

On the cover:

Sisyrinchium micranthum
in Tunisia, is reported by
El Mokni and Verloove
(pp. 133-137)



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Observations on the marine benthic diatom *Halamphora yundangensis* W.W.Wu et al., a species originally described from China, in the Mediterranean Region

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Abstract – During light and scanning electron microscope observations of marine benthic diatoms on various substrates in the eastern Adriatic Sea and the western Black Sea, we discovered a peculiarly structured amphoroid diatom species. The species was characterised by semi-elliptic to semi-lanceolate valves with distinctly protracted capitate to occasionally rostrate apices, a dorsal valve side crossed by transapical slits, a wide dorsal marginal ridge, biseriate dorsal striae internally, and usually lacking ventral striae externally, except for one or two poroids at the apices. Following a search in the literature, this taxon could only be identified as *Halamphora yundangensis*, a recently described species from the southeastern coasts of China. Here we present the first verified observation of this species in the Mediterranean Region. After thorough analyses of the observed populations in scanning electron microscopy and a literature overview, we provide an emended description of the species, and additional information enabling/justifying its separation from similar taxa occurring in the Mediterranean Region. Notes on the ecology and distribution of the species based on the new observations are also given.

Keywords: Adriatic Sea, Bacillariophyta, Black Sea, diatom diversity, Europe, morphology

Introduction

Halamphora yundangensis W.W.Wu, C.P.Chen & Y.H.Gao in Wu et al. (2020: p. 210) was described as an epiphyte on green “leafy” seaweeds (i.e., *Ulva lactuca* Linnaeus) from the saltwater Yundang Lake located near Xiamen, southeastern China. This is a shallow, alkaline, artificial lagoon connected to the South China Sea, and due to the urbanization of the Xiamen area, subjected to eutrophication, chemical pollution, algal blooms and changes in salinity (Wang et al. 2020 and references therein). The species, *Halamphora yundangensis*, possesses a unique combination of features distinguishing it from other *Halamphora* taxa. When observed in scanning electron microscope (SEM), it shows a broad dorsal marginal ridge, a large, flat, unornamented area along the valve face/mantle junction, and dorsal striae appear externally as transapical

slits crossing the valve face, continuing onto the mantle after the wide marginal ridge (Wu et al. 2020). In contrast to all other *Halamphora* taxa studied in SEM, the valves of *H. yundangensis* observed by Wu et al. (2020) from the type population in China lacked ventral striae, apart from one to three poroids at the apices. Later, on hermit crabs from the coasts of Xiaodeng Island in southeastern China, valves similar in morphology to *H. yundangensis* were discovered by Huang et al. (2023a). These valves presented ventral striae, as a row of small, single areolae on the ventral side (Huang et al. 2023a, b). Based on the presence of ventral striae, Huang et al. (2023a, b) recognised the observed taxon as a new variety of *H. yundangensis* (“var. *foramina*”, *nomen nudum*), not validly published under Article 29 and Article 38.1 of the International Code for Botanical Nomenclature (Turland et al. 2025).

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During a survey of the marine benthic *Halamphora* flora in diatom samples from the Adriatic Sea, the northernmost arm of the Mediterranean Sea, and from the Black Sea, the latter a marginal sea to the Mediterranean Sea, we discovered valves with morphology similar to that of *H. yundangensis*. The valves usually lacked ventral striae, with the exclusion of one or two poroids at the apices, but sometimes the ventral side was ornamented by a short row of irregularly spaced, single, small, rounded areolae externally and by “ghost” striae internally. Following light microscopy (LM) and scanning electron microscopy (SEM) observations and comparisons with the type of *H. yundangensis* in Wu et al. (2020), we could only identify the observed valves as belonging to the latter species. Here we present the first verified record of *H. yundangensis* in Europe, more specifically from the Mediterranean Region. The species was found living both epizoically on the carapace of an injured loggerhead sea turtle admitted for recovery at the Sea Turtle Rescue Center of Aquarium Pula, Croatia, and as an epiphyte on various macroalgae from off the coast of Verudela, Croatia, the Northern Adriatic Sea. A few valves were also discovered on plexiglass substrates placed near the western coast of the Black Sea in Bulgaria.

After its description in 2020, *H. yundangensis* has also been reported outside China from the Revillagigedo Archipelago in the Eastern Pacific Ocean (Siqueiros Beltrones et al. 2021) and from coral reefs in Indonesia (Risjani et al. 2021). A single record exists from the western Mediterranean Sea near Spain (Pérez Burillo 2022). However, in all these studies the species was identified based solely on LM observations, which are often not sufficient for the correct separation of small *Halamphora* taxa (Levkov 2009, Stepanek and Kociolek 2018, Pottiez et al. 2025). From the Revillagigedo Archipelago, Siqueiros Beltrones et al. (2021) illustrated two very similar valves in LM: the valve shown on their figure 26j was reported as *H. yundangensis*, but the valve illustrated on their figure 26k, likely belonging to the same taxon, was identified as *H. coffeiformis* (C. Agardh) Levkov (as *coffeaeformis*). The latter species has been largely misinterpreted, and past reports of it often included other and different taxa (Archibald and Schoeman 1984, Levkov 2009). In addition, from the estuary of the Indian River in Florida, USA, Navarro (1982: Pl. XX, Figs. 5–7) illustrated a taxon very similar to *H. yundangensis* in SEM. However, they misidentified and reported this taxon as *Amphora costata* W. Smith (currently *Halamphora costata* (W. Smith) Levkov), a little known, but larger and coarsely striated species from the marine coasts of Northern Europe (Smith 1853, Levkov 2009). These observations suggest that *H. yundangensis* might be another species hidden within the records of *H. coffeiformis* or other *Halamphora* taxa in the Mediterranean Region. Finally, during the literature overview we discovered that the external dorsal structure of *H. yundangensis* in SEM is very similar to that of *H. cejudoe* Álvarez-Blanco and S. Blanco (2014: p. 54), a taxon described from the Mediterranean Sea, but overlooked by Wu et al. (2020) when comparing *H. yundangensis* with

other taxa. Hence, with the lack of recently developed marine benthic diatom floras for the studied areas, we felt it necessary to share our findings and observations. Along with the clear separation from *H. cejudoe* and a few other morphologically similar taxa present in the Mediterranean Region, we provide additional information concerning the unique morphology of *H. yundangensis* and its ecology.

Material and methods

Samples for the study originate from the Croatian (eastern) coast of the Adriatic Sea and the Bulgarian (western) coast of the Black Sea. The following samples, slides and stubs, kept in the BR Collection, Meise Botanic Garden, Belgium, are included in the study:

1. Sample TB225: diatom sample from the carapace of a loggerhead sea turtle superficially cleaned of barnacles and macroalgae. The injured turtle had been rescued northeast of Korčula Island (42°58' N, 16°53' E), Croatia, on 17. 07. 2021, and was then admitted to the Sea Turtle Rescue Center of Aquarium Pula. Sample collected after the turtle had spent one month in a recovery tank at the Center, coll. L. Kanjer, 16. 08. 2021. Slide BR-4915, stub BR-4916.

2. Sample ENV10: diatoms on young thalli of various macroalgae, Verudela, Istria Peninsula, Adriatic Sea (44°50'00" N, 13°49'27" E), coll. L. Kanjer, 27. 02. 2022. Slide s.n., stub s.n.

3. Sample ENV14: diatoms from brown spots on young red calcifying macroalgae (Corallinales), Verudela, Istria Peninsula, Adriatic Sea (44°50'00" N, 13°49'27" E), coll. L. Kanjer, 27. 02. 2022. Slide s.n., stub BR-4873.

4. Sample C3.3: biofilm on artificial substrate (plexiglass) submerged at 3 m below sea surface for a period of 25 days, Sozopol Bay, Bulgaria, Black Sea (42°25'05" N, 27°41'20" E), coll. R. Zidarova & P. Ivanov, 30. 08. 2020. Slide BR-4880, stub BR-4881.

Sampling methods are given in detail by Pottiez et al. (2025) and Zidarova et al. (2022, 2023, 2025). Environmental parameters (water temperature, salinity, pH) were measured during sampling in the Black Sea using a HI98194 Multi-parameter field meter (Hanna Instruments), and are summarised in Zidarova et al. (2023, see Tab. 1 therein). Parameters of the water in the tank housing the turtle in Pula were also taken upon sampling, using Multifunction pH Meter PCE-PHD 1 (PCE Instruments).

For LM analyses, samples were treated either by the method of van der Werff (1955), or by the method described in Hasle and Fryxell (1970), after pretreatment with a few drops of concentrated HCl to dissolve carbonates. Slides were prepared with Naphrax® and studied at 1000x magnification (N.A. 1.30) using an Olympus BX51 light microscope, equipped with Differential Interference Contrast (DIC) optics and Olympus digital imaging system. For SEM observations, a small amount of the pretreated samples was filtered through polycarbonate membrane filters (Whatman™) with a pore size of 2 µm or 5 µm. After airdrying, pieces of

the filters were affixed on aluminium stubs, coated with a platinum/palladium layer of 20 nm, and studied using a JEOL-JSM-7100F field emission SEM at Meise Botanic Garden (Belgium). Identification of the species is based on the recent work by Wu et al. (2020), where it was described. The European populations are compared to both the type of the species in Wu et al. (2020) and the observations of Huang et al. (2023a, b) on similar populations from China. For the description of the morphological features of the species, we followed Levkov (2009) and Stepanek and Kociolek (2018).

Results

Table 1 shows the morphological comparisons between the European populations of *H. yundangensis*, its type population from China (Wu et al. 2020: p. 210, Figs. 1–25), and the population identified as a variety of the latter from China (Huang et al. 2023a, b).

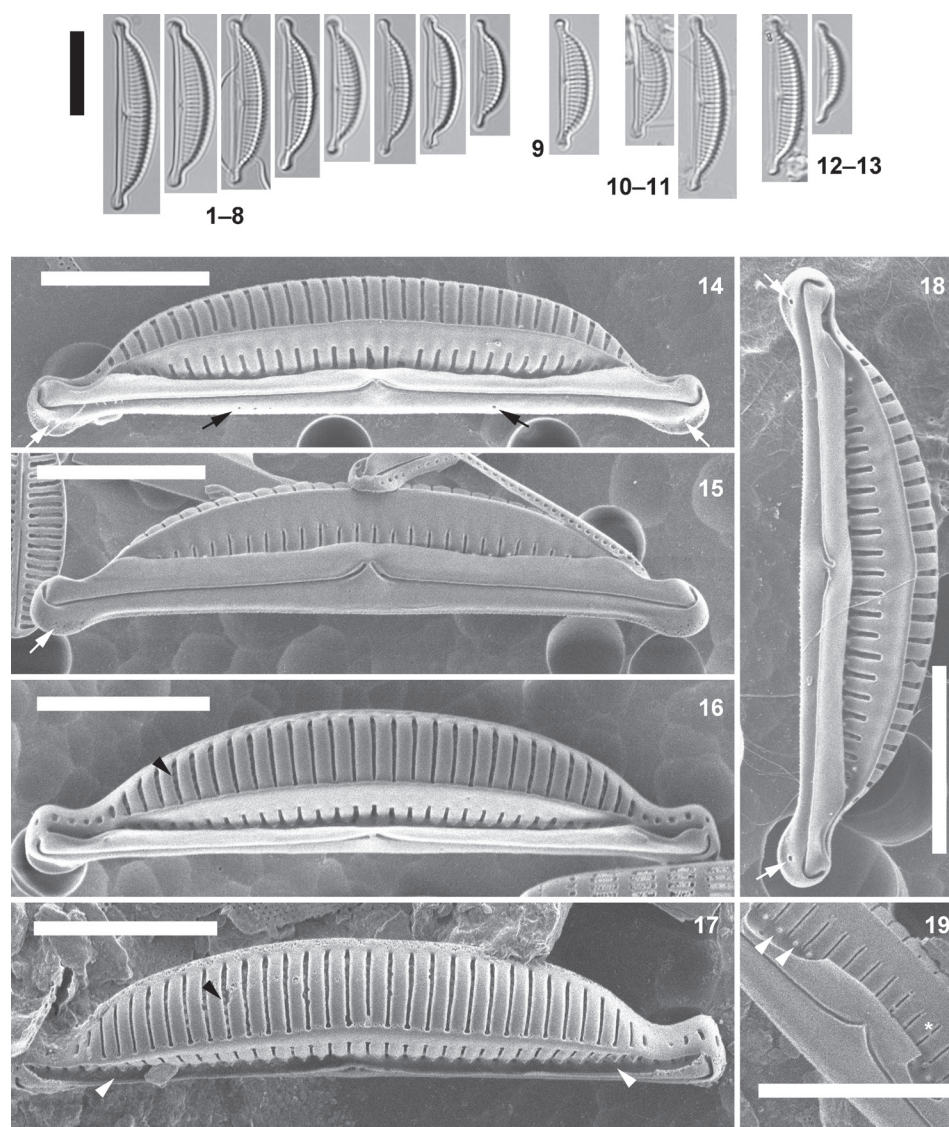
Valves from the European populations are illustrated here in LM on Pl. 1 (Figs. 1–13) and in SEM on Pl. 1 (Fig. 14–19) and Pl. 2 (Figs. 20–23). All valves observed in the samples from both the Adriatic Sea and the Black Sea fit well the type of *H. yundangensis* in Wu et al. (2020) in valve outline, being semi-elliptic to semi-lanceolate with capitate to rostrate protracted apices (Pl. 1: Figs. 1–13, Tab. 1 here;

Wu et al. 2020: Figs. 1–12). The European populations are, however, mostly on the smaller side of the range of dimensions given for the type population: length 11.0–28.0 µm and width 3.0–4.5 µm vs length 13.0–39.0 µm and width 3.5–5.5 µm in the type population (Tab. 1). The dorsal stria densities of the type population and the European populations are also comparable: 17–21 dorsal striae in 10 µm were reported for the type, and 18–22 dorsal striae in 10 µm were present in both the Adriatic Sea and the Black Sea populations (Tab. 1). No morphological differences could be found among the different populations from Europe and Asia in the external structure of the dorsal valve side and striae, dorsal marginal ridge, raphe ledge, external and internal central raphe endings and terminal fissures (Pl. 1: Figs. 14–19 here; Wu et al. 2020: Figs. 13, 17–20). Both the type of *H. yundangensis* from China and its European populations also present small papulae-like structures under the raphe ledge (Pl. 1: Figs. 17, 19, white arrows). The latter, although not mentioned in the original description of the species, are visible in the original illustrations of it (Wu et al. 2020: Figs. 15, 18).

Regarding the ventral striae, the European populations usually lack them (Pl.1: Figs. 15, 18), but occasionally possess a short row of small rounded areolae on the ventral side externally (Pl. 1: Fig. 14, black arrows) and “ghost” striae internally (Pl. 2: Fig. 21). Based on this feature, they take an

Tab. 1. Morphological comparisons between the different populations of *Halamphora yundangensis*, and comparisons with *Halamphora coffeiformis* and other similar taxa present in the Mediterranean Region. Entries marked with an asterisk (*) represent author interpretations based on published figures.

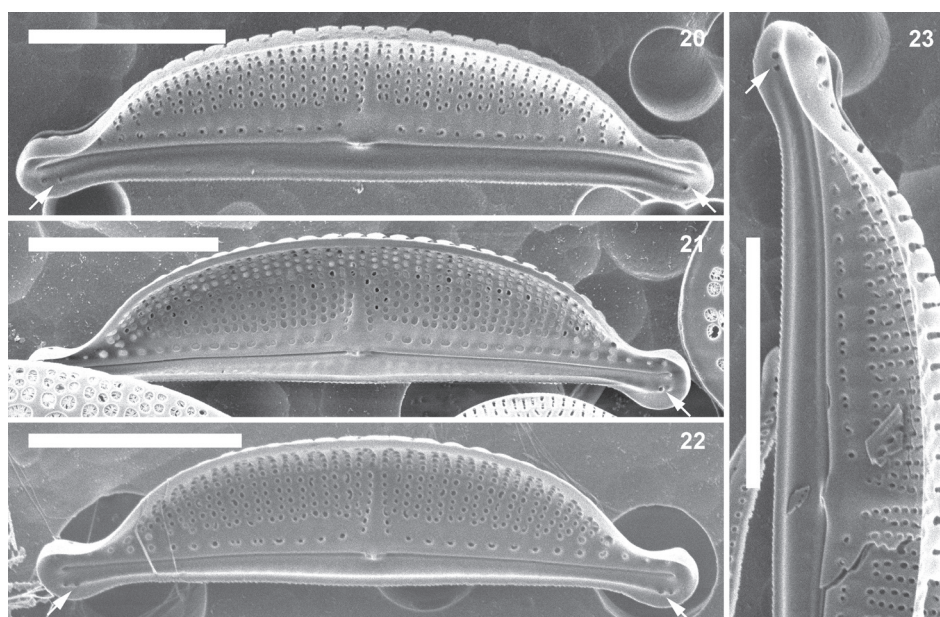
Feature/taxon	<i>H. yundangensis</i>	<i>H. yundangensis</i> (type)	<i>H. yundangensis</i> var. <i>foramina</i> (invalid)	<i>H. cejudoe</i> (type)	<i>H. coffeiformis</i> (type and other populations)	<i>H. tenuicostata</i> (type)
reference	this study	Wu et al. 2020	Huang et al. 2023b	Álvarez-Blanco and Blanco 2014	Archibald and Schoeman 1984, Levkov 2009, Stepanek and Kociolek 2018 (all as <i>coffeaeformis</i>)	Stepanek and Kociolek 2018 (as <i>tenuicostata</i>)
valve outline	semi-elliptic to semi-lanceolate	semi-elliptic to semi-lanceolate	semi-lanceolate*	semi-lanceolate	semi-lanceolate	semi-elliptic
valve apices	protracted capitate to rostrate	protracted capitate to rostrate	protracted capitate*	subcapitate	protracted capitate, rostrate to subrostrate	protracted capitate to subcapitate
valve length, µm	11.0–28.0	13.0–39.5	12.0–16.2	16.0–33.0	14.0–55.0	23.0–30.0
valve width, µm	3.0–4.5	3.5–5.5	3.7–4.3	3.8–5.5	3.5–7.5	4.5–5.0
dorsal striae in 10 µm	18–22	17–21	21–24	16–19	(16)17–24(26)	17–19
slits dorsally externally	present	present	present*	present*	absent	absent
dorsal striae internally	biseriate	biseriate	biseriate*	biseriate	biseriate	biseriate
ventral striae in 10 µm	usually absent, “ghost” striae (if present) ca 30	absent	27–28	20–22	21–36	30–31
dorsal marginal ridge	wide	wide	wide*	wide*	thin	thin
poroids at the apices	present, 1–2	present, 1–3	present*	absent	absent	absent
semi-stauros	present	n/a	n/a	absent	absent	absent
axial silica band	present	present*	present*	absent	absent	present



Pl. 1. LM views (Figs. 1–13) and SEM external views (Figs. 14–19) of *Halamphora yundangensis* from the Adriatic Sea and the Black Sea: 1–8 – LM views of valves observed on the carapace of a loggerhead sea turtle (Adriatic Sea, sample TB225, slide BR-4915). 9–11 – LM views of valves from samples from macroalgae off the coast of Verudela, Adriatic Sea (Fig. 9 from sample ENV10, s.n., Figs. 10–11 from sample ENV14, s.n.). 12–13 – LM views of two valves from artificial substrates at Sozopol Bay (Black Sea, sample C3.3, slide BR-4880). 14–15 – SEM external views valves observed on the carapace of a loggerhead sea turtle (Adriatic Sea, sample TB225, stub BR-4916). White arrows on Figs. 14–15 indicate the poroids at the apices, black arrows on Fig. 14 indicate the several small areolae on the ventral side. 16–17 – SEM external views of the mantle of valves from the Adriatic Sea (Fig. 16, stub BR-4916) and the Black Sea (Fig. 17, stub BR-4881). Black arrowheads on Figs. 16–17 show the external openings of the areolae inside the slits, white arrowheads on Fig. 17 indicate the papulae-like projections under the raphe ledge. 18 – SEM external view of a valve from the thalli of red calcifying algae (Adriatic Sea, sample ENV14, stub BR-4873). 19 – SEM detail from the central part of a valve from the Black Sea externally showing the dorsally bent central raphe endings (sample C3.3, stub BR-4881). White arrowheads on Fig. 19 indicate the papulae-like projections under the raphe ledge, asterisk points to the occasional interruption of the slits. Scale bars = 10 µm (Figs. 1–13) and 5 µm (Figs. 14–19). LM – Light microscopy, SEM – Scanning electron microscopy.

intermediate position between the type population lacking any ventral striae (Wu et al. 2020), and the second population from China that was recognised by Huang et al. (2023a) as a variety of *H. yundangensis* due to the presence of ventral striae. Further, the ventral striae in the valves reported as *H. yundangensis* var. *foramina* by Huang et al. (2023a) from China, are represented by a single row of small areolae on the ventral side, ranging around 27–28 in 10 µm (Huang et al. 2023b). This number is comparable to the number of the “ghost” striae (ca 30 in 10 µm) present on the ventral side of

the valves internally in the European populations (Pl. 2: Fig. 21, Tab.1). Therefore, in our opinion, the valves recognised as *H. yundangensis* var. *foramina* by Huang et al. (2023a, b) should be considered to be *H. yundangensis*. Moreover, the valve dimensions given by Huang et al. (2023b) for their variety (length 12.0–16.2 µm, width 3.7–4.3 µm) are also in the range of those of the European populations (length 11.0–28.0 µm, width 3.0–4.5 µm), and of the type population of *H. yundangensis* (length 13.0–39.0 µm, width 3.5–4.5 µm) (Tab. 1). Dorsal striae in all populations are also similarly



Pl. 2. SEM internal views of *Halamphora yundangensis* (Figs. 20–23) from populations on a loggerhead sea turtle's carapace (Figs. 20, 23, Adriatic Sea, sample TB225, stub BR-4916) and on macroalgae (Figs. 21, 22, Adriatic Sea, samples ENV10, s.n., and ENV14, stub BR-4873, respectively). White arrows indicate the poroids at the apices. Scale bars = 5 µm. SEM – Scanning electron microscopy.

spaced (17–21 in 10 µm in the type in Wu et al. 2020; 18–22 in 10 µm in the European populations reported here), however, they are slightly denser in the small valves presenting ventral striae, 21–24 in 10 µm (Huang et al. 2023b) (Tab. 1).

Minor differences were observed among the populations in the inner structure of the valves. All populations have biseriate striae internally, but in the type population from China the biseriate dorsal striae internally are composed of smaller areolae (Wu et al. 2020: Figs. 21–25), whereas the European populations show larger areolae with narrower virgae internally (Pl. 2: Figs. 20–23). All populations also have an axial silica band internally, but the areolae separated by the axial silica band near the raphe internally are usually larger in the European populations (Pl. 2: Figs. 20–23 here; Wu et al. 2020: Figs. 21, 25). In the European populations, sometimes, two small adjacent areolae are present in this row (Pl. 2: Fig. 23), whereas all valves illustrated from the type population from China have a single areola along the entire length of the row (Wu et al. 2020: Figs. 21–25). Wu et al. (2020) also did not mention a thickening in the central part of the valves internally (see the central part of the valves from the European populations on Pl. 2: Figs. 20–23). However, in the Chinese population areolae of the first striae near the valve center are similarly more spaced (Wu et al. 2020: Figs. 21, 24), as in the European populations (Pl. 2: Figs. 21–23), suggesting that a small thickening is present. Finally, in the European populations the first rows of areolae at the valve center internally are sometimes longer (Pl. 2: Figs. 21, 22) than those of the type population (Wu et al. 2020: Figs. 21, 24). Nevertheless, at present, we do not find the minor differences observed internally sufficient for the morphological separation of the European populations as another taxon.

Following the observations on the populations of *H. yundangensis* from the Adriatic Sea and the Black Sea and their comparisons with the type of the species in Wu et al. (2020) and the second population from China (Huang et al. 2023a, b), below we provide an emended description of the species, including the following features: the slightly larger range of valve dimensions and dorsal stria numbers; the occasional presence of ventral striae externally and “ghost” striae on the ventral side internally; the papulae like-structures under the raphe ledge; the semi-stauros-like thickening in the middle of the dorsal side of the valves internally. In addition, we further discuss the dorsal slit-like stria structure of the species.

