed visiting *L. aureus* umbels. Low visitation rate of lepidopterans can be explained by the length of their proboscis, which is more adapted to tubular corollas, or by weaker attractiveness of flowers which can be attributed to the scent or floral nectar components. *L. aureus* could be considered an ecologically generalized species in terms of the need for specialized pollinators. There are many reasons for this. Although *L. aureus* like many other umbellifers lack any visible or invisible floral signs such as honey guides leading to nectar, the flowers are slightly aromatic and could emit some chemical signals that attract specific groups of pollinators, as previously reported for this family (Tollsten et al. 1994, Tollsten and Øvstedal 1994, Niemirski and Zych 2011). Regardless of their floral uniformity, some umbellifers are suggested to exhibit cryptic flower specialization enabling oligolectic relationships with bee pollinators (Lindsey 1984, Lindsey and Bell 1985, Niemirski and Zych 2011). Unlike Zych (2006) who did not observe any honey bee on *Heracleum sphondylium*, *L. aureus* flowers were very frequently visited, which is in line with the findings of other authors who pointed out the importance of honey bees in pollinating umbellifers (Langenberger and Davis 2002b, Davila and Wardle 2002). Following the diurnal dynamics of honey bee visits, plants were the most frequently visited about midday. Considering the frequency of honey bee visits *L. aureus* could be regarded as a melliferous plant contributing to honey bee pasture. However, regarding its rather "unspecialized" floral morphology and its being visited by numerous species of flower visitors, this plant species is without ecological specialization to particular insect species.

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Effect of excess boron on growth, membrane stability, and functional groups of tomato seedlings

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Abstract - With the scarcity of good quality water, plants like tomatoes will be more susceptible to excess boron (EB) in Mediterranean regions. The effects of EB on the growth, free, semi-bound, and bound boron (B) concentrations, and macromolecules of the *Solanum lycopersicum* L. cultivar Castle Rock, were investigated in this study. Seedlings were exposed to four levels of EB using boric acid. The results showed that EB inhibited tomato growth, total water content, and photosynthetic pigments. EB harmed membrane stability, as seen by increased potassium (K) leakage, UV absorbance metabolites, and electrolyte conductivity (EC) in leaf disc solution. EB raised concentrations of free, semi-bound, and bound forms of B in seedlings. Fourier-transform infrared spectroscopy (FTIR) data revealed that EB induced uneven wax deposition, altered the shape of cell walls, and lowered cellulose synthesis in seedlings. EB affected the amide I and amide II proteins indicating damage to the protein pools. These results provide new insights into understanding the specific effects of EB on the functional groups of different macromolecules of tomato seedlings.

Keywords: excess boron, FTIR analysis, membrane stability, photosynthetic pigments, plant growth, tomato

Introduction

Boron (B) performs vital tasks in plant life at ideal levels, whereas excess boron (EB) has negative consequences. The difference in B insufficiency and toxicity levels is minimal (Fang et al. 2016). B-rich soils can be found all over the world and are prevalent in arid and semi-arid areas (Ardıc et al. 2009). With reduced precipitation in the Mediterranean area (Cervilla et al. 2012) and irrigation water shortage due to new dams, demand for desalinated water for agriculture is expected to rise, potentially raising the level of B in irrigation water. Moreover, rising sea levels (Mediterranean Sea) can pollute groundwater, resulting in higher B levels in irrigation water (Princi et al. 2016). In Egypt the cultivated area is suitable for intensive cultivation and this, along with anthropogenic activity, may lead to B contamination (Elbehiry et al. 2017). EB produces different physiological and morphological changes in plants, resulting in decreased plant growth, leaf chlorophyll, membrane stability (El-Shazoly et al. 2019), and ultimately reduced production (Metwally et al. 2018).

Tomatoes are grown all over the Mediterranean region, where there is a disturbance with EB in the soil. Tomatoes are among the most important vegetable crops in Egypt throughout the year, with a total production of 6,729,004 tons and a total cultivation area of 166,206 hectares (FAOSTAT 2017). EB has led to alterations in tomatoes, including biomass, membranes, photosynthetic pigments, phenolic compounds, and antioxidant enzymes (Cervilla et al. 2012, Farghaly et al. 2022b), leading to reduced yields (Kaya et al. 2009).

The advantage of Fourier-transform infrared spectroscopy (FTIR) is its ability to produce spectra on different samples such as powders and liquids with minimal sample preparation, which reduces analysis time (Canteri et al. 2019). FTIR is an appropriate analytical tool for biological macromolecules, assessing the composition of organic components (Wu et al. 2017). Absorption outlines show fixed peaks area that identifies modest modifications of metabolites related to physiological processes after infrared spectra (400–4000 cm^{-1}) pass through plant samples (Renuka et al. 2016). The peak areas, positions, and bandwidth values are critical to changes in plant macromolecules (Renuka et al. 2016). However, to our knowledge, there are no reports on the use of this technique to assess physiological changes produced by excess boron on tomato seedlings.

In this study, we aimed to focus on how EB treatments alter tomato macromolecules, assessing the composition of

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organic components using FTIR analysis. Additionally, we measured the growth, photosynthetic pigments, membrane stability, and B forms concentration in tomato seedlings. The findings reveal a fresh understanding of the various structural responses of tomato seedlings when exposed to EB.

Materials and methods

Growth conditions

Vegetables Department, Faculty of Agriculture, Assiut University gave seeds of *Solanum lycopersicum* L. (tomato), cultivar Castle Rock. Under a laminar airflow hood, seeds were surface sterilized for 15 minutes with a 5% NaClO solution and rinsed four times with sterile water. We wanted to achieve data without any extraneous influences and repeat the experiment under the same settings, so we did it *in vitro*. Sterilized seeds were grown on sterile, half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962). The medium was supplemented with 2.2 g L^{-1} MS, 3% sucrose, various concentrations (0, 2, 4, and 6 mM) of H_3BO_3, and 0.3% gelrite added after adjusting the pH to 5.7. The medium was sterilized for 15 min at 121 °C, pressed at 105 kPa, and allowed to cool to room temperature. Seedlings were cultured in a growth chamber at 25 ± 1 °C, 65–70% relative humidity, and a photoperiod of 16/8 h with 30 $\mu M\ m^{-2}\ S^{-1}$ illumination.

After 20 days, some seedlings were separated into shoots and roots, weighed quickly to estimate the fresh weight (FW), and stored at –80 °C. Other seedlings were oven-dried at 60 °C to determine the dry weight (DW). The total water content (TWC) of shoots and roots was determined using the following formula:

$$\mathrm{TWC} = \mathrm{FW} - \mathrm{DW}$$

Photosynthetic pigments

Using a spectroscopic approach, photosynthetic pigments, including chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), and carotenoids (cars), were determined (Lichtenthaler 1987). 0.1 g of a fresh leaf was dropped in 5 mL of 95% ethanol at 60 °C until colorless, and the volume was then finished to 10 mL using 95% ethanol. Using a spectrophotometer (Unico UV-2100), the concentrations of carotenoids and chlorophylls were determined using formulas:

$$\text{chl } a = (13.36 \times A_{663}) - (5.19 \times A_{644})$$

$$\text{chl } b = (27.49 \times A_{644}) - (8.12 \times A_{663})$$

$$\text{cars} = \{(1000 \times A_{452}) - (2.13 \times \text{chl } a) - (9.76 \times \text{chl } b)\}/209.$$

Results were expressed as mg g^{-1} FW.

Cell membrane stability

Different parameters were assessed, including electrical conductivity (EC%), potassium leakage (K leakage), and UV-absorbing metabolites (metabolite leakage) for determining the cell membrane stability.

The percentage of injury (electrical conductivity; EC) was measured according to the Premachandra et al. (1992) method. Fresh leaf discs (2.1 cm) were soaked in 10 mL of distilled water for 24 h at 10 °C. After measuring the initial electrical conductivity (EC_1) of all test tubes at 25 °C, the leaf discs were autoclaved for 15 min, cooled to 25 °C, and then the last EC_2 was measured again. The cell membrane stability index was estimated using a percentage of damage:

$$\text{Electrical conductivity (\%)} = (EC_1/EC_2) \times 100$$

Potassium leakage was measured using a flame photometer in the same conductivity solution before and after sterilization (Williams and Twine 1960).

Metabolite leakage was assessed using the Navari-lzzo et al. (1989) method in the same solution of conductivity measurements.

Boron analysis

Boron forms were extracted, according to Du et al. (2002) and Li et al. (2017). 5 mL of distilled water was added to 0.2 g of powdered dry seedlings, shaken at 25 °C for 24 h, filtered, and the free B was measured. The residue was shaken at 25 °C for 24 h in a plastic tube with 1 M NaCl, filtered, and then semi-bound B was quantified in the filtrate. Finally, the residue was shaken at 25 °C for 24 hours in a plastic tube with HCl (1 M), filtered, and then the bound B was quantified in the filtrate.

According to Mohan and Jones (2018), B concentration was quantified using the curcumin-acetic acid method and detected at 550 nm. The curcumin-acetic acid (1 mL) solution was added to 1 mL of filtrates and 0.25 mL of concentrated H_2SO_4, shaken for 30 min, and diluted with 95% ethanol to 5 mL after 30 min read at 550 nm using H_3BO_3 as a reference.

Fourier-transform infrared spectroscopy (FTIR) analysis

To analyze macromolecular alteration, we employed Fourier-transform infrared spectroscopy (Nicolet IS 10 FTIR) in Chemistry Department. A translucent, homogeneous tablet was prepared by a tablet-making machine using a little amount of the finely powdered sample (approximately 100 µg) mixed with KBr (1: 100 p/p). The absorbance of spectra was measured (400–4000 cm^{-1}) against an ordinary KBr pellet (blank), then the resolution was 4 cm^{-1}. The functional groups of the sample were determined by comparison of the spectroscopic result with a reference.

Statistical analysis

The studies (25 jars/each treatment) were repeated at least twice, with the findings being an average ± standard deviation (SD) of four biological replicates. Charts were generated by Origin 8.6 and Microsoft Excel 2010. Using SPSS

Statistical Package 22.0, a one-way analysis of variance test was performed and followed by a Tukey's test for significant differences ($P \leq 0.05$) to compare the means. The correlation between the mean values of different parameters of tomatoes under EB treatments was determined using Pearson's correlation analysis.

Results

Growth

EB affected all growth parameters of the tested tomato seedlings, including FW, DW, and TWC of shoots and roots (Figs. 1A-D, On-line Suppl. Fig. 1). Compared to control, tomatoes treated with 2 mM B showed a slight or considerable increase in FW, DW, and TWC of shoots and roots. In contrast, treatments with 4 and 6 mM B lowered FW, DW, and TWC of shoots and roots. After exposure to 6 mM B, the highest DW reductions of 56.90% and 86.52% were recorded in shoots and roots, respectively. Further, treatment with EB showed a significant negative association between shoots and roots, FW, DW, TWC, and an increase in free, semi, and bound B contents. However, the shoot/root ratio was positively and strongly associated with free, semi-bound, and bound B concentrations in the shoots (0.907**, 0.922**, and 0.646*, respectively, On-line Suppl. Tab. 1 and Tab. 2). Although the correlations between bound B and growth parameters were significant, they were the weakest of all the growth criteria associations.

Photosynthetic pigments

EB had varied effects on the contents of chl *a*, *b*, *a*+*b*, and cars pigments in leaves (Fig. 2A). Compared to control, EB at a low-level (2 mM) stimulated chl *a* content in leaves by 31.28%, but at a high-level (6 mM), it significantly reduced it by 48.34%. In the shoots, there were strong negative associations between chl *a* and free, semi-bound, and bound B content (–0.768**, –0.822**, and –0.812**, respectively, On-line Suppl. Tab. 1).

Low EB treatments promoted the synthesis of chl *b* in tomato leaves. Compared to control, the rise in chlorophyll *b* content at low EB levels (2 and 4 mM) was considerable (129.45% and 66.23%, respectively), but there was no significant increase at a high EB level (6 mM). Insignificant relationships between chl *b* and free, semi-bound, and bound B levels in shoots confirmed these findings (–0.224, –0.311, and –0.341, respectively, On-line Suppl. Tab. 1).

6 mM B reduced chl *a* + *b* concentration by 42.69% compared to control. The moderate treatment (4 mM B) showed a lower increase in the chl *a* + *b* content (0.63%) than exposure to 2 mM B, which resulted in a 40.66% rise compared to control. Moreover, results revealed a strong negative association between chl *a* + *b* and free, semi-bound, and bound B content in shoots (–0.704*, –0.767**, and –0.675**, respectively, On-line Suppl. Tab. 1).

Regarding carotenoids, EB boosted cars content by 37.18% at a low-level (2 mM) but lowered it by 44.72% at a

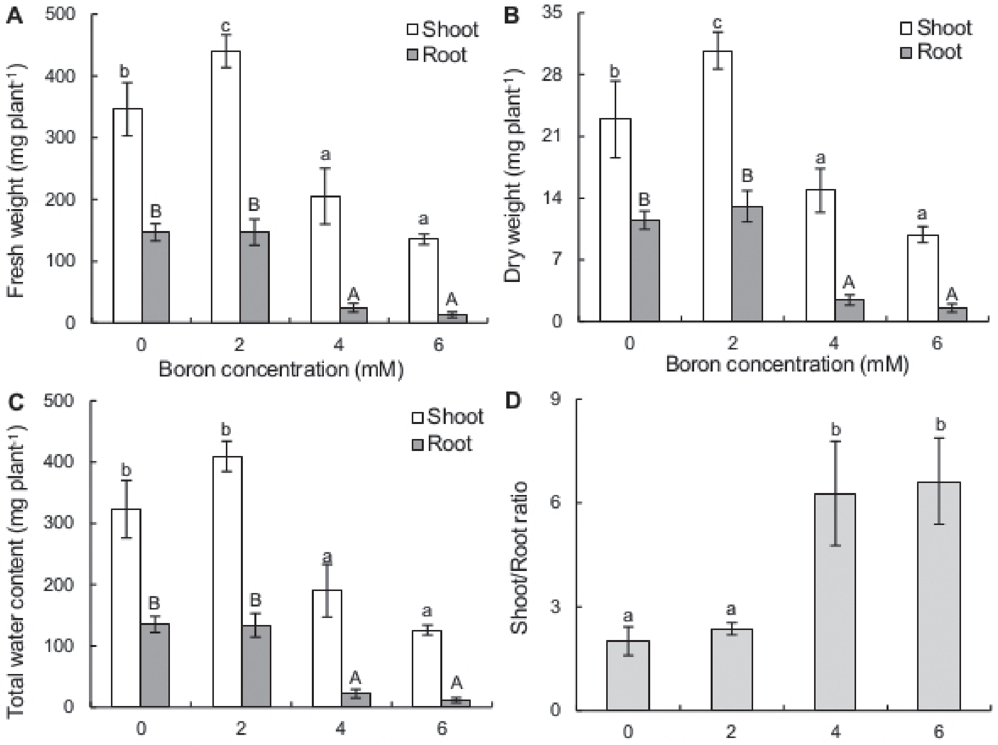

Fig. 1. Fresh (A), dry weight (B), total water content (C), and shoot/root ratio (D) in tomato seedlings grown under 0, 2, 4, and 6 mM boron for 20 days. The data are means ± SD (n = 4). Different letters, capital for roots and small for shoots, indicate statistically significant differences ($P \leq 0.05$).

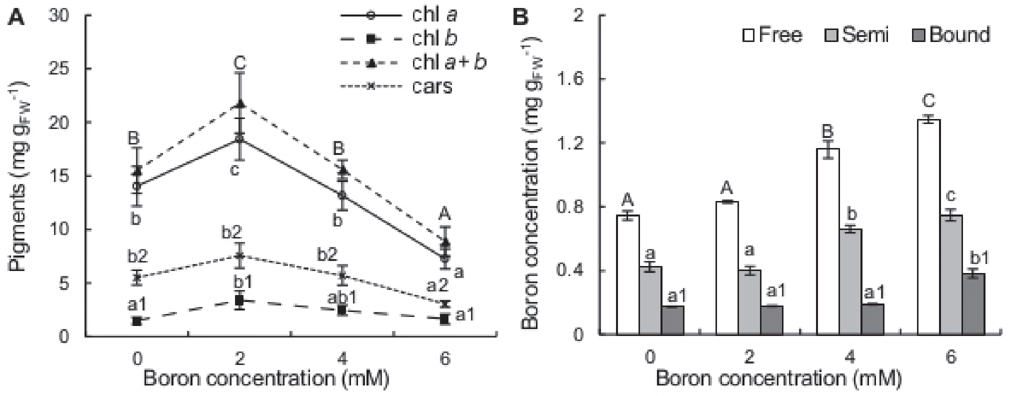

Fig. 2. Photosynthetic pigments (chl *a*; chl *b*; chl *a* + *b*; cars; A) and boron concentration (free, semi-bound; and bound B) in tomato seedlings grown under 0, 2, 4, and 6 mM boron for 20 days. The data are means ± SD (n = 4). Different letters, capital for chl *a*, free B and small for chl *b*, semi-bound B, small^1 for chl *a* + *b*, bound B, small^2 for cars, indicate statistically significant differences (P ≤ 0.05).

high level (6 mM) compared to control. Carotenoids and free, semi-bound, and bound B levels had negative associations (–0.655*, –0.593*, and –0.686*, respectively), like chlorophylls (On-line Suppl. Tab. 1). Moreover, the data revealed a significant and positive relationship between chl *a*, *a* + *b*, cars, and shoot DW (0.669*, 0.734**, and 0.785**, respectively), except for chl *b*, which was not.

Boron concentrations

The most important factor in measuring a plant's tolerance to EB is the B concentration in its tissues. Therefore, the B forms in tomatoes grown under various EB treatments were measured (Fig. 2B). Our results indicated that free B content was higher than the content of semi-bound and bound B content in seedlings. Our results indicated that free B content was higher than semi-bound and bound B content in seedlings. With increasing EB concentrations, the accumulation of all B forms also increased. Compared with optimal B concentration, EB at the low level (2 mM) increased free, semi-, and bound B by 25.41%, 37.40%, and 88.61%, while the high level (6 mM) increased it by 149.69%, 134.98%, and 367.93%, respectively.

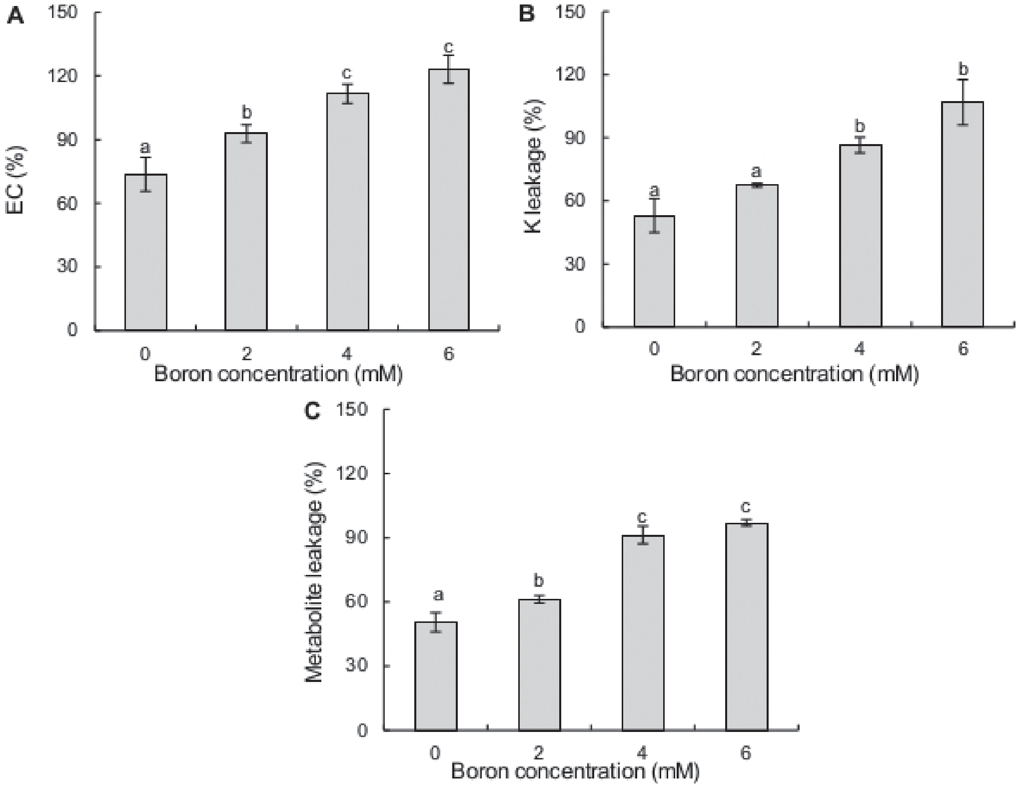

Fig. 3. Electrical conductivity (EC; A), potassium leakage (K leakage; B), and UV absorbing metabolites (metabolite leakage; C) in tomato seedlings grown under 0, 2, 4, and 6 mM boron for 20 days. The data are means ± SD (n = 4). Different letters indicate statistically significant differences (P ≤ 0.05).

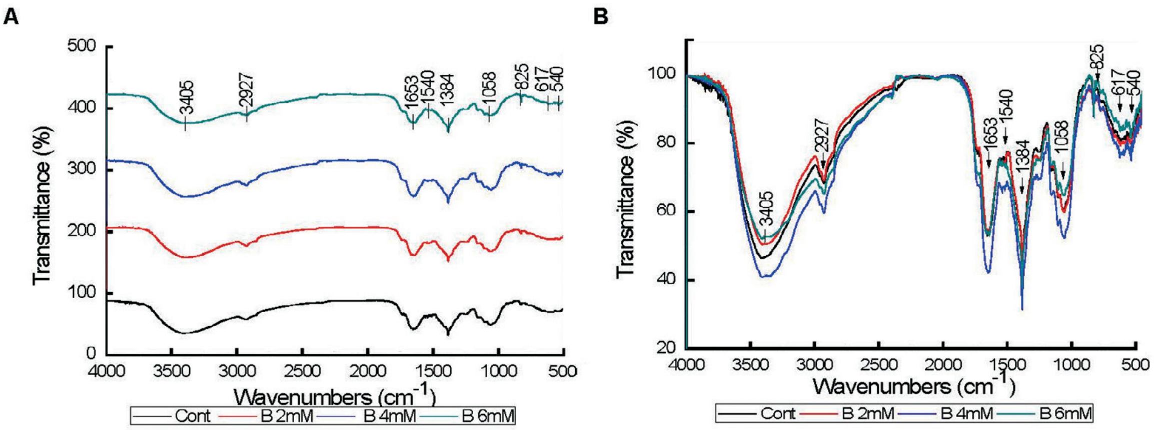

Fig. 4. Fourier-transform infrared spectroscopy (FTIR) spectra (range 4000–400 cm^{-1}; A, and 0–500 cm^{-1} region expanded; B) of tomato seedlings grown under 0, 2, 4, and 6 mM boron for 20 days.

Membrane stability

To quantify the degree of membrane integrity under EB stress, the EC, K leakage, and metabolite leakages in seedlings undergoing various treatments were measured (Figs. 3A-C and On-line Suppl. Tab. 1 and Tab. 2). 6 mM B raised the EC and incidence of K and UV metabolites in leaves by 67.22%, 101.54%, and 91.99%, respectively. Furthermore, EC (0.931**, 890**, and 0.724**, respectively), K (0.943**, 0.915**, and 0.828**, respectively), and metabolite leakages (0.971**, 0.937**, and 0.687*, respectively) were shown to be strongly connected with free, semi-bound, and bound B levels in shoots.

FTIR analysis

We employed FTIR analysis to assess the effect of EB on seedling ultrastructure (Fig. 4 and Tab. 1). EB did not induce extensive alterations within the four peaks at 3405.17 cm^{-1}, 2927.25 cm^{-1}, 1384.45 cm^{-1}, and 825.42 cm^{-1}. Treatment with 4 mM B raised the peak intensity of 3405.17 cm^{-1}, but exposure to 2 and 6 mM B lowered it compared to control. Moreover, treatments with 4 and 6 mM B raised the peak intensity at 2927.25 cm^{-1} and 1384.45 cm^{-1}, respectively, while exposure to 2 mM B lowered them compared to control. However, EB levels raised the peak intensity of 825.45 cm^{-1} relative to control.

Regarding the peak at 1653.21 cm^{-1} (control), 2 mM B treatment did not significantly alter its transmission, while 4 and 6 mM concentrations lowered it by –6.34 cm^{-1} and –12.48 cm^{-1}, respectively. Moreover, 4 mM B raised this peak intensity, although other B treatments did not. Meanwhile, the peak at 1540.60 cm^{-1} (control) disappeared under B treatments (2, 4, and 6 mM).

The peak recorded at 1058.10 cm^{-1} (control) was negatively shifted by –3.20 cm^{-1}, –4.27 cm^{-1}, and –4.18 cm^{-1} upon exposure to 2, 4, and 6 mM B, respectively. Moreover, treatments with 2 mM and 4 mM B stimulated this peak intensity, but treatment with 6 mM EB reduced it.

Tab. 1. Fourier-transform infrared spectroscopy (FTIR) spectra showing observed peaks in tomato seedlings grown under 0, 2, 4, and 6 mM boron for 20 days.

	Frequency (cm^{-1})			
	Excess boron (mM)			
Peak	0	2	4	6
1	3405.17	3404.98	3405.43	3406.01
2	2927.25	2927.25	2926.60	2926.75
3	1653.21	1656.79	1646.87	1640.73
4	1540.60	–	–	–
5	1384.45	1384.28	1384.34	1384.30
6	1058.10	1054.9	1053.83	1053.92
7	825.42	825.55	825.68	825.42
8	617.04	618.08	622.35	620.79
9	–	540.52	536.78	537.63
10	483.52	484.26	–	–
11	459.94	464.20	–	–
12	–	453.29	–	453.74

Compared to the peak recorded at 617.04 cm^{-1} (control), EB treatments increased from the transmission area by 1.04 cm^{-1}, 5.31 cm^{-1}, and 3.75 cm^{-1} at 2, 4 and 6 mM concentrations, respectively. Furthermore, low levels of EB (2 and 4 mM) increased the peak intensity, while the treatment with 6 mM B decreased it.

The peaks recorded at 540.52 cm^{-1}, 536.78 cm^{-1}, and 537.63 cm^{-1} appeared under treatments with 2, 4, and 6 mM B, respectively. Under the high levels of EB (4 and 6 mM), the 483.52 cm^{-1} and 459.94 cm^{-1} peaks disappeared, while the exposure to 2 mM B stimulated their values by 0.74 cm^{-1} and 4.26 cm^{-1}, respectively.

Discussion

The shoot/root ratio of seedlings was enhanced at the lowest EB concentration, indicating the higher growth (Figs. 1A-D). Moreover, lower growth at high levels of EB was linked to the concentration of B forms within the seedlings, demonstrating that B buildup was limiting growth. As demonstrated by the strong positive relationships between shoot/root ratio and B level within shoots, the negative B effect was more evident in tomato roots than in shoots. EB has a comparable negative effect on tomato growth, according to Kaya et al. (2020). Cell division (Liu and Yang 2000), cell expansion, cell numbers (Choi et al. 2007), water content (Metwally et al. 2018), and cell wall matrix stiffness (Farghaly et al. 2022b) are all linked to decreased seedling growth. Conversely, promoting growth at a low EB level may be associated with active B influx, which lowers intracellular B levels (Reid et al. 2004, Ardic et al. 2009).

The primary organelles damaged by EB are the chloroplasts (Landi et al. 2019). The deficiency of photosynthetic pigments found in this study (Fig. 2A) could be due to a structural damage to thylakoids as a result of abnormal spongy parenchyma growth (Papadakis et al. 2004), oxidation of chlorophyll and chloroplast membranes (Aftab et al. 2012), and a reduction in three types of thylakoid-related proteins (Sang et al. 2015). Our results match the findings of wheat and tomato, which are vulnerable to EB (El-Shazoly et al. 2019, Kaya et al. 2020). Thus, EB has a variety of consequences on photosynthetic processes, including changes in photosynthetic pigment levels, lower CO_2 assimilation, impaired photosystem II performance, and a decreased electron transport rate (Landi et al. 2019).

High content of photosynthetic pigments at a low EB level suggests seedling tolerance. Furthermore, the chloroplasts were less vulnerable to EB since the DW was high at this level of 2 mM EB. Additionally, strong positive relationships between pigment contents and shoot DW revealed that pigment preservation is necessary to stimulate seedling growth. Accordingly, Eraslan et al. (2007) found no significant changes in chl *a* and *b* concentration in carrot plants when exposed to EB.

Boron amount in plants is considered the main physiological feature utilized to examine tolerance to EB in an environment. Our findings revealed that all B forms were significantly increased in EB-stressed seedlings, explaining the symptoms of increased EB (Fig. 2B). Likewise, during exposure to EB, an accumulation of B forms was previously observed in tomato calli (Farghaly et al. 2021, 2022b). Free B demonstrated the ability to cross cell membranes and showed promise as being immediately accessible for potential physiological roles in the cell, according to Dannel et al. (1998). In this study, the content of semi-bound and bound B forms varied from about 6%–76% and 2%–119%, respectively. These data may reveal that a small amount of EB was attached to the cell walls in exchange for increased B availability, but this amount was too low to actively participate in EB detoxification, as indicated by increased free B (Dannel et al. 1998, Farghaly et al. 2022b).

Ionic solutes and cellular metabolites are widely applied to assess membrane integrity (Palta et al. 1977, Navari-Izzo et al. 1993). According to our findings presented in Figs. 3A-C, the membrane damage was more severe as EB levels in the medium increased. These findings showed that EB had a significant impact on the permeability of tomato membranes, revealed by the EC and leakage of K and UV metabolites, which were all confirmed in a prior work with wheat (Metwally et al. 2012).

FTIR spectra revealed further information about the influence of EB on seedling macromolecules (Fig. 4 and Tab. 1). Türker-Kaya and Huck (2017) correlate the first peak, recorded at 3405.17 cm^{-1} in control, with O-H and N-H related to alcohol, carbohydrates, phenols, and proteins. EB did not affect the wavenumber, indicating that the lack of alterations in cell wall components and the reduction in bound-B in seedlings may clarify these findings. Furthermore, EB lowered peak intensity, suggesting that EB may change the pattern in binding between alcohols, carbohydrates, proteins, phenols, and components of walls. Riaz et al. (2021) demonstrated that EB increased lignin and suberin levels in rice plants, perhaps leading to cell wall stiffness. Otherwise, changes in peak intensity can be referred to as changes in cell wall shape (Zuverza-Mena et al. 2016).

The peak found around 3000–2800 cm^{-1} was assigned the C-H stretching area of lipids, wax, and fats (Legner et al. 2018). EB did not affect this peak's value (3000–2800 cm^{-1}), but the intensity of the peak increased at 4 and 6 mM EB. These data indicate that no changes were made to wall wax amount, while the shape of wall wax may only change under EB (Morales et al. 2013). Mesquita et al. (2016) found irregular wax deposition on the surface of citrus leaves under EB, which might support our findings.

The peak in the region of 1700–1600 cm^{-1} is characteristic of the C=O of the amide I (proteins) (Dumas and Miller 2003). The amide II peak in the region of 1480–1580 cm^{-1} is a mixture of N-H and C-N vibrations that aid in ionic reaction response, although it is less well understood (Zhao and Wang 2016). The amide I peak value was reduced by high levels of EB (4 and 6 mM), demonstrating that the protein's structure is changed to chelate EB, and this explanation might be confirmed by the finding of Farghaly et al. (2022a). According to Riaz et al. (2021), EB significantly affected the amide protein, amide II, and amide III, indicating damage to the protein pools. The disappearance of the amide II peak under EB treatments can disclose the binding of B ions to nitrogen amide to chelate the EB, and plants can use this claw to withstand B toxicity. Dunbar et al. (2012) reported the disappearance of amide II (around 1550 cm^{-1}; bending of amide N-H) owing to iminol structural coordination between the amide II and magnesium, nickel, and cobalt.

Wei et al. (2015) assigned the peaks between 1500–1000 cm^{-1} fingerprint regions of the amide III, nucleic acid functional groups, and carbohydrates. Our results revealed that EB treatments induce a change in the intensity of the peak recorded at 1384.45 cm^{-1}. The increasing peak intensity might reveal that the additional B has changed the fingerprint region's components and linked EB with proteins.