***Halamphora yundangensis* W.W.Wu, C.P.Chen & Y.H.Gao emend. Pottiez, Zidarova, P. Ivanov, Kanjer & Bosak**

References: Wu et al. (2020: p. 210, Figs. 1–25), Huang et al. (2023a, b)

Pl. 1 (Figs. 1–19) and Pl. 2 (Figs. 20–23)

Light microscopy description (Pl. 1: Figs. 1–13):

Valves semi-elliptic to semi-lanceolate. Dorsal margin with coarsely jagged outline (Pl. 1: Figs. 1–3, 11, 12), moderately convex, in the middle slightly flattened, forming small “shoulders” before the apices. Apices distinctly protracted capitate, in smaller valves protracted rostrate (Pl. 1: Fig. 13). Ventral margin straight, sometimes slightly inflated in the middle (Pl. 1: Figs. 1, 12). Valve dimensions: length 11.0–39.5 µm, 3.0–5.5 µm, up to 5.5 µm. Raphe very close to the ventral margin, straight to weakly arched. Central raphe endings slightly enlarged, bent to the dorsal side (Pl. 1: Figs. 4, 13). Raphe ledge as a thin line running parallel to

the raphe (Pl. 1: Figs. 2, 7). Dorsal striae not punctate, parallel in the middle, becoming slightly radiate towards the apices, usually ca 20 in 10 μm , but ranging 17–24. Dorsal marginal ridge as a thin line near the dorsal margin (Pl. 1: Figs. 1, 9). Ventral striae not visible.

Scanning electron microscopy description (Pl. 1: Figs. 14–19 and Pl. 2: Figs. 20–23):

Valves heavily silicified. Externally, dorsal valve face flat, crossed by thin transapical slits, occasionally interrupted (Pl. 1: Fig. 19, asterisk). On the valve face, slits parallel in the middle, radiate at the apices (Pl. 1: Figs. 14, 15). A prominent, wide dorsal marginal ridge present at the valve face/mantle junction (Pl. 1: Figs. 15, 18), interrupting the slits that continue onto the mantle (Pl. 1: Figs. 16, 17). Raphe ledge moderately wide, following the valve outline: slightly expanded in the valve middle, at the constriction at apices pinched, at the apices once again widened, and not narrowed at the ends (Pl. 1: Figs. 15, 18). Under the edge of the raphe ledge, on the virgae between the slits, small papulae-like structures present (Pl. 1: Figs. 17, 19, white arrowheads). Raphe almost straight. External central raphe endings bent towards the dorsal side, closely positioned (Pl. 1: Figs. 15, 19). External distal fissures strongly hooked to the dorsal side, slightly enlarged (Pl. 1: Fig. 18), finishing under the end of the raphe ledge (Pl. 1: Figs. 16, 17). Internally (Pl. 2: Figs. 20–23), valves with thickened central part into a weakly developed semi-stauros dorsally, separating the dorsal striae in the valve middle. Internal central raphe endings finishing onto small, protruded, fused central helictoglossae (Pl. 2: Figs. 20–23). Distal raphe endings internally terminating onto small helictoglossae. Dorsal striae clearly biseriate, composed of two rows of rounded areolae internally (Pl. 2: Figs. 20–23). Externally, areolae discretely open into the slits' foramina (Pl. 1: Figs. 16, 17, black arrowheads). Ventral striae usually absent externally (Pl. 1: Figs. 15, 18, 19), occasionally, a row of a few, irregularly spaced, rounded areolae present on the ventral side (Pl. 1: Fig. 14, black arrows), areolae 27–28 in 10 μm . Internally, ventral striae usually absent, but "ghost" striae possibly present on the ventral side, ca 30 in 10 μm (Pl. 2: Fig. 21). Axial silica band running along the length of the valve internally, near the raphe separating a row of single (Pl. 2: Figs. 20, 21), or of a series of two adjacent, rounded to elliptic areolae (Pl. 2: Fig. 23). Row of areolae near the raphe interrupted in the valve center (Pl. 2: Figs. 20–23). One to three poroids with rounded external and internal openings present at the apices (Pl. 1: Figs. 14, 18 and Pl. 2: 20–23, white arrows). Occasionally, poroids at the apices with very small openings externally, only faintly visible (Pl. 1: Fig. 15, white arrow).

Ecology and associated diatom flora:

The largest population of *H. yundangensis* in the studied area was found on the carapace of an injured loggerhead sea turtle admitted for recovery at the Sea Turtle Rescue Center of Aquarium Pula in summer (sample TB225). The turtle had been residing for a month inside an individual recovery

tank when the sample containing *H. yundangensis* was taken. The sea water in the tank, as measured upon sampling, was weakly alkaline (pH of 7.9) with a temperature of 21.5 °C and salinity of 34.3. The sample was dominated by *Halamphora kolbei* (Aleem) Álvarez-Blanco & S. Blanco and *Tabularia* spp., and contained several other *Halamphora* taxa, such as *H. bosakiana* Pottiez, M. de Haan & Zidarova, *H. capitata* (R. Hagelstein) Álvarez-Blanco & S. Blanco, and *H. cf. isumiensis* J. Stepanek, Mayama & Kociolek. Small populations of *H. yundangensis* were also present during winter in the Northern Adriatic Sea, where it was living epiphytically on various macroalgae, including calcifying red seaweeds. In the Black Sea, the species was observed only occasionally in the biofilm formed on plexiglass substrates during summer months near the western coast of the sea. There it was accompanied by various small-celled *Halamphora* and *Nitzschia* Hassall taxa. The measured environmental parameters at the sampling site in the Black Sea during the sampling period showed weakly alkaline waters (mean pH 8.3) with a temperature of ca 26 °C and salinity of 17–18 (Zidarova et al. 2023, see Tab. 1 therein).

Discussion

With its dorsal valve side structure and the usual absence of ventral striae, *Halamphora yundangensis* is difficult to confuse in SEM. The absence of ventral striae is uncommon in amphoroid diatoms, but it has been observed in some *Amphora* Ehrenberg ex Kützinger taxa, such as *Amphora aliformis* J. Stepanek, Mayam & Kociolek (Stepanek et al. 2015). According to Stepanek et al. (2015), in *A. aliformis* ventral virgae are initially formed during early valve development but are later filled. This may also be the case in *Halamphora yundangensis*, considering the "ghost" striae observed internally (Pl. 2: Fig. 21), as well as the occasional row of poroids ornamenting the ventral surface in some valves externally (Pl. 1: Fig. 14 and Huang et al. 2023a, b).

Along with the usual absence of ventral striae, the presence of one or a few poroids at the apices is another unusual feature of *H. yundangensis* (Pl. 1: Figs. 14, 18 and Pl. 2: 20–23, white arrows here; Wu et al., 2020: Figs. 20, 22–23 therein). To our knowledge, in the genus *Halamphora* such apertures on the ventral side of the valves had not been reported in any other taxon. Apertures at the apices, known as portulae, had been observed particularly in *Amphora micrometra* Giffen, a species doubtfully positioned within the genus *Amphora* (Ács et al. 2011, Zidarova et al. 2023). More recently, valves presenting portulae at the apices were identified by López-Fuerte et al. (2024) as *Halamphora adumbratoides* J. Stepanek & Kociolek. The latter has a morphology very similar to that of *A. micrometra*, but was originally described as a species without any portulae (Stepanek and Kociolek 2018). However, the portulae in the populations identified as either *A. micrometra* or *H. adumbratoides* are located on the dorsal side of the valves, contrary to the poroids in *H. yundangensis*, located on the ventral side of the valves. Among amphoroid diatoms, two

Amphora species, *A. aliformis* (in Stepanek et al. 2015) and *A. commutata* Grunow (in Sato et al. 2013), present pores at the apices on the ventral side. In both taxa these apertures are occluded internally by sieve plates (Stepanek et al. 2015). We did not observe any occlusions in *H. yundangensis* (Pl. 2: Figs. 20–23). The former two *Amphora* taxa also show unique structures among other amphoroid diatoms, and we doubt that there is any relation between these taxa and *H. yundangensis*, even if they present pores at the apices ventrally.

Wu et al. (2020) provided a comprehensive comparison of *H. yundangensis* with a number of amphoroid taxa from the genera *Amphora* and *Halamphora*. They found the dorsal structure of its valves similar to that of several *Amphora* taxa bearing slits on the valve face and mantle, including *A. ovalis* (Kützing) Kützing, *A. eximia* J.R.Carter, *A. pseudoeximia* Levkov, and *A. mongolica* Østrup. However, as noted by Wu et al. (2020: p. 215), all these taxa also have a narrower dorsal marginal ridge and uniseriate, not biseriate, striae internally. Therefore, their structure is not exactly homologous to that of *H. yundangensis*. Based on our observations on the European populations of *H. yundangensis* in SEM and the illustrations in Wu et al. (2020), the striae externally in *H. yundangensis* are not simple slits, but they have a rather complex structure. The slits seem to be formed in between bold, thickened virgae on the valve face and mantle, which, with their edges, shade the openings of areolae externally (Pl. 1: Figs. 16, 17, black arrowheads). Virgae merge at the dorsal margin into the broad marginal ridge, and then continue onto the mantle (Pl. 1: Figs. 16–18). This way, the valves of *H. yundangensis* externally look like double-layered thick “shells” with slits. Regarding the dorsal marginal ridge and external dorsal stria structure, *H. yundangensis* is very similar to *H. cejudoe*, a taxon discovered in the western Mediterranean Sea near El Mojón, Murcia, Spain (Álvarez-Blanco and Blanco 2014). As in *H. yundangensis*, its dorsal valve side possesses a wide, distinct dorsal marginal ridge, slits on the valve face and on the valve mantle externally, and biseriate striae internally (Álvarez-Blanco and Blanco 2014: Pl. 79, Figs. 1–5). Areolae of its biseriate striae are sometimes also visible externally, located just under the edge of the slits (Álvarez-Blanco and Blanco 2014: Pl. 79, Figs. 3, 4), as in *H. yundangensis* (e.g. Pl. 1: Figs. 16, 17 here; Wu et al. 2020: Fig. 18).

Halamphora cejudoe and *H. yundangensis* have similar valve outlines, and they also have comparable dimensions: length 16.0–33.0 µm and width 3.8–5.5 in *H. cejudoe*, overlapping with the range in *H. yundangensis* from all known populations (length 11.0–39.0 µm, width 3.0–5.5) (Tab. 1). They both also have comparable dorsal stria numbers, however, striae are denser in *H. yundangensis* (17–24 in 10 µm in all populations of *H. yundangensis*, vs 16–19 in *H. cejudoe*, Tab. 1). In LM, these two taxa can be separated by: 1) the valve apices, that are distinctly protracted capitate or rostrate in *H. yundangensis*, but only weakly, smoothly protracted subcapitate in *H. cejudoe*, and 2) the always present ventral striae, only 20–22 in 10 µm in *H. cejudoe*, vs 27–30 in 10 µm,

if present, in *H. yundangensis* (Tab. 1). In SEM, the two taxa are easily differentiated by: 1) the usually absent ventral striae and the presence of one or a few poroids at the apices in *H. yundangensis*, as well as by 2) the axial silica band internally, a feature present in *H. yundangensis*, but absent in *H. cejudoe* (Tab. 1). Further, unlike *H. yundangensis*, *H. cejudoe* lacks any semi-stauros or unilateral thickening in the central area internally, but possesses prominent virgae between the two rows of areolae and distinct, fused central helictoglossae (Álvarez-Blanco and Blanco 2014: Pl. 79, Figs. 1, 5).

Halamphora coffeiformis, a species with biseriate dorsal striae composed of small rounded poroids, as defined in Archibald and Schoeman (1984) and later in Levkov (2009) and Stepanek and Kociolek (2018), is only slightly similar to *H. yundangensis*. The similarities between them include the valve outline, and their range of dimensions and dorsal stria density (Tab. 1). In LM, *Halamphora coffeiformis* can be differentiated by its smoothly arched dorsal margin lacking “shoulders” before the apices, as well as by the arched raphe (Archibald and Schoeman 1984: Figs. 1–34, 45, 46, 49, 50, 61–66, Levkov 2009: Pl. 94, Figs. 20–27, Pl. 99, Figs. 15–23, Stepanek and Kociolek 2018: Pl. 45, Figs. 1–8). In SEM, due to the complex structure of *H. yundangensis*, the two taxa cannot be confused.

In LM, in valve outline, dimensions and dorsal stria numbers, *H. yundangensis* is also comparable to *H. tenuicostata* J. Stepanek & Kociolek (2018: p. 42, as *tenuicostata*) (Tab. 1), a species described from Florida, USA, and recently also recorded in the Adriatic Sea (Zidarova et al. 2025). The main difference in LM between the two taxa is the more smoothly arched dorsal valve margin in *H. tenuicostata* and its visible ventral striae in LM (Stepanek and Kociolek 2018: Pl. 45, Figs. 9–12). Finally, due to the robust dorsal structure, the dorsal margin of *H. yundangensis* often appears distinctly and coarsely jagged in LM (Pl. 1: Figs. 1–3). A coarsely jagged dorsal margin in LM is also present in the similarly structured *H. cejudoe* (Álvarez-Blanco and Blanco 2014: Pl. 34, Figs. 3, 6), but not in *H. coffeiformis* and *H. tenuicostata* (e.g. Stepanek and Kociolek 2018: Figs. 1–8 and 9–12).

With the small number of records of *H. yundangensis* so far, little is known about its ecology. Originally, *H. yundangensis* was described from circumneutral to slightly alkaline waters with a temperature of ca 15 °C and moderately high salinity (30–31, Wu et al. 2020). Its presence in the more saline waters of the Adriatic Sea during winter, as well as in the less saline warm waters of the Black Sea during summer, suggests that it has broader temperature and salinity tolerances. In conclusion, *H. yundangensis* is a brackish/marine diatom species, present year-round in the temperate zones.

The currently known and verified distribution of *H. yundangensis* includes isolated locations in two quite distantly located regions, the southern Chinese coasts and the Mediterranean region. The records of this taxon from Indonesia (Risjani et al. 2021) and the Eastern Pacific Ocean (Siqueiros Beltrones et al. 2021) suggest it may have a wider

distribution, but these records still need to be verified. It is at present unclear if the species is indeed native to China and whether it has been transported among the different distant locations. Little is known about the dispersal capabilities of marine benthic diatoms and the vectors for their transportation between continents, such as ship transport or ship maintenance procedures, for instance. The recent discovery and establishment of the Pacific large-celled diatom species *Diademoides luxuriosa* (Greville) K.-D. Kemp & Paddock on the well-studied coastline at Edinburgh, Scotland, indicate that the long-distance dispersal and new introduction of marine benthic diatom species is possible (Mann 2025). *Halumphora* species, in particular, were reported as excellent biofoulers and dominant diatom species on ships' hulls (Park et al. 2025 and references therein). Experimentally, Park et al. (2025) also demonstrated that some *Halumphora* species present in unfiltered water from in-water hull cleaning can remain viable for at least 21 days, and once released into the water column, they may reattach to substrates in the new environment. However, there is no evidence that this is the case with *H. yundangensis*. In the Black Sea, the species was found near a small local port accommodating mostly small pleasure craft and local fishing boats that do not cover large distances. There are no specific ship maintenance services at the port, apart from basic pleasure craft maintenance (RZ, *pers. observ.*). Loggerhead sea turtles, on whose carapaces the largest population of *H. yundangensis* was observed in the Adriatic Sea, could serve as a possible vector for diatom species dispersal (Van de Vijver et al. 2020). In the Black Sea, however, loggerhead sea turtles are extremely rarely seen, though the number of records seems to increase (see Zinenko et al. 2021 for summary of the data). Nevertheless, the presence of *H. yundangensis* in samples collected during two different seasons from different localities and substrates in the Adriatic Sea and the Black Sea shows that the species is established there, and it could have been long present, but overlooked or misidentified.

Finally, most of the species are usually described based on a single population from a single locality of a given region. When species are only recently described, as in the case of *H. yundangensis*, until enough data for their distribution is collected, we can only make assumptions about their distributional range. The comparatively smaller number of studies on marine benthic diatoms, the still small knowledge of marine benthic *Halumphora* taxa and the lack of recently developed regional diatom floras hinders taxa identification. This poses a risk of incorrect species assignments and distributional records. Perhaps the best example is *H. coffeiformis*, but many other small *Halumphora* taxa can be easily misidentified in the lack of comprehensive regional guides, or due to technical insufficiencies of the studies. For the species of the genus *Halumphora*, the use of adequate techniques for their study, such as combining light and scanning electron microscopy (e.g. Levkov 2009, Stepanek and Kociolek 2018, Zidarova et al. 2025, Pottiez et al. 2025), and whenever possible adding molecular data

(Stepanek and Kociolek 2018), is crucial for their correct identification, and for understanding their distribution and dispersal.

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Author contribution statement

M.P. – LM and SEM analyses, writing, review, R.Z. – funding, sample collection, LM analyses, writing, review, P.I. – sample collection, editing, review, L.K. – sample collection, editing, review, S.B. – funding, sample collection, editing, review.

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Research Article

Remarks on *Acalypha rhomboidea* in Europe, with a fifth record at the continental level

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Abstract – *Acalypha* is a genus native to tropical and subtropical regions of the world and eastern North America and Asia, with some species considered as aliens in Eurasia. The European flora is reported to include four species, all occurring in Italy according to Plant of the World Online database. As part of the ongoing studies of the flora of Treviso Province (Veneto region, north-eastern Italy), we came across to a population of *A. rhomboidea* Raf. which represents not only the first record for Veneto and the second one for the Italian flora, but also the fifth discovery of the species out of its native range; a description of the species (the first one for Italy), ecological remarks, and a map of the European occurrences are provided.

Keywords: *Acalypha persimilis*, *Acalypha rhomboidea*, *Acalypha virginica*, Europe, exotic species, Italy, new record

Introduction

Acalypha L. (Euphorbiaceae Juss.) is a genus comprising 429 species naturally distributed across tropical and subtropical regions of the world as well as eastern North America and Asia; some species are considered aliens in Europe, Caucasus, Turkey, Tadjikistan, and west Siberia (POWO 2025).

According to POWO (2025), the flora of Europe includes four *Acalypha* species, i.e. *A. australis* L., *A. persimilis* Müll. Arg., *A. rhomboidea* Raf. and *A. virginica* L. A proposal to conserve the name *A. ostryifolia* Riddell ex J.M. Coult. (published in 1894) against *A. persimilis* (published in 1865) has been made by Levin et al. (2025) to preserve a well-established name (*A. persimilis*) that has been in use for almost two centuries (so following Recommendation 14A of *Madrid Code*; Turland et al. 2025); we will refer to this species hereafter as *A. ostryifolia*.

During field surveys in Treviso Province (Veneto region, north-eastern Italy) to investigate the flora of this area, we discovered a population of *A. rhomboidea*, representing not only the first one for Veneto and the second one for Italy but also the fifth record of the species out of its native range, after De Almeida and Costa Mato (2006) for Portugal, Di Pietro et al. (2021) for Italy, Borovečki-Voska et al. (2023) for Croatia, and Sirbu (2023) for Romania. In Italy, three additional species have been recorded, i.e. *A. australis*, *A. virginica* and *A. ostryifolia* (see Del Guacchio 2005, Portal to the Flora of Italy 2025). Since there are difficulties in the

identification of *Acalypha* species as well as taxonomic difficulties in the genus as a whole (see e.g., Levin 2016, Levin et al. 2022, Martínez-Gordillo et al. 2022), we also investigate Rafinesque's name in order to verify and clarify its identity.

Material and methods

The present research was based on personal field investigations in Veneto during the spring and summer of 2024, and examination of exsiccata kept in Herbaria BM, BR, CLF, GH, HFLA, LINN, LZ, P, PH, PI, RO, and W (*sensu* Thiers 2026). Relevant literature (protologues included) was also analyzed.

The description of the species is based on observed plants in field and the alien status follows Pyšek et al. (2004).

The articles cited through the text follow the International Code of Nomenclature for algae, fungi, and plants (Turland et al. 2025, hereafter reported as "ICN").

Results

Acalypha rhomboidea Raf., New Fl. [Rafinesque] 1: 45. 1836 ≡ *Acalypha virginica* var. *rhomboidea* (Raf.) Cooperr. in Michigan Bot. 23: 165. 1984.

Lectotype (designated by Weatherby 1940: 46): U.S.A., South Carolina [labelled by Rafinesque] (G, *non vidi fide* Weatherby 1940: 46).

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Description: Herbs, annual, 1.5–6 dm, monoecious. Stems usually erect, sometimes ascending, usually sparsely pubescent, rarely sparsely hirsute. Leaves: petiole 0.5–8.1 cm; blade ovate to rhombic, 2.1–9.5 × 0.8–4.5 cm, base attenuate, margins crenate to serrate, apex acute to slightly acuminate. Inflorescences bisexual, axillary; peduncle 0.1–0.55 cm, pistillate portion 0.7–1.5 × 1–2 cm or pistillate bract solitary, staminate portion 0.2–1 cm; allomorphic pistillate flowers absent. Pistillate bracts loosely arranged to crowded (inflorescence axis scarcely visible between bracts), 6–15 × 9–23 mm, abaxial surface sparsely pubescent and stipitate-glandular; lobes 6–8, lanceolate to triangular, 1/3–2/3 bract length. Pistillate flowers: pistil 3-carpellate; styles multifid or lacinate. Capsules pubescent. Seeds 1.5–1.7 mm, minutely pitted.

Flowering and fruiting times: June to September.

Native distribution area: *Acalypha rhomboidea* is a species native to eastern Canada and central-eastern United States of America (Levin 2016, POWO 2025).

Occurrence of *Acalypha rhomboidea* in Europe:

Acalypha rhomboidea has been recorded only three times outside of North America, all in European sites (Fig. 1):

> Portugal (De Almeida and Costa Mato 2006): introduced by culture in the Botanical Garden of Coimbra, from which it has escaped, becoming a naturalized alien weed in ruderal communities of gardens and roadsides (70 m a.s.l.).

The species is considered naturalized. First introduction: October 2005;

> Italy (Di Pietro et al. 2021): inside and in outer parts of the nursery garden of Aurunci Mountains Natural Park (not there cultivated) at 215 m a.s.l. The species is considered naturalized. First introduction: October 2017;

> Croatia (Borovečki-Voska et al. 2023): in two sites on north-Croatia, i.e. arable land in Karlovac Municipality (at 111 m a.s.l.) and on a gravelled parking in Hrvatsko zagorje (Kostel) at 288 m a.s.l. No specific indication of the status of naturalization was reported by Borovečki-Voska et al. (2023), but the authors stated that “... the plants in Karlovac are numerous and robust, while those in Kostel are stunted. The abundance in the former locality [Karlovac] might indicate that the plants have been present there for a longer time.”; so, we can consider the species as casual in Kostel and naturalized in Karlovac. First introduction at Kostel: October 2022.

> Romania (Sirbu 2023): in the central area of Iași city (north-eastern Romania), as a weed in a garden of ornamental shrubs and trees (50–60 m a.s.l.). The species is considered naturalized. First introduction: August 2021.

The population found in the present research (Fig. 2) grows along banks of the river Sile (not far from the city of Treviso, Veneto region, northeastern Italy); it is represented by about 100 individuals, which occupy an area of about 4 m². *Acalypha rhomboidea* is strictly associated with *Persicaria lapathifolia* (L.) Delabre s.l. which is dominant in the plant

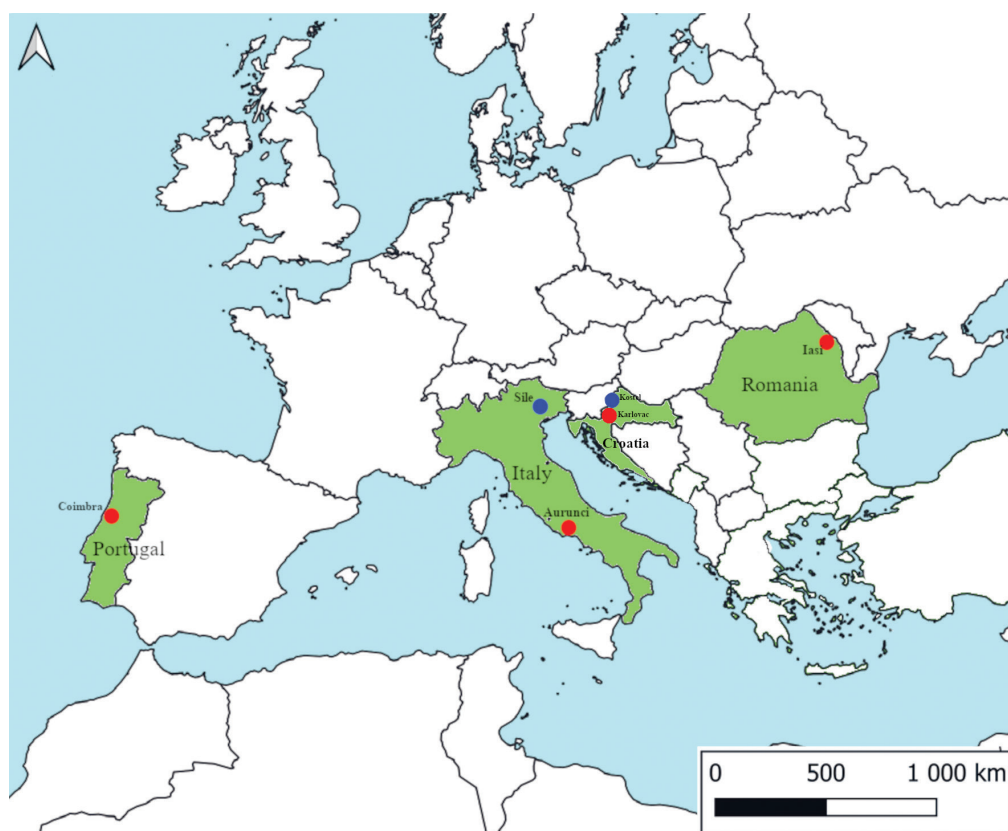


Fig. 1. Distribution map of *Acalypha rhomboidea* Raf. (Euphorbiaceae Juss.) in Europe. Symbols: red dots = naturalized populations (previous reports); blue dot = casual population (new report).



Fig. 2. Habitat where *Acalypha rhomboidea* Raf. (Euphorbiaceae Juss.) grows along river Sile (Treviso Municipality, Veneto region, north-eastern Italy). A – leaf, B – bract of pistillate flower.

community (alliance *Bidenton tripartitae* Nordhagen 1940 ex Klika et Hadač 1944, according to Biondi and Blasi 2015). A further common species is *Paspalum dilatatum* Poir., an invasive alien in Veneto region, but in general throughout Italy (excluding Val d'Aosta where it is absent) (Portal to the Flora of Italy 2025). Finally, some individuals of *Convolvulus sepium* L., *Mentha longifolia* (L.) L., and *Rubus caesius* L. occur.

The occurrence of this population does not appear to be linked to *in situ* human activities, while the river plays clearly a fundamental role as pathway of introduction of the species, as highlighted by the first author (DI) for other aliens growing along the banks (see e.g., Iamónico et al. 2010, Ceschin et al. 2016, Iamónico 2021, Iamónico and Nicoletta 2023, 2024). Despite this and the high number of individuals found in a restricted area (see above), we were not able to verify its occurrence during the years. Consequently, we prefer to consider *A. rhomboidea* a casual alien in Veneto. Our discovery is not only the first one at the regional level and for northern Italy, but also the second record for Italy and the fourth one out of its native range (Fig. 1).

Typification of the name *Acalypha rhomboidea*: *A. rhomboidea* was firstly described by Rafinesque (1836: 45) in the 1st volume of his *New flora of North America* where a short description was given; the provenance was also reported as “Pennsylvania, to Ohio, Alleghanies &c.” and two figures (part of the original material) were cited. The first was “Autikon Raf. 24.” This refers to Rafinesque’s (1840) *Autikon Botanikon*, where the author included only 1500 of his proposed 2500 species, but no actual illustrations were prepared (Warren 2004: 180). Thus, illustration no. 24 by Rafinesque does not exist. The second cited figure was “Lamarck tab 780,” which refers to Lamarck’s (1823) *Tableau encyclopédique et méthodique des trois règnes de la nature. Botanique* where, however, the *Acalypha* illustration is no.

789 (image available at <https://www.biodiversitylibrary.org/page/33443015>), not “780” as reported by Rafinesque (1836: 45; probably an orthographic error). Note that Lamarck’s *Tabula* 789 includes two taxa, i.e. *A. spiciflora* Burm.f. (no. 1 under *Acalypha* in Rafinesque’s work) and *A. virginica* L. (no. 2 plus magnifications “f-p”). The Burman name is currently recognized as a distinct species of *Claoxylon* A.Juss. [as *C. spiciflorum* (Burm.f.) A.Juss; see Montero Muñoz et al. (2022)]. Furthermore, Rafinesque (1836: 45) stated that *A. rhomboidea* is the same as *A. caroliniana* Walter cited by “... Michaux and all our Botanists except Walter and Elliot”. In other words, Rafinesque (1836: 45) reconsidered most of the citations of *A. caroliniana* (e.g. that by Michaux [1803: 216] in his *Flora Boreali-Americana*), finding them different from *A. caroliniana* as originally published by Walter (1788: 238).

According to Stafleu and Cowan (1983) and HUH-Index of Botanists (2013), Rafinesque’s herbarium and types are mainly preserved at P (duplicates in some European and American herbaria). No specimen useful for the lectotypification purpose was traced at P. However, Weatherby (1940: 96) briefly discussed some of Rafinesque’s names in *Acalypha* and proposed a lectotype for *A. rhomboidea*, choosing a Rafinesque specimen deposited at G.

Selected specimens examined: *Acalypha rhomboidea*.

CANADA: Québec, Sherbrooke, mauvaise herbe dans mon Jardin, 22 August 1974, *A. Legault* 9635 (P04786297); Québec, La Trappe, comté Deux-Montagnes, vieux paturages, 7 September 1955, *P. Louise-Marie s.n.* (CLF093404). ITALY: Lazio, Itri municipality, 41°18'16" N, 13°31'59" E, 215 m, inside and in outer parts of nursery garden of Aurunci Mountains Natural Park (where *Acalypha rhomboidea* is not cultivated), 2 October 2018, *M. R. Perna s.n.* (HFLA); Veneto region, Treviso Province, banks of the river Sile, 4 m, 27 July 2024, *M. Crosato s.n.* (RO). U.S.A.: Pennsylvania, Beaver, 1 mile E of Route 30 bridge over Raccoon Creek, 11 September 1951, *M. Henrici s.n.* (PH00456890); North Carolina, Gaston, 4.1 miles east of Gastonia just north of U.S. 29-74 on Ranlo Road, wooded stream, 17 September 1956, *H. E. Ahles* 18864 (P05547201); Massachusetts, Hampshire, Mt. Horse, West Hatfield, disturbed areas, 20 August 1975, *H. E. Ahles* 81274 (P05547200); Pennsylvania, Berks, Hopewell Furnace National Historic Site, S of furnace near S side of French Creek, edge of woods, 13 September 1986, *A. E. Schuyler* 6794 (PH00459471); Connecticut, Fairfield, Newtown, 5 August 2010, *M. A. Vincent & M. W. Vincent* 15141 (GH01069425); Massachusetts, Duke, Thimble Farm, Island Grown Initiative, path between moist woods and farmland on south side of property, 25 September 2019, *G. Palermo & M. Curtin* 19-227 (PHARB00993).

***Acalypha virginica*.** ITALY: Italy. Veneto. Venetia. Patavium (Padova), in marginibus herbosis fossarum prope Porta Ponte Corvo, 25 June 1908, *A. Béguinot s.n.* (PI032585); Veneto, Zenson del Piave, digue du fleuve, 15 September 1978, *S. Pignatti* 9411 (P05082481); Piemonte, Provinz Novara, zwischen Novara und Ticino, ca. 4 km E

Gallia, unmittelbar N der Autobahn, 145 m, 26 October 1996, R. Karl s.n. (W2016-0005483); Friuli-Venezia Giulia, Provinz Pordenone, ca. 3 km N Sacile und E Fiaschetti, Umgebung der Brücke über die Livenza, ca. 45 m, 02 September 2002, R. Karl s.n. (W2016-0005483). SLOVENIA: Slovenia. Primorska. Provinz Görz: unterhalb des Franziskanerklosters Castagnevizza bei Görz, am Wege nach Panowitz, July 1947, C. Zirnich 22095 (LZ227256). U.S.A.: Texas, Bowie County, near Texarkana, 25 August 1898, 300 feet, A. A. & E. Gertrude Heller 4151 (P04836907); Arkansas, old fallow bottom field near the Duck Pond, 380 ft., 23 October 1968, D. Demaree 59595 (P04786299).

Discussion

Acalypha rhomboidea has been often called *A. virginica* in North America, primarily due to controversy about the typification of that name (Levin 2016). Conservation of the type of *A. virginica* by Reveal et al. (1990) resolved the issue. These authors highlighted that Weatherby's (1927) proposed lectotype of *A. virginica* (a Kalm specimen, LINN no. 1139.1; image available at <https://linnean-online.org/11787/#?s=0&cv=0>) had been examined and circumscribed by Müller (1865) as the broad-leaved *A. virginica* var. *genuina*. On the other hand, Weatherby (1927) considered Müller's narrow-leaved *A. virginica* var. *intermedia* to be a distinct species, i.e., *Acalypha digyneia* Raf. Weatherby's choice was rejected by Mackenzie (1928), who lectotypified *A. virginica* on the specimen Clayton 201 (BM00038872; image of the lectotype available at <https://data.nhm.ac.uk/record/51e7a60c-cbda-4e88-8a68-ef93442643e6/1106/1595951462363>), while he referred the Kalm specimen to *A. rhomboidea*. After a detailed discussion, Reveal et al. (1990) reached the conclusion that Weatherby's lectotype of *A. virginica*, if maintained, would cause the application of broad-leaved plants to Linnaean species, placing *A. rhomboidea* in synonymy and taking up *A. digyneia* for what generally had been treated as *A. virginica*. Therefore, they proposed the conservation of *A. virginica* using Clayton 201, which represents the narrow-leaved plants (= *A. digyneia*; see e.g., POWO 2025). This nomenclatural problem, combined with the use of inappropriate characters to distinguish *A. rhomboidea* and *A. virginica*, has resulted in considerable confusion between these amply distinct species and the clarification of the identity of Rafinesque's *Acalypha* comes full the circle.

Based on literature data, as well as the examination of type material of *Acalyphainica* (lectotype BM00038872) and other specimens collected in both North America and Italy, our opinion is that these two species were (and are still) confused in Europe, as happened in America (see Levin 2016 and Reveal et al. 1990). Therefore, we wonder if at least some European collections of *A. virginica* are actually to be referred to *A. rhomboidea* and we suggest their identifications should be checked. The description given in the present paper is the first one for Italy and Europe [*A. rhomboidea* appeared neither in the recent 2nd edition of *Flora d'Italia* (Pignatti et al. 2017) nor in *Flora*

Europaea (Tutin 1968)] so it could be useful to verify European populations currently known as *A. virginica*.

A diagnostic key for *Acalypha* species occurring in Europe, is presented below:

1. Leaf bases cordate *Acalypha ostryfolia*
– Leaf bases cuneate 2
2. Pistillate bract lobes rounded, 1/20 as long as the bract
..... *Acalypha australis*
– Pistillate bract lobes triangular or lanceolate, at least 1/4 as long as the bract 3
3. Pistillate bract abaxial surface hirsute, lobes triangular, (9–)10–14(–16), 1/10–1/4 as long as the bract
..... *Acalypha virginica*
– Pistillate bract abaxial surface sparsely pubescent and stipitate-glandular, lobes lanceolate, 6–8, 1/3–2/3 times as long as the bract *Acalypha rhomboidea*

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Research Article

The genus *Carex* (Cyperaceae) in Kosovo

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Abstract – This study presents the first verified checklist of *Carex* taxa in Kosovo, based on field surveys (2022–2024), herbarium revisions and literature analyses. We recorded 52 species, including 19 subspecies and 1 variety, as well as 2 hybrids. Among them are 15 taxa newly reported for the flora of Kosovo. Most of the records come from the Sharri Mountains and the Albanian Alps of Kosovo, both national parks, although significant populations occur throughout the country. Several taxa are restricted to single localities, emphasising the urgent need for conservation efforts. These results refine the taxonomy and distribution of *Carex* in Kosovo and provide an important basis for future molecular and conservation studies in the Balkans.

Keywords: Balkans, *Carex*, checklist, conservation, endangered flora, Kosovo, taxonomy

Introduction

The Balkan Peninsula is a biodiversity hotspot and one of the richest floristic regions in Europe, characterised by exceptional species diversity (Hewitt 2011, Nieto Feliner 2014, Španiel and Rešetnik 2022). Its unique geographical location and historical role as a refuge during climatic fluctuations have significantly shaped biodiversity patterns in the region (Mittermeier et al. 2011). In addition, the Balkans harbour refugia that serve as reservoirs of genetic diversity and evolutionary potential due to the long-term persistence of species (Médail and Quézel 1999, Médail and Diadema 2009). This combination of high endemism and ecological importance makes the region a priority area for nature conservation (Griffiths et al. 2004).

Among the diverse plant taxa of the Balkans, the genus *Carex* L. (Cyperaceae) is of particular interest due to its ecological importance and taxonomic complexity. However, the floristic richness of Kosovo, a central part of the Balkans, is still relatively understudied compared to neighbouring countries, partly due to historical challenges and a lack of comprehensive botanical surveys (Berisha et al. 2020). Preliminary results indicate a high diversity of *Carex* species in Kosovo, but inconsistencies in records and reliance on

outdated data hinder accurate assessment. In addition, many *Carex* specimens in the Kosovo herbarium have remained unidentified or misclassified so far, emphasising the need for taxonomic revision.

The genus *Carex* is one of the largest and most diverse genera of vascular plants, with around 2,000 species distributed worldwide, covering a wide range of ecological habitats (Jiménez-Mejías and Larridon 2021, POWO 2024). Recent phylogenetic studies have significantly reshaped the classification of *Carex*, recognising six major subgeneric lineages: *Siderostictae* Franch. ex Ohwi, *Carex*, *Euthyceras* Peterm., *Psyllophora* (Degland) Peterm., *Uncinia* (Pers.) Peterm., *Vignea* (P.Beauv. ex T.Lestib.) Heer (Villaverde et al. 2020). These subgenera are supported by molecular data, refining the previously used infrageneric classifications that often did not reflect monophyletic groups (Roalson et al. 2021). Despite its high species diversity, many subgenera and sections within *Carex* have been shown to be polyphyletic, necessitating a revised classification framework that incorporates both molecular and morphological traits to provide a more accurate systematic organisation (Villaverde et al. 2020, Roalson et al. 2021). This refined classification does not only aid taxonomic clarity but also

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enhances our understanding of evolutionary relationships and species identification within this complex genus.

Several *Carex* species are known for their important role in wetland ecosystems, contributing to soil stabilisation, water filtration and biodiversity conservation. Their wide distribution in temperate and boreal regions underlines their ecological adaptability and evolutionary success (Martín-Bravo et al. 2019). Taxonomically, *Carex* is one of the most difficult genera to study due to its highly morphological variability (e.g. Więclaw et al. 2021, Więclaw et al. 2022), complex hybridisation patterns (Więclaw and Wilhelm 2014) and cryptic diversity (Chater 1980, Maguilla and Escudero 2016). Despite these challenges, the genus is of great importance for ecological research, habitat restoration and climate change studies as it occurs in sensitive ecosystems such as alpine, subalpine and boreal wetlands (Bernard 1990). In Europe, *Carex* is an important genus within plant diversity, comprising 235 species, some of which are endemic or highly protected (Kukkonen 1998, Martín-Bravo et al. 2019, Koopman 2022). The genus is particularly well represented in mountainous regions and wetlands, where it plays a central role in the formation of vegetation communities and the maintenance of ecosystem functions. Several European countries have made considerable efforts to document the diversity of *Carex*, but there are

still gaps, especially in less studied regions of the continent, such as the Balkans (Martín-Bravo et al. 2019).

The genus *Carex* is an example of the diversity on the Balkan Peninsula, where 108 native *Carex* species have been documented (Koopman 2022, Govaerts et al. 2021). Nevertheless, there are still many uncertainties, especially in areas such as Kosovo, where data on *Carex* remain sparse and fragmented. The lack of detailed studies, compounded by historical taxonomic ambiguities, leaves many question marks regarding the exact number of *Carex* taxa present, their distribution and ecological role. This issue highlights the urgent need for targeted floristic and taxonomic studies in Kosovo to clarify the status of *Carex* taxa and contribute to broader regional biodiversity assessments.

This study aims to provide a comprehensive and updated account of the genus *Carex* in Kosovo, filling significant knowledge gaps and resolving taxonomic ambiguities. The main objectives are to document the diversity of *Carex* taxa through detailed floristic inventories and herbarium revisions, to clarify taxonomic ambiguities by resolving misidentifications and discrepancies in existing records of *Carex* taxa in different habitats, especially in wetlands and in subalpine and alpine ecosystems. In addition, the study aims to identify rare, endemic and potentially threatened *Carex* taxa, assess their conservation status and propose

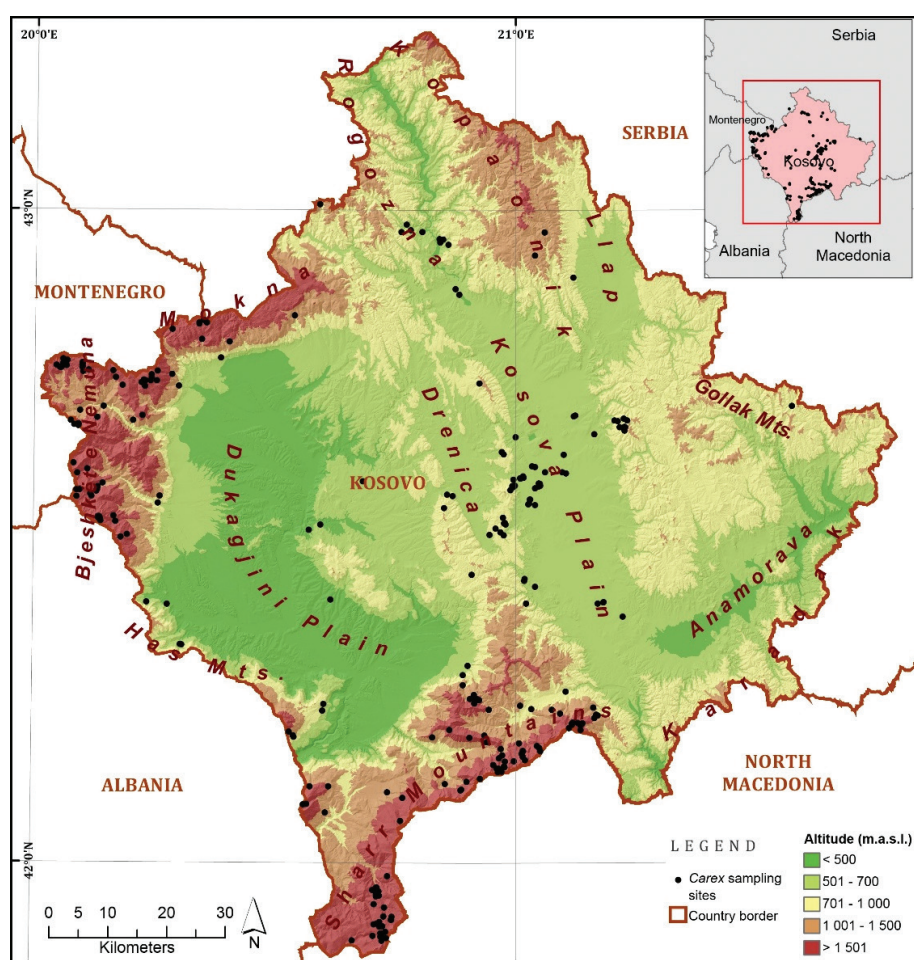


Fig. 1. Map of Kosovo showing sampling sites of *Carex* taxa.

measures for their protection. By placing these results in a broader Balkanic and European context, this research aims to improve the regional understanding of *Carex* diversity and fill floristic gaps in this region. Ultimately, this work will support long-term monitoring, management and conservation of sensitive ecosystems in Kosovo.

Material and methods

Sampling

During 2022–2024, extensive field expeditions were carried out in various habitats throughout Kosovo (Fig. 1) during three consecutive vegetative periods. The main objective of these expeditions was to document and collect as many *Carex* taxa as possible. All collected specimens were carefully processed and deposited in the Herbarium of the Faculty of Mathematics and Natural Sciences (UPH) at the University of Prishtina. In addition to this recently collected material, existing *Carex* herbarium vouchers were thoroughly examined. Many of these specimens had previously been either unidentified or misidentified, so a systematic taxonomic revision was required to ensure the accuracy and reliability of the documentation of *Carex* diversity.

This work is part of a broader research initiative focusing on the genus *Carex* (Cyperaceae) in the Balkans, aiming to comprehensively document and analyse its diversity in the region. Kosovo is involved in this large-scale project and is making a valuable contribution to ongoing efforts to refine the taxonomy, distribution and conservation status of *Carex* taxa in the Balkans.

Taxonomic study

Species identification was primarily based on an upgraded key of *Carex* (Koopman et al. 2025), as well as on established taxonomic keys, including those from the Flora of Albania (Pils 2016), Field guide of Spanish and Portuguese sedges (Luceño et al. 2023), the Excursion Flora of Germany (Jäger et al. 2017) and the Sedges of the British Isles (Jermy et al. 2007), along with other relevant literature sources. Morphological analyses of the plant material were performed under a stereozoom microscope (KERN OZM-5) to ensure accurate identification. All specimens were deposited in the Herbarium of the University of Prishtina (UPH), Kosovo. In certain cases, which required detailed morphological comparison, scanned herbarium specimens from the Herbarium of Vienna (JACQ, <https://www.jacq.org/>) were used. In addition, herbarium material from the Macedonian National Herbarium (MKNH), at the Faculty of Natural Sciences, Institute of Biology Skopje, North Macedonia, Herbarium Stetinense (SZUB) at University of Szczecin, Poland, and specimens from the private herbarium of Jac. Koopman were used to resolve taxonomic uncertainties. In addition, selected vouchers from the Herbarium of the Natural History Museum in Belgrade, Serbia, were examined on request in order to support species identifica-

tion and confirm the occurrence of certain *Carex* taxa in the flora of Kosovo. The nomenclature of *Carex* taxa in this study follows *Carex Europaea* (Koopman 2022).

Literature sources

A wide range of literature sources mentioning *Carex* taxa from Kosovo were consulted for this study. They included both floristic and phytosociological publications mentioning *Carex* taxa. From these sources we were able to extract valuable information such as the reported locations, habitat types and ecological relationships of the taxa. Using this information, we were able to visit the reported sites, collect specimens and verify their identity in the field. Our approach was to include in the final checklist only those *Carex* taxa for which we have had the opportunity to observe specimens and identify them confidently to species, subspecies or variety level. Taxa that are mentioned in the literature but for which no plant material was available, or cases where the collected specimens did not match the taxa given in the literature, are discussed separately in this article to eliminate inconsistencies and clarify the entries.

Results

Confirmed *Carex* taxa

Our study has identified a total of 52 *Carex* species in the flora of Kosovo, including 19 subspecies and 1 variety, as well as 2 hybrids. There are 15 new taxa for the flora of Kosovo (Tab. 1).

An alphabetical summary of all the recorded *Carex* taxa, along with their distribution in Kosovo, is provided in the Supplementary material (On-line Suppl. Tab. 1). In the following, we present taxonomic and detailed distributional notes for all documented taxa, including those recorded during field surveys as well as those verified from herbarium specimens.

Tab. 1. Newly recorded *Carex* taxa in Kosovo.