EB reduced the 1058.10 cm^{-1} peak, which was attributed to cellulose (Wu et al. 2017), indicating a reduction in cellulose production in seedlings under EB. This peak intensity was lowered by EB at its maximum level, indicating a decrease in cellulose synthesis. Similarly, Farghaly et al. (2022b) discovered that EB decreased the cellulose content of tomato calli in their study.

The peak recorded at 825.42 cm^{-1} in control, which was not affected by EB treatments, was assigned to the trisaccharide (D-(+)-raffinose pentahydrate) with α-glycosidic bonds (Wiercigroch et al. 2017). The intensity of this peak was increased, indicating B binding to the pentahydrate. EB also boosted the 617.04 cm^{-1} peak, which was assigned to D-(+)-glucose (Wiercigroch et al. 2017), showing the degradation of cellulose or sucrose into simple monosaccharides. This explanation might confirm a decrease in the cellulose wavelengths. Under EB, the appearance of additional peaks, recorded at 540.52 cm^{-1}, 536.78 cm^{-1}, and 537.63 cm^{-1}, may also demonstrate glucose buildup (Farghaly et al. 2022b). Furthermore, at high EB levels, the disappearance of ribose peaks (484 cm^{-1} and 460 cm^{-1}; Wiercigroch et al. 2017) suggested EB binding to ribose, and this confirmed the ability of EB to stabilize ribose to create a nucleotide of a borate ester (Grew et al. 2011, Scorei 2012).

In conclusion, EB treatments exhibited unfavorable influences on FW, DW, TWC, and photosynthetic pigments of tomato seedlings. EB also caused a reduction in membrane integrity, as seen by higher EC, and K and UV-metabolite leakage. B absorption matched the B content in the nutritional medium, resulting in increased accumulation of various B forms in seedlings. EB inhibited cellulose synthesis in seedlings and altered wax deposition in cell walls. Moreover, EB affected the amide I and amide II indicating damage to the protein pools. Finally, our results reveal that decreased tomato growth under EB might be related to alterations in photosynthetic pigments, membrane stability, and macromolecules.

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Acer velutinum Bioss. (velvet maple) seedlings are more tolerant to water deficit than *Alnus subcordata* C.A. Mey. (Caucasian alder) seedlings

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Abstract – Drought stress is a major environmental factor limiting plant growth. Selection of drought-tolerant plants is of critical importance in vegetation restoration and forestation programs. *Alnus subcordata* and *Acer velutinum* are two valuable, dominant, and endemic species in the Hyrcanian forests. There are fast-growing species and significant diffuse-porous hardwood in afforestation and reforestation. One-year old seedlings of both species were exposed to four water shortage treatments (100, 75, 50 and 25% of field capacity (FC) chosen as control, mild, moderate, and severe) for 12 weeks. Thereafter, their morphological characteristics such as height and basal area, total and organ biomass (root, stem, and leaf), leaf area (LA), specific leaf area (SLA), leaf area ratio (LAR), as well as physiological and biochemical characteristics such as relative water content (RWC), content of chlorophyll, free proline and malondialdehyde (MDA), and superoxide dismutase (SOD) and peroxidase (POD) activity were measured. The results showed that when exposed to reduced water availability, plant height, basal diameter, total and organ biomass, LA, LAR, RWC and chlorophyll content decreased, but their proline concentration, MDA content, SOD, and POD activity increased in both species. The root to shoot ratio (R/S) and root mass ratio (RMR) increased at 50 and 25% FC treatments in *A. subcordata*, whereas no significant difference was found in *A. velutinum* under drought treatments. SLA increased significantly at 50% FC in *A. velutinum* and decreased in *A. subcordata* under drought treatments compared to control treatment. *A. velutinum* showed more proline content, RWC, POD, and lower increase in MDA content than *A. subcordata* under moderate treatment. Therefore, *A. velutinum* appears to possess a better mechanism to cope with drought stress. The drought tolerance of *A. velutinum* may enhance its potential for climatic adaptations under drier conditions with the ongoing climatic change.

Keywords: *Alnus subcordata*, *Acer velutinum,* antioxidant enzymes, biomass, growth, water deficit

Introduction

The impacts of climate change on vegetation will appear as a combination of stress factors, including high temperatures, reduction of rainfall, and alterations in wildfire regimes. The principal aspect of global climate change, the frequency, and intensity of drought stress will increase in the future (Wu et al. 2017). Drought can damage afforestation and reforestation programs because seedlings are more prone to drought than mature trees. Drought-tolerant species should be considered so as to contribute to sustainable forest ecosystems (Bhusal et al. 2020). Selection of drought-tolerance plants has a critical role in vegetation restoration and silvicultural strategies (Khaleghi et al. 2019).

Drought affects various aspects of the plant; the roots are the first part to be affected in the face of drought. The chemical signals (abscisic acid) produced in the roots along with decreased leaf turgor and atmospheric vapor pressure can reduce stomatal conductance. The limitation associated with increased stomatal resistance (under mild to moderate water deficit), is known as a stomatal limitation. Limitation due to non-stomatal disturbance under severe drought stress (non-stomatal limitation) can be induced by the limited diffusion of CO_2 from the intercellular spaces to the chloroplasts or by metabolic factors such as a decrease in Rubisco activity, disturbances in the regeneration of ribulose diphosphate and reactive oxygen species (ROS) production from the excess excitation energy. Low growth can be

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due to suppression of the photosynthetic process that eventually reduces biomass (Du et al. 2010, Dulai et al. 2014). Chlorophyll content can directly influence photosynthetic potential and primary production. Reduction in chlorophyll content under water deficit has been regarded as a typical feature of oxidative stress (Liu et al. 2019). Photosynthetic pigment stabilization under stress conditions increases resistance to drought stress (Ge et al. 2014). Decreased chlorophyll content under water deficit was reported in such tree species as *Fagus sylvatica* (Gallé and Feller, 2007), *Quercus variabilis* (Wu et al. 2013), *Alnus cremastogyne* (Tariq et al. 2018), and *Acer davidii* (Guo et al. 2019), while no change in chlorophyll content was found in *Melia azedarach* (Dias et al. 2014).

Relative water content (RWC) is a key indicator of degree of hydration and vital for optimal physiological functions and growth processes. RWC in woody and shrubby species reached 50 to 40% and seldom was it as low as 30 to 20% under severe water stress, which eventually causes leaf senescence (Wu et al. 2013). Relatively high RWC maintenance in water shortage is an indicator of drought tolerance (Ying et al. 2015, Toscano et al. 2016). *Quercus variabilis* seedlings could maintain sufficient RWC and slight growth at 40% field capacity (FC) (Wu et al. 2013). RWC of *Alnus cremastogyne* signifcantly decreased by 32.6% under drought (Tariq et al. 2018). Decrease of RWC in response to moderate (50% FC) and severe (30% FC) drought treatment in *Maclura pomifera* has been reported (Khaleghi et al. 2019).

Resistance to biotic and abiotic stress in plants increases by the accumulation of significant amounts of free proline, soluble sugars (sucrose, glucose and fructose), and soluble proteins (maturation proteins). These compatible solutes are able to maintain the concentration of cell sap and prevent the loss of water in plasma (Mohammadkhani and Heidari 2008, Farooq et al. 2009, Guo et al. 2018). Proline functions not only as an osmolyte, but also as an antioxidant, thus helping ROS detoxification by membrane integrity protection and enzyme/protein stabilization (Ghaffari et al. 2019, Khaleghi et al. 2019).

The intercellular concentration of malondialdehyde (MDA), a breakdown product of lipid peroxidation, has been measured as an indicator of oxidative damage (Ge et al. 2014, Abid et al. 2018). To scavenge ROS, plants maintain an efficient antioxidant defense system including non-enzymatic antioxidants and antioxidant enzymes (Khaleghi et al. 2019). Peroxidase (POD) and superoxide dismutase (SOD) disintegrate ROS, and therefore, protect plants from drought stress (Geng et al. 2019). SOD catalyzes the conversion of superoxide radical ($O_2^{\bullet-}$) to molecular oxgen (O_2) and hydrogen peroxide (H_2O_2). This H_2O_2 is detoxified to O_2 and H_2O through the activities of catalase (CAT) and POD as well as the ascorbate-glutathione (AsA-GSH) cycle (Wang et al. 2012, Abid et al. 2018).

Based on climate modeling, the air temperature in Iran will rise by 2.7 °C up to 2050, which will increase the water needs of plants (Attarod et al. 2017). The Caspian forest climate has become warmer and the vegetation growth trend has been upwards of about one hundred meters in the last half-century (Taleshi et al. 2018). Reforestation by *Alnus subcordata* C.A. Mey. (Caucasian alder), and *Acer velutinum* Bioss. (Persian or velvet maple) to increase production capacity reduced the pressure of wood exploitation on Hyrcanian forests (Abdolahi et al. 2017). *A. subcordata* and *A. velutinum* are the most valuable endemic species and are indigenous to the Hyrcanian province in the Euro-Siberian region. Due to their importance, numerous studies have done on the quantitative and qualitative characteristics of the species, mechanical properties of wood and nutrient elements (Naghdi et al. 2016, Naji et al. 2016, Tavankar et al. 2017, Ghorbani et al. 2018, Jourgholami et al. 2020).

According to a few recent studies, nano priming technique increased drought tolerance of *A. subcordata* seeds (Rahimi et al. 2016). *A. subcordata* as an urban tree showed limited tolerance to water deficit by determination of midday leaf water potential (Ψ_L) and stomatal conductance (g_s) (Sjöman et al. 2021). However, their response to drought and the mechanism of these two species in artificial cultivation are still unclear and poorly understood. Therefore, the objectives of the present study were (i) to evaluate the effects of drought stress on *A. subcordata* and *A. velutinum* seedlings which are dominant species in Hyrcanian forests and have a high commercial value in wood industries, to discover their capacity to handle water deficit in the initial vegetative growth period by morphological, physiological and biochemical responses; and (ii) to determine these two species' different adaptive responses to drought stress.

Materials and methods

Plant material and drought treatments

The experiment was carried out in a greenhouse at University of Guilan, Iran (37°15′ N, 49°36′ E). The average annual temperature was 15.9 °C and cumulative precipitation 1329.1 mm (Allahyari et al. 2016). One-year-old *A. subcordata* C.A. Mey. and *A. velutinum* Boiss. seedlings were obtained from a local nursery called Pilambara (37°35′ N, 49°05′ E) in Resvanshahr, Guilan Province, Iran. The seedlings were transplanted to 9 L plastic pots filled with homogenized topsoil. The plants were grown in a naturally lit greenhouse (temperature range: 18–28 °C; relative humidity range 73–94%) in a semi-controlled environment (only sheltered from rainfall) from July 10 to October 10, 2019. The greenhouse was well ventilated by plastic side films being rolled around it (Guo et al. 2013).

Drought treatments were performed three months after the planting of the seedlings (an acclimatization period, and when plants had produced fully expanded leaves) (Guo et al. 2013, Medeiros et al. 2013, Meng et al. 2013). A randomized complete design with two factors (two species and four watering regimes) was employed with three replications for four water shortage treatments (100, 75, 50 and 25% of field capacity performed as a control, mild, moderate, and severe, respectively). Using a scale with a capacity of 40 kg, transpiration water loss was measured gravimetrically by weighing all pots and re-watering with tap water every two days. The water added to each pot during the experimental period was 27, 18, 10.8 and 6.75 L for control, mild, moderate, and severe treatments respectively for seedlings of *A. subcordata* and 22.5, 15, 9, and 6 L for seedlings of *A. velutinum*. The evaluation was performed after three months at the end of the experiment.

Growth parameters

Seedling height (cm) was measured from the soil surface to the terminal bud of the main stem using a measuring tape; also, the basal diameter (mm) was measured at the ground line by electronic calipers. Plant height, basal diameter and biomass (total dry mass) were recorded at the end of the experiments. Three seedlings were harvested randomly from each treatment. The leaves, stems, and roots were cut and dried in an oven at 65 °C for 48 hours to calculate root, stem, and leaf biomass (the average weight of three samples per treatment). Biomass contribution including leaf mass ratio (LMR), stem mass ratio (SMR) and root mass ratio (RMR) was calculated by dividing the stem, leaf, and root biomass by the total biomass (root, stem, and leaf), respectively. Root: shoot ratio (R/S) was calculated using root biomass by total leaf and stem biomass in percentage. Leaf area (LA) was determined with a leaf scanner (model A3 Light box GCL Bubble Etch Tanks), and WinDIAS 3.2. software. Specific leaf area (SLA) was estimated by dividing the leaf area by leaf biomass, while leaf area ratio (LAR) was determined by dividing the total leaf area by every seedling total biomass (Wu et al. 2017, Zhang et al. 2019).

Relative water content

Ten leaf discs with a diameter of 5 mm were cut from the interveinal parts of each plant and fresh weight (FW) was determined. After that, turgor weight (TW) was calculated by weighing discs dipped in water for 24 hours in the dark. Finally, leaf discs were oven dried for 24 hours at 65 °C to determine dry weight (DW). Relative water content (RWC) was measured as follows: RWC (%) = (FW-DW) / (TW-DW) × 100 (Toscano et al. 2016).

Photosynthetic pigment content

For the extraction of photosynthetic pigments, 200 mg liquid nitrogen frozen tissue was ground by pestle and mortar and pigments were extracted by adding 10 mL of 80% cold acetone. The content of chlorophyll *a* (chl *a*) and *b* (chl *b*), total chlorophyll (chl *a*+*b*) and carotenoids was measured spectrophotometrically at 663, 645 and 470 nm respectively by spectrophotometer (Ltd T80 + UV/VIS; PG Instruments, Leicestershire, UK) according to Lichtenthaler (1987). The chlorophyll and carotenoid concentrations expressed as mg $g^{-1}{}_{FW}$ were calculated as:

$$\text{chl } a = \left[(12.7 \times A_{663}) - (2.69 \times A_{645})\right] \times V / 1000 \times W$$

$$\text{chl } b = \left[(22.9 \times A_{645}) - (4.68 \times A_{663})\right] \times V / 1000 \times W$$

$$\text{chl } a + b = \left[(20.2 \times A_{645} + 8.02 \times A_{663}) \times V\right] / (1000 \times W)$$

$$\text{carotenoids} = \frac{1000 \times A_{470} - 2.27 \times \text{chl } a - 81.4 \times \text{chl } b}{227} \times \frac{V}{1000 \times W}$$

where:

A – absorbance at specific wavelengh
V – final volume of chlolophyll extract in 80% acetone
W – fresh weight of tissue extracted

Free proline concentration

Free proline concentration was estimated according to Bates et al. (1973). In this method, 0.5 g of frozen leaf samples was extracted with 10 mL of 3% (w/v) sulfosalicylic acid; 2 mL of an aliquot of the supernatant was mixed with 2 mL of acetic acid and 2 mL of ninhydrin acid incubated for 40 minutes at 100 °C. The reaction was stopped in an ice bath and the reaction mixture was obtained with 4 mL of toluene and absorbance of the top layer was measured at 520 nm. Proline concentration was calculated by a standard curve, ranging from 0 to 400 µg mL^{-1} that was plotted with L-proline. Free proline concentration in tissue was calculated as:

$$\text{proline } (\mu\text{mol g}^{-1}) = (\mu\text{mol proline/mL}) \times (\text{mL toluene}/115) \times 5/W$$

Malondialdehyde (MDA) content

The extent of lipid peroxidation was evaluated as malondialdehyde (MDA) content. 100 mg leaf tissue was extracted in 2 mL 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12000 *g* for 15 min and then 0.5 mL of the upper phase was mixed with 1.5 mL TCA 20% (w/v) containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated for 90 min at 90 °C and then rapidly cooled in an ice bath. Afterwards, the mixture was centrifuged at 10000 *g* for 5 min and the absorbance (A) of the supernatant was recorded at 532 and 600 nm. The MDA content in tissue was calculated by an extinction coefficient of 155 mM^{-1} cm^{-1} as nmol g^{-1} (Chakhchar et al. 2015):

$$\text{MDA (nmol g}^{-1}\text{ FW)} = \frac{A_{532} - A_{600}}{155} \times 1000 \times \frac{V}{W} \times D$$

where:

V – final volume of extract
W – fresh weight of tissue extracted
D – dilution factor

Enzyme activities

100 mg fresh leaves was ground in liquid nitrogen using a mortar and pestle, and the ground samples were homogenized with 1 mL 50 mM sodium phosphate buffer at neutral pH containing 2 mM α-dithiothreitol, 2 mM EDTA, 0.2% Triton X-100, 50 mM Tris-hydrochloric acid and 2% polyvinylpyrrolidone. The homogenate was centrifuged at

14000 *g* for 30 min at 4 °C and the supernatant was collected and stored at −80 °C for SOD and POD activity analysis (Yang and Miao 2010, Ghaffari et al. 2019). SOD activity (EC 1.15.1.1) was evaluated by inhibition ability of the photochemical reduction of nitroblue tetrazolium (NBT) reduction to formazan by $O_2^{\bullet-}$. One unit of SOD was considered as the amount of enzyme required to cause 50% inhibition of NBT photochemical reduction which can be measured at 560 nm (Giannopolitis et al. 1977). Guaiacol peroxidase activity (POD) (EC 1.11.1.7) was assayed according to the guaiacol method (Plewa et al. 1991). POD catalyzes guaiacol to tetraguaiacol by H_2O_2. Absorbance was read at 465 nm for 2 min. The calculation were done through the following formulas:

$$\text{POD activity}\left(\frac{\mu\text{mol}}{\text{g FW min}}\right)=\frac{\left|A_{465}(t2)-A_{465}(t1)\right|}{t2-t1}\times\frac{V_t}{E\times V_s\times W}$$

where:

A – absorbance at specific wavelength
V_t – total volume
V_s – enzyme volume
E – extinction coefficient

$$\text{SOD activity}\left(\frac{U}{\text{g FW}}\right)=\frac{100-\left[\frac{(\text{OD control}-\text{OD sample})}{\text{OD control}}\times 100\right]}{50\times W}$$

where:

OD control- absorbance in the absence of SOD
OD sample- absorbance in the presence of SOD.