No.	Taxon
1.	<i>Carex acutiformis</i> Ehrh.
2.	<i>Carex brizoides</i> L.
3.	<i>Carex castroviejoi</i> Luceño & Jim.-Mejías
4.	<i>Carex depressa</i> subsp. <i>transsilvanica</i> (Schur) K.Richt.
5.	<i>Carex distachya</i> Desf. subsp. <i>distachya</i>
6.	<i>Carex ferruginea</i> Scop.
7.	<i>Carex flacca</i> subsp. <i>erythrostachys</i> (Hoppe) Holub
8.	<i>Carex macrolepis</i> Bertol.
9.	<i>Carex muricata</i> L. subsp. <i>muricata</i>
10.	<i>Carex pairae</i> F.W.Schultz
11.	<i>Carex sempervirens</i> subsp. <i>pseudotristis</i> (Domin) Pawł.
12.	<i>Carex umbrosa</i> Host subsp. <i>umbrosa</i>
13.	<i>Carex vesicaria</i> L.
14.	<i>Carex</i> × <i>ruedtii</i> Kneuck. [<i>C. flava</i> × <i>C. lepidocarpa</i>]
15.	<i>Carex</i> × <i>subviridula</i> Fernald [<i>C. flava</i> × <i>C. oederi</i>]

1. *Carex acuta* L., Sp. Pl.: 978 (1753).

A widespread species found mainly in wetlands of Europe, including floodplains, riverbanks and marshes (Müller et al. 2021). In Kosovo it thrives in riparian zones and wet meadows at low to medium altitudes.

Specimens: – Albanian Alps of Kosovo: Liqenat (Kuqishtë), at the vicinity of Lumbardhi i Pejës - 42°41.488' N, 20°5.707' E, 939 m, 29 May 2022, *Leg. Berisha, N.*; – Central Kosovo: Llapçevë (Rajoni i Mirushës), 42°30.600' N, 20°34.237' E, 461 m, 22 Jun 2023, *Leg. Krasniqi, E.*; – Lumi Drenicë (Bardh i Vogël), Fushë Kosovë, 42°36.424' N, 21°2.382' E, 533 m, 19 Jun 2024, *Leg. Krasniqi, E.*

2. *Carex acutiformis* Ehrh., Beitr. Naturk. 4: 43 (1789).

A widespread species that occurs mainly in wetlands in Europe, specifically in nutrient-rich marshes, fens, river valleys, and along watercourses (Müller et al. 2021). In Kosovo, it grows in riparian habitats at low altitudes. This is a new species for the flora of Kosovo.

Specimens: – Ligatina e Hencit (Fushë Kosovë), 42°34.869' N, 21°2.926' E, 537 m, 12 May 2024, *Leg. Berisha, N* [UPH – 00002035].

3. *Carex aterrima* Hoppe, Caricolog. Germ.: 51 (1826).**3.1. – *Carex aterrima* subsp. *aterrima***

A mountainous taxon that occurs mainly in the montane, subalpine to alpine regions of Europe, often in association with moist habitats such as wooded slopes and alpine meadows (Konrad et al. 2018). In Kosovo it grows in high-altitude areas where it thrives in moist microhabitats.

Specimens: – Albanian Alps of Kosovo: Hajlë, 42°45.195' N, 20°6.697' E, 1779 m, 15 Jul 1987, *Leg. Krivošej, Z.*; – Bogičë, 42°34.175' N, 20°5.687' E, 1946 m, 29 Jun 1988, *Leg. Millaku, F.*; – Hajlë, 42°45.422' N, 20°5.897' E, 1709 m, 01 Jul 1988, *Leg. Millaku, F.*; – Sharri Mts.: Bresanë – Opojë, 42°6.020' N, 20°45.964' E, 1634 m, 18 Jun 2018, *Leg. Mala, Xh.*; – Rrasa e Zogut, 42°30.124' N, 20°11.623' E, 1821 m, 29 Jun 2021, *Leg. Berisha, N.*; – Sharri Mts.: Vracë, 41°54.456' N, 20°42.829' E, 1961 m, 27 Jul 2023, *Leg. Millaku, F.*; – Marjash, 42°35.745' N, 20°5.417' E, 1968 m, 20 May 2024, *Leg. Berisha, N.*

4. *Carex atrata* L., Sp. Pl.: 976 (1753).**4.1. *Carex atrata* subsp. *atrata***

A widespread species that occurs throughout Europe in montane, subalpine and alpine habitats and is specifically found in wet meadows, fens, and rocky slopes with sufficient moisture (Konrad et al. 2018). In the high mountains of southern Europe, this taxon behaves as a chionophile, preferring habitats with prolonged snow cover. Similarly, in Kosovo, it is found in high-altitude grasslands, alpine meadows, and damp rocky crevices.

Specimens: – Albanian Alps of Kosovo: Gjeravicë, 42°31.708' N, 20°8.182' E, 2307 m, 22 Jun 2011, *Leg. Millaku, F.*; – Gjeravicë, 42°31.747' N, 20°8.035' E, 2310 m, 23 Jul

2023, *Leg. Berisha, N.*; – Sharri Mts.: Gryka e Jezercës, 42°9.506' N, 21°1.125' E, 2303 m, 29 Jun 2002, *Leg. Millaku, F.*; – Vracë, 41°54.193' N, 20°43.186' E, 2009 m, 27 Jul 2023, *Leg. Millaku, F.*; – Bistër, 42°8.890' N, 20°58.510' E, 2088 m, 09 Aug 2023, *Leg. Krasniqi, E.*

5. *Carex brizoides* L., Cent. Pl. 1: 31 (1755).

A rhizomatous species native to central, south-western and eastern Europe, usually found in mesic to moist habitats, including grasslands, forest clearings and roadsides (Müller et al. 2021, Luceño et al. 2023). In Kosovo, it thrives in open forest clearings, meadows and disturbed sites with moderate moisture. This is a new species for the flora of Kosovo.

Specimens: – Albanian Alps of Kosovo: Hajlë, 42°45.870' N, 20°5.958' E, 1859 m, 15 Jul 1987, *Leg. Krivošej, Z.* [UPH – 00002049]; – Hajlë, 42°45.055' N, 20°6.010' E, 1522 m, 03 Jul 1998 [UPH – 00002050], *Leg. Millaku, F.*; – Bjeshka e Rusolisë, 42°44.563' N, 20°14.566' E, 2201 m, 07 Aug 1987, *Leg. Millaku, F.* [UPH – 00002051]; – Bjeshka e Rusolisë, 42°44.270' N, 20°13.817' E, 1969 m, 02 Jul 2014, *Leg. Berisha, N.* [UPH – 00002052]; – Sharri Mts.: Bistër, 42°8.842' N, 20°58.590' E, 2088 m, 24 Jul 2021, *Leg. Berisha, N.* [UPH – 00002053]; – Brod, 41°57.141' N, 20°42.742' E, 1791 m, 20 Aug 2023, *Leg. Krasniqi, E.* [UPH – 00002054]; – Central Kosovo: Mali Drenicë (Llapçevë), 42°31.104' N, 20°35.702' E, 497 m, 30 Apr 2024, *Leg. Krasniqi, E.* [UPH – 00002055]; – Northern Kosovo: Banjskë (Zvečan), 42°58.698' N, 20°46.338' E, 641 m, 14 Jun 1986, *Leg. Krivošej, Z.* [UPH – 00002056].

6. *Carex canescens* L., Sp. Pl.: 974 (1753).**6.1. *Carex canescens* subsp. *canescens***

This species is widespread across boreal, temperate, and montane regions of Europe, while it is absent or extremely rare in Mediterranean areas. It specifically grows in moist, acidic habitats such as bogs, fens, peatlands and on the edges of lakes and streams (Müller et al. 2021). In Kosovo, it is found in high-montane wetlands, nutrient-poor bogs and on the edges of water bodies, often in association with peat mosses and other moisture-dependent vegetation.

Specimens: – Sharri Mts.: Bistër, 42°8.919' N, 20°58.531' E, 2087 m, 24 Jul 2021, *Leg. Berisha, N.*; – Brod, 41°55.949' N, 20°43.747' E, 1942 m, 01 Aug 2020, *Leg. Mala, Xh.*

7. *Carex caryophyllea* Latourr., Chlor. Lugd.: 27 (1785).

A widespread species found in dry to mesic grasslands, open woodlands and meadows throughout Europe. It thrives in well-drained, nutrient-poor soils in calcareous and sandy habitats (Müller et al. 2021). In Kosovo, it is found in dry grasslands, on forest edges and in sparse forests, especially in sunny or semi-shady locations.

Specimens: – Albanian Alps of Kosovo: Bjeshka e Rusolisë, 42°44.315' N, 20°13.587' E, 1889 m, 19 Jun 1988, *Leg. Millaku, F.*; – Bjeshka e Rusolisë, 42°44.298' N, 20°13.608' E, 1901 m, 23 Jun 2012, *Leg. Millaku, F.*; – Peklen, 42°41.051' N, 20°13.469' E, 1754 m, 29 Jun 2022, *Leg. Berisha,*

N.; – Central Kosovo: Blinajë, 42°30.416' N, 20°58.068' E, 701 m, 13 May 2024, *Leg. Berisha, N.*; – Golesh Mt., 42°34.303' N, 20°59.314' E, 906 m, 01 Jun 2024, *Leg. Berisha, N.*; – Llapushnik, 42°32.647' N, 20°51.083' E, 951 m, 30 Apr 2024, *Leg. Berisha, N.*; – Sharri Mts.: Vracë, 41°54.242' N, 20°43.522' E, 2190 m, 19 Jun 2023, *Leg. Millaku, F.*; – Luboten Mt., 42°12.679' N, 21°8.000' E, 1799 m, 23 Jun 2018, *Leg. Berisha, N.*; – Konjushë Mt., 42°8.714' N, 20°57.845' E, 2198 m, 23 Jun 2024, *Leg. Berisha, N.*; – Shtërpçë, 42°14.193' N, 21°1.890' E, 954 m, 01 Jul 2020, *Leg. Mala, Xh.*; – South-Western Kosovo: Pashtrik Mt., 42°11.600' N, 20°32.500' E, 949 m, 16 Jun 2012, *Leg. Berisha, N.*; – North Kosovo: Sërboc – Zvečan, 42°57.350' N, 20°50.932' E, 732 m, 03 Jul 1989, *Leg. Krivošej, Z.*

8. *Carex castroviejoi* Luceño & Jim.Mejías, *Acta Bot. Malac.* 34: 231 (2008).

A species endemic to the Balkans, occurring in montane, marshy soils over ophiolitic rocks (Jiménez-Mejías and Luceño 2009). It has recently been reported from Albania (Martín-Bravo et al. 2022). In Kosovo it has been found on serpentine and silicate substrates in mountain and high mountain regions. This is a newly reported species for the flora of Kosovo.

Specimens: – North Kosovo: Rogozna Mt., 43°0.565' N, 20°35.489' E, 1060 m, 10 Jun 1994, *Leg. Rexhepi, F.* [UPH – 00002037]; – Sharri Mts.: "Vahufloc.", no coord., 09 Jul 1979, *Leg. Pichler, A.* [UPH – 00002038]; – Maja e Zezë, 42°7.517' N, 20°53.897' E, 1527 m, 30 May 1998, *Leg. Rexhepi, F.* [UPH – 00002039]; – Albanian Alps of Kosovo: Bogičë, 42°33.559' N, 20°5.359' E, 2097 m, 19 Jul 1979, *Leg. Lindtner, V.* [UPH – 00002040].

9. *Carex curvula* All., *Fl. Pedem.* 2: 264 (1785).

9.1. *Carex curvula* subsp. *curvula*

A species widespread in the high mountains of Europe. It is specifically found in alpine grasslands, rocky slopes and windswept ridges and thrives on acidic, nutrient-poor soils (Konrad et al. 2018). In Kosovo, it is found in the alpine zones of the Sharri Mountains.

Specimens: – Sharri Mts.: Pribreg Mt., 42°10.626' N, 21°2.860' E, 2121 m, 10 Aug 2023, *Leg. Millaku, F.*; – Shutman – Brod, 41°55.074' N, 20°44.692' E, 2166m, 31 Jul 2023, *Leg. Krasniqi, E.*; – Konjushë Mt., 42°8.736' N, 20°57.627' E, 2127 m, 23 Jun 2024, *Leg. Berisha, N.*

10. *Carex davalliana* Sm. in *Trans. Linn. Soc. London* 5: 266 (1800).

A dioecious species native to continental Europe, specifically found in calcareous fens and wet meadows (Müller et al. 2021). It thrives on alkaline, carbonate-rich soils, often in small spring bogs. In Kosovo, it has been found in similar habitats, in nutrient-rich, subalpine calcareous fens.

Specimens: – Sharri Mts.: Konjushë Mt., 42°8.664' N, 20°57.750' E, 2155m, 23 Jun 2024, *Leg. Berisha, N.*; –

Vracë, 41°54.598' N, 20°43.155' E, 2049 m, 19 Jun 2023, *Leg. Millaku, F.*

11. *Carex depauperata* Curtis ex Woodw. in W.Withering., *Bot. Arr. Brit. Pl.*, ed. 2, 2: 1049 (1787).

A sedge native to Europe. It thrives on semi-shady, dry, well-drained soils of calcareous as well as silicate substrates, on forest edges (Müller et al. 2021). In Kosovo, it has been found in similar habitats in the edges of *Quercus pubescens* Willd. forest.

Specimens: – Central Kosovo: Golesh Mt., 42°34.708' N, 20°59.886' E, 736 m, 12 May 2024, *Leg. Berisha, N.*

12. *Carex depressa* Link in *J. Bot. (Schrader)* 1799(2): 309 (1800).

12.1. *Carex depressa* subsp. *transsilvanica* (Schur) T.V.Egorova, *Novosti Sist. Vysš. Rast.* 9: 80 (1972).

A densely tufted subspecies with short rhizomes that grows in forests and dry grasslands. It is widespread in south-east Europe and the Carpathians (Koopman 2022). The species grows on well-drained soils, often in open or semi-shaded habitats (Konrad et al. 2018). In Kosovo, it has been found in the vicinity of forest vegetation. It is a newly recorded taxon for Kosovo.

Specimens: – Sharri Mts.: Pashallore Mt., 42°14.716' N, 20°54.839' E, 1709 m, 27 Jul 2023, *Leg. Berisha, N.* [UPH – 00002041].

13. *Carex digitata* L., *Sp. Pl.*: 975 (1753).

A widespread Eurasian species that occurs in dry to mesic forests, on forest edges and rocky slopes (Nikolić, 2020). In Kosovo it has been found in montane and subalpine regions, where it grows on calcareous substrates.

Specimens: – Sharri Mts.: Gllboqicë, 42°10.671' N, 21°9.534' E, 1170 m, 02 Jul 2009, *Leg. Mala, Xh.*; – Gërmi (Prishtinë), 42°40.087' N, 21°12.937' E, 819 m, 14 Jun 2023, *Leg. Berisha, N.*

14. *Carex distachya* Desf., *Fl. Atlant.* 2: 336 (1799).

14.1. *Carex distachya* subsp. *distachya*

Primarily a Mediterranean species, often found in dry woodland, scrub and on rocky slopes, on calcareous and siliceous soils (Nikolić 2020, Koopman et al. 2024). In Kosovo, it thrives in *Quercus pubescens* forests as well as in their clearings. This is a new (sub)species for Kosovo.

Specimens: – Gërmi (Prishtinë), 42°40.068' N, 21°13.616' E, 822 m, 24 May 2024, *Leg. Berisha, N.* [UPH - 00002042]; – Llapashticë (Podujevë), 42°53.827' N, 21°7.179' E, 708 m, 30 May 2022, *Leg. Berisha, N.* [UPH - 00002043].

15. *Carex distans* L., *Syst. Nat.*, ed. 10, 2: 1263 (1759).

A widespread Eurasian species found in wet meadows, marshes and along riverbanks, often on saline or nutrient-rich soils (Müller et al. 2021). In Kosovo, it has been found in lowland and mountainous regions, specifically in wet meadows and near water bodies.

Specimens: – Central Kosovo: Golesh Mt., 42°34.670' N, 20°59.542' E, 731 m, 13 May 2023, *Leg. Berisha, N.*; – Miradi e Epërme (Fushë Kosovë), 42°35.940' N, 21°5.775' E, 533 m, 20 May 2024, *Leg. Berisha, N.*; – Gazimestan, 42°41.167' N, 21°7.395' E, 584 m, 02 Jul 2022, *Leg. Berisha, N.*; – Harilaç (Fushë Kosovë), 42°35.449' N, 21°0.946' E, 551 m, 05 May 2024, *Leg. Berisha, N.*; – Ligatina e Hencit (Fushë Kosovë), 42°34.702' N, 21°3.041' E, 531 m, 12 May 2024, *Leg. Berisha, N.*; – Dobrajë (Lipjan), 42°32.935' N, 21°2.398' E, 555 m, 12 May 2024, *Leg. Berisha, N.*; – Shtime, 42°25.424' N, 21°2.379' E, 604 m, 10 Jun 2019, *Leg. Mala, Xh.*; – Sojevë (Ferizaj), 42°22.658' N, 21°13.252' E, 549 m, 02 Jul 2024, *Leg. Berisha, N.*; – Blinajë, 42°30.165' N, 20°58.510' E, 651 m, 13 May 2024, *Leg. Krasniqi, E.*; – Sharri Mts.: Shutman, 41°54.832' N, 20°44.606' E, 2161 m, 31 Jul 2023, *Leg. Millaku, F.*; – Albanian Alps of Kosovo: Zalli i Rupës, 42°34.072' N, 20°7.789' E, 1504 m, 19 May 2024, *Leg. Berisha, N.*; – Bjeshka e Rusolisë, 42°43.815' N, 20°17.982' E, 882 m, 30 Jun 1987, *Leg. Millaku, F.*; – Mokna, 42°46.399' N, 20°23.214' E, 641 m, 19 Jul 1989, *Leg. Krivošej, Z.*; – Northern Kosovo: Banjskë (Zvečan), 42°58.184' N, 20°46.868' E, 644 m, 14 Jun 1986, *Leg. Krivošej, Z.*; – Sërboc (Zvečan), 42°57.061' N, 20°50.551' E, 539 m, 16 May 1986, *Leg. Krivošej, Z.*; – Zhazhë (Zvečan), 42°56.856' N, 20°51.561' E, 725 m, 16 May 1986, *Leg. Krivošej, Z.*; – Jashevik (Zvečan), 42°58.011' N, 20°48.316' E, 540 m, 14 May 1986, *Leg. Krivošej, Z.*; – South-Western Kosovo: Malësi e Gjakovës, 42°20.030' N, 20°18.534' E, 551 m, 29 Jun 2024, *Leg. Berisha, N.*; – Potaqan (Rahovec), 42°24.227' N, 20°36.964' E, 409 m, 20 Jun 1979, *Leg. Hundozi, B.*

16. *Carex divisa* Huds., Fl. Angl.: 348 (1762).

A species native to Europe, Asia and North Africa. It specifically inhabits seasonally moist meadows, marshes and riverbanks; in much of Europe it behaves as a soil indifferent, from siliceous to saline soils (Jermy et al. 2007). In Kosovo, it has been found in moist meadows at low altitudes.

Specimens: – Central Kosovo: Miradi i Poshtme (Fushë Kosovë), 42°35.944' N, 21°3.626' E, 519 m, 14 May 2024, *Leg. Berisha, N.*; – Dobrajë (Lipjan), 42°32.987' N, 21°1.547' E, 553 m, 12 May 2024, *Leg. Berisha, N.*

17. *Carex divulsa* Stokes in W. Withering, Bot. Arr. Brit. Pl., ed. 2, 2: 1035 (1787).

A widespread species native to Europe and Asia, specifically found in grasslands, forest edges and roadsides, often on nutrient-rich soils (Nikolić 2020). In Kosovo, it has been found in moist grassland and forest clearings from the montane to the high mountain zone.

Specimens: – Central Kosovo: Blinajë, 42°31.750' N, 20°58.411' E, 704 m, 13 May 2024, *Leg. Berisha, N.*; – Albanian Alps of Kosovo: Gjeravicë, 42°31.296' N, 20°10.204' E, 1701 m, 10 Jul 2022, *Leg. Berisha, N.*; – Zalli i Rupës: 42°34.244' N, 20°7.872' E, 1398 m, 19 May 2024, *Leg. Berisha, N.*; – Marjash: 42°36.122' N, 20°6.639' E, 1680 m, 30 Jun 2024, *Leg. Berisha, N.*; – Sharri Mts.: Luboten Mt., 42°14.337' N, 21°9.571' E, 1096 m, 24 Jun 2017, *Leg. Berisha, N.*; –

Northern Kosovo: Iberane (Zvečan), No coord., 650 m, 15 May 1986, *Leg. Krivošej, Z.*

18. *Carex echinata* Murray, Prodr. Stirp. Gott.: 76 (1770).

18.1. *Carex echinata* subsp. *echinata*

A widespread species native to Europe, Asia and North America, specifically found in wet meadows, bogs and acidic swamps (Jermy et al. 2007). In Kosovo it has been found in montane and subalpine regions, where it grows on moist grasslands, fens and nutrient-poor soils.

Specimens: – Albanian Alps of Kosovo: Ishtedim, 42°44.089' N, 20°13.325' E, 1820 m, 10 Jul 1987, *Leg. Krivošej, Z.*; – Hajlë, 42°45.688' N, 20°5.901' E, 1784 m, 15 Jul 1987, *Leg. Millaku, F.*; – Mokna, 42°49.623' N, 20°20.457' E, 1869 m, 21 Jun 2012, *Leg. Millaku, F.*; – Gjeravicë, 42°31.580' N, 20°8.286' E, 2148 m, 23 Jul 2023, *Leg. Berisha, N.*; – Marjash, 42°36.634' N, 20°4.925' E, 2021 m, 10 Jul 2024, *Leg. Berisha, N.*; – Sharri Mts.: Përrojet e Durlës, 42°10.376' N, 21°0.860' E, 1631 m, 30 Jul 2022, *Leg. Berisha, N.*; – Luboten Mt., 42°12.818' N, 21°6.899' E, 1935 m, 26 Jun 2018, *Leg. Berisha, N.*; – Vracë, 41°53.587' N, 20°43.295' E, 2027 m, 09 Jul 2023, *Leg. Millaku, F.*; – Ujërat e Qeta, 42°6.792' N, 20°53.256' E, 1762 m, 30 Jun 2014, *Leg. Mala, Xh.*; – Pashalllore Mt., 42°14.718' N, 20°54.856' E, 1774 m, 27 Jul 2023, *Leg. Berisha, N.*

19. *Carex ferruginea* Scop., Fl. Carniol., ed. 2, 2: 225 (1772).

A montane to alpine species native to central and southern Europe, specifically found in subalpine and alpine grasslands, on rocky slopes and in open coniferous forests (Müller et al. 2021). In Kosovo it has been found in high mountain regions of Sharri Mts., where it grows on calcareous substrates. Given its previously unverified occurrence, this record represents a species new to Kosovo.

Specimens: – Sharri Mts.: Brod, 41°58.831' N, 20°44.095' E, 2009 m, 20 Aug 2023, *Leg. Berisha, N.* [UPH - 00002044]; – Pashalllore Mt., 42°15.037' N, 20°55.467' E, 1540 m, 27 Jul 2023, *Leg. Berisha, N.* [UPH - 00002045]; – Përrojet e Durlës, 42°10.093' N, 21°0.695' E, 1774 m, 30 Jul 2022, *Leg. Berisha, N.* [UPH - 00002046]; – Vracë, 41°53.267' N, 20°43.309' E, 2087 m, 09 Jul 2023, *Leg. Millaku, F.* [UPH - 00002047]; – Maja e Zezë, 42°7.071' N, 20°55.415' E, 2104 m, 17 Jun 2021, *Leg. Krasniqi, E.* [UPH - 00002048].

20. *Carex flacca* Schreb., Spic. Fl. Lips.: App. 178 (1771).

A widespread Eurasian species, often found in wet meadows, fens, and calcareous grasslands. In Kosovo it has been found in montane to high montane regions, where it specifically grows on moist, calcareous soils. It is represented in Kosovo by the following two subspecies:

20.1. *Carex flacca* subsp. *flacca*

Specimens: – Albanian Alps of Kosovo: Shtupeç i Vogël, 42°40.614' N, 20°12.291' E, 1204 m, 30 Jun 2022, *Leg. Berisha, N.*; – Bogë, 42°45.416' N, 20°3.114' E, 1521 m, 08 Jun 2024, *Leg. Berisha, N.*; – Rekë e Allagës, 42°44.541' N, 20°10.105' E, 1637 m, 19 Jun 2024, *Leg. Berisha, N.*; – Kuqishtë, 42°40.251' N, 20°5.449' E, 1858 m, 14 Jun 2024, *Leg. Berisha, N.*

N.; – Northern Kosovo: Banjskë (Zvečan), 42°58.022' N, 20°45.671' E, 852 m, 14 Jun 1986, *Leg. Krivošej, Z.*

20.2. *Carex flacca* subsp. *erythrostachys* (Hoppe) Holub, *Folia Geobot. Phytotax.* 23: 413 (1988).

This subspecies is a newly reported taxon for Kosovo.

Specimens: – Central Kosovo: Goleš Mt., 42°34.584' N, 20°59.656' E, 752 m, 12 May 2024, *Leg. Berisha, N.* [UPH – 00002036].

21. *Carex flava* L., *Sp. Pl.*: 975 (1753).

A widespread species native to Europe, Asia and North America. It occurs in wet meadows, fens, and marshes, often on calcareous and nutrient-rich soils (Jermy et al. 2007). In Kosovo it has been found in high montane regions, where it thrives in moist grasslands and fens.

Specimens: – Sharri Mts.: Brod, 41°57.761' N, 20°42.837' E, 1570 m, 20 Aug 2023, *Leg. Millaku, F.*; – Përrojet e Durlës: 42°9.811' N, 21°0.840' E, 2016 m, 03 Jul 2017, *Leg. Berisha, N.*; – Vracë, 41°53.347' N, 20°42.358' E, 1916 m, 27 Jul 2023, *Leg. Krasniqi, E.*; – Luboten Mt., 42°12.965' N, 21°7.028' E, 1887 m, 09 Jul 2018, *Leg. Berisha, N.*; – Lumi i Murzhicës (Shtërpçë), 42°14.514' N, 21°0.525' E, 945 m, 29 May 1973, *Leg. Kirković, D.*; – Maja e Zezë, 42°7.804' N, 20°55.534' E, 2351 m, 06 Jul 2011, *Leg. Millaku, F.*; – Albanian Alps of Kosovo: Mokna, 42°50.310' N, 20°32.404' E, 1176 m, 27 Jun 1996, *Leg. Krivošej, Z.*; – Bogë, 42°45.539' N, 20°3.464' E, 1551 m, 08 Jun 2024, *Leg. Berisha, N.*; – Rekë e Allagës, 42°45.171' N, 20°9.795' E, 1874 m, 19 Jun 2024, *Leg. Berisha, N.*

22. *Carex halleriana* Asso, *Syn. Stirp. Aragon.*: 133 (1779).

A species native to southern and central Europe, North Africa and Western Asia. It specifically inhabits rocky and grassy slopes, often on calcareous soils (Müller et al. 2021). In Kosovo, it has been found in dry grasslands and open forests, especially in mountainous regions.

Specimens: – Koritnik Mt.: Breznë, 42°7.068' N, 20°36.810' E, 1526 m, 11 Jul 2012, *Leg. Krasniqi, E.*; – Kërsteç, 42°4.671' N, 20°36.401' E, 1187 m, 29 Apr 2024, *Leg. Berisha, N.*; – Shalë e Bajgorës: Vesekovc, 42°55.863' N, 21°2.388' E, 1243 m, 27 Jun 2009, *Leg. Berisha, N.*; – Iberane (Zvečan), No coord., 671 m, 15 May 1986, *Leg. Krivošej, Z.*; – Mazrek (Has), 42°14.028' N, 20°36.024' E, 623 m, 10 Sep 2023, *Leg. Berisha, N.*; – Llapushnik, 42°14.657' N, 20°36.163' E, 790 m, 30 Apr 2024, *Leg. Berisha, N.*; – Gërmi (Prishtinë), 42°40.633' N, 21°12.718' E, 788 m, 24 May 2024, *Leg. Berisha, N.*

23. *Carex hirta* L., *Sp. Pl.*: 975 (1753).

A widespread Eurasian species, often found in grasslands, riverbanks, ditches and disturbed habitats, preferably on nutrient-rich soils (Müller et al. 2021). In Kosovo, it is one of the most widespread *Carex* species, occurring from lowlands to mountainous regions in a variety of moist and mesic environments.

Specimens: – Albanian Alps of Kosovo: Bogë, 42°45.745' N, 20°3.483' E, 1584 m, 19 Jun 2024, *Leg. Berisha, N.*; – Gjeravicë, 42°31.813' N, 20°9.950' E, 1901 m, 23 Jul 2023,

Leg. Berisha, N.; – Mali Drenicë, 42°30.167' N, 20°56.729' E, 669 m, 01 Jul 2008, *Leg. Krasniqi, E.*; – Blinajë, 42°31.152' N, 20°58.706' E, 648 m, 13 May 2024, *Leg. Krasniqi, E.*; – Vasilevë, 42°37.793' N, 20°58.240' E, 719 m, 17 Jun 2024, *Leg. Berisha, N.*; – Prishtinë, 42°39.426' N, 21°9.787' E, 584 m, 16 May 2024, *Leg. Berisha, N.*; – Mushtisht, 42°17.333' N, 20°53.401' E, 698 m, 21 Jun 2005, *Leg. Mala, Xh.*; – Harilaç (Fushë Kosovë), 42°35.479' N, 21°0.950' E, 551 m, 05 May 2024, *Leg. Berisha, N.*; – Çiçavicë, 42°44.107' N, 20°55.460' E, 931 m, 04 Jun 1987, *Leg. Rexhepi, F.*; – Sharri Mts.: Prevallë, 42°10.546' N, 20°58.118' E, 1450 m, 22 Jul 2011, *Leg. Berisha, N.*

24. *Carex hordeistichos* Vill., *Hist. Pl. Dauphiné* 2: 221 (1787).

A species native to Europe and western Asia, specifically found in wet meadows, marshes and seasonally flooded grasslands (Müller et al. 2021). In Kosovo it was found in lowland meadows and also thrived in temporarily wet habitats near an artificial lake.

Specimens: – Ligatina e Hencit (Fushë Kosovë), 42°34.889' N, 21°2.966' E, 537 m, 12 May 2024, *Leg. Berisha, N.*

25. *Carex humilis* Leyss., *Fl. Halens.*: 175 (1761).

A species native to Europe, specifically found in dry grasslands, rocky slopes and open forests, preferably on calcareous soils (Konrad et al. 2018). In Kosovo it occurs in montane regions and thrives in dry, well-drained habitats.

Specimens: – Albanian Alps of Kosovo: Bjeshka e Rusolisë, 42°44.254' N, 20°15.219' E, 2053 m, 21 Jul 2010, *Leg. Millaku, F.*; – Pashtrik Mt., 42°11.756' N, 20°32.419' E, 1066 m, 16 Jun 2012, *Leg. Berisha, N.*; – Gllarevë, 42°35.110' N, 20°40.946' E, 608 m, 03 Apr 2023, *Leg. Berisha, N.*; – Mushtisht, 42°18.173' N, 20°54.036' E, 708 m, 19 May 2020, *Leg. Mala, Xh.*

26. *Carex kitaibeliana* Bech. in *Ber. Schweiz. Bot. Ges.* 70: 178 (1960).

26.1. *Carex kitaibeliana* var. *kitaibeliana*

A species native to south-eastern and south-central Europe, as well as western Asia (Türkiye), specifically inhabiting dry, rocky slopes, high mountain grasslands and open forests, often on calcareous soils (Pignatti et al. 2018). In Kosovo, it has been found in high montane, subalpine regions where it thrives in rocky grasslands. Further research should reveal whether the variety *capillata*, known from the Balkans in Bulgaria and Greece, occurs in Kosovo, too.

Specimens: – Sharri Mts.: Vracë, 41°54.405' N, 20°42.479' E, 1970 m, 27 Jul 2023, *Leg. Millaku, F.*; – Luboten Mt., 42°12.281' N, 21°7.999' E, 1879 m, 02 Jul 2017, *Leg. Berisha, N.*; – Konjushë Mt., 42°8.713' N, 20°57.868' E, 2171 m, 23 Jun 2024, *Leg. Berisha, N.*; – Shutman, 41°55.116' N, 20°43.671' E, 2164 m, 30 Jun 2011, *Leg. Millaku, F.*; – Oshlak, 42°11.562' N, 20°54.249' E, 1956 m, 24 Jun 2019, *Leg. Berisha, N.*; – Albanian Alps of Kosovo: Mokna, 42°49.505' N, 20°20.461'

E, 1888 m, 21 Jun 2012, *Leg. Millaku, F.*; – Bjeshka e Rusolisë, 42°44.805' N, 20°14.578' E, 2263 m, 21 Jun 2011, *Leg. Millaku, F.*; – Ishtedim, 42°43.753' N, 20°13.737' E, 1931 m, 28 Jun 2011, *Leg. Millaku, F.*; – Rrasa e Zogut, 42°29.877' N, 20°10.842' E, 2060 m, 27 Jun 2021, *Leg. Berisha, N.*; – Pashtrik Mt., 42°12.082' N, 20°31.875' E, 1426 m, 20 Jun 2019, *Leg. Berisha, N.*; – Shalë e Bajgorës: Kaçandoll, 42°58.000' N, 21°3.605' E, 1321 m, 03 Jul 2010, *Leg. Berisha, N.*

27. *Carex lazarei* Jac. Koopman, Niketić, Więclaw & Govaerts, *Phytotaxa* 422: 296 (2019).

A species endemic to the Balkan Mountains, specifically found on acidic grasslands and rocky slopes (Koopman et al. 2019). In Kosovo it has been found in high-montane and subalpine regions, where it grows on siliceous substrates in dry, open habitats.

Specimens: – Sharri Mts.: Konjushë Mt., 42°09'07" N, 20°57'23" E, 1846 m, 23 Jun 2024, *Leg. Berisha, N.*; – Vracë, 41°53'13" N, 20°44'01" E, 2168 m, 29 Jun 2011, *Leg. Berisha, N.*; – Bistër, 42°08'44" N, 20°58'24" E, 2082 m, 24 Jul 2021, *Leg. Berisha, N.*; – Brezovicë, 42°10'33" N, 21°03'01" E, 2048 m, 03 Jul 2007, *Leg. Krasniqi, E.*; – Albanian Alps of Kosovo: Bjeshka e Rusolisë, 42°44'27" N, 20°14'44" E, 2229 m, 24 Jun 2010, *Leg. Millaku, F.*; – Gjeravicë, 42°31'36" N, 20°08'34" E, 2085 m, 22 Jun 2011, *Leg. Rexhepi, F.*

28. *Carex lepidocarpa* Tausch, *Flora* 17: 179 (1834).

28.1. *Carex lepidocarpa* subsp. *lepidocarpa*

A species native to Europe and eastern Canada, specifically found in base-rich fens, calcareous swamps and lakeshores (Nikolić 2020). In Kosovo it was found in moist, calcareous habitats in the montane regions.

Specimens: – Malësi e Gjakovës, 42°23'45" N, 20°16'40" E, 561 m, 29 Jun 2024, *Leg. Berisha, N.*; – Bjeshka e Morinë, 42°23'56" N, 20°14'07" E, 512 m, 02 Jul 2021, *Leg. Millaku, F.*; – Sharri Mts.: Brod, 41°56'15" N, 20°42'28" E, 1901 m, 20 Aug 2023, *Leg. Berisha, N.*

29. *Carex leporina* L., *Sp. Pl.*: 973 (1753).

A species native to Eurasia, North Africa and parts of North America, specifically found in seasonally moist to wet habitats such as meadows, fields and open areas (Konrad et al. 2018). In Kosovo, it has been found in lowland to mountainous regions where it thrives in moist grasslands and open woodlands.

Specimens: – Sharri Mts.: Luboten Mt., 42°13'23" N, 21°09'43" E, 1378 m, 23 Jun 2016, *Leg. Berisha, N.*; – Firajë, 42°13'48" N, 21°05'27" E, 1368 m, 02 Jul 2020, *Leg. Berisha, N.*; – Brod, 41°57'26" N, 20°43'03" E, 1735 m, 14 Jul 2016, *Leg. Berisha, N.*; – Vracë, 41°53'43" N, 20°43'54" E, 2096 m, 09 Jul 2023, *Leg. Millaku, F.*; – Bistër, 42°09'23" N, 20°58'37" E, 1815 m, 24 Jul 2021, *Leg. Berisha, N.*; – Konjushë Mt., 42°09'40" N, 20°57'53" E, 1592 m, 23 Jun 2024, *Leg. Berisha, N.*; – Përrojet e Durlës, 42°11'04" N, 21°00'23" E, 1432 m, 03 Jul 2017, *Leg. Berisha, N.*; – Pashallorë Mt., 42°15'08" N, 20°54'19" E, 1652 m, 27 Jul 2023, *Leg. Millaku, F.*; –

Mushtisht, 42°16'24" N, 20°53'26" E, 1115 m, 21 Jun 2005, *Leg. Mala, Xh.*; – Bresanë (Opojë), 42°06'32" N, 20°44'04" E, 1240 m, 21 Jun 2007, *Leg. Mala, Xh.*; – Albanian Alps of Kosovo: Bogë, 42°45'37" N, 20°02'42" E, 1590 m, 09 Jun 2024, *Leg. Berisha, N.*; – Mokna, 42°48'07" N, 20°20'48" E, 1556 m, 21 Jun 2012, *Leg. Millaku, F.*; – Central Kosovo: Blinajë, 42°30'47" N, 20°57'27" E, 788 m, 13 May 2024, *Leg. Berisha, N.*; – Vasilevë, 42°37'35" N, 20°58'29" E, 726 m, 17 Jun 2024, *Leg. Berisha, N.*; – North Kosovo: Sërboc – Zvečan, 42°57'21" N, 20°50'26" E, 596 m, 03 Jul 1989, *Leg. Krivošej, Z.*

30. *Carex macrolepis* DC., *Cat. Pl. Horti Monsp.*: 89 (1813).

A sedge species native to parts of southern Europe, including Italy, Albania, Greece and Turkey. This species thrives in moist meadows, grasslands and near forests (Pignatti et al. 2018). In Kosovo, it was found in a white oak forest (*Quercus pubescens*) in a moist habitat on a serpentine substrate. This is a new species for Kosovo.

Specimens: – Malësi e Gjakovës, 42°20'02" N, 20°18'21" E, 528 m, 29 Jun 2024, *Leg. Berisha, N.* [UPH – 00002057].

31. *Carex melanostachya* Willd., *Sp. Pl.* 4: 299 (1805).

A species native to Central and Eastern Europe and Western Asia, specifically found in wet grasslands, marshes and riverbanks, often on slightly saline or calcareous soils (Konrad et al. 2018). In Kosovo, it has been recorded in lowland regions and thrives in moist, open habitats.

Specimens: – Ligatina e Hencit (Fushë Kosovë), 42°34'39" N, 21°02'52" E, 536 m, 12 May 2024, *Leg. Berisha, N.*; – Harilaç (Fushë Kosovë), 42°35'34" N, 21°00'55" E, 560 m, 05 May 2024, *Leg. Berisha, N.*; – Talinoc (Ferizaj), 42°23'52" N, 21°10'10" E, 569 m, 09 May 2024, *Leg. Berisha, N.*

32. *Carex michelii* Host, *Syn. Pl.*: 507 (1797).

A species native to Central, Southern and Eastern Europe, extending as far as Turkey, the Caucasus and Iran. It specifically inhabits semi-arid grasslands, often on calcareous soils. In Kosovo, it has been found in forest clearings in montane regions.

Specimens: – Gërmi (Prishtinë), 42°40'52" N, 21°13'28" E, 826 m, 29 May 1962, *Leg. Nimolić, V.*

33. *Carex muricata* L., *Sp. Pl.*: 974 (1753).

33.1. *Carex muricata* subsp. *muricata*

A species native to Europe and western Asia, specifically found in calcareous grasslands and forests (Müller et al. 2021). In Kosovo it has been found in similar habitats and thrives on well-drained soils in montane to high montane regions. This is a new taxon for Kosovo.

Specimens: – Albanian Alps of Kosovo: Bjeshka e Deçanit, 42°33'40" N, 20°15'42" E, 873 m, 04 Jul 2023, *Leg. Berisha, N.* [UPH – 00002058]; – Liqenat, 42°40'33" N, 20°04'32" E, 1633 m, 28 Jun 2020, *Leg. Berisha, N.* [UPH – 00002059]; – Zalli i Rupës, 42°34'50" N, 20°08'34" E, 1301 m, 25 Jun 2024, *Leg. Berisha, N.* [UPH – 00002060]; – Mokna, 42°47'53" N, 20°24'17" E, 1415 m, 06 Jul 2012, *Leg.*

Millaku, F. [UPH – 00002061]; – Bjeshka e Rusolisë, 42°44'51" N, 20°17'07" E, 1361 m, 29 Jun 1987, *Leg. Millaku, F.* [UPH – 00002062]; – Bogë, 42°45'35" N, 20°03'58" E, 1620 m, 08 Jun 2024, *Leg. Berisha, N.* [UPH – 00002063]; – Sharri Mts.: Brod, 41°56.981' N, 20°42.744' E, 1842 m, 01 Aug 2023, *Leg. Berisha, N.* [UPH – 00002064]; – Central Kosovo: Blinajë, 42°31.259' N, 20°58.525' E, 682 m, 13 May 2024, *Leg. Berisha, N.* [UPH – 00002065]; – Gërmi (Prishtinë), 42°39.759' N, 21°13.553' E, 931 m, 24 May 2024, *Leg. Berisha, N.* [UPH – 00002066]

34. *Carex nigra* (L.) Reichard, Fl. Moeno-francof. 2: 96 (1778).

34.1. *Carex nigra* subsp. *nigra*

A species native to Europe, western Asia and North America, occurring in a variety of habitats, in wetlands, marshes, near water sources, in *Sphagnum*-dominated springs and along river banks, often on calcareous or acidic soils (Konrad et al. 2018). In Kosovo, it has been found in high montane to subalpine regions, where it thrives in fens and near high-mountain lakes. *Carex nigra* subsp. *nigra* is epistomatic (sometimes with a few stomata on the abaxial surface of the leaves), whereas the subspecies *intricata* (Tineo ex Guss.) Rivas Mart. (mainly distributed in the western Mediterranean region) and *transcaucasica* (T.V.Egorova) Jim.Mejías, G.E.Rodr.-Pal., Amini Rad & Martin-Bravo (from the Balkans to Iran) are both amphis-tomatic. Further research should reveal whether subsp. *transcaucasica* occurs also in Kosovo, as genomic studies suggest that at least some of the Balkan *C. nigra* material should be assigned to the subsp. *transcaucasica* (Valdés-Florido et al. 2024).

Specimens: – Albanian Alps of Kosovo: Gjeravicë, 42°31.733' N, 20°8.163' E, 2288 m, 23 Jul 2023, *Leg. Berisha, N.*; – Bogë, 42°46.217' N, 20°3.512' E, 1618 m, 08 Jun 2024, *Leg. Berisha, N.*; – Hajlë, 42°45.782' N, 20°5.927' E, 1811 m, 09 Jul 2007, *Leg. Millaku, F.*; – Bjeshka e Rusolisë, 42°45.148' N, 20°15.287' E, 2051 m, 21 Jul 2010, *Leg. Millaku, F.*; – Bjeshka e Dobroshit, 42°34.168' N, 20°5.310' E, 2041 m, 25 Jun 2024, *Leg. Berisha, N.*; – Sharri Mts.: Luboten Mt., 42°12.448' N, 21°6.551' E, 2072 m, 24 Jun 2017, *Leg. Berisha, N.*; – Konjushë Mt., 42°8.423' N, 20°57.756' E, 2248 m, 23 Jun 2024, *Leg. Berisha, N.*; – Shutman, 41°54.818' N, 20°44.629' E, 2161 m, 31 Jul 2023, *Leg. Millaku, F.*; – Brod, 41°57.553' N, 20°42.281' E, 1769 m, 01 Aug 2020, *Leg. Krasniqi, E.*; – Bresanë (Opojë), 42°3.892' N, 20°45.696' E, 1982 m, 21 Jun 2007, *Leg. Mala, Xh.*; – Ujërat e Qeta, 42°6.711' N, 20°53.147' E, 1756 m, 30 Jun 2014, *Leg. Mala, Xh.*

35. *Carex ornithopoda* Willd., Sp. Pl. 4: 255 (1805).

35.1. *Carex ornithopoda* subsp. *ornithopoda*

A taxon native to Europe spreading as far as Anatolia. It is specifically found on dry grasslands, in open woodlands, on calcareous as well as siliceous grasslands and on rocky slopes (Jermy et al. 2007). In Kosovo, it has been found in high montane regions and thrives in well-drained, and well insolate habitats.

Specimens: – Sharri Mts.: Oshlak, 42°11.147' N, 20°56.389' E, 1491 m, 30 May 2018, *Leg. Krasniqi, E.*; – Albanian Alps of Kosovo: Ujerat e Kuqe, spruce meadows forest clearings, no. coord., 1791 m, 03 Jul 1952, *Leg. Rudski, J.*; – Bogiqevicë, calc. grassland, no. coord., 1350 m, 08 Jul 1979, *Leg. Rexhepi, F.*; – Sejnovë, 42°48.995' N, 20°17.123' E, 1708 m, 28 Jul 1969, *Leg. Anonymous*; – Zalli i Rupës, 42°33.835' N, 20°7.047' E, 1721 m, 19 May 2024, *Leg. Berisha, N.*; – Koritnik Mt., no coord., 14 Jul 1936, *Leg. Oelizm, H.*; Koritnik Mt., 42°7.076' N, 20°34.500' E, 1497 m, 29 May 2012, *Leg. Millaku, F.*

36. *Carex otomana* Molina & Llamas, Bot. J. Linn. Soc. 156: 404 (2008).

A species native to Europe spreading as far as Central Asia. It is specifically found in anthropogenically managed pastures, meadows and tall herbaceous meadows on fertile, deep soils (Molina et al. 2008). In Kosovo, it has been found in montane regions, where it thrives in damp meadows and open forests. In Kosovo, this species was previously erroneously known as *Carex leersii* F.W.Schultz.

Specimens: – Sharri Mts.: Brod, 41°57.043' N, 20°43.133' E, 1644 m, 20 Aug 2023, *Leg. Berisha, N.*; – Albanian Alps of Kosovo: Gryka e Rugovës, 42°41.906' N, 20°8.618' E, 1019 m, 09 Jun 2008, *Leg. Rexhepi, F.*; – Lumbardhi i Deçanit, 42°33.015' N, 20°15.457' E, 682 m, 19 Aug 2023, *Leg. Berisha, N.*; – Zalli i Rupës, 42°34.485' N, 20°7.978' E, 1472 m, 19 May 2024, *Leg. Berisha, N.*

37. *Carex otrubae* Podp. in Spisy Prír. Fak. Masarykovy Univ. 12: 15 (1922).

A species native to Europe, parts of Asia and North Africa, specifically found in moist habitats such as marshes, ditches, riverbanks and wet meadows (Müller et al. 2021). In Kosovo, it has been found in lowland to mountainous regions and thrives in moist, nutrient-rich soils.

Specimens: – Central Kosovo: Ligatina e Hencit (Fushë Kosovë), 42°34.681' N, 21°2.921' E, 536 m, 12 May 2024, *Leg. Berisha, N.*; – Lumi Drenicë (Bardh i Vogël), 42°36.510' N, 21°2.026' E, 533 m, 09 Jun 2024, *Leg. Krasniqi, E.*; – Golesh Mt., 42°35.244' N, 20°59.691' E, 681 m, 12 Jun 2015, *Leg. Krasniqi, E.*; – Vasilevë, 42°37.797' N, 20°58.257' E, 723 m, 17 Jun 2024, *Leg. Berisha, N.*; – Harilaç (Fushë Kosovë), 42°35.348' N, 21°0.569' E, 566 m, 05 May 2024, *Leg. Berisha, N.*; – Shtime, 42°25.981' N, 21°1.056' E, 591 m, 24 Jun 2017, *Leg. Mala, Xh.*; – Talinoc (Ferizaj), 42°23.973' N, 21°10.836' E, 566 m, 28 May 2023, *Leg. Mala, Xh.*; – Gazimestan, 42°41.100' N, 21°7.228' E, 582 m, 02 Jul 2022, *Leg. Berisha, N.*; – North Kosovo: Mitrovicë, 42°52.766' N, 20°52.458' E, 508 m, 17 May 2022, *Leg. Berisha, N.*

38. *Carex pairae* F.W.Schultz, Flora 51: 303 (1868).