Statistical analysis

A randomized complete design was employed with three replications (n = 3). First, the variables were analyzed using one-way ANOVA (analysis of variances) with water supply regimes as factors for each species, then the main effects of drought stress and species and their interactions were determined by two-way ANOVA. When significant differences occurred among treatments, means were separated by Duncan's multiple range tests at $P \le 0.05$. Pearson's correlation coefficients were used to calculate the bivariate relationships between some morphophysiological and biochemical traits.

Results

Growth parameters

The highest plant growth parameters (height, basal diameter, total and organ biomass and leaf area) were observed in the well-watered 100% FC treatment, while drought treatments significantly decreased plant height, basal diameter, total and organ biomass in both species ($P \le 0.05$). Plant height decreased by 30.9, 26.6 and 16.9% when exposed to 25, 50 and 75% FC in *A. subcordata* respectively, and 23.3 and 17.8% in *A. velutinum* at 25 and 50% FC treatments, respectively in comparison with control treatment. Basal diameter decreased by 29.2, 32.7 and 13.8% at 25, 50 and 75% FC treatments in *A. subcordata* respectively, and 19.8% at 25% FC in *A. velutinum,* compared to control condition. Biomass traits showed a decreasing trend in both species under water treatment; namely, leaf biomass reduction was 79.1 and 80.8%, that of stem biomass was 40.5 and 75.8%, root biomass 60.9 and 64.2%, and finally total biomass 61.6 and 64.2% at 25% FC in *A. velutinum* and *A. subcordata* respectively compared to control condition (Tab. 1 and Tab. 2).

Drought stress significantly decreased leaf area in both species. Leaf area decreased 71.9 and 83.6% in *A. velutinum* and *A. subcordata,* respectively, when exposed to 25% FC. Specific leaf area tended to increase with decreasing soil water contents and significantly increased by 70.9% when exposed to 50% FC in *A. velutinum*. In contrast, it decreased 19.3, 26.3 and 49.6% in *A. subcordata* at 75, 50 and 25% FC,

Tab. 1. Effect of drought stress on height, basal diameter, leaf area (LA), special leaf area (SLA), and leaf area ratio (LAR) of *A. velutinum* and *A. subcordata* seedlings. Values are means of three replicates ± standard deviation (SD). Different capital letters indicate significant ($P \le 0.05$) differences between *A. velutinum* and *A. subcordata* subjected to the same treatment. Different lowercase letters indicate significant ($P \le 0.05$) differences among different treatments applied to the same species. F_S: species effect, F_D: drought effect, $F_S \times F_D$: species × drought interaction effect. *, **, and ***: significant at $P \le 0.05$, 0.01, and 0.001, respectively.

	Field capacity (FC, %)	Plant height (cm)	Basal diameter (mm)	Leaf area (cm²)	Special leaf area (cm² g⁻¹)	Leaf area ratio (cm² g⁻¹)
Acer velutinum	100	52.75 ± 1.96^{Da}	14.51 ± 0.37^{BCa}	123.40 ± 11.21^{Ba}	117.23 ± 5.22^{Eb}	30.51 ± 1.53^{Ba}
	75	50.75 ± 1.24^{Da}	14.06 ± 0.52^{Ca}	78.55 ± 3.41^{Cb}	131.84 ± 10.34^{DEb}	24.49 ± 0.85^{BCb}
	50	43.33 ± 1.44^{Db}	13.35 ± 0.59^{CDa}	60.80 ± 4.30^{Cb}	200.35 ± 14.53^{Ca}	29.00 ± 0.21^{Ba}
	25	40.44 ± 2.23^{Db}	11.63 ± 0.38^{Db}	34.62 ± 0.81^{Dc}	126.79 ± 11.11^{DEb}	17.74 ± 1.49^{Cc}
Alnus subcordata	100	132.67 ± 7.97^{Aa}	18.69 ± 0.80^{Aa}	153.66 ± 12.50^{Aa}	356.28 ± 22.99^{Aa}	57.95 ± 3.74^{Aa}
	75	110.12 ± 5.54^{Bb}	16.11 ± 0.87^{Bb}	78.94 ± 7.08^{Cb}	287.35 ± 5.33^{Bab}	57.46 ± 2.35^{Aa}
	50	97.33 ± 6.32^{Cab}	12.58 ± 0.62^{CDc}	37.36 ± 3.90^{Dc}	262.53 ± 30.17^{Bb}	31.78 ± 7.17^{Bb}
	25	90.89 ± 5.37^{Cc}	13.23 ± 0.70^{CDc}	25.13 ± 2.39^{Dc}	179.33 ± 0.05^{CDc}	18.70 ± 0.90^{Cb}
F_S		328.49^{***}	15.95^{***}	0.01^{ns}	98.39^{***}	53.38^{***}
F_D		13.68^{***}	65.12^{***}	93.90^{***}	8.89^{**}	28.64^{***}
$F_S \times F_D$		3.98^{*}	18.00^{**}	5.40^{**}	11.69^{***}	14.17^{***}

Tab. 2. Effect of drought stress on biomass in *A. velutinum* and *A. subcordata* seedlings. Values are means of three replicates ± standard deviation (SD). Different capital letters indicate significant ($P \leq 0.05$) differences between *A. velutinum* and *A. subcordata* applied to the same treatment. Different lowercase letters indicate significant ($P \leq 0.05$) differences among different treatments applied to the same species. F_S: species effect, F_D: drought effect, $F_S \times F_D$: species × drought interaction effect. *, **, and ***: significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

	Field capacity (FC, %)	Root biomass (g)	Leaf biomass (g)	Stem biomass (g)	Total biomass (g)
Acer velutinum	100	33.33 ± 3.38^{Ba}	16.00 ± 0.58^{Ba}	12.33 ± 1.20^{DEa}	61.67 ± 3.76^{Ca}
	75	22.67 ± 1.45^{CDb}	8.00 ± 0.58^{Cb}	12.00 ± 0.58^{DEa}	42.67 ± 1.45^{DEb}
	50	13.00 ± 0.58^{Ec}	3.67 ± 0.33^{Dc}	8.33 ± 0.33^{DEb}	25.00 ± 0.58^{FGc}
	25	13.00 ± 0.58^{Ec}	3.33 ± 0.88^{Dc}	7.33 ± 0.33^{Eb}	23.67 ± 1.45^{Gc}
Alnus subcordata	100	46.66 ± 2.90^{Aa}	20.33 ± 0.66^{Aa}	58.00 ± 4.16^{Aa}	125.00 ± 4.00^{Aa}
	75	27.33 ± 1.66^{Bb}	14.33 ± 1.20^{Bb}	30.00 ± 3.05^{Bb}	71.66 ± 5.48^{Bb}
	50	21.33 ± 2.02^{CDbc}	5.33 ± 0.33^{Dc}	19.00 ± 1.15^{Cc}	45.66 ± 2.40^{Dc}
	25	16.66 ± 2.18^{DEc}	3.90 ± 0.92^{Dc}	14.00 ± 0.57^{CDc}	34.56 ± 3.47^{EFc}
F_S		26.21^{***}	44.43^{***}	211.64^{***}	185.72^{***}
F_D		60.19^{***}	195.72^{***}	61.62^{***}	162.89^{***}
$F_S \times F_D$		2.23^{ns}	7.25^{**}	39.88^{***}	25.16^{***}

respectively. Leaf area ratio significantly decreased by 41.85 and 67.7% at 25% FC in *A. velutinum* and *A. subcordata,* respectively (Tab. 1).

The biomass contribution was significantly affected by changes in water availability. R/S increased by 45 and 53.3% in *A. subcordata* under moderate and severe treatments, while no significant difference among drought treatments was found in *A. velutinum.* RMR increased with reduced water availability in *A. subcordata*. The enhancement was 24.4% at 50% FC and 28.2% at 25% FC in comparison with control treatment, whereas no significant diffrence was observed in *A. velutinum*. Drought stress markedly decreased LMR by 45.9 and 44.1% when exposed to 25 and 50% FC in *A. velutinum* respectively, and 32.1 and 27.3% in *A. subcordata* in the 25 and 50% FC treatments, respectively in comparison with control treatment. SMR in *A. velutinum* significantly increased in all treatments in comparison with control treatment, while it showed a reduction tendency in *A. subcordata* (Tab. 3).

Relative water content and photosynthetic pigment content

RWC showed significant decreases of 24.9 and 33.5% respectively at 50 and 25% FC in *A. subcordata,* whereas in *A. velutinum* the only significant decrease was of 27.3% at 25% FC compared with the well-watered seedlings (Tab. 4).

Chl *a* content was reduced by 24 and 28% at 50 and 25% FC in *A. velutinum*, respectively, and 21.9, 60.9 and 53.3% in *A. subcordata* in the 75, 50 and 25% FC treatments, respectively, compared to control condition. Chl *b* content de-

Tab. 3. Effect of drought stress on biomass partitioning rate of *A. velutinum* and *A. subcordata* seedlings. Values are means of three replicates ± standard deviation (SD). Different capital letters indicate significant ($P \leq 0.05$) differences between *A. velutinum* and *A. subcordata* subjected to the same treatment. Different lowercase letters indicate significant ($P \leq 0.05$) differences among different treatments applied to the same species. F_S: species effect, F_D: drought effect, $F_S \times F_D$: species × drought interaction effect. *, **, and ***: significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

	Field capacity (%)	Root to shoot ratio (R/S)	Leaf mass ratio (LMR)	Stem mass ratio (SMR)	Root mass ratio (RMR)
Acer velutinum	100	1.18 ± 0.12^{Aa}	26.19 ± 2.12^{Aa}	19.96 ± 1.33^{Ca}	53.85 ± 2.60^{ABa}
	75	1.13 ± 0.07^{ABa}	18.73 ± 1.09^{BCb}	28.25 ± 2.14^{Bb}	53.01 ± 1.62^{ABa}
	50	1.09 ± 0.08^{ABCa}	14.62 ± 1.07^{CDEb}	33.36 ± 1.50^{Bb}	52.01 ± 2.00^{ABCa}
	25	1.22 ± 0.05^{Aa}	14.16 ± 1.48^{DEb}	30.78 ± 1.92^{Bb}	55.05 ± 1.04^{Aa}
Alnus subcordata	100	0.60 ± 0.06^{Db}	16.26 ± 0.01^{BCDa}	46.33 ± 2.42^{Aa}	37.39 ± 2.43^{Db}
	75	0.62 ± 0.03^{Db}	19.97 ± 0.44^{Ba}	41.75 ± 1.60^{Aa}	38.26 ± 1.31^{Db}
	50	0.87 ± 0.08^{Ca}	11.82 ± 1.40^{Eb}	41.64 ± 2.25^{Aa}	46.53 ± 2.25^{Ca}
	25	0.92 ± 0.05^{BCa}	11.03 ± 1.80^{Eb}	41.03 ± 2.91^{Aa}	47.94 ± 1.37^{BCa}
Fs		56.18^{***}	14.74^{***}	105.59^{***}	65.76^{***}
F_D		2.90^{ns}	20.74^{***}	1.63^{ns}	4.58^{*}
$F_S \times F_D$		2.58^{ns}	5.90^{**}	8.20^{**}	4.09^{*}

Tab. 4. Effect of drought stress on photosynthetic pigments content, and RWC of *A. velutinum* and *A. subcordata* seedlings. Values are means of three replicates ± standard deviation (SD). Different capital letters indicate significant ($P \leq 0.05$) differences between *A. velutinum* and *A. subcordata* subjected to the same treatment. Different lowercase letters indicate significant ($P \leq 0.05$) differences among different treatments applied to the same species. FW: fresh weight, RWC: relative water content; Fs: species effect, F_D: drought effect, $F_S \times F_D$: species × drought interaction effect. *, **, and ***: significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

	Field capacity (%)	Chlorophyll *a* ($mg\ g^{-1}{}_{FW}$)	Chlorophyll *b* ($mg\ g^{-1}{}_{FW}$)	Total chlorophyll ($mg\ g^{-1}{}_{FW}$)	Total carotenoids ($mg\ g^{-1}{}_{FW}$)	RWC (%)
Acer velutinum	100	1.00 ± 0.05^{ABab}	0.49 ± 0.13^{Aab}	1.49 ± 0.07^{ABab}	0.18 ± 0.02^{BCDa}	72.58 ± 3.82^{Aa}
	75	1.30 ± 0.20^{Aa}	0.53 ± 0.03^{Aa}	1.84 ± 0.23^{Aa}	0.22 ± 0.03^{ABCa}	75.08 ± 2.66^{Aa}
	50	0.76 ± 0.07^{BCb}	0.39 ± 0.09^{ABCab}	1.11 ± 0.01^{Cbc}	0.25 ± 0.02^{ABa}	69.20 ± 3.82^{Aa}
	25	0.72 ± 0.10^{BCDb}	0.23 ± 0.03^{BCb}	0.95 ± 0.12^{CDc}	0.25 ± 0.02^{ABa}	52.77 ± 1.36^{Bb}
Alnus subcordata	100	1.05 ± 0.08^{ABa}	0.44 ± 0.03^{Aa}	1.50 ± 0.06^{Aa}	0.26 ± 0.02^{Aa}	70.38 ± 3.00^{Aa}
	75	0.82 ± 0.12^{BCb}	0.40 ± 0.06^{Aba}	1.23 ± 0.11^{BCb}	0.21 ± 0.00^{ABCb}	67.61 ± 2.73^{Aa}
	50	0.41 ± 0.00^{Dc}	0.19 ± 0.01^{Cb}	0.60 ± 0.02^{Dc}	$0.13\pm0.0^{\ Dc}$	52.83 ± 3.08^{Bb}
	25	0.49 ± 0.04^{CDc}	0.21 ± 0.02^{BCb}	0.70 ± 0.07^{Dc}	0.16 ± 0.00^{CDc}	46.77 ± 3.08^{Bb}
Fs		11.55^{**}	5.21^{*}	18.36^{**}	5.54^{*}	13.99^{**}
F_D		12.69^{***}	8.13^{**}	23.72^{***}	0.82^{ns}	23.10^{***}
$F_S \times F_D$		2.42^{ns}	0.81^{ns}	3.03^{ns}	7.69^{**}	1.94^{ns}

creased 20.4 and 53% in *A. velutinum* and 56.8 and 52% in *A. subcordata* at 50 and 25% FC respectively. Chl *a+b* decreased by 20, 60 and 53.3% when exposed to 75, 50 and 25% FC in *A. subcordata*, respectively, and 25.5 and 36.2% in *A. velutinum* in the 25 and 50% FC treatments respectively, in comparison with control treatment. The content of carotenoids significantly decreased under drought in *A. subcordata,* where the reduction was 50 and 38.5% at 50 and 25% FC, whereas *A. velutinum* showed a tendency to increase in carotenoids under drought stress (Tab. 4).