A species native to Europe and north-west Africa, specifically found in forest borders, meadows and disturbed areas (Jermy et al. 2007). In Kosovo, it has been recorded in a montane region, thriving in thermophilous forest borders. This is a new species for Kosovo.

Specimens: – Gërmi (Prishtinë), 42°40.686' N, 21°12.268' E, 760 m, 01 Jun 2024, *Leg. Berisha, N.* [UPH – 00002067].

39. *Carex pallescens* L., Sp. Pl.: 977 (1753).

A species native to the northern hemisphere, including Europe, Asia and North America. It specifically inhabits moist meadows, open woodlands and grassy clearings and often favours acidic to neutral soils (Müller et al. 2021) though present also on calcareous soils (Luceño et al. 2023). In Kosovo, it has been found in high mountain regions where it thrives in semi-shaded, damp environments.

Specimens: – Sharri Mts.: Përrojet e Durlës, 42°10.339' N, 21°0.877' E, 1649 m, 30 Jul 2022, *Leg. Berisha, N.*; – Luboten Mt., 42°13.691' N, 21°9.927' E, 1209 m, 24 Jun 2017, *Leg. Berisha, N.*; – Oshlak, 42°11.786' N, 20°56.119' E, 1409 m, 02 Jun 2017, *Leg. Berisha, N.*; – Pashallore Mt., 42°15.032' N, 20°54.897' E, 1659 m, 27 Jul 2023, *Leg. Berisha, N.*; – Bistër, 42°9.971' N, 20°58.320' E, 1504 m, 24 Jul 2021, *Leg. Berisha, N.*; – Albanian Alps of Kosovo: Hajlë, 42°45.548' N, 20°5.973' E, 1769 m, 09 Jul 2007, *Leg. Millaku, F.*; – Mokna, 42°49.850' N, 20°21.008' E, 2019 m, 21 Jun 2012, *Leg. Millaku, F.*; – Bogë, 42°45.683' N, 20°3.577' E, 1609 m, 08 Jun 2024, *Leg. Berisha, N.*; – Rekë e Allagës, 42°43.817' N, 20°10.947' E, 1741 m, 17 Jun 2023, *Leg. Berisha, N.*

40. *Carex paniculata* L., Cent. Pl. 1: 31 (1755).

40.1. *Carex paniculata* subsp. *paniculata*

A species native to Europe, western Asia, and parts of North America. It specifically inhabits wet, often more or less shady areas such as peaty swamps, marshes, and the edges of lakes, canals, and streams (Jermy et al. 2007). In Kosovo, it has been recorded in similar habitats, thriving in moist, nutrient-rich soils of high-mountain regions.

Specimens: – Sharri Mts.: Pashallore Mt., 42°15.065' N, 20°54.845' E, 1679 m, 27 Jul 2023, *Leg. Berisha, N.*; – Brezovicë, 42°10.814' N, 21°2.608' E, 1900 m, 17 Jul 2020, *Leg. Krasniqi, E.*; – Vracë, 41°52.924' N, 20°39.789' E, 1722 m, 19 Jun 2023, *Leg. Millaku, F.*; – Oshlak, 42°12.278' N, 20°51.780' E, 1752 m, 24 Jun 2019, *Leg. Krasniqi, E.*; – Luboten Mt., 42°12.902' N, 21°8.248' E, 1715 m, 24 Jun 2017, *Leg. Berisha, N.*; – Brod, 41°57.441' N, 20°42.928' E, 1601 m, 14 Jul 2016, *Leg. Mala, Xh.*; – Përrojet e Durlës, 42°10.344' N, 21°0.868' E, 1630 m, 03 Jul 2017, *Leg. Berisha, N.*; – Albanian Alps of Kosovo: Bogë, 42°46.077' N, 20°4.033' E, 1656 m, 08 Jun 2024, *Leg. Berisha, N.*

41. *Carex pendula* Huds., Fl. Angl.: 352 (1762).

A species native to western, central and southern Europe and extending to north-west Africa and parts of the Middle East. It specifically inhabits moist, shady environments such as woodlands, stream banks and hedgerows and often favours heavy clay soils (Müller et al. 2021). In Kosovo, it has been recorded in similar habitats and thrives on moist, nutrient-rich soils from the lowlands to the mountainous regions.

Specimens: – Zalli i Rupës, 42°34.188' N, 20° 7.868' E, 1488 m, 25 Jun 2024, *Leg. Berisha, N.*; – Gërmi (Prishtinë), 42°40.681' N, 21°13.875' E, 931 m, 25 May 1989, *Leg. Anonymous*; – Sharri Mts.: Gotovushë, 42°14.153' N, 21°4.428' E, 1071 m, 10 Aug 2023, *Leg. Millaku, F.*

42. *Carex pyrenaica* Wahlenb. in Kongl. Vetensk. Acad. Nya Handl., nov. Ser. 24: 139 (1803).

42.1. *Carex pyrenaica* subsp. *pyrenaica*

A species native to the mountainous regions of Europe. It specifically inhabits moist meadows and stream banks, often at higher altitudes (Davis, 1985). In Kosovo it has been found in subalpine zones and thrives on moist, well-drained soils, also near snow-bed vegetation.

Specimens: – Sharri Mts.: Liqeni i Jazhincës, 42°9.408' N, 20°59.738' E, 2070 m, 10 Jul 2013, *Leg. Berisha, N.*; – Bistër, 42°8.714' N, 20°57.745' E, 2268 m, 24 Jul 2021, *Leg. Berisha, N.*

43. *Carex remota* L., Fl. Angl.: 24 (1754).

A species native to Europe, West Asia and parts of North Africa, specifically found in moist forests, riparian forests, and shady stream banks (Müller et al. 2021). In Kosovo, it has been found in mountainous regions where it thrives in moist gallery forests and well-drained soils.

Specimens: – Sharri Mts.: Firajë: 42°15.822' N, 21°6.160' E, 731 m, 23 Aug 2022, *Leg. Mala, Xh.*; – Lubinjë e Poshtme, 42°7.305' N, 20°51.226' E, 1294 m, 01 Aug 2023, *Leg. Krasniqi, E.*; – Malet e Jezercës, Petrovë: 42°23.884' N, 21°1.288' E, 770 m, 20 May 2020, *Leg. Berisha, N.*; – Central Kosovo: Blinajë, 42°31.278' N, 20°58.646' E, 690 m, 13 May 2024, *Leg. Berisha, N.*

44. *Carex riparia* Curtis, Fl. Londin. 2(4): t. 60 (1783).

A robust, rhizomatous species native to Europe and parts of Asia and North Africa. It is specifically found in moist habitats such as marshes, swamps, riverbanks and the edges of lakes (Jermy et al. 2007). In Kosovo, it has been found in lowland regions where it thrives in nutrient-rich, waterlogged soils in swamps and on riverbanks.

Specimens: – Ligatina e Hencit (Fushë Kosovë), 42°34.917' N, 21°2.835' E, 536 m, 12 May 2024, *Leg. Berisha, N.*; – Dobrajë (Lipjan), 42°33.049' N, 21°1.636' E, 551 m, 12 May 2024, *Leg. Berisha, N.*; – Graboc i Epërm (Obiliç), 42°39.166' N, 20°59.964' E, 551 m, 13 May 2024, *Leg. Krasniqi, E.*; – Miradi e Epërme (Fushë Kosovë), 42°35.825' N, 21°6.224' E, 548 m, 10 May 2024, *Leg. Berisha, N.*; – Medvec (Lipjan), 42°33.441' N, 21°1.738' E, 548 m, 12 May 2024, *Leg. Berisha, N.*; – Jezercë (Ferizaj), 42°26.507' N, 20° 54.508' E, 794 m, 10 Jul 2023, *Leg. Berisha, N.*; – Talinoc (Ferizaj), 42°24.009' N, 21°10.903' E, 566 m, 09 May 2024, *Leg. Berisha, N.*; – Harilaç (Fushë Kosovë), 42°35.416' N, 21°0.929' E, 550 m, 05 May 2024, *Leg. Berisha, N.*; Mitrovicë, 42°52.268' N, 20°52.951' E, 503 m, 11 May 2024, *Leg. Berisha, N.*; – Shtime, 42°26.148' N, 21°1.083' E, 594 m, 09 May 2024, *Leg. Berisha, N.*

45. *Carex rostrata* Stokes in W. Withering, Bot. Arr. Brit. Pl., ed. 2, 2: 1059 (1787) nom. cons.

A rhizomatous taxon native to the Holarctic of Europe, Asia and North America. It specifically inhabits wetlands such as marshes, bogs, fens and the margins of lakes and rivers (Müller et al. 2021). In Kosovo, it has been recorded in similar habitats and thrives in nutrient-rich, waterlogged soils in high montane regions.

Specimens: – Sharri Mts.: Bistër, 42°8.572' N, 20°57.818' E, 2198 m, 09 Jul 2018, *Leg. Berisha, N.*; – Ujërat e Qeta, no coord., 1977 m, 29 Jun 1980, *Leg. Rexhepi, F.*; – Vracë, 41°53.367' N, 20°43.405' E, 2084 m, 29 Jun 2011, *Leg. Millaku, F.*; – Koritnik Mt., 42°5.461' N, 20°34.098' E, 1981 m, 29 May 2012, *Leg. Krasniqi, E.*; – Albanian Alps of Kosovo: Mokna, 42°49.605' N, 20°21.409' E, 1969 m, 21 Jun 2012, *Leg. Millaku, F.*; – Ligenat (Kuşishtë), 42°40.173' N, 20°5.526' E, 1869 m, 28 Jun 2020, *Leg. Berisha, N.*

46. *Carex rupestris* All., Fl. Pedem. 2: 264 (1785).

A rhizomatous species native to the subarctic and temperate regions of the northern hemisphere of Europe, Asia, and North America. It specifically inhabits dry to mesic heaths, meadows, and rocky outcrops (Müller et al. 2021). In Kosovo, it has been recorded in alpine and subalpine zones, thriving in well-drained, rocky soils and wind-exposed short base-rich grasslands.

Specimens: – Albanian Alps of Kosovo: Ligenat (Kuşishtë), 42°40.187' N, 20°4.945' E, 2011 m, 28 Jun 2020, *Leg. Berisha, N.*; – Koritnik Mt., 42°5.419' N, 20°33.705' E, 2109 m, 28 Jul 2013, *Leg. Krasniqi, E.*

47. *Carex sempervirens* Vill., Hist. Pl. Dauphiné 2: 214 (1787).

47.1. *Carex sempervirens* subsp. *pseudotrists* (Domni) Pawł., Publ. Inst. Bot. Univ. Jagell. Cracov. 1937: 5 (1937).

A tussock-forming species native to the mountainous regions of Europe, specifically found in nutrient-poor grasslands, rocky slopes and alpine meadows at high altitudes (Luceño 2008). In Kosovo, it has been found in subalpine and alpine zones, where it thrives in well-drained, silicate grasslands. This is a newly recorded species for Kosovo.

Specimens: – Sharri Mts.: Luboten Mt., 42°12.949' N, 21°7.611' E, 1989 m, 27 Jun 2019, *Leg. Berisha, N.* [UPH - 00002068]; – Vracë, 41°52.884' N, 20°43.448' E, 2151 m, 29 Jun 2011, *Leg. Millaku, F.* [UPH - 00002069]; – Bjeshka e Lumbardhit të Prizrenit, 42°11.576' N, 20°49.640' E, 1284 m, 12 Aug 2023, *Leg. Berisha, N.* [UPH - 00002070]; – Albanian Alps of Kosovo: Gjeravicë, 42°31.596' N, 20°8.054' E, 2210 m, 23 Jul 2023, *Leg. Berisha, N.* [UPH - 00002071].

48. *Carex spicata* Huds., Fl. Angl.: 349 (1762).

48.1. *Carex spicata* subsp. *spicata*

A caespitose species native to Europe, parts of Western Asia and North Africa. It usually grows in grasslands, meadows, roadsides and disturbed areas and often favours heavy, slightly alkaline soils (Jermy et al. 2007). In Kosovo it has been recorded in similar habitats. It thrives on well-

drained, nutrient-rich soils from the lowlands to the mountain regions.

Specimens: – Ligatina e Hencit (Fushë Kosovë), 42°34.457' N, 21°2.364' E, 538 m, 12 May 2024, *Leg. Berisha, N.*; – Dobrajë (Lipjan), 42°33.455' N, 21°1.776' E, 547 m, 12 May 2024, *Leg. Berisha, N.*; – Koretin (Kamenicë), no coord., 808 m, 11 Jul 1990, *Leg. Pajazitaj, Q.*

49. *Carex sylvatica* Huds., Fl. Angl.: 353 (1762).

49.1. *Carex sylvatica* subsp. *sylvatica*

A densely tufted species native to deciduous forests throughout Europe and parts of Asia as far as north Iran. It specifically inhabits moist, shady environments and thrives in heavy, humus-rich soils (Jermy et al. 2007). In Kosovo, it has been recorded in similar habitats, favouring moist, shady forest soils.

Specimens: – Albanian Alps of Kosovo: Bogë, 42°45.413' N, 20°3.320' E, 1501 m, 09 Jun 2024, *Leg. Berisha, N.*; – Zalli i Rupës (Deçan), 42°33.619' N, 20°7.201' E, 1553 m, 19 May 2024, *Leg. Berisha, N.*; – Drelaj, 42°40.855' N, 20°7.825' E, 1660 m, 10 Jun 2024, *Leg. Berisha, N.*; – Sharri Mts.: Pashallore, 42°15.520' N, 20°54.825' E, 1570 m, 27 Jul 2023, *Leg. Berisha, N.*; – Luboten Mt., 42°13.601' N, 21°10.102' E, 1249 m, 20 Jun 2019, *Leg. Berisha, N.*; – Vërbeshticë (Shtërpçë), 42°14.124' N, 20°56.649' E, 1289 m, 11 Jul 2023, *Leg. Mala, Xh.*

50. *Carex tomentosa* L., Mant. Pl. 1: 123 (1767).

A species native to Europe and parts of Asia, specifically found in seasonally moist meadows, ditch margins and light, slightly moist deciduous forests (Müller et al. 2021). In Kosovo, it has been recorded in similar habitats and thrives on calcareous, nutrient-poor soils in partially shaded areas.

Specimens: – Gërmi (Prishtinë), 42°40.120' N, 21°13.518' E, 830 m, 24 May 2024, *Leg. Berisha, N.*; – Llapushnik, 42°33.897' N, 20°51.418' E, 711 m, 30 Apr 2024, *Leg. Berisha, N.*; – Harilaç (Fushë Kosovë), 42°35.394' N, 21°0.523' E, 568 m, 05 May 2024, *Leg. Berisha, N.*; – Bresje (Fushë Kosovë), 42°37.557' N, 21°5.939' E, 529 m, 06 May 2024, *Leg. Berisha, N.*

51. *Carex umbrosa* Host, Icon. Descr. Gram. Austriac. 1: 52 (1801).

51.1. *Carex umbrosa* subsp. *umbrosa*

A caespitose taxon native to Europe and parts of Asia, specifically found in grassy areas of deciduous forests, where it thrives in moist, shady environments (Konrad et al. 2018). In Kosovo it has been recorded in similar habitats, favouring well-drained, calcareous soils of the montane zone. This species is newly recorded for Kosovo.

Specimens: – Svirçë (Kamenicë), 42°41.947' N, 21°34.421' E, 819 m, 07 May 2023, *Leg. Berisha, N.* [UPH - 00002072]; – Llapushnik, 42°33.761' N, 20°52.128' E, 733 m, 30 Apr 2024, *Leg. Berisha, N.* [UPH - 00002073]; – Sharri Mts.: Pashallore, 42°15.119' N, 20°55.150' E, 1589 m, 27 Jul 202, *Leg. Berisha, N.* [UPH - 00002074]; – Albanian Alps of

Kosovo: Bogë, 42°45.525' N, 20°3.409' E, 1550 m, 08 Jun 2024, *Leg. Berisha, N.* [UPH - 00002075].

52. *Carex vesicaria* L., Sp. Pl.: 979 (1753).

A species native to temperate regions of the northern hemisphere of Europe, Asia and North America. It is specifically found in wetlands such as marshes, swamps, lake and pond shores and wet meadows, in areas that are seasonally flooded in spring but can dry out later in the year (Müller et al. 2021). In Kosovo, it has been recorded in a similar habitat, thriving in moist, nutrient-rich soil nearby a lake in the high mountainous region. This species is newly recorded for Kosovo.

Specimens: – Albanian Alps of Kosovo: Liqenat (Kuqishtë), 42°40.097' N, 20°5.483' E, 1861 m, 28 Jun 2020, *Leg. Berisha, N.* [UPH - 00002086].

53. *Carex* × *ruedtii* Kneuck., in M.A.Seubert, Excurs.-Fl. Baden, ed. 5: 67 (1891).

[*C. flava* × *C. lepidocarpa*]

Carex × *ruedtii* is a rather rare hybrid, documented in various parts of Europe. It prefers mineral-rich bogs where there is a regular through-flow of water (Koopman 2010, 2022). In Kosovo it was recorded in a wet meadow. It is a newly recorded hybrid for Kosovo.

Specimens: – Sharri Mts.: Brod, 41°57.800' N, 20°42.806' E, 1526 m, 20 Aug 2023, *Leg. Berisha, N.* [UPH - 00002087].

54. *Carex* × *subviridula* Fernald, Rhodora 35: 231 (1933).

[*C. flava* × *C. oederi*]

Carex × *subviridula* is a rather rare hybrid, specifically found in habitats similar to its parent species, such as wetlands, fens, and moist meadows, often in areas with calcareous or slightly acidic soils (Więclaw 2014, Koopman 2022). In Kosovo, *C. × subviridula* has been observed in the subalpine region, where it thrives in fen vegetation of slightly acidic fens. It is a newly recorded hybrid for Kosovo. However, despite the presence of its hybrid, we have not recorded the parental species *Carex oederi* Retz. in this area. Nevertheless, we expect that additional field surveys will confirm its presence.

Specimens: – Sharri Mts.: Luboten Mt., 42°12.773' N, 21°6.938' E, 1969 m, 08 Jul 2018, *Leg. Berisha, N.* [UPH - 00002088].

Discussion

This study has resulted in the most comprehensive and taxonomically verified checklist of *Carex* taxa in Kosovo to date. The recorded 52 species, 19 subspecies, 1 variety and 2 hybrids constitute a considerable increase in the known *Carex* diversity in the region. The confirmation of 15 taxa newly recorded for the flora of Kosovo underlines the floristic gaps that still exist in this Balkan country.

The high representation of *Carex* taxa in subalpine and alpine ecosystems, especially in the Sharri Mountains and the Albanian Alps, confirms the ecological importance and

habitat specificity of this genus. Many taxa, such as *C. castroviejoi*, *C. sempervirens* subsp. *pseudotristsis* and *C. lazareii*, are restricted to serpentine or silicate substrates, emphasizing their importance as habitat specialists and potential indicators of habitat integrity.

Compared to neighbouring countries such as Albania (Martín-Bravo et al. 2022), Croatia (Nikolić 2020) and Serbia (Niketić and Tomović 2018), the diversity of the genus *Carex* in Kosovo is now well documented and more accurately represented due to this comprehensive study. While the country has reached a comparable level of knowledge regarding the richness of *Carex*, further field research in underexplored regions could reveal additional taxa. In particular, some species already reported (e.g. *Carex arenaria*, *C. tricolor*) have not yet been confirmed, underlining the need for more targeted surveys.

The taxonomic challenges within the genus, such as hybridisation (e.g. *C. × ruedtii* and *C. × subviridula*) and morphological plasticity, have been addressed by using modern identification keys and comparative herbarium studies. This approach has not only corrected numerous misidentifications but also improved our understanding of infraspecific variation in the region. *Carex* hybrids can usually be recognised by empty utricles and anthers that do not dehisce, hidden under the male glumes (Jermy et al. 2007). Moreover, hybrids like *C. × ruedtii* and *C. × subviridula* are generally morphologically intermediate compared to their parental taxa or they show a mosaic of parental, intermediate and unique characters (Więclaw and Wilhelm 2014).

The discovery of narrowly distributed and habitat-specific taxa also has conservation implications. Many of these sites are located in national parks but are not actively monitored.

This updated checklist and distribution assessment contributes significantly to the understanding of *Carex* diversity in the Balkans. It provides a taxonomic basis for future ecological, biogeographical and conservation research. It also emphasizes the need for systematic botanical inventories, especially in areas such as Kosovo that are rich in biodiversity but have historically been unexplored.

Non-confirmed records of *Carex* taxa

Drawing on several key sources for the genus in our region (Josifović 1976, Sarić and Diklić 1986, Niketić and Tomović 2018), we have not been able to confirm the presence of the 16 *Carex* taxa previously reported for Kosovo in Tab. 2.

Our review of all available herbarium specimens and field observations revealed that these records were either misidentifications (*C. appropinquata*, *C. leersii*, *C. montana*, *C. pilosa*, *C. praecox* and *C. vulpina*), the specimens being assigned erroneously to other *Carex* taxa, or were entirely absent from their reported habitats in Kosovo.

Accurate taxonomic identification is essential for understanding regional species diversity. In the case of *Carex*, a genus known for its morphological complexity, misidenti-

Tab. 2. Non-confirmed *Carex* taxa in Kosovo.

No.	Taxon
1.	<i>Carex appropinquata</i> Schumach.
2.	<i>Carex brevicollis</i> DC.
3.	<i>Carex elongata</i> L.
4.	<i>Carex foetida</i> All.
5.	<i>Carex fuliginosa</i> Schkuhr
6.	<i>Carex leersii</i> F.W.Schultz
7.	<i>Carex limosa</i> L.
8.	<i>Carex montana</i> L.
9.	<i>Carex myosuroides</i> Vill.
10.	<i>Carex oederi</i> Retz.
11.	<i>Carex ornithopoda</i> subsp. <i>ornithopodioides</i> (Hausm.) Nyman
12.	<i>Carex panicea</i> L.
13.	<i>Carex pilosa</i> Scop.
14.	<i>Carex praecox</i> Schreb.
15.	<i>Carex strigosa</i> Huds.
16.	<i>Carex vulpina</i> L.

fications are not uncommon (Clerc-Blain et al. 2009). The results of this study emphasise the need to regularly review historical records and herbarium specimens with taxonomic expertise (Więclaw et al. 2021). Although future field expeditions and broader surveys may reveal some of these *Carex* taxa in Kosovo, our current evidence indicates that, to date, they remain unverified.

Diversity, distribution, and rarity of *Carex* taxa in Kosovo

Carex taxa play a central role in maintaining wetland ecosystem functions. Their dense, fibrous root systems stabilise soil by reducing erosion and promoting sediment retention along riparian zones. Moreover, these taxa enhance water infiltration and retention, moderate runoff patterns, and thereby promote nutrient cycling and overall water quality. Given their sensitivity to hydrological changes and pollutant inputs, *Carex* taxa are frequently used as bio-indicators in wetland monitoring programmes, offering valuable insights into ecosystem health (Yuan et al. 2019).

The *Carex* flora of Kosovo exhibits notable diversity, with currently 52 species within 4 different subgenera. Geographically, the documentation of these taxa spans various regions of Kosovo. The Sharri Mountains and the Albanian Alps, both national parks, are particularly significant, with 36 taxa and 90 records, and 25 taxa and 62 records, respectively. These figures underscore their roles as major reservoirs for *Carex* diversity, although the high record counts may include multiple observations from the same localities. Additionally, substantial records originate from other areas, such as Pashallor Mt., low meadows of Kosovo plain and Malësia e Gjakovës, demonstrating that *Carex* diversity is widespread and not confined solely to the two major mountain ranges. Within these broader regions, several hotspots emerge: Vracë and Brod each host 13 taxa,

followed by Luboten Mt. with 12, Bogë with 10, and Bistër with 9 taxa. Other localities, including Bjeshka e Rusolisë, Pashallor Mt., Gërmi, Mokna, Ligatina e Hencit, Gjeravicë Mt., Blinajë, Zalli i Rupës, and Përrojet e Durlës, each record 7 or 8 taxa, indicating a clustered pattern of species richness.