Biochemical responses

In the leaves of both species, increase in proline content was recorded upon stress treatments. Proline content in *A. velutinum* leaves increased 22.1 and 132.6% at 75 and 50% FC, respectively and 136.8% at 25% FC. In *A. subcordata* the increase was 34.9 and 62.2% at 75 and 50% FC, respectiely and 169.8% at 25% FC in comparison with control treatment (Fig. 1A). The MDA content increased substantially as drought stress progressed in both species. In *A. subcordata* the increase was 93.7 and 133.8% at 75 and 50% FC, respec-

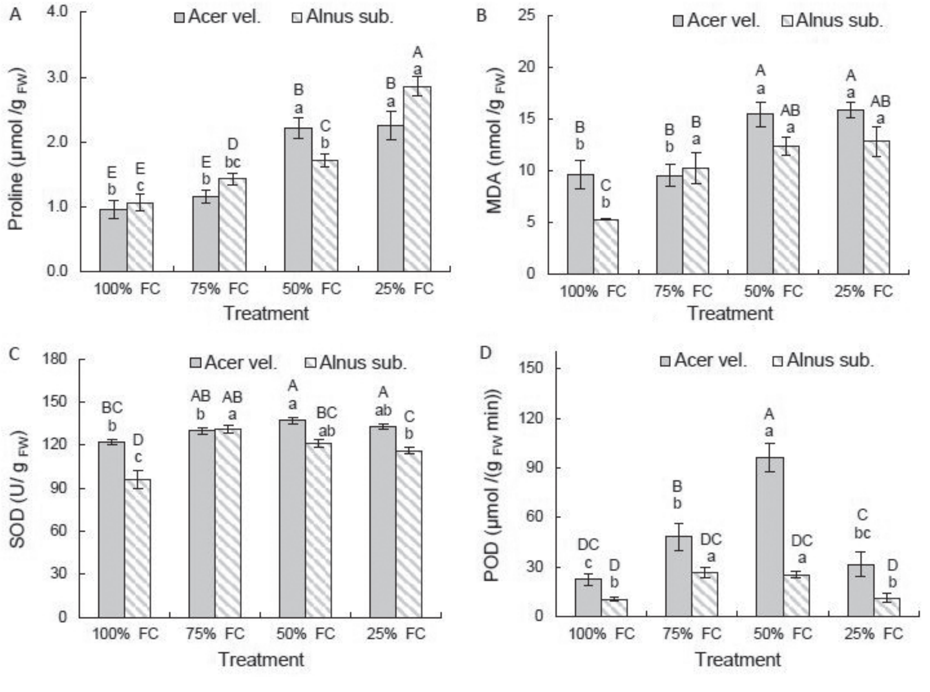

Fig. 1. Changes in proline (A), malondialdehyde (MDA) (B), superoxide dismutase (SOD) (C) and guaiacol peroxidase (POD) measured in leaves from *A. velutinum* and *A. subcordata* seedlings subjected to four drought treatments (100, 75, 50 and 25% of field capacity – FC). Values are means of three replicates ± standard deviation (SD). Different capital letters indicate significant ($P \leq 0.05$) differences between *A. velutinum* and *A. subcordata* subjected to the same treatment. Different lowercase letters indicate significant ($P \leq 0.05$) differences among the different treatments to which the same species were subjected.

Tab. 5. Correlation analysis among some morphophysiological and biochemical traits in *Acer velutinum* under drought stress conditions. Each square indicates the Pearson correlation coefficient of a pair of parameters. Leaf area: LA, specific leaf area: SLA, relative water content: RWC, chlorophyll *a*: chl *a*, chlorophyll *b*: chl *b*, total chlorophyll: chl *a+b*, and carotenoids: car, free proline: pro, malondialdehyde: MDA, peroxidase: POD, superoxide dismutase: SOD. ** and * indicate a significant correlation between control and drought treatments at P ≤ 0.01 and P ≤ 0.05, respectively.

	LA	SLA	car	SOD	POD	MDA	pro	RWC	chl *a*	chl *b*	chl *a+b*
LA	1.000	–0.293	–0.476	–0.695*	–0.306	–0.719**	–0.803**	0.607*	0.466	0.426	0.524
SLA		1.000	0.300	0.632*	0.779**	0.323	0.427	0.047	–0.256	–0.196	–0.294
car			1.000	0.605*	0.365	0.343	0.415	–0.213	–0.096	–0.229	–0.181
SOD				1.000	0.624*	0.638*	0.771**	–0.150	–0.224	–0.298	–0.314
POD					1.000	0.366	0.487	0.151	–0.332	–0.042	–0.293
MDA						1.000	0.910**	–0.524	–0.585*	–0.523	–0.651*
pro							1.000	–0.540	–0.651*	–0.420	–0.667*
RWC								1.000	0.596*	0.768**	0.735**
chl *a*									1.000	0.473	0.939**
chl *b*										1.000	0.745**
chl *a+b*											1.000

tively and 142.7% at 25%, whereas in *A. velutinum* the increase was 60.5 and 65% at 50 and 25% FC (Fig. 1B).

In *A. velutinum*, SOD activity increased 12 and 8.9% at 50 and 25% FC, respectively. In *A. subcordata,* SOD activity was significantly increased by 36, 25 and 20.9% at 75, 50 and 25% FC, respectively (Fig. 1C). POD activity in *A. velutinum* increased by 113 and 327% at 75 and 50% FC , respectively and 40% at 25% FC, whereas the values in *A. subcordata* were increased by 148 and 140% at 75 and 50% FC, respectively (Fig. 1D).

Correlation analysis

Correlation analysis indicated that there was a significant and positive correlation between SLA and chl *a*, chl *b* and chl *a+b* in *A. subcordata*, but there was no significant correlation between SLA and chl concentration in *A. velutinum*. Correlation analysis revealed that there was a significant and positive correlation between SOD and POD activities also, between proline and chl *a*, chl *a+b* in both species. According to correlation analysis there was no significant correlation between RWC and proline in *A. velutinum* but also, there was a negative correlation between RWC and proline in *A. subcordata*. Correlation analysis also revealed that there was a significant and positive correlation between carotenoid content and SOD activity in *A. velutinum* (Tab. 5 and Tab. 6).

Discussion

Drought stress is the most adverse abiotic stress to plant growth. Permanent or temporary water shortage causes detrimental effects on plant growth and development (Tariq et al. 2018; Du et al. 2019). Height, total and organ biomass of both species signifcantly declined under moderate and severe treatments (50 and 25% FC) in comparison with control treatment. Basal diameter signifcantly decreased under moderate and severe treatments (50 and 25% FC) in *A. subcordata* and just reduced under severe treatments (25% FC) in *A. velutinum.* These results are in accordance with

Tab. 6. Correlation analysis among some morphophysiological and biochemical traits in *Alnus subcordata* under drought. Each square indicates the Pearson correlation coefficient of a pair of parameters. Leaf area: LA, specific leaf area: SLA, relative water content: RWC, chlorophyll *a*: chl *a*, chlorophyll *b*: chl *b*, total chlorophyll: chl *a+b*, and carotenoids: car, free proline: pro, malondialdehyde: MDA, peroxidase: POD, superoxide dismutase: SOD. ** and * indicate a significant correlation between control and drought treatments at P ≤ 0.01 and P ≤ 0.05, respectively.

	LA	SLA	car	SOD	POD	MDA	pro	RWC	chl *a*	chl *b*	chl *a+b*
LA	1.000	0.836**	0.873**	–0.566	–0.314	–0.826**	–0.757**	0.775**	0.869**	0.758**	0.889**
SLA		1.000	0.648*	–0.300	0.094	–0.797**	–0.746**	0.746**	0.632*	0.607*	0.665*
car				–0.559	–0.330	–0.716**	–0.626*	0.799**	0.944**	0.698*	0.925**
SOD				1.000	0.705*	0.444	0.295	–0.250	–0.538	–0.210	–0.466
POD					1.000	0.281	–0.191	0.113	–0.309	–0.182	–0.288
MDA						1.000	0.543	–0.702*	–0.630*	–0.697*	–0.692*
pro							1.000	–0.812**	–0.688*	–0.655*	–0.721**
RWC								1.000	0.749**	0.684*	0.776**
chl *a*									1.000	0.725**	0.974**
chl *b*										1.000	0.861**
chl *a+b*											1.000

previous studies on *Salix paraqplesia* and *Hippophae rhamnoides* (Fang et al. 2012) as well as *Prunus sargentii* and *Larix kaempferi* seedlings (Bhusal et al. 2020) which demonstrated that drought significantly reduced seedling growth and biomass.

We found that drought treatment significantly increased the R/S and RMR in *A. subcordata*. It was statistically ineffective in *A. velutinum*. The increase in R/S is the result of declining growth rate and biomass production and increased water uptake (Wu et al. 2008, Du et al. 2010). Many studies have shown that there is an increase in R/S ratio under water stress (Fang et al. 2012; Guo et al. 2019, Zhang et al. 2019). More biomass allocation to belowground organs and maintainance of higher R/S can be indicated as an important adaptive trait (Fang et al. 2012).

In the present study, drought decreased LA in both species under drought stress. SLA showed an increasing trend in *A. velutinum* under drought stress treatments. However, it decreased in all drought treatments in *A. subcordata*. Also, LAR significantly decreased under drought conditions in both species. Decreased LA usually occurs due to inhibition of leaf development, loss of access to photosynthetic products to make new cells (Tariq et al. 2018). Some plant species adjust LA to prevent transpiration or a relative increase in root water uptake capacity (Guo et al. 2019). SLA and LAR increased under severe stress compared to the control in *Jatropha curcas* seedlings, which is considered a drought-tolerant plant (Díaz-lópez et al. 2012). In our study, *A. velutinum* significantly increased the SLA under moderate treatment (50% FC), which indicates that it probably has been able to cope with drought stress by increasing photosynthetic capacity and carbon assimilation (Wu *e*t al. 2017, Barros et al. 2020). Correlation analysis indicated that there was a significant and positive correlation between SLA and chl *a*, chl *b* and chl *a+b* in *A. subcordata,* but there was no significant correlation between SLA and chl concentration in *A.velutinum*.

We found that chl *a*, chl *b*, and chl *a+b* content significantly decreased under drought stress in both species. *A. velutinum* had a higher chlorophyll content (chl *a*, chl *b*, and chl *a+b*) than *A. subcordata* under moderate and severe treatment (50 nd 25% FC). According Lei et al. (2006), the dry climate population of *Populus przewalskii* had higher chlorophyll content than the wet climate population under the drought treatment. Drought stress also significantly decreased chlorophyll content of *Juglans mandshurica*, *Juglans nigra* and *Juglans regia* seedlings (Liu et al. 2019). Our results also showed that the carotenoid content was not significantly increased by drought in *A. velutinum*, while it was significantly decreased under moderate and severe treatment (50 and 25% FC) in *A. subcordata*. Reduction of carotenoids suggested that drought stress caused noticeable oxidative stress by ROS accumulation (Lei et al. 2006). The slight increase in carotenoid content in *A. velutinum* could suppress photosynthetic apparatus damage by oxygen consumption in xanthophyll cycle or detoxification of ROS (Ashraf and Harris, 2013, Medeiros et al. 2013). Correlation analysis also revealed that there was a significant and positive correlation between carotenoids content and SOD activity in *A. velutinum.*

In our study, *A. velutinum* seedlings showed a decline in RWC only under the severe treatment (25% FC), whereas *A. subcordata* showed a significant decrease in the moderate and severe treatments (50 and 25% FC, respectively). Díaz-López et al. (2012) indicated that *Jatropha curcas* can be considered a drought-resistant species as it has been able to sustain its RWC level under mild to severe stress drought treatments. Moreover, Ying et al. (2015) suggested that provenance Kunming (KM) had higher RWC than provenance Nanchang (NC) of *Camptotheca acuminate* under moderate and severe treatments (50 and 30% FC) and exhibited greater drought stress tolerance as expected given the natural habitat of this provenance. Proline content of both the species, investigated in this study, was significantly increased under drought treatments with respect to the well-watered plants although the higher increase was recorded in *A. velutinum* comparied to *A. subcordata* under moderate treatment (50% FC), whereas the increment was significantly greater in *A. subcordata* than in *A. velutinum* under the severe treatment (25% FC). According to correlation analysis, there was no significant correlation between RWC and proline content in *A. velutinum*, while negative correlation between RWC and proline was recorded in *A. subcordata*. Ashrafi et al. (2018) reported a negative correlation between RWC and osmoprotectants in *Thymus vulgaris* and *T. kotschyanus*, and found that osmoprotectants accumulate by reduction of RWC to maintain plant water. Similarly, Bangar et al. (2019) found that proline content was negatively associated with RWC in *Vigna radiate*.

MDA is a product of poly-unsaturated fatty acid degeneration in phospholipids of cellular membrane, and is used as an index of oxidative stress magnitude under drought (Wang et al. 2012, Guo et al. 2018). MDA content increased along with the drought stress in both species in this study. The significant increase of MDA content with progressive drought stress, suggests that drought stress caused oxidative damage. Our results, according to Wu et al. (2013) in *Quercus variabilis* and Tariq et al. (2018) in *Alnus cremastogyne* subjected to drought stress, showed an increase of MDA content. In *A. velutinum*, the values increased under moderate and severe treatment (50 and 25% FC), while in *A. subcordata* MDA content was elevated upon all drought treatments. The increases in MDA content in *A. velutinum* were lower than those in *A. subcordata.* This indicated that drought led to more damage in the cellular membranes under stress treatments in *A. subcordata*. Similarly, Ying et al. (2015) found that drought stress significantly increased MDA content in *Camptotheca acuminata* provenance KM and NC and the increases in MDA content in provenance KM were lower than those in provenance NC. They suggested that the less production of ROS in provenance KM under water deficit led to better membrane integrity.

The ability of antioxidant enzymes to eliminate ROS and reduce its harmful effects may be related to plant drought resistance (Anjum et al. 2011). High accumulation of ROS initiated and accelerated lipid peroxidation. POD plays an essential role in reducing the accumulation of H_2O_2, reducing MDA content and maintaining cell membrane integrity. Increased SOD and POD activity in stress treatments reflects an increase in ROS removal capacity and thus a reduction in membrane lipid damage (Ge et al. 2014, Guo et al. 2018). Toscano et al. (2016) suggested that *Eugenia uniflora* and *Photinia × fraseri* subjected to mild and moderate water stress showed increasing activities of antioxidant enzymes. We found that drought stress induced POD and SOD activity in both species under drought treatments in our study, although the highest activities were measured under mild and moderate treatments (75 and 50% FC) compared to the control. Our results are in good accordance with those published by Ge et al. (2014), who reported an increase of POD and SOD activities in *Phoebe bournei* subjected to mild and moderate water stress and a decrease under severe drought. In addition, Ge et al. (2014) demonstrated that the increase in MDA content acts as a feedback mechanism to control the activities of antioxidant enzymes. In our study, *A. veltinum* showed higher POD activity and lower increment of MDA than *A. subcordata* under the moderate and severe treatments. Similarly, Wang et al. (2012) found that a stronger protective mechanism by a drought-tolerant apple rootstock (*Malus prunifolia*) than in a sensitive-tolerant apple rootstock (*Malus hupehensis*) can be ascribed to lower MDA content, higher values for leaf RWC, and greater antioxidative defense system. Wu et al. (2013) has also shown that the MDA content at 60% FC treatment kept a lower increase compared with 40 and 20% FC treatments, indicating better protection against membranes lipid peroxidation, more efficient repairing mechanisms, including the antioxidative system, osmotic adjustment, and photosynthetic pigments in *Quercus variabilis* seedlings.

Conclusion

The present study concluded that although there were common responses in investigated parameters between two Hyrcanian endemic species i.e., *A. velutinum* and *A. subcordata*, certain different responses were also recorded under drought stress. Our results demonstrated that drought stress significantly reduced growth, biomass and photosynthetic pigment content, but increased free proline content, POD and SOD activities in both species. *A. velutinum* showed a slight reduction in seedlings height, basal diameter, biomass and had higher RWC and photosynthetic pigment than *A. subcordata*. *A. velutinum* also showed more efficient antioxidant systems with higher activities of POD, and a lower increase in MDA content under drought stress. Our results highlight that *A. velutinum* maintained stronger drought tolerance based on the measured parameters. According to these findings, it is recommended that *A. velutinum* plantation should have priority over *A. subcordata* in water deficit regions.

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Effects of exogenous NO on the growth and photosynthetic fluorescence characteristics of ryegrass seedlings under B[a]P stress

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Abstract –Benzoapyrene (B[a]P) pollution poses a threat to the environment and the food chain and consequently to human health. However, the alleviation of the harmful effects of B[a]P pollution in perennial ryegrass (*Lolium perenne* L.) by the application of exogenous nitric oxide (NO) has been ignored. Thus, in this paper the effects of exogenous sodium nitroprusside (SNP, a NO donor) on the growth, photosynthetic fluorescence characteristics, and antioxidant enzyme activity of ryegrass exposed to B[a]P stress are investigated. B[a]P stress induced the reduction of the aboveground and belowground dry weights, chlorophyll (*a*, *b*), the total chlorophyll contents, the carotenoid content, the net photosynthetic rate (Pn), the intercellular carbon dioxide concentration (Ci), the water use efficiency (WUE), the photosystem II (PSII) potential activity (Fv/F0), the maximum quantum yield of PSII photochemistry (Fv/Fm), the steady-state fluorescence yield (Fs), and the non-photochemical quenching (qN), while enhancement was recorded in response to the foliar spray of SNP at 200 and 300 μmol L^{-1} under B[a]P stress. Gray correlation and principal component analyses show that 200 μmol L^{-1} of SNP more drastically alleviated the damage caused by B[a]P stress than 300 μmol L^{-1} of SNP. The exogenous NO-mediated alleviation of B[a]P toxicity in ryegrass was associated with preserved photosynthetic characteristics and activation of antioxidant enzymes.

Keywords: NO, B[a]P stress, ryegrass, growth, photosynthesis, chlorophyll fluorescence parameters

Introduction

Benzo(a)pyrene (B[a]P), a typical polycyclic aromatic hydrocarbon (PAH) organic compound commonly found in the natural environment, has been recognized as one of the three major carcinogens by the World Health Organization and is often used as a representative indicator for determining PAHs (Ye et al. 2019). B[a]P has a high octanol-water partition coefficient and high vapor pressure, so it is difficult to degrade in the natural environment and can easily accumulate in the atmosphere, water bodies, and soil and cause serious environmental pollution (Ncube et al. 2017). B[a]P contaminated soil, which is primarily distributed in industrially contaminated sites, such as in Northeast and North China, is a major concern in several regions in China, with the B[a]P content of the soils of industrial areas peaking at over 1500 μg kg^{-1}. The average B[a]P content of the soil in the Yangtze River delta region of China exceeds 200 μg kg^{-1} (Fismes et al. 2002).

Nitric oxide (NO), which is a reactive nitrogen species, is recognized to play a very important signaling role in plants and has been reported to be involved in plant growth processes (Dai et al. 2020) and responses to various environmental stresses, including salinity (Ali et al. 2017), UV light (Yan et al. 2016), water deficit (Silveira et al. 2016), heat (Song et al. 2013), and heavy metals (He et al. 2014). Reportedly, exogenous NO application is involved in various physiological mechanisms that improve plant tolerance to various stresses, including metal toxicity, by increasing the activity of antioxidant enzymes and subsequently reducing the accumulation of reactive oxygen species (ROS) (Nagel et al. 2019). Foliar spray of SNP can enhance metal transporters and reduce As uptake while inducing new adventitious root formation and enhancing antioxidant and defense capacities (Souri et al. 2020), indicating the positive role of exogenous NO in As detoxification. NO also plays a key role in regulating plant stomatal movement and maintaining chlorophyll content under environmental stress. The application of NO synthesis promotes the production of plant carotenoids and enhances photosynthetic capacity by increasing the quantum production of photosystem II (PSII) in stressed plants (Tiwari et al. 2019). However, very

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few studies have focused on the influence of exogenous NO on a series of physiological changes and growth abnormalities in plants under B[a]P toxicity.

Perennial ryegrass (*Lolium perenne* L.) is a commonly used turfgrass species in the urban areas of Central and Western Europe and China and has a very wide scientific value (Ding et al. 2002). Given its high biomass production rates, ryegrass has been selected as a ditch plant for domestic wastewater treatment and plays an important role in nitrogen removal from wastewater (Duan et al. 2017). Ryegrass is also used as a forage crop and exhibits easy germination (Dąbrowski et al. 2015). Recent research confirms ryegrass' potential in phytoextraction for single metal Cd pollution or combined Cd and Zn pollution (Zhang et al. 2019). Ryegrass can be used as a material for the remediation of contaminated soil and is widely used for phytoremediation (Xie et al. 2021). However, little information is available regarding the alleviation of the adverse effects of B[a]P stress in ryegrass through seeding treatment with exogenous NO. Therefore, the present study aims to explore (1) whether exogenous sodium nitroprusside (SNP, an exogenous NO donor) treatment ameliorates B[a]P toxicity in ryegrass and (2) the mechanisms by which NO improves B[a]P tolerance, by investigating the growth parameters, photosynthetic characteristics, chlorophyll fluorescence properties, and antioxidant enzyme activities.

Materials and methods

Plant material and treatment

Seeds of ryegrass were available from Liaoning Fuyou Seed Co. Ltd. in Shenyang, China. SNP and B[a]P were purchased from Sigma-Aldrich. All reagents used in this study were of analytical grade.

Ryegrass seeds of uniform size were selected and surface sterilized with 5% H_2O_2 for 3 min. After soaking in distilled water for 12 h, the seeds of uniform size and shape were pregerminated on a double layer of moist filter paper for 48 h. The germinated seeds were transferred to plastic pots (22 cm in height, 20 cm in diameter) with 2 kg of nutrient soil. The measured soil parameters were as follows: soil pH 6.25, organic matter 20.27 g kg^{-1}, and cation exchange capacity 20.54 cmol kg^{-1}, total nitrogen and total phosphorus 3.66 and 3.79 g kg^{-1}, respectively. The soil was sieved through a 3-mm sieve, and the uncontaminated soil was air-dried and mixed thoroughly with the base fertilizer. The soil pH, organic matter, alkaline soluble nitrogen, available phosphorus, and available potassium after mixing were 5.56, 25.3 g kg^{-1}, 5.37 g kg^{-1}, 6.22 g kg^{-1} and 5.93 g kg^{-1}, respectively. The pots were placed in an experimental field in the Ecological Research Center of Liaoning University in a controlled environment, with an environmental temperature of 29 ± 5 °C/22 ± 3 °C (12 h day/ 12 h night), relative humidity of 62%~76%, and a photosynthetic photon flux density (PPFD) of 625 μmol m^{-2} s^{-1} provided by the LED light source. Appropriate soil moisture was maintained by watering every four days throughout the experimental duration. Excess seedlings were removed, leaving thirty ryegrass seedlings for growth in each pot.

Pre-experiments were conducted to analyze the inhibition rate and growth of ryegrass seedlings at different concentrations of B[a]P applied foliarly, and 30 μmol L^{-1} was identified as the B[a]P stress concentration (data not shown). In this experiment, these treatments were applied via leaf spraying: the control was sprayed with deionized water; B[a]P 30 was sprayed with a solution with 30 μmol L^{-1} B[a]P; SNP 100 + B[a]P 30, SNP 200 + B[a]P 30, SNP 300 + B[a]P 30, and SNP 400 + B[a]P 30 were sprayed with solutions with 30 μmol L^{-1} B[a]P plus 100, 200, 300, and 400 μmol L^{-1} SNP, respectively. When the ryegrass plants reached approximately 15 cm in height, 100 mL of the prepared treatment solution was sprayed uniformly per pot every other day by foliar spraying at 16:00. These treatments were arranged in a randomized complete block design with at least three pots per treatment. The investigated parameters were measured and analyzed when the plants reached approximately 22 cm in height after 14 days of SNP treatments under B[a]P stress.

Growth measurements

Ten seedlings were harvested and divided into roots and leaves. Root length and aboveground plant height of ryegrass seedlings (five plants of each treatment) were measured with a sliding caliper. Then they were rinsed with tap water and distilled water three times, blotted with filter paper to dry the surface water, weighed immediately for belowground and aboveground fresh weight. The fresh sample materials were dried at 85 °C for 60 hours and weighed for aboveground dry weight and belowground dry weight.

Photosynthetic pigment content measurements

Photosynthetic pigments contents were determined using fresh leaves according to the method described by Lichtenthaler (1987). Ryegrass leaves were accurately weighed to 0.5 g, and soaked in extraction solution for 24 h in the dark. The extraction solution was composed of 10 mL of 80% acetone and 5 mL of 95% ethyl alcohol. The absorbance of the extract was recorded at 663, 645 and 470 nm, and the contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid were obtained using the equations described by Arnon (1949).

Photosynthetic parameters measurements

Five leaves of similar height and shape were selected from each pot to measure net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular carbon dioxide concentration (Ci), transpiration rate (Tr) and water use efficiency (WUE) measurements were made using a LI-6400XT (LI-COR, USA) portable photosynthesis meter in each application. Measurements were performed from 9:00 to 12:30 in sunny weather conditions. To ensure that the measurements were carried out under approximately ideal photosynthetic conditions, the leaf surface temperature was controlled at 25 °C, the relative humidity was controlled at about 60%, the photosynthetically active radiation (PAR) was set to 1400 mol m^{-2} s^{-1}, the anaerobic conditions were set to T = 27 ± 2 °C, and airborne CO_2 concentration was 430 ± 20 μmol mol^{-1}.

Chlorophyll fluorescence measurements

Five leaves of similar height and shape were selected from each pot to measure chlorophyll fluorescence parameters using a Li-6400 portable photo synthesizer equipped with a pulse-modulated-fluorescent leaf chamber (6400-30, LI-COR Inc., USA). Before fluorometer measurements, plants were dark-adapted for 6 hours with leaf clips. The dark-adapted minimal fluorescence (F_0) and maximal fluorescence (F_m) were measured by applying a saturating actinic pulse of 8000 μmol m^{-2} s^{-1} for 1 s. The variable fluorescence ($F_v = F_m - F_0$), PSII potential activity (F_v/F_0) and maximum quantum yield of PSII photochemistry (F_v / F_m) were calculated from F_m, F_v and F_0. Steady-state fluorescence yield (F_s) was recorded in the light. A saturating actinic pulse of 8000 μmol m^{-2} s^{-1} for 1 s was applied to produce maximum fluorescence yield in the light-adapted state (F'_m). The actual quantum yield of PSII photochemistry (Φ PSII), minimum fluorescence value in the light (F'_0) (Murchie and Lawson 2013), photochemical quenching (qP) and non-photochemical quenching (qN) were calculated as follows (Schreiber et al. 1995):

$$F_v/F_0 = (F_m - F_0) / F_0,$$

$$F_v/F_m = (F_m - F_0) / F_m,$$

$$\Phi\ PSII = (F'_m - F_s) / F'_m,$$

$$qP = (F'_m - F_s) / (F'_m - F'_0),$$

$$qN = 1 - (F'_m - F'_0) / (F_m - F_0).$$

Determination of antioxidant enzyme activity

Leaf samples (0.6 g) were ground with 10 mL 50 mmol L^{-1} phosphate buffer (pH 7.0). Then the homogenate was centrifuged (13,000 *g*, 20 min), and the supernatant was used to determine antioxidant enzyme activity. SOD activity was defined by measuring the inhibition of nitro blue tetrazolium (NBT) photochemical reduction (Tandy et al. 1989). POD activity was determined by monitoring guaiacol oxidation using the method described by Pinhero et al. (1997). CAT activity was assayed in a reaction mixture containing 50 mmol L^{-1} sodium phosphate buffer (pH 7.0), 0.2 μmol L^{-1} H_2O_2 and a suitable aliquot of enzyme extract (Dai et al. 2020).

Data analysis

All experimental data were expressed as mean ± SD of at least three replicates. All figures were plotted by using Origin PRO 8.5. Statistical significance analysis was performed by Duncan's multiple range test at 0.05 probability level using computer Software SPSS 24.0 (SPSS Inc, Chicago, IL, USA). The value of $P < 0.01$ or $P < 0.05$ represented a very significant difference or remarkable variance, respectively. Significant differences at the $P < 0.05$ level were indicated by different lower-case letters.

As a systemic analysis method, gray correlation degree theory is often used to measure the correlation degree between each factor according to the similar degree or different degree of their development situation. The comprehensive evaluation of evaluated parameters of ryegrass under B[a]P stress after SNP application was carried out according to the equations of grey correlation degree and entropy weight method using SPSS 24.0.

Principal component analysis (PCA) using Origin PRO 8.5 and SPSS 24.0 was performed to further evaluate the responses of growth, photosynthetic characteristics and chlorophyll fluorescence parameters and antioxidant enzymes activities of ryegrass to different SNP treatments under B[a]P stress. The PCA allowed the ordination of the parameters to discover potential groupings within the parameters. Plots were generated using principal components (PC) 1, 2 and 3 as axes. Therefore, PCA can be used to determine the most appropriate exogenous NO concentration which can alleviate B[a]P stress of ryegrass.

Results

Effect of exogenous NO on the growth characters of ryegrass under B[a]P stress

B[a]P stress significantly reduced the underground dry weights ($P < 0.05$) of the ryegrass plants compared with the control (Fig. 1). In contrast, the aboveground fresh weight and the belowground root length significantly increased by 14.16% and 26.17%, respectively, in the B[a]P treatment compared with the control. B[a]P stress did not affect the aboveground dry weight and plant height and the belowground fresh weight. The exogenous application of 200 μmol L^{-1} SNP considerably enhanced the belowground dry and fresh weights, the root length, and the aboveground plant height by 78.67%, 86.07%, 7.78%, and 14.38% compared with those under B[a]P stress, respectively. The foliar application of 200 μmol L^{-1} SNP showed more pronounced results than that of the other three concentrations of SNP treatments on ryegrass plants under B[a]P stress.

Effect of exogenous NO on photosynthetic pigment contents of ryegrass under B[a]P stress

The chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents of the ryegrass under 30 μmol L^{-1} B[a]P stress drastically decreased ($P < 0.05$) and the carotenoid content declined but not radically compared with the control (Fig. 2). Compared with the sample under 30 μmol L^{-1} B[a]P stress, the chlorophyll *b* contents of ryegrass under the 100, 200, 300, and 400 μmol L^{-1} SNP treatments were notably boosted by 60.92%, 72.73%, 161.11%, and 110.16%, respectively. Similarly, the carotenoid contents dramatically increased by 71.29%, 91.72%, 94.24%, and 71.16% compared with those under B[a]P stress. The total chlorophyll content also increased following the 200 μmol L^{-1} SNP treatment.