Another important result of this study is the establishment of the rarity of some taxa. An analysis of locality data shows that several *Carex* taxa are known only from a single locality. For example, *C. depressa*, *C. digitata*, *C. distachya*, *C. macrolepis*, *C. michelii*, *C. pairae*, and *C. vesicaria* have all been recorded from single sites. These restricted occurrences may indicate true rarity – due to specific habitat requirements or historical isolation – or could reflect gaps in sampling. Nevertheless, taxa with such a narrow distribution are of particular conservation interest, as they are more vulnerable to environmental disturbance.

In view of these findings, targeted conservation measures are urgently needed. For instance, *C. vesicaria* is only known from Liqenat, where it thrives along the lake shore, a popular destination for tourists and mountain hikers – potentially increasing habitat disturbance. Similarly, *C. pairae* and *C. michelii*, recorded in the bushy vegetation and forest clearings of Gërmi, underscore the importance of designating Gërmi a regional park with unique conservation value. *Carex macrolepis*, found in Malësia e Gjakovës on serpentine soils near *Quercus* forests, is confined to a limited natural habitat that is subject to military exercises and is expected to become a strict military zone, further jeopardising its survival. Additionally, *C. depressa* from Pashallor Mt. and *C. flacca* subsp. *erythrostachys*, documented only in Golesh Mt. of central Kosovo, highlight the vulnerability of species with a very narrow distribution. Monitoring population trends and implementing targeted conservation measures in these areas are crucial for preserving these taxa, their habitats, and the overall ecological integrity of Kosovo's landscapes (van Diggelen et al. 2006).

Finally, the patterns observed in Kosovo reflect broader trends in the Balkan region, where complex topography and diverse microhabitats have fostered high levels of endemism and floristic turnover (Martín-Bravo et al. 2022, Więclaw et al. 2019). Continued field research, particularly in understudied areas, is essential to refine our understanding of the distributional limits and conservation status of these taxa. Such efforts, coupled with molecular studies to resolve taxonomic ambiguities, will provide deeper insights into the evolutionary and ecological processes shaping sedge diversity in Kosovo and the Balkans.

Conclusions

This study provides the first verified checklist of *Carex* taxa for Kosovo. It confirms the occurrence of 52 species, including 19 subspecies and one variety, and two hybrids. There are 15 taxa newly reported for the flora of Kosovo; earlier reports of another 10 taxa remaining are refuted as

so far unconfirmed. Although our extensive field surveys and herbarium reviews have provided an important basis for regional *Carex* diversity, further fieldwork is needed to update these results and ensure that all details are accurately documented. In addition, molecular studies would be invaluable for clarifying the phylogenetic relationships among these taxa, clarifying taxonomic uncertainties and prioritising conservation efforts. It is essential that government agencies conduct systematic assessments of the conservation status of these taxa, especially those with restricted distributions, in order to develop effective management and conservation strategies.

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Author contribution statement

N.B. designed the research, obtained the research grant, conducted and organised the field surveys and sample collections, performed taxonomic work, prepared the first draft of the manuscript and the final version; F.M. conducted the field surveys and sample collections, worked on the taxonomy of taxa, examined and prepared herbarium specimens, and wrote/edited parts of the manuscript; E.K. conducted the field surveys and sample collections, performed taxonomic work, and wrote parts of the manuscript; R.C. examined and prepared herbarium specimens at different stages of the work, performed taxonomic work, and wrote/edited parts of the manuscript; X.M. conducted the field surveys and sample collections, performed taxonomic work, and assisted in herbarium specimen collections; H.W. performed taxonomic work, verified the final taxa identifications, and contributed to the manuscript structure and writing; J.K. performed taxonomic work, verified the final taxa identifications, and contributed to the manuscript structure and writing. All authors contributed to the concept and implementation of the study and took part in the final revision, discussion, and approval of the manuscript.

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Research Article

Spontaneous spread of the alien species *Nepeta racemosa* (Lamiaceae) in Ukraine

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Abstract – In the flora of Ukraine, 48 findings of the spontaneous spread of the alien species *Nepeta racemosa* Lam. were recorded: 30 records within the introduction centers and 18 outside the boundaries of cultivation. A brief morphological description of the plants is provided, and their ecological and biological features are investigated. It was found that all the habitats of this species in Ukraine were confined to synanthropic biotopes: decorative cultivated biotopes (parks, squares, lawns, flower gardens), residential and technotopes (biotope complexes of built-up areas, garbage and solid waste landfills). Most often, the plants grow in cracks in sidewalks and asphalt. According to time of introduction, *N. racemosa* belongs to neophytes, and according to method of introduction to ergasiophytes. The species requires further monitoring to assess the likelihood of its further spread within and beyond urban environments.

Keywords: alien species, flora, introduction, ornamental plants, ruderal vegetation

Introduction

The leading role in the processes of anthropogenic transformation of the urban environment's vegetation cover is played by non-native species, the appearance of which is due to many factors: transport flows, significant concentration of people, increase in built-up areas, infrastructure development and so on (Arianoutsou et al. 2021, Haubrock et al. 2021, Rodríguez et al. 2024). A separate factor is the purposeful or spontaneous introduction of plants which not only contributes to the preservation of plant diversity and the selection of promising species to replenish plant resources (decorative, medicinal, food, fodder, etc.) but can also become a source of phytointvasions. Recent observations of new alien species growing spontaneously in Europe have revealed a current trend for them to emerge in consequence of their use in ornamental horticulture and commerce via the Internet, retail outlets, specialist nurseries, etc. (Ööpik et al. 2013, Hulme et al. 2017, Arianoutsou et al. 2021). Introductions often escape from their cultivation sites and become wild in a new region, achieve varying degrees of naturalization, and contribute to the replenishment of the spontaneous flora with alien plant species (Pyšek et al. 2022, Iamónico and Nicolella 2024, Koniakin et al. 2024).

Climate change will only exacerbate the problem of biological invasions, which will damage the environment and bring about undesirable economic and social consequences.

Therefore, the European Union and Ukraine have adopted a strategy for biodiversity conservation, where special attention is focused on limiting the introduction of alien plant species with uncertain adaptive potential; ongoing monitoring studies of the adventitious fraction of the flora are being conducted (Burda and Ignatyuk 2011, Genovesi et al. 2015, European Commission 2022).

During floristic surveys of Kyiv, we recorded for the first time outside the place of cultivation the foci of the alien species *Nepeta racemosa* Lam. (Lamiaceae), which has not been previously reported by previous researchers into this area (Mosyakin and Yavorska 2002, Yavorska 2004). This species belongs to the genus *Nepeta* L. (subfamily Nepetoideae (Dumort.) Luer.), which has 295 accepted species, common in Southwest and Central Asia, North and Central America, North Africa, Southeastern Europe, Korea, Japan (POWO 2024). Representatives of this genus are characterized by high decorative qualities and valuable economic properties due to the presence of various bioactive compounds. For practical use, they are grown in many countries around the world – Great Britain, Netherlands, Romania, Czech Republic, USA, Argentina, New Zealand and others (Salehi et al. 2018, POWO 2024).

There are four taxa of the genus *Nepeta* growing in Ukraine: two native species – *N. nuda* L. subsp. *nuda* (syn. *N. pannonica* L.), *N. ucranica* L. subsp. *parviflora* (M. Bieb.) M. Masclans (syn. *N. parviflora* M. Bieb.) and two alien

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species – *N. cataria* L., *N. grandiflora* M. Bieb. (Klokov 1960, GBIF 2025, POWO 2024). There are 14 taxa of this genus that have been introduced in the botanical gardens and arboretums of Ukraine (Mashkovskaya 2015).

Nepeta racemosa is an ornamental, medicinal, melliferous, food, and essential oil species (Hawke 2007, Baser et al. 2011, Lungoci et al. 2023). According to time of introduction, it is a neophyte, and to method of introduction an ergasiophyte (species that are wild near their cultivation). The plant is entomophilous and it spreads anemochorically. Branched stems with fruiting inflorescences easily break off at their base and are rolled by the wind, gradually scattering seeds (Takhtadzhyan 1981). The aerial parts of *N. racemosa* secrete essential oil, the main components of which are biologically active substances – monoterpenoids (non-petalactones), which have a wide range of uses. It is used as an antibacterial, antifungal, antitumor, antiviral, antioxidant, sedative, and insecticidal agent (Baser et al. 2011, Salehi et al. 2018). This species is considered to have a potential for improving the structure of saline soils while obtaining higher concentrations of bioactive substances (Lungoci et al. 2023). It is also used in the food industry as a powerful preservative and a source of aromatic raw materials for winemaking. In the world, according to the needs of society, varieties have been invented for various applications (phytodesign, pharmaceutical, medicinal, in food, etc.) *N. racemosa* – ‘Walker's Low’, ‘Blue Ice’, ‘Grog’, ‘Superba’, ‘Posvyata Meysu’ and others (Hawke 2007, Kovtun-Vodyanitska and Rakhmetov 2018). The species has a wide ecological range of growth, easily adapts to new conditions, is resistant to frost and drought, and can also be self-seeding in culture (Bojňanský and Fargašová 2007, Hawke 2007, Rigó et al. 2023).

The native range of *N. racemosa* includes the mountainous regions of Turkey, Iran, Iraq, and Transcaucasia (Azerbaijan, Armenia, and Georgia), where it grows as part of the composition of upland xerophytic and mountain meadow-steppe vegetation (POWO 2024). Due to its valuable economic qualities, it is cultivated outside its native range – Austria, Switzerland, Czech Republic, Hungary, Spain, Great Britain, Finland, Australia, New Zealand South, USA (POWO 2024, GBIF 2025). As a result of introduction, it has been naturalized in temperate countries – the United States of America (states Wyoming, Wisconsin, New York), Canada and Europe (Kartesz 2015, Pyšek et al. 2022, GBIF 2025).

In Ukraine, *N. racemosa* was introduced in 12 research centers with various natural and climatic conditions (in the zone of Mixed Forests, Forest-Steppe and Steppe, Ukrainian Carpathians) (Mashkovskaya 2015). This species is also used in the phytodesign of cities and towns, and is grown in nurseries and on homestead plots.

Recently, according to various information sources and our own observations, the escape of this species from cultivation sites has been recorded (Shynder et al. 2018, Chorna et al. 2021, iNaturalist 2025). In view of this, there is a need to study the foci of spontaneous spread of *N. racemosa* in

Ukraine, as well as to investigate its ecological and biological traits and biotope location to assess the further spread of the species in the urban environment.

Material and methods

Data on the spontaneous spread of *Nepeta racemosa* were collected during field surveys in Kyiv, a review of literary sources and from Internet open access resources – iNaturalist, GBIF and UkrBIN. The nomenclature follows Plants of the World Online (POWO 2024). Morphometric measurements of *Nepeta racemosa* were conducted in Kyiv. A total of 20 plant specimens from different localities were examined. Descriptive statistics (mean $\pm$ standard deviation) were calculated. The P values were considered significant at < 0.05 . Statistical analyses were performed using Microsoft Excel 2016. The seeds of the local reproduction were photographed with a Canon EOS600D digital camera mounted on an Olympus SZX 12. Biotopes are defined according to the European Nature Information System (EUNIS) (Chytrý et al. 2020). Naturalization status was assessed and defined according to Pyšek et al. (2022). The research was conducted during 2021 – 2024. Own findings are uploaded to the iNaturalist database.

Results

According to our observations, scientific publications, and Internet resources, 48 occurrences of the spontaneous spread of *Nepeta racemosa* have been identified in Ukraine (Shynder et al. 2018, Chorna et al. 2021, iNaturalist 2025, UkrBIN 2025). Depending on their habitat, these occurrences can be conditionally divided into two groups: those resulting from escape from the initial point of introduction within the introduction centers – 30 localities and those that emerged as a result of leaving cultivation sites and settled outside cultivation in various anthropogenic places – 18 localities.

The spontaneous spread of *N. racemosa* in Ukraine within the introduction centers began in 2011 in Kyiv (Kyiv region), where the largest number of localities is currently located – 24. Subsequently, *N. racemosa* has continue to spread spontaneously in introduction institutions of the central, eastern, and southern regions of Ukraine. During the period from 2021 to 2024, its settlement was observed in the following locations: National Dendrological Park "Sofiivka" (Uman, Cherkasy region, 2021 – 2023); Botanical Garden of the Mechnikov National University of Ukraine (Odesa, Odesa region, 2022); Kryvyi Rih Botanical Garden of the National Academy of Sciences of Ukraine (Kryvyi Rih, Dnipropetrovsk region, 2024); Children's Botanical Garden (Zaporizhzhia, Zaporizhzhia region, 2024) (Figs. 1, 2).

Eight years after the first settlement of *N. racemosa* within the boundaries of introductory institutions, in 2019, the species was first observed wild, outside of cultivation (Dnipro city). In the future, the number of such finds in

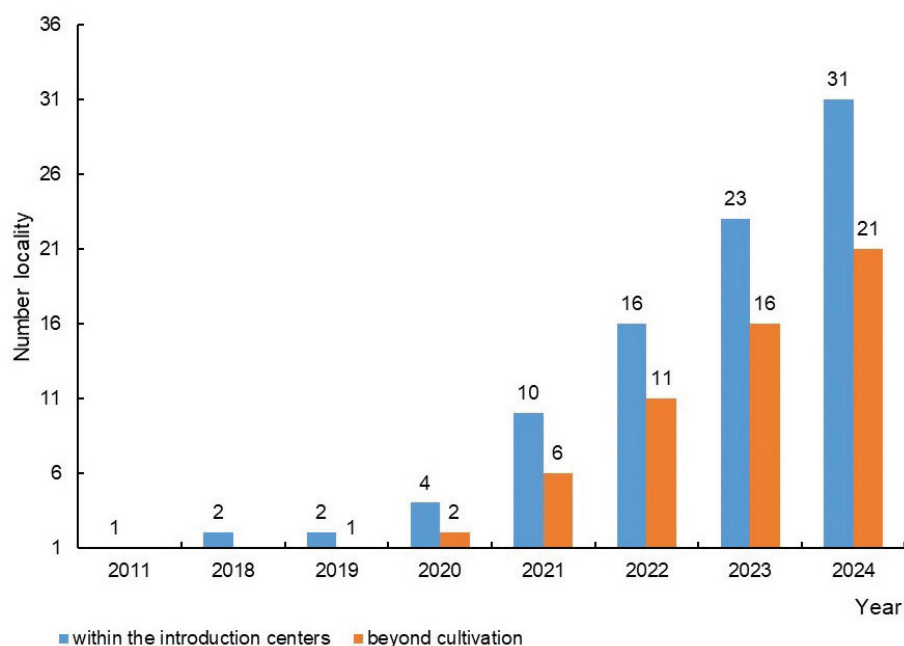


Fig. 1. Temporal dynamics (2011 – 2024) of recorded localities of spontaneous spread of *Nepeta racemosa* in Ukraine, showing cumulative numbers within cultivation sites (introduction centres) and outside cultivation.

Ukraine will increase annually. The largest number of spontaneous growths of this alien species is currently recorded in Kyiv and the Kyiv region – 14. In addition, in 2023 – 2024, *N. racemosa* was noted wild and outside cultivation in the central (Smila city, Cherkasy region) and

southern regions of Ukraine (in Odesa and Odesa region) (Figs. 2, 3).

An analysis of the nature of the spontaneous spread of *N. racemosa* in Ukraine outside the boundaries of culture found that all the finds are located near introduction centers

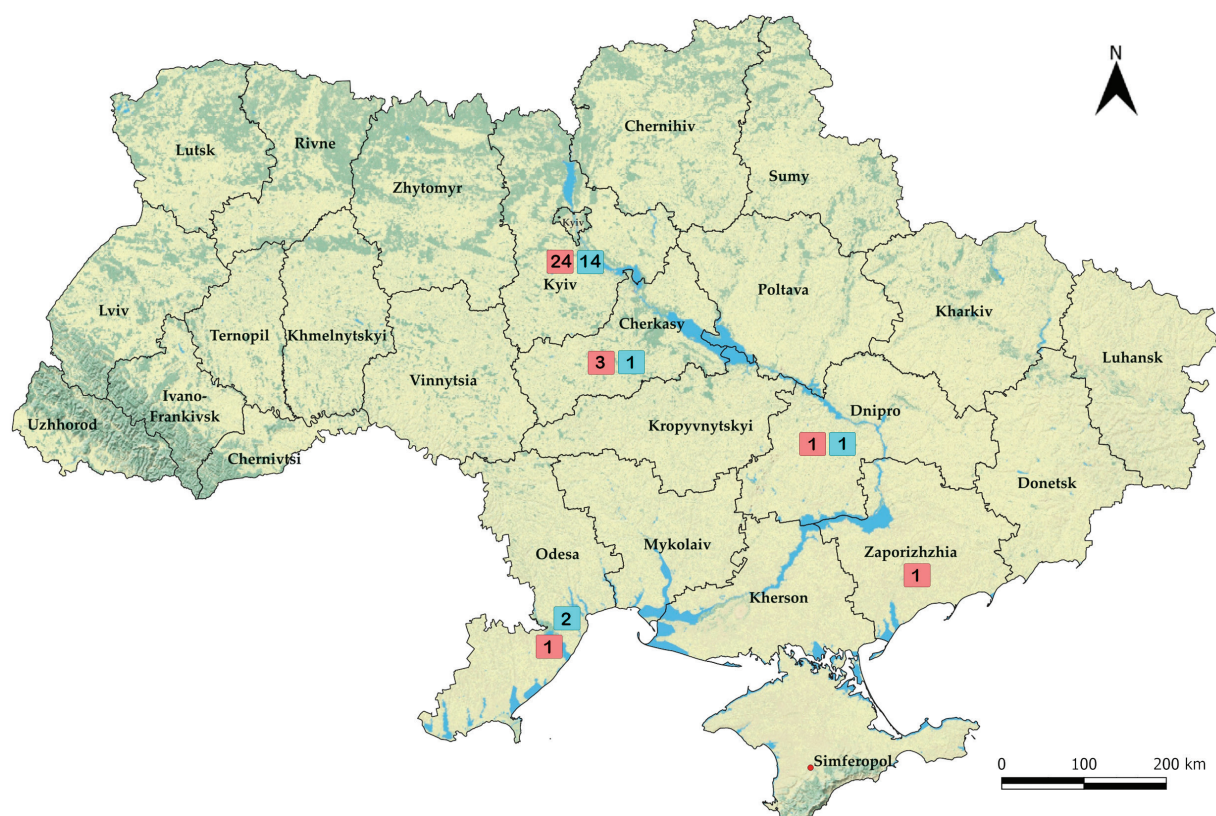


Fig. 2. Occurrence of *Nepeta racemosa* in Ukraine: records from cultivation (red) and records outside cultivation (blue); numbers indicate the number of localities.

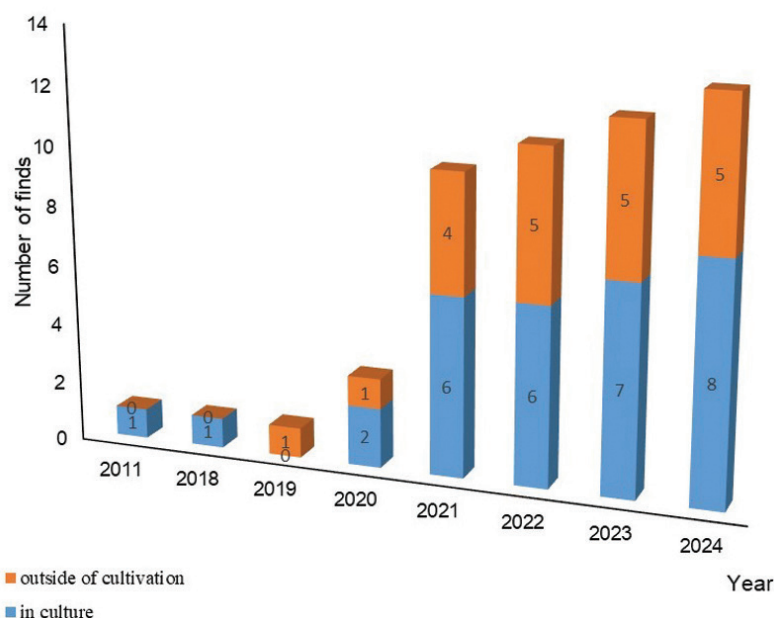


Fig. 3. Annual records of spontaneous spread of *Nepeta racemosa* in culture and outside cultivation for the period 2011 – 2024 in Ukraine.

or places of cultivation – cultural phytocenoses of various types. It was established that the spread of *N. racemosa* outside cultivation is closely related to its spontaneous growth in centers of introduction ($r = 0.84$, $P < 0.05$).

Most localities in both cases were found at the side of paths, in cracks in the pavement and asphalt, less often – in grass and lawns (Fig. 4).

Also, outside the cultivation area, we found *N. racemosa* in a natural dump of solid construction materials (crushed concrete, bricks, glass, asphalt residues, etc.; 50°22'01.4" N, 30°28'04.1" E, Kyiv, 2021) and the remains of garden waste

near the fallow of the archaeological site "Khotiv hillfort" (50°20'00.2" N 30°29'34.3" E; Khotiv village, Kyiv region, 2024). To determine the invasive potential of this alien species in our conditions, we investigated its ecological and biological features.

Plant habit: 42 – 48 cm in height and 70 – 90 cm in diameter (Fig. 5A). Leaves are short-petiolate, ovate, heart-shaped at the base, and bluntly toothed. The length of the leaf blade of the average formation is 1.9 – 3.4 (2.6 ± 0.9) cm, width 1.5 – 3.1 (2.1 ± 0.1) cm. Flowers are lilac, 1.2 – 1.7 (1.5 ± 0.01) cm long, collected in 6 – 8 (12) verticils that form spike-



Fig. 4. Habitat of *Nepeta racemosa*: A – a crack in an asphalt path (Children's Botanical Garden, Zaporizhzhia, photo: O. Shnyder), B – a crack in a curb, Bastionna Street, Kyiv, photo A. Levon, C – gap between stone fence and paving slabs (Sadovo-Botanicna Street, Kyiv) (photo: S. Koniakin).



Fig. 5. *Nepeta racemosa* outside the place of cultivation ("Expocenter of Ukraine", Kyiv): A – habit, B – inflorescence and flowers (photo: O. Leshcheniuk, S. Koniakin), C – seeds (photo: O. Bidzilya).

shaped inflorescences (Fig. 5B). Inflorescence length 8 – 31 (23.7 ± 1.2) cm, number of flowers per inflorescence 34 – 42. Fruit four-nut, 4.6 – 8.2 (6.5 ± 0.2) mm long, 2.1 – 3.0 (2.3 ± 0.1) mm wide. Nuts are dark brown, broadly elliptical, slightly flattened, 1.8 – 2.0 (1.7 ± 0.1) mm long, 0.9 – 1.5 (1.3 ± 0.01) mm wide (Fig. 5C).

Phenological observations of *N. racemosa* individuals in Kyiv during 2021 – 2024 revealed that their annual development cycle was consistent with the weather and climatic conditions of a particular year. The beginning of regrowth occurred in the second–third decade of March. The budding phase occurred in the second decade of April–first decade of May. The duration of this phase was 6 – 8 days. Flowering began in the second decade of April–second decade of May and ended in the first – second decade of July. The flowering period lasted 54 – 70 days and was limited by dry and hot conditions. This phase was resumed each time after precipitation. Regular fruiting of *N. racemosa* individuals was noted in the third decade of May – the second decade of June. The end of the growing season was noted in the third decade of October – the second – third decade of November. The duration of the growing season was 224 – 246 days.

We investigated the quality indicators of local reproduction seeds – germination energy, dormancy period and seed germination (laboratory and soil), since seed characteristics are directly related to the preservation and growth of plant populations, and each seed is of great importance in the restoration and spread of the species (Misnik 1976, Baskin and Baskin 2014). The specific state of seed dormancy depends on genetics, growth pattern, and environmental conditions (Kildisheva et al. 2020). It was established that in laboratory conditions at a temperature of +25 °C, seed germination began on the 6th – 8th day and ended on the 19th – 21st day from the beginning of the emergence of shoots. The germination energy, which characterizes the simultaneous germination of seeds, is 9.3%. Seed dormancy lasted approximately 13.5 days; laboratory seed germination was 36 – 44%. In the soil, seedlings appeared on the 12th – 15th

day after sowing. Soil seed germination is much lower, at 8 – 12%. In addition, we investigated the features of ontogenesis of *N. racemosa* in culture. It was found that in one year the plants go through all phases of ontogenetic development – from seed to fruiting with the formation of viable seeds.