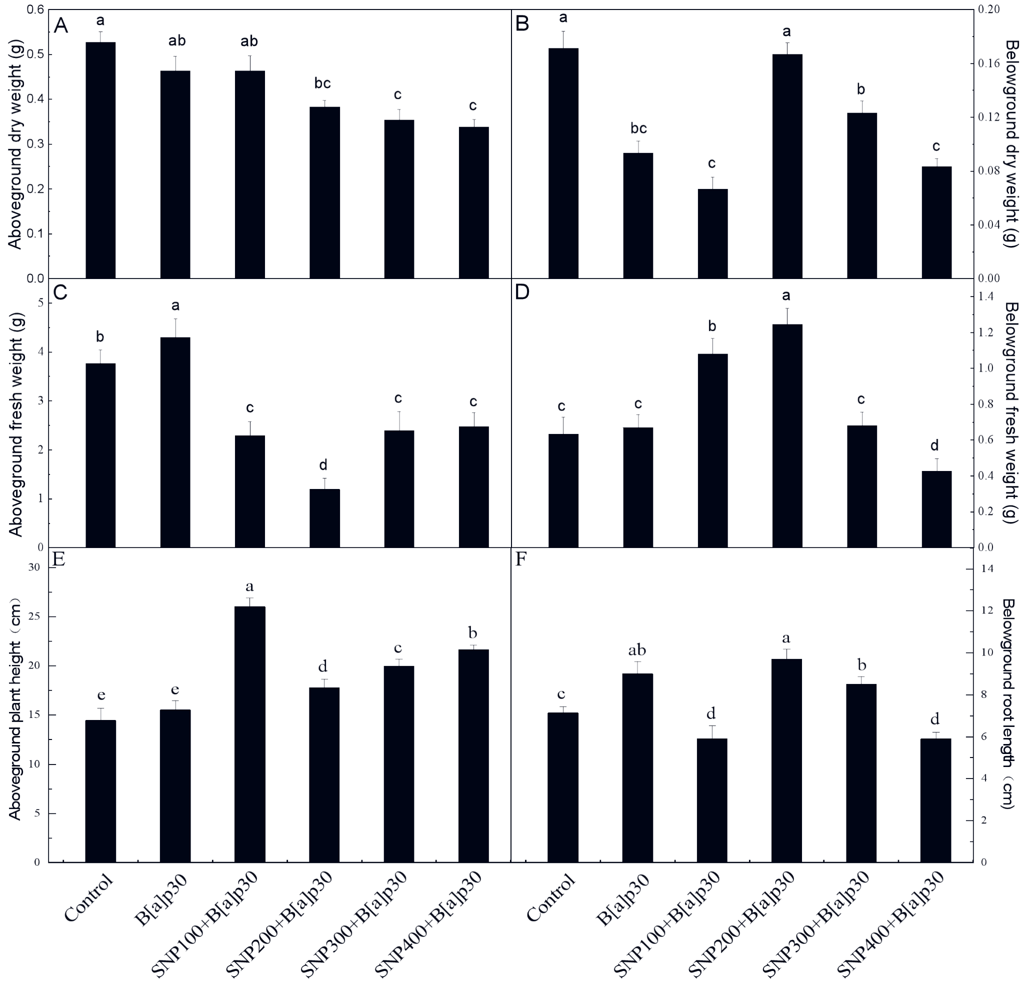

Fig. 1. Effect of different concentrations (100, 200, 300 and 400 μmol L^{-1}) of sodium nitroprusside (SNP), a NO donor, on the biomass of ryegrass seedlings under benzoapyrene (B[a]P) stress. (A) Aboveground dry weight, (B) Belowground dry weight, (C) Aboveground fresh weight, (D) Belowground fresh weight, (E) Aboveground plant height, (F) Belowground root length. Data are mean ± standard deviation, n = 3. Different letters indicate significant differences at P < 0.05.

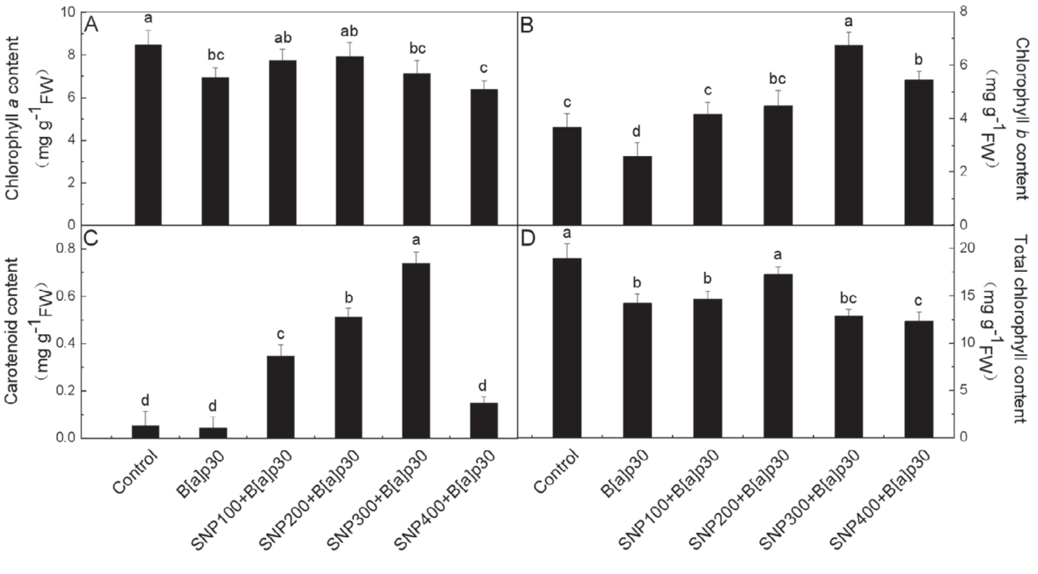

Fig. 2. Effect of different concentrations (100, 200, 300 and 400 μmol L^{-1}) of sodium nitroprusside (SNP), a NO donor, on the photosynthetic pigment content of ryegrass seedlings under benzoapyrene (B[a]P) stress. (A) Chlorophyll *a* content, (B) Chlorophyll *b* content, (C) Carotenoid content, (D) Total chlorophyll content. FW – fresh weight. Data are mean ± standard deviation, n = 3. Different letters indicate significant differences at P < 0.05.

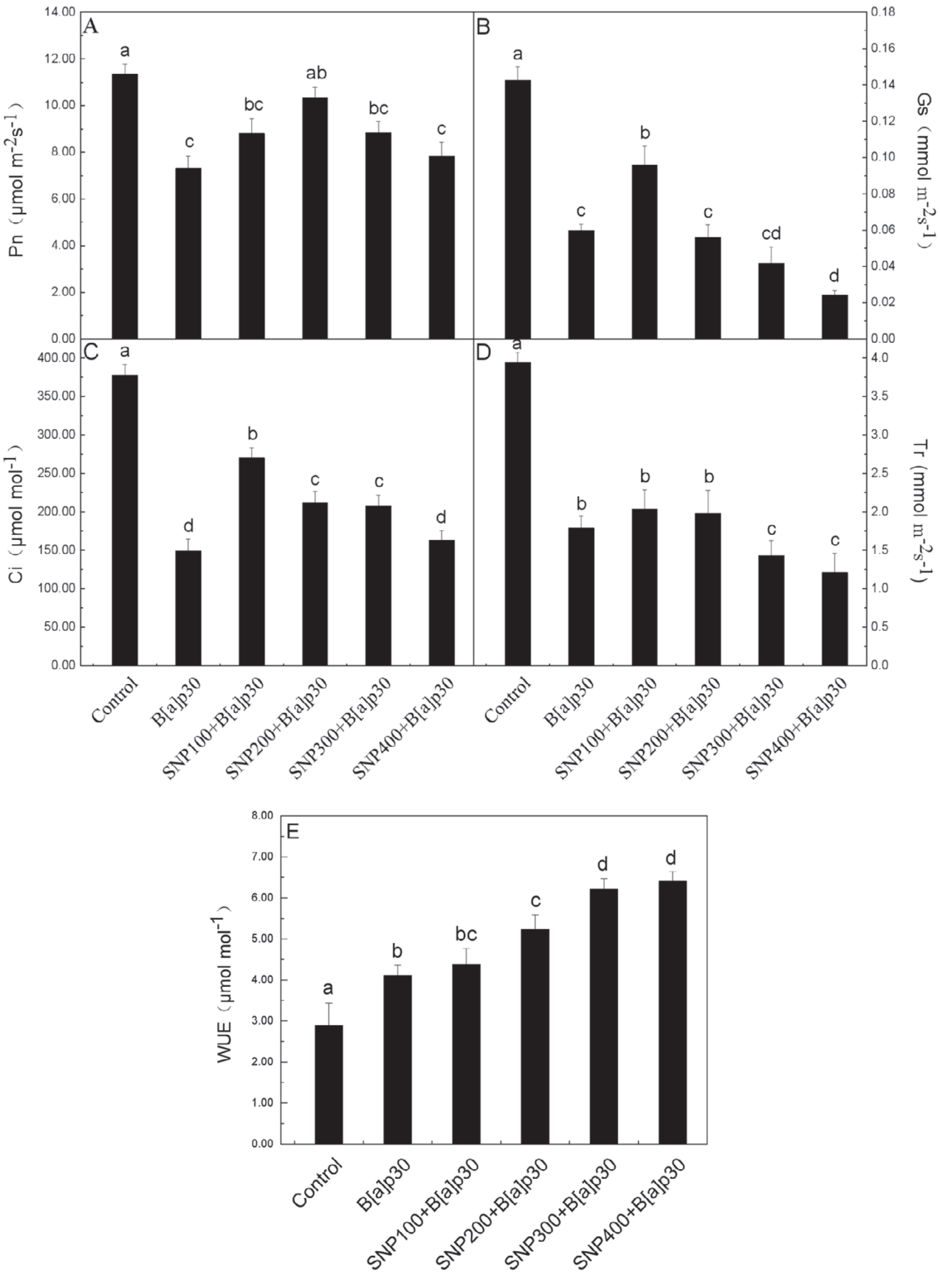

Fig. 3. Effect of different concentrations (100, 200, 300 and 400 μmol L^{-1}) of sodium nitroprusside (SNP), a NO donor, on photosynthetic gas exchange parameters of ryegrass seedlings under benzoapyrene (B[a]P) stress. (A) Net photosynthetic rate (Pn), (B) Stomatal conductance (Gs), (C) Intercellular carbon dioxide concentration (Ci), (D) Transpiration rate (Tr), (E) Water use efficiency (WUE). Data are mean ± standard deviation, n = 3. Different letters indicate significant differences at $P < 0.05$.

Effect of exogenous NO on photosynthetic gas exchange parameters in ryegrass under B[a]P stress

The Pn, Ci, and Tr in ryegrass radically decreased under 30 μmol L^{-1} B[a]P stress compared with the control, but WUE increased considerably by 42.21% (Fig. 3). The exogenous SNP application mostly alleviated the photosynthetic inhibition caused by B[a]P stress. The Pn significantly increased when the concentration of SNP was 200 μmol L^{-1} compared with that in the B[a]P treatment. Under 100 μmol L^{-1} SNP treatment, the Gs and the Ci significantly increased with the B[a]P treatment, indicating that 100 μmol L^{-1} SNP had the best effect in alleviating B[a]P stress on photosynthetic gas exchange parameters.

Effect of exogenous NO on chlorophyll fluorescence parameters of ryegrass under B[a]P stress

Compared with the control, the F_v/F_0 and ΦPSII decreased considerably due to B[a]P stress, whereas the qP and qN increased in ryegrass leaves under B[a]P stress conditions (Fig. 4). The F_v/F_0, Fs, and qN further increased in various degrees under low SNP concentrations compared with the B[a]P stress, and the most significant effects were observed in 200 μmol L^{-1} SNP treatment. After the application of 200 μmol L^{-1} SNP significantly improved the Fs and qN of ryegrass plants under 30 μmol L^{-1} B[a]P stress, the Fs and qN were at their highest, namely, 116.20% and 24.37%, respectively, higher than those under B[a]P treatment.

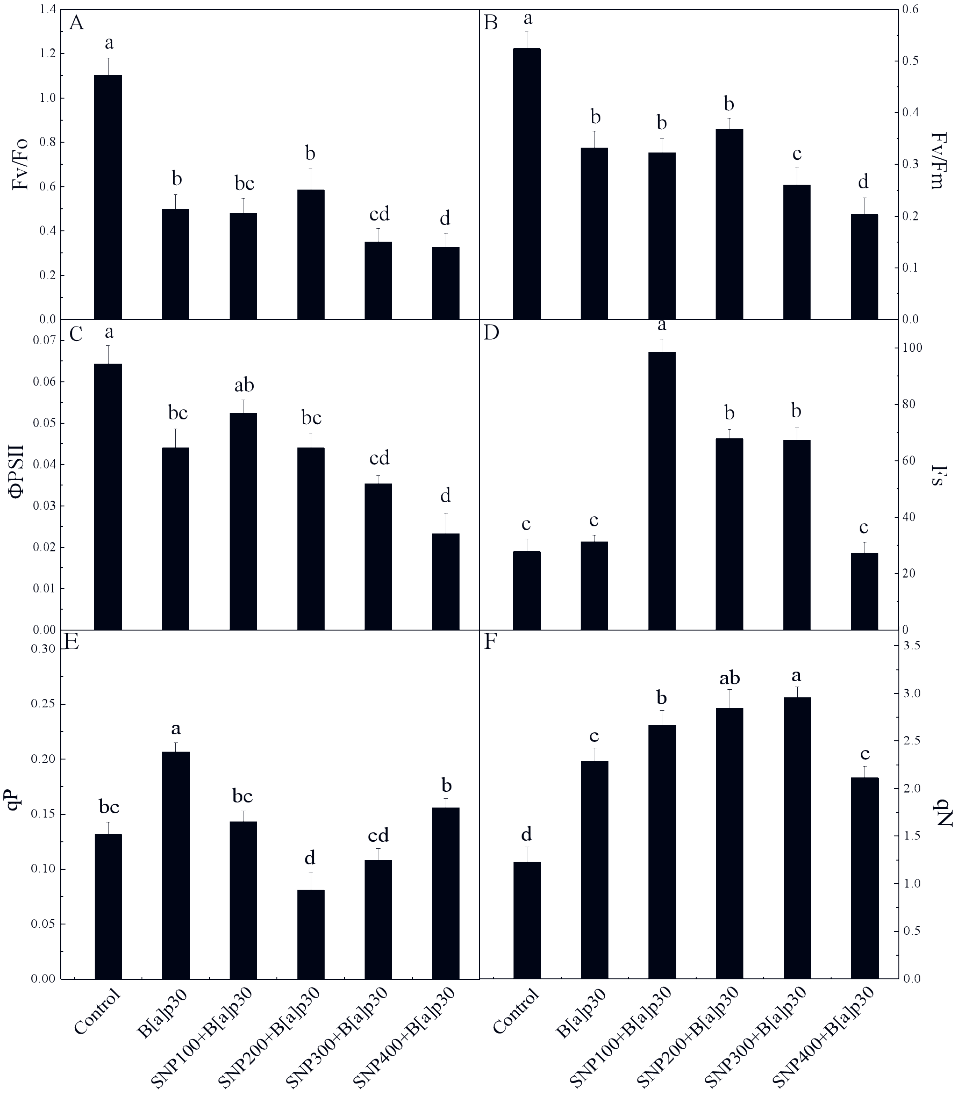

Fig. 4. Effect of different concentrations (100, 200, 300 and 400 μmol L^{-1}) of sodium nitroprusside (SNP), a NO donor, on chlorophyll fluorescence parameters of ryegrass seedlings under benzoapyrene (B[a]P) stress. (A) PSII potential activity (F_v/F_0), (B) PSII maximum light energy conversion efficiency (F_v/F_m), (C) Actual quantum yield of PSII photochemistry (ΦPSII), (D) Steady-state fluorescence (F_s), (E) Photochemical quenching coefficient (qP), (f) Non-photochemical quenching coefficient (qN). Data are mean ± standard deviation, n = 3. Different letters indicate significant differences at $P < 0.05$.

Effect of exogenous NO on antioxidant enzyme activity of ryegrass under B[a]P stress

The SOD, POD and CAT activities in the presence of 30 μmol L^{-1} B[a]P increased by 6.29%, 56.33% and 26.73%, respectively, compared with those in the untreated control plants (Fig. 5). However, no significant differences were found in the SOD between the 30 μmol L^{-1} B[a]P treatment and the control. Compared with the 30 μmol L^{-1} B[a]P treatment alone, the 100, 200, 300, and 400 μmol L^{-1} SNP treatments increased the SOD, POD and CAT activities. Among them, the 200 μmol L^{-1} SNP supplementation of the 30 μmol L^{-1} B[a]P stress boosted the SOD, POD and CAT activities by 17.85%, 14.74% and 53.58%, respectively. Meanwhile, the application of 300 μmol L^{-1} SNP under B[a]P stress further boosted the SOD, POD and CAT activities by 27.36%, 10.16% and 63.60%, respectively.

Gray correlation analysis and PCA

We analyzed the investigated parameters of ryegrass under B[a]P stress after SNP application using the gray correlation degree (Tab. 1). Our results show drastic differences in the 24 parameters among the different SNP treatments. The associative order of first six parameters is as follows: chlorophyll *a* content > belowground root length > aboveground dry weight > net photosynthetic rate > total chlorophyll content > qP.

The PCA revealed that the first three components with eigenvalues could explain more than 70.4% of the total variation (Fig. 6). PC1, PC2, and PC3 respectively account for 46.2%, 24.2%, and 14.6% of the physiological indexes. The PCA provided a simplified classification of the growth, photosynthesis, fluorescence, and antioxidant enzyme activities of ryegrass for different SNP treatments under B[a]P stress. PC1 tended to separate the effects of B[a]P

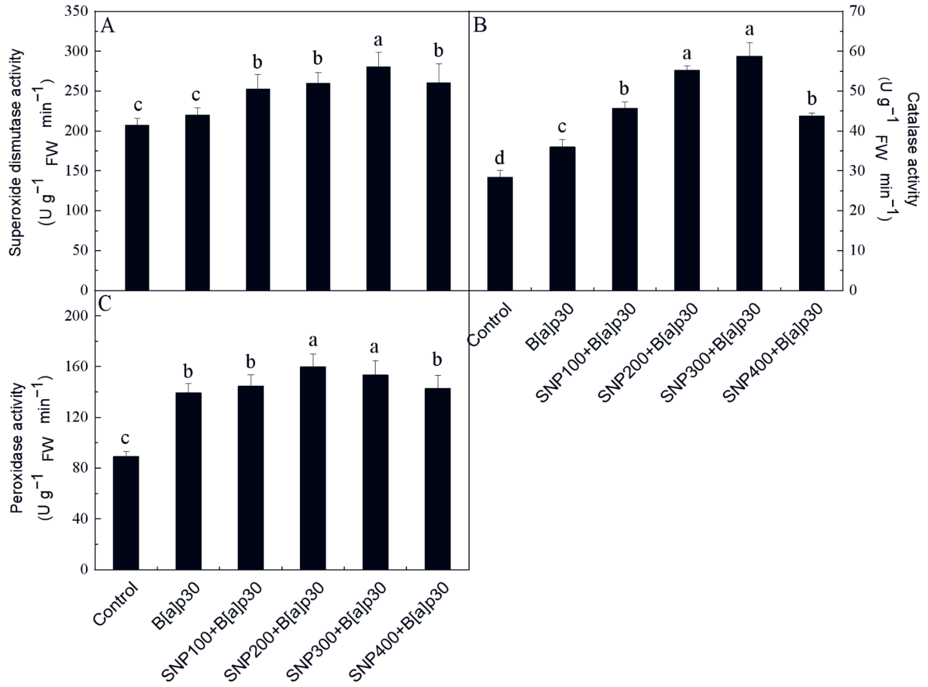

Fig. 5. Effect of different concentrations (100, 200, 300 and 400 μmol L^{-1}) of sodium nitroprusside (SNP), a NO donor, on antioxidant enzyme activity of ryegrass seedlings under benzoapyrene (B[a]P) stress. (A) Superoxide dismutase (SOD) activity. (B) Catalase (CAT) activity. (C) Peroxidase (POD) activity. Data are mean ± standard deviation, n = 3. U – unit of enzyme activity, FW – fresh weight. Different letters indicate significant differences at $P < 0.05$.

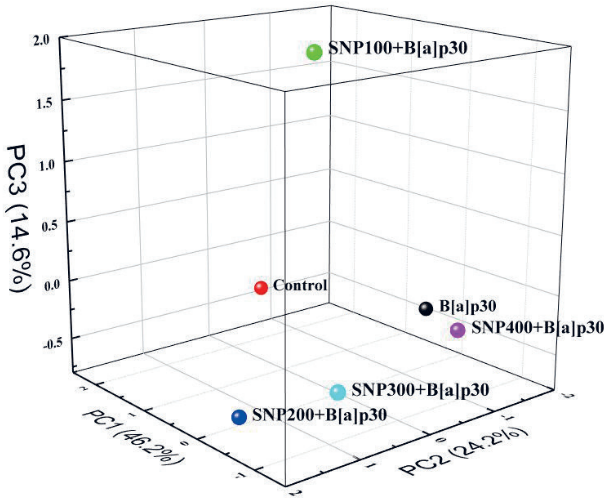

Fig. 6. Principal component analysis (PCA) plots of growth, photosynthetic characteristics and chlorophyll fluorescence parameters and antioxidant enzyme activity of ryegrass exposed to different SNP treatments (100, 200, 00 and 400 μmol L^{-1}) under benzoapyrene B[a]P stress.

stress and different concentrations of SNP, and PC2 further segregated the differences of SNP. According to the results, the application of 200 μmol L^{-1} SNP had a greater alleviative effect on B[a]P stress than the other three concentrations of SNP.

Discussion

B[a]P stress on plants causes reduced growth and photosynthesis, posing a threat to plant life due to metabolic

Tab. 1. Gray correlation degree and correlation sequence of indexes investigated in ryegrass under benzoapyrene stress after application of sodium nitroprusside (SNP) as a NO donor.

Index	Correlation	Associative order
Chlorophyll *a* content	0.981	1
Belowground root length	0.971	2
Aboveground dry weight	0.970	3
Net photosynthetic rate (P_n)	0.970	4
Total chlorophyll content	0.969	5
Photochemical quenching coefficient (qP)	0.965	6
Belowground dry weight	0.954	7
Actual quantum yield of PSII photochemistry (ΦPSII)	0.953	8
Peroxidase (POD) activity	0.953	9
Chlorophyll *b* content	0.953	10
Aboveground fresh weight	0.953	11
Aboveground plant height	0.952	12
Catalase (CAT) activity	0.950	13
Belowground fresh weight	0.949	14
PSII maximum light energy conversion efficiency (F_v/F_m)	0.947	15
Superoxide dismutase (SOD)	0.945	16
Intercellular carbon dioxide concentration (C_i)	0.942	17
Transpiration rate (T_r)	0.931	18
PSII potential activity (F_v/F_0)	0.928	19
Stomatal conductance (Gs)	0.927	20
Water use efficiency (WUE)	0.905	21
Steady-state fluorescence (F_s)	0.887	22
Non-photochemical quenching coefficient (qN)	0.877	23
Carotenoid content	0.675	24

disorders (He et al. 2014). NO, which is a small ubiquitous signaling molecule, plays a vital role in the response to abiotic stress in many plants (Gadelha et al. 2017, Fancy et al. 2017, Li et al. 2018, Wei et al. 2020). In this study, compared with the control, the growth and photosynthesis of ryegrass was inhibited as shown by the decreased aboveground and underground dry weights, and the photosynthetic characteristics. The decrease in ryegrass' growth caused by B[a]P pollution could be attributed to the decrease in the leaf photosynthetic pigment content and the photosynthetic gas exchange parameters. Moreover, B[a]P stress caused a decrease in F_v/F_0, F_v/F_m and ΦPSII suggesting a decline in PSII function, and indicating that photosynthetic units and membrane-bound electron transfer processes were disrupted under this stress.

After SNP application, the chlorophyll *b,* total chlorophyll, and carotenoid contents increased compared with those under B[a]P stress, implying that the release of NO from SNP increased the photosynthetic pigments of ryegrass and maintained a high photosynthetic rate. The protective effect of SNP on photosynthesis had a positive effect on plant growth as shown by the increased plant height, underground dry weight, and fresh weight. This result is consistent with the work of Ahanger et al. (2019) who reported that the exogenous application of NO evidently contributed to the improved growth and photosynthetic parameters of salt-stressed *Vigna angularis.* Moreover, the exogenous application of NO reportedly enhances the carotenoid synthesis, effectively channels additional energy, and increases the quantum production of PSII in stressed plants, resulting in enhanced photosynthesis and growth (Ahmad et al. 2021). In this study, 200 μmol L^{-1} SNP treatment increased F_v/F_0, F_v/F_m, and F_s, implying that SNP alleviated the disruption of PSII caused by 30 μmol L^{-1} B[a]P stress.

The exogenous application of SNP was able to increase the SOD, POD and CAT activities. Ahmad et al. (2018) reported that the observed decline in lipid peroxidation and membrane leakage in NO-treated tomato plants can be attributed to the upregulation of the antioxidant system, which rapidly eliminates ROS, including H_2O_2. Our results show that SNP treatments increased activities of CAT and POD thus alleviating oxidative stress and preventing damage of the photosynthetic apparatus.

Grey correlation analysis is one of the most commonly used multivariable statistical methods (Xiao et al. 2021). The gray correlation analysis enabled the combined evaluation value of 24 measured indicators, thus avoiding the limitation of using individual trait indicators to describe the response of ryegrass. According to the comparison of gray correlation, the chlorophyll *a* content, the underground root length, and the above ground dry weight have the highest correlation with resistance to B[a]P stress in ryegrass, which can be used as the analysis index for reflecting the response effect of ryegrass to stress. The results show that the comprehensive evaluation of ryegrass indicators using gray system theory is practical and feasible. The PCA showed that the application of 200 μmol L^{-1} SNP had an obvious alleviating effect on ryegrass' growth and physiological properties under 30 μmol L^{-1} B[a]P stress. NO could exert protective effects by increasing the plant tolerance to stress conditions, but high concentrations of NO may be toxic to plants due to their high reactivity (Reda et al. 2018).

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Short communication

New bryophyte taxa for Bosnia and Herzegovina

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Abstract – Bosnia and Herzegovina has a long history of bryophyte flora research. However, it is still considered insufficiently investigated, and until recently bryophyte investigations were completely neglected. Hence new records for the country are expected with novel explorations. Here, we report one liverwort (*Porella obtusata)* and four moss species (*Bryum klinggraeffii, Cinclidotus danubicus, Habrodon perpusillus and Imbribryum subapiculatum*) new for the country's bryophyte flora. With these new records, the bryoflora of Bosnia and Herzegovina numbers 673 taxa (no hornworts, 134 liverworts and 539 mosses).

Keywords: Balkans, bryoflora, liverwort, mosses, new records

Introduction

The Balkan Peninsula is characterized by a diversity of geological history, climate and habitat types, which all resulted in a diverse and rich bryophyte flora in a relatively small area (Sabovljević et al. 2011). Lately, numerous new records have been published within the Balkan region, i.e. Albania, Croatia, North Macedonia, Montenegro and Serbia (e.g., Sabovljević et al. 2010). In spite of that, distributional data of many species are still incomplete, especially for ephemeral species and those that are taxonomically difficult and unresolved. Furthermore, certain parts of many regions and countries are to date completely unexplored in terms of bryology. Bryological research in Bosnia and Herzegovina started with Sendtner in the middle of the 19th century (Kummer and Sendtner 1849). However, explorations were sporadic, with long interruptions (Grgić 1985), hence there is a lack of recent and updated floristic data, in particular of certain areas. For example, only a few studies were published recently for Bosnia and Herzegovina (e.g., Pantović et al. 2016, 2017). At present, the bryophyte flora of the country numbers 133 liverworts and 535 moss taxa (Hodgetts and Lockhart 2020, Ellis et al. 2021a,b).

Materials and methods

The subject of the bryological research was the tributaries of the lower course of the Neretva River in the region of southern Herzegovina (Bosnia and Herzegovina, SE Europe), namely the rivers Buna, Bunica, Bregava, Tihaljina and Trebižat (Fig. 1). The area of southern Herzegovina is influenced by a Mediterranean and sub-Mediterranean climate. This area has approximately 2,291 hours of sunshine per year, while the vegetation period lasts around or more than 240 days. Although the precipitation is high, with an average rainfall of 1,515 mm y^{-1}, owing to the porous nature of its karstic soil, there is a general lack of surface water (Galić 2011). The main features of the investigated area result in the richness of natural geomorphological, hydrological and biological values (Redžić et al. 2008, Lasić and Jasprica 2016).

The bryophyte samples were collected in August 2020. All main habitat types alongside the river courses were investigated, and specimens were collected from various substrata, e.g. soil, rocks, and tree bark. The list of species localities with details is given below. Voucher specimens were deposited in the Bryophyte Collection of the Herbarium of University of Belgrade (BEOU). Nomenclature for liverworts and mosses follows Hodgetts and Lockhart (2020).

The investigated sites include: 1 – Peć Mlini, 43.33747 N, 17.32592 E, 143 m a.s.l., date 11.08.20., leg: Jovana Pantović (JP) & Gordana Čokanović (GČ), det: JP, 2 – Peć Mlini, 43.33698 N, 17.32373 E, 137 m, 11.08.20., leg: JP & GČ, det: Marko Sabovljević (MS), 3 – Bagin most, Humac, 43.18675 N, 17.51575 E, 69 m, 11.08.20., leg: JP & GČ, det: MS, 4 – Struge, 43.09229 N, 17.69733 E, 7 m, 12.08.20., leg: JP & GČ,

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det: MS, 5 – Trebižat River, near Hacijenda bar, 43.12312 N, 17.67339 E, 17 m, 12.08.20., leg: JP & GČ, det: MS, 6 – Bagin most, 43.18342 N, 17.52270 E, 64 m, 13.08.2020., leg: JP & GČ, det: JP, 7 – Ljubuški, Baščine, 43.18040 N, 17.52745 E, 65 m, 13.08.20., leg: JP & GČ, det: JP, 8 – Bregava River, near the confluence, 43.10134 N, 17.73029 E, 8 m, 13.08.20., leg: JP & GČ, det: JP, 9 – Bregava River, near mini hydroelectric power plant "Do", 43.08491 N, 18.00514 E, 94 m, 14.08.20., leg: JP & GČ, det: JP, 10 – Buna and Neretva confluence, 43.23527 N, 17.83394 E, 25 m, 15.08.20., leg: JP & GČ, det: JP, 11 – Buna and Neretva confluence, 43.23570 N, 17.83407 E, 30 m, 15.08.20., leg: JP & GČ, det: JP, 12 – Buna River, Dokića pond, 43.24556 N, 17.84653 E, 367 m, 15.08.20., leg: JP & GČ, det: MS, 13 – Bunica River, Malo Polje, 43.23239 N, 17.88093 E, 42 m, 15.08.20., leg: JP & GČ, det: JP, 14 – Bunica River, 43.23667 N, 17.86907 E, 38 m, 15.08.20., leg: JP & GČ, det: JP, 15 – Tekija, Buna River source, 43.25667 N, 17.90298 E, 38 m, 15.08.20., leg: JP & GČ, det: JP.

Results and discussion

Here we report five new species for the bryophyte flora of Bosnia and Herzegovina: one liverwort and four mosses (number referring to sites given in text of Materials and methods).

Bryum klinggraeffii Schimp.: site 13: wet rocks by the river.

The ruderal moss *B. klinggraeffii* is widespread through Europe, but it is red-listed in some countries like Portugal (Critically Endangered – CR), Romania (Endangered – EN), and Slovenia (Data Deficient – DD) (Hodgetts and Lockhart 2020).

Cinclidotus danubicus Schiffn. & Baumgartner: site: 3: rocks in the water; site 4: the *Platanus* sp. roots by the water; site 6: rock by the river; site 7: roots by the water; site 8: limestone in dry riverbed; site 10: rocks in the water; site 11: *Salix* sp. bark; site 13: rocks in the water; site 15: rocks in the water.

This species is endemic for Europe, and in the Balkan region is known only from Croatia and Slovenia as well as from Hungary. (Hodgetts and Lockhart 2020).

Habrodon perpusillus (De Not.) Lindb.: site: 5: *Fraxinus* sp. bark; site 9: *Populus nigra* bark.

This species is common in the Mediterranean region; however, it is rare and red-listed in some non-Mediterranean countries, e.g. Romania (CR), Norway, Great Britain, Slovenia (EN), Canary Islands and Switzerland (Vulnerable – VU) (Hodgetts and Lockhart 2020).

Imbribryum subapiculatum (Hampe) D.Bell & Holyoak: site 1: rock crevice by the water; site 2: tufa; site 13: wet rocks by the river; site 14: wet soil by the river.

Imbribryum subapiculatum is a temperate species somewhat less frequent in the Balkan peninsula, probably due to misidentification with other species of small tuber-bearing *Bryum* species.

Porella obtusata (Taylor) Trevis.: site 12: *Populus nigra* bark.

Porella obtusata is a liverwort with a southwestern distribution in Europe. It is considered a threatened species in some European countries, for example it is endangered (EN) in Norway, vulnerable (VU) in Serbia and near threatened (NT) in Italy and the Canary Islands (Hodgetts and Lockhart 2020).

The bryoflora of Bosnia and Herzegovina, together with the new records reported here numbers 673 taxa (134 liverworts and 539 mosses). Further new findings of bryophytes are expected with the intensification of field investigation.

Fig. 1. Position of the investigated area of the lower course of the Neretva River within Bosnia and Herzegovina. All recorded localities of five new species records for the country (liverwoth *Porella obtusata* and mosses *Bryum klinggraeffii*, *Cinclidotus danubicus*, *Habrodon perpusillus* and *Imbribryum subapiculatum*) are marked on the map with a unique symbol.

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