The plasticity of the phenorhythm, the completeness of all phenological phases of development, the rate of ontogenesis, and the viability of seeds of local reproduction confirmed the high adaptive potential of this alien species in our conditions.

Based on the analysis of our own observations, literary sources, and Internet resources, it was determined that all findings of spontaneous growth of *N. racemosa* in Ukraine, according to the European Nature Information System, are confined to synanthropic biotopes: decorative cultivated biotopes (parks, squares, lawns, flower beds), residential and technotopes (biotope complexes of built-up areas, garbage and solid waste landfills).

Discussion

The spread of this species in Ukraine is most facilitated by the presence of a significant number of introduction centers from which alien species most often escape and by their use in phytodesign. For example, the locality of *N. racemosa* studied in Kyiv and the region could have appeared as a result of escape from flower and decorative exhibitions, or as a result of their reconstruction, or due to accidental introduction of seeds or parts of plants by people or the wind. We also believe that the emergence of such habitats is facilitated by non-compliance with the rules and regulations for the storage and disposal of plant waste.

It should be noted that in the urban flora of other European countries, *N. racemosa* is distributed in biotopes and habitats similar to ours. For example, in Budapest (Hungary) this species is reported as naturalized, mostly growing in pavement cracks and along roadsides (Rigó et al. 2023). Pyšek et al. (2022) report on the naturalization of

this neophyte in the Czech Republic, where its spontaneous growth was recorded in the cracks of sidewalks and asphalt pavements, near the foundations of buildings, etc. in Prague, Borno, Kutná Hora, Kyjov and other cities (Pyšek et al. 2022, iNaturalist 2025). In Finland, as in our country, *N. racemosa* has been found in garden waste dumps and landfills (Kurtto et al. 2019). In general, currently the largest number of findings has been recorded in Sweden (1446 finds), Belgium (661), Austria (187), and the Czech Republic (172) (Kartesz 2015, Pyšek et al. 2022, GBIF 2025).

Predicting the further spread of introduced species beyond their primary cultivation areas is an important contemporary task. Based on the dynamics of newly recorded localities of *N. racemosa* in Ukraine, we estimated the potential future spread of this species in urban environments over the next 20 years. An annual increase in cases of spontaneous spread is expected, from 7 in 2025 to 17 in 2045 under conditions of introduction, and from 5 to 12, respectively, outside cultivation. We believe that the spontaneous spread of this alien species in Ukraine is not yet of a threatening nature. But the high adaptive potential of *N. racemosa* and its wide involvement in landscape design may provoke an increase in the number of spontaneous localities of the species in anthropogenically transformed territories in the near future, because in cultural phytocenoses, self-seeding is observed in many cases, and plants are able to go through all phases of ontogenetic development with the formation of viable seeds in one growing season. Also, climate warming will contribute to the further spread of this species, since weather and climatic conditions will likely approach those of the original range, which will increase the competitiveness of the species.

Therefore, the species studied has a wide ecological amplitude of growth in the secondary range and is gradually increasing its presence in the urban environment of many European countries, including Ukraine, where it is naturalizing in synanthropic biotopes. *N. racemosa* requires ongoing monitoring and control of its spread to prevent possible invasion into new territories. If the species shows signs of becoming invasive, its use in ornamental gardening should be limited.

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Author contribution statement

Both authors conducted the study, analyzed the statistical data, worked on the manuscript, and approved the submitted version of the manuscript.

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Research Article

Sisyrinchium micranthum (Iridaceae, Iridoideae, Sisyrinchieae) in Tunisia, a first record for the African continent

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Abstract – This study reports the first discovery of *Sisyrinchium micranthum* in Tunisia, marking the species' inaugural record on the African mainland and along the southern Mediterranean coast. The paper provides a concise morphological description of the species, an account of its phenology, and detailed information on its distribution, habitat, and ecological context in Tunisia. Additionally, we present original photographs of this diminutive annual species. An updated key to the diversity of the Iridaceae family in North Africa is also included.

Keywords: alien, floristics, North Africa, *Sisyrinchium rosulatum*, Tunisia

Introduction

The genus *Sisyrinchium* L., which is native to the Hawaiian Islands, temperate and subtropical regions of the Americas, and the Falkland Islands, encompasses approximately 212–232 accepted species (WPO Plant List 2025, POWO 2025). Several of these species have become cosmopolitan weeds across Europe, Asia, Russia, and Australia. The genus is considered one of the largest and most taxonomically complex within the Iridaceae family, due to frequent polyploidy, overlapping morphological traits, and the difficulty in identifying reliable distinguishing features between closely related species. This has made taxonomic classification and delimitation challenging (see e.g. Chauveau et al. 2011, Karst and Wilson 2012, Tacuatí et al. 2012, 2017, Alves et al. 2014, Shin et al. 2016, Burchardt et al. 2018, Weakley et al. 2023).

In the Mediterranean region of Europe, *Sisyrinchium* is represented by approximately seven species and one variety: *S. californicum* (Ker Gawl.) Dryand., *S. angustifolium* Mill., *S. montanum* Greene var. *crebrum* Fernald, *S. micranthum* Cav. (including *S. rosulatum* E.P.Bicknell), *S. platense* I.M.Johnston, *S. septentrionale* E.P.Bicknell, and *S. striatum* Sm., with varying statuses of naturalization (World Checklist of Selected Plant Families 2010, POWO 2025). To date,

no records of *Sisyrinchium* have been reported along the southern Mediterranean coast, nor elsewhere in Africa, except in Madagascar, Réunion, and Mauritius, where *S. micranthum* has been recorded as introduced, although its precise status remains unclear (POWO 2025).

This study identifies *Sisyrinchium micranthum* as the first documented occurrence in Tunisia and the continental interior of Africa among the vascular monocotyledonous flora, where it is now established as a naturalized alien species. Furthermore, we provide an updated account of the generic diversity within the Iridaceae family in North Africa, building upon previously published records.

Material and Methods

Extensive botanical surveys were conducted across various regions of northeastern Tunisia (specifically the Mogods area) over the past two years (since August 2023). The primary objective was to update the records of the wild and non-native flora, particularly focusing on undocumented species. These surveys led to the discovery of a previously unrecorded, widespread population of an unfamiliar annual, *Iris*-like plant characterized by typical sword-shaped leaves, which overlap at the base and are oriented in a single plane.

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Data on the plant population and its habitat were systematically compiled.

For species identification, the relevant literature was consulted (e.g. Shin et al. 2016, Stoyanov et al. 2023, under *Sisyrinchium rosulatum* E.P.Bicknell; Eminağaoğlu & Akyildirim Beğen 2024), and specimens were examined at several accessible herbaria (LY, MPU, P; herbarium acronyms follow Thiers 2025+). The collected specimens have been deposited in the personal herbarium of the first author (Herb. R. El Mokni) at the Faculty of Pharmacy, Monastir University (not listed in Index Herbariorum), with duplicates stored at BR. Nomenclature follows POWO (2025). Habitat descriptions are based on the first author's personal observations (REM).

Results

Sisyrinchium micranthum Cav., Diss. 6: 345 (1788)

≡ *S. iridifolium* Kunth, F.W.H.von Humboldt, A.J.A. Bonpland & C.S.Kunth, Nov. Gen. Sp. 1: 324 (1816), *Marica micrantha* (Cav.) Ker Gawl. in Irid. Gen.: 22 (1827), *Bermudiana micrantha* (Cav.) Kuntze in Revis. Gen. Pl. 2: 700 (1891), *Sisyrinchium rosulatum* E.P.Bicknell, Bull. Torrey Bot. Club 26: 228. (1899), *S. exile* E.P.Bicknell, Bull. Torrey Bot. Club 28: 573 (1901).

Description (Figs. 1-3)

An herbaceous grass-like annual plant (Fig. 1C) with branched, flattened to winged stems. Stems 10–18(–25) cm, compressed, usually 2–3 in a tuft, up to 10 in more vigorous individuals (Fig. 2A), rarely simple, glabrous, with 1–2 nodes. Leaf blades 15–50(–70) × 1–2.5 mm, linear-lanceolate, glabrous, straight, acuminate, sparsely scabrous on margins (Fig. 1A, B). Inflorescences terminal, with 2–5 pedunculate flowers (Fig. 3A), spathes 15–25(–30) mm long, conspicuously compressed, keeled, glabrous, usually entire, with thin, 0.2–0.3 mm wide, scarious margins (Fig. 2B–E). Perianth urceolate-campanulate basally, spreading in the upper half, tepals 5–10 mm long, acute to aristate, the spreading part pale lavender, tinged purple at the base, urceolate part yellowish to ochroleucous with purple patterns or strips, hairy outside (Fig. 3B–E), filaments connate basally, occasionally to 1/2 their length, the ovary ovate, up to 1 mm long, green, patent-hairy. Capsules 2–3 mm, globose, tan with purplish sutures, pedicels spreading to arcuate (Fig. 3F). Seeds 0.5–1 mm, ± globose to slightly compressed, black, surface rugulose to finely alveolate (see also, Shin et al. 2016: 296–299, Stoyanov et al. 2023: 495–496, sub *Sisyrinchium rosulatum*, Eminağaoğlu and Akyildirim Beğen 2024: 714–716, Weakley et al. 2023: 215–218).

Phenology

Flowering and fruiting from May–June.



Fig. 1. *Sisyrinchium micranthum* in Tunisia. A–B – General habit of an individual before flowering period, C – General habit of an individual during flowering period, scale bar = 20 mm (Sejnane, 06.08.2024 and 03.06.2025, photos: R. El Mokni).



Fig. 2. *Sisyrinchium micranthum* in Tunisia. A – Habit during flowering period, B–E – Different steps of first blooming, scale bar = 10 mm (Sejnane, 21.05–03.06.2025, photos: R. El Mokni).

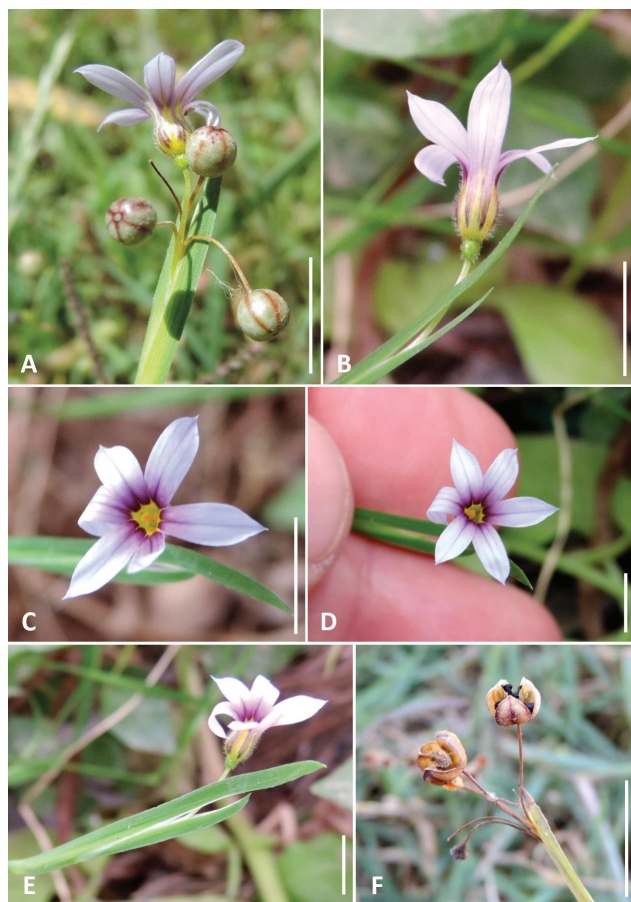


Fig. 3. *Sisyrrinchium micranthum* in Tunisia. A – Terminal inflorescence, B-E – Profile view and front view of single flowers with basally urceolate-campanulate perianth, and acute to aristate tepals, F – Dehiscent capsule, scale bar = 10 mm (Sejnane, 21.05-03.06.2025, photos: R. El Mokni).

Iconography

Shin et al. (2016: 298, Fig. 4.).

Habitat

In its native range, *Sisyrrinchium micranthum* is typically found in roadsides, old fields, disturbed areas, stream banks, and wet zones bordering woodlands. Elsewhere, the species has been reported from disturbed areas, including lawns, roadsides, village greens, construction sites, and sunny road verges in Korea (Shin et al. 2016) and Turkey (Eminağaoğlu and Akyildirim Beğen 2024).

General distribution

Sisyrrinchium micranthum is native to regions from Mexico to Argentina, spanning much of Central and South America, while *S. rosulatum*—now considered conspecific – was originally recognized as a North American species. In many parts of its native range, the species has become a widespread and often weedy presence in disturbed areas, including roadsides, meadows, and wetland margins. Beyond its native distribution, *S. micranthum* has been introduced and naturalized in various regions worldwide. It has been reported as introduced in several European

countries, such as France (Parent 1977), Italy (Nicolella and Ardenghi 2013), Spain (Verloove and Sánchez Gullón 2012), and Portugal (Verloove and Gonçalves Silva 2022). More recent records also include Bulgaria (Stoyanov et al. 2023) and Türkiye (Eminağaoğlu and Akyildirim Beğen 2024). Additionally, it has been reported as introduced in parts of Asia (including Southeast China and Tibet), as well as in Queensland, South Australia, and New Zealand (POWO 2025). The species is known to have naturalized in various regions, including Japan, Korea, New Zealand, Samoa, and South Africa (Shin et al. 2016).

Habitat and distribution in Tunisia

In Tunisia, *Sisyrrinchium micranthum* has been found in several sites, particularly in littoral wet meadows, disturbed wet grassy areas, and wet depressions within cork oak forests in the Sejnane (including mainly Cap-Serrat and Oued Ziatine) region (Bizerte, northeastern Tunisia). These habitats are typically characterized by the presence of wet, nutrient-rich soils in association with diverse wetland species.

Ecology

Sisyrrinchium micranthum grows on peaty, sandy-loam soils at elevations between 10-20 m a.s.l., often in association with species including: *Alnus glutinosa* (L.) Gaertn., *Alopecurus bulbosus* Gouan, *Arctotheca calendula* (L.) K.Lewin (invasive alien), *Baldellia ranunculoides* (L.) Parl., *Briza minor* L., *Cladanthus mixtus* (L.) Chevall., *Cynodon dactylon* (L.) Pers., *Cynosurus polybracteatus* Poir., *Elatine macropoda* Guss., *Eryngium pusillum* L., *Eudianthe laeta* (Aiton) Rchb. ex Willk., *Falona echinata* (L.) Dumort., *Hypericum afrum* Lam. (an Algerian-Tunisian endemic), *Hypochaeris achyrophorus* L., *H. radicata* L. *Isolepis cernua* (Vahl.) Roemer & Schultes, *Juncus bufonius* L., *J. tenageia* Ehrh. ex L. f., *Lotus angustissimus* L., *L. hispidus* Desf. ex DC., *Lysimachia loeflingii* F.J.Jiménez & M.Talavera, *Lythrum hyssopifolia* L., *L. junceum* Banks & Sol., *L. portula* (L.) D.A.Webb, *Medicago polymorpha* L., *Mentha pulegium* L., *Myosotis arvensis* (L.) Hill, *Nerium oleander* L., *Persicaria decipiens* (R.Br.) K.L.Wilson, *Pteridium aquilinum* (L.) Kuhn, *Phyla nodiflora* (L.) Greene var. *nodiflora*, *Plantago coronopus* subsp. *cupanii* (Guss.) Nyman, *Poa trivialis* L., *Polypogon maritimus* Willd., *Quercus suber* L., *Rubus ulmifolius* Schott, *Rumex dentatus* L. subsp. *dentatus*, *Silene gallica* L., *Solenopsis laurentia* (L.) C.Presl, *Sparganium erectum* L., *Trifolium campestre* Schreb., *T. tomentosum* L., and *T. subterraneum* L.

Pathways of introduction and current naturalization status in Tunisia

The exact pathway through which *Sisyrrinchium micranthum* was introduced to Tunisia and continental Africa remains unclear. However, horticulture is probably one of the primary pathways for the introduction of many alien species worldwide (Reichard and White 2001,

Dehnen-Schmutz et al. 2007a, b, Hulme 2011, Richardson and Rejmánek 2011, Hulme et al. 2018). The escape of ornamental plants from cultivation is a well-documented phenomenon (Kowarik 2005, Dehnen-Schmutz et al. 2007b), and *S. micranthum* may have dispersed in this way, through wind, water, or animal-mediated seed dispersal (Kowarik, 2005). Currently, the presence of *S. micranthum* with hundreds of individuals in several wet depressions within the Sejnane region suggests an established, long-term introduction, following the terminology of Pyšek et al. (2004). The species now forms part of the early summer floristic vegetation in these wetland areas within cork oak forests.

Notes

Sisyrinchium micranthum (including *S. laxum* Otto ex Sims, as per Tacuatiá et al. 2017, and *S. rosulatum* E.P. Bicknell) forms a monophyletic group (Chauveau et al. 2011), all belonging to sect. *Morphanthus* C.D.Inácio, Chauveau & L.Eggers. This species can easily be confused with other closely related species of the genus, especially those with similarly colored perigones, such as *S. angustifolium* Mill. and *S. minutiflorum* Klatt. However, these species belong to different sections.

- *S. micranthum* is part of sect. *Morphanthus*, characterized by annual plants with fibrous and slender roots (never fleshy), a cup-shaped perigon, and connate filaments of varying lengths (but never reaching the top).

- *S. angustifolium* belongs to sect. *Sisyrinchium* Lem. ex Klatt, a group of perennial plants that may exhibit fleshy roots, a disk-shaped perigon, and connate filaments nearly reaching the top.

- *S. minutiflorum* is classified in sect. *Rhizilium* C.D. Inácio, Chauveau & L.Eggers.

This group consists of annual, erect plants with obviously flat leaves, never setaceous, and a disk-shaped perigon.

Further distinctive characters between *S. angustifolium* and *S. micranthum* can be found in Eminağaoğlu & Akyildirim Beğen (2024: Tab. 1, p. 716), while more details on the differences between *S. angustifolium* and *S. minutiflorum* are available in Johnston (1938: 378–379).

Over the past two decades, numerous amendments and additions to the generic diversity of the Iridaceae family (Monocots, Ord. Asparagales sensu APGIII) in North Africa have been made (see e.g., El Mokni and El Aouni 2011, El Mokni and Hadj Khalifa 2020). The genus *Sisyrinchium*, reported here for the first time, represents one such addition. An updated analytical key to the generic diversity of the family in North Africa is provided below (adopted from New South Wales Flora Online (2025) and Fennane et al. (2014: 423)).

Revised dichotomous key to the genera of Iridaceae in North Africa

1. Inflorescence with 1(–2) flowers 8
 - Inflorescence with 2 or more flowers 2

2. Inflorescence cymose; each pair of spathe bracts enclosing 2 or more usually pedicellate flowers; flowers wither and fall off quickly 3
 - Inflorescence spicate; each pair of spathe bracts enclosing a single sessile flower; flower persistent, not withering quickly 5
3. Style with petaloid branches, each covering a stamen, and bearing two erect crests exceeding the flap-like stigma (*Iris*-type flower) 4
 - Style without prominently crested petaloid branches; stamens exposed *Sisyrinchium* L. (*S. micranthum* Cav.)
4. Plant evergreen, with rhizomes; leaves several, erect; stamens free *Iris* Tourn. ex L.
 - Plant deciduous, with corms; leaf usually solitary and lax; stamens fused *Moraea* Mill.
5. Style branches deeply bifid; stigmas 6 or more *Freesia* Ecklon ex Klatt (*F. alba* (G.L.Mey.) Gumbel.)
 - Style branches entire; stigmas 3 6
6. Perianth tube straight; flowers more or less erect *Sparaxis* Ker Gawl. (*S. tricolor* (Curt.) Ker-Gawl.)
 - Perianth tube curved; flowers spreading, often at right angles to the axis 7
7. Perianth tube swollen on one side near the base, abruptly dilated c. 10 mm above the ovary *Chasmanthe* N.E.Br. (*Ch. floribunda* (Salisb.) N.E.Br. var. *floribunda*)
 - Perianth tube gradually widening, never swollen on one side near the base *Gladiolus* L.
8. Floral tube (hypanthium) sunken into the ground; perianth tube greyish, usually longer than the lobes .. *Crocus* L.
 - Floral tube ± elongate above ground; perianth tube shorter than the lobes *Romulea* Maratti

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Research Article

Ultrastructural features of cypselsae in selected *Senecio* L. (Asteraceae) species

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Abstract – In this study, the cypselae features of eight *Senecio* species in Türkiye (*Senecio olympicus* Boiss., *S. paludosus* L., *S. tauricolus* V.A. Matthews, *S. salsuginea* H. Duman & Vural, *S. castagneanus* DC., *S. fluviatilis* Wallr., *S. pseudo-orientalis* Schischk., and *S. racemosus* (M. Bieb.) DC.) were investigated in detail using light microscopy (LM) and transmission electron microscopy (TEM). A very thick and darkly stained cuticle layer was observed on the epidermal cells of all the species examined. Papillae were observed only in epidermal cells of *S. olympicus* Boiss., and sclerenchyma cells were less developed in the cypselae. In the other seven species, well-developed sclerenchyma tissue was found along the pericarp wall in the form of a semicircle, in bundles, or around the secretory ducts. Although secretion ducts were observed along the cypselae wall in the mid-layer cells of *S. olympicus*, *S. tauricolus*, *S. castagneanus*, and *S. fluviatilis*, no secretion ducts were observed in the other four species. Although prismatic calcium oxalate crystals were observed in the intermediate (mid) layer of *S. tauricolus*, *S. castagneanus*, and *S. pseudo-orientalis*, no crystals were found in the other five species. Phytomelanin accumulation was found in the schizogen spaces between the outer and intermediate layers of *S. olympicus*, *S. paludosus*, *S. tauricolus*, *S. castagneanus*, *S. pseudo-orientalis*, and *S. racemosus*, but not in *S. salsuginea* and *S. fluviatilis*.

Keywords: anatomy, cypselae, cytology, *Senecio*, transmission electron microscopy (TEM)

Introduction

The genus *Senecio* L. belongs to the *Asteraceae* (Compositae) family and the *Senecioneae* Cass tribe. *Senecioneae*, the largest tribe of the *Asteraceae* family, has a worldwide distribution with 150 genera and approximately 1200 species (Nordenstam 2007, Pelser et al. 2007). Additionally, described is the genus *Turanecio*, originating from Türkiye (Hamzaoglu and Çetin 2016). It has been very difficult to classify the species within this widely distributed genus and its taxonomic positions have been revised multiple times.

Comprehensive and consistent morphological studies contribute to both a better understanding of the biology of the species and a correct taxonomic and phylogenetic classification of species with similar characters (Reis et al. 2016). Phylogenetic studies attempt to determine the boundaries of the genus and the relationships between species by including sister groups (Bozkurt et al. 2024). Many researchers have reported that the morphological and anatomical features of fruits and seeds are important criteria in taxonomic, phylogenetic, and ecophysiological studies (Soares et al. 2019). These data were also used in the classification of the *Asteraceae* (Zarembo and Boyko 2008,

Leszek and Marek 2013, Jana and Mukherjee 2014, Talukdar 2015a, b, Sukhorukov and Nilova 2015, Karanović et al. 2016, Shil et al. 2016, Zareh et al. 2016, Paul 2017, Paul and Mukherjee 2017, Özcan 2017, Kürşat et al. 2023, Naderifar et al. 2023).

There are many published studies on achene, cypselae (pericarp+testa), and seed morphology or micromorphology in different *Asteraceae* taxa. Findings based on anatomical and ultrastructural features of cypselas, such as the organization of sclerenchyma tissue in cypselae, the presence of papillae or trichomes in epidermal cells, the presence of secretion ducts along the cypselae wall, the localization of prismatic-shaped crystals in the mid-layer cells, and the presence of phytomelanin in the intercellular spaces in the mesocarp and mid-layer are valuable data for taxon classification. According to a literature survey, there is no record regarding the seed anatomy, histology, and cytology of the *Senecio* genus. This study aimed to contribute to the taxonomic problems of the genus by examining the cypselae features of eight *Senecio* species, using light microscopy (LM) and transmission electron microscopy (TEM), and revealing their histological and cytological features.

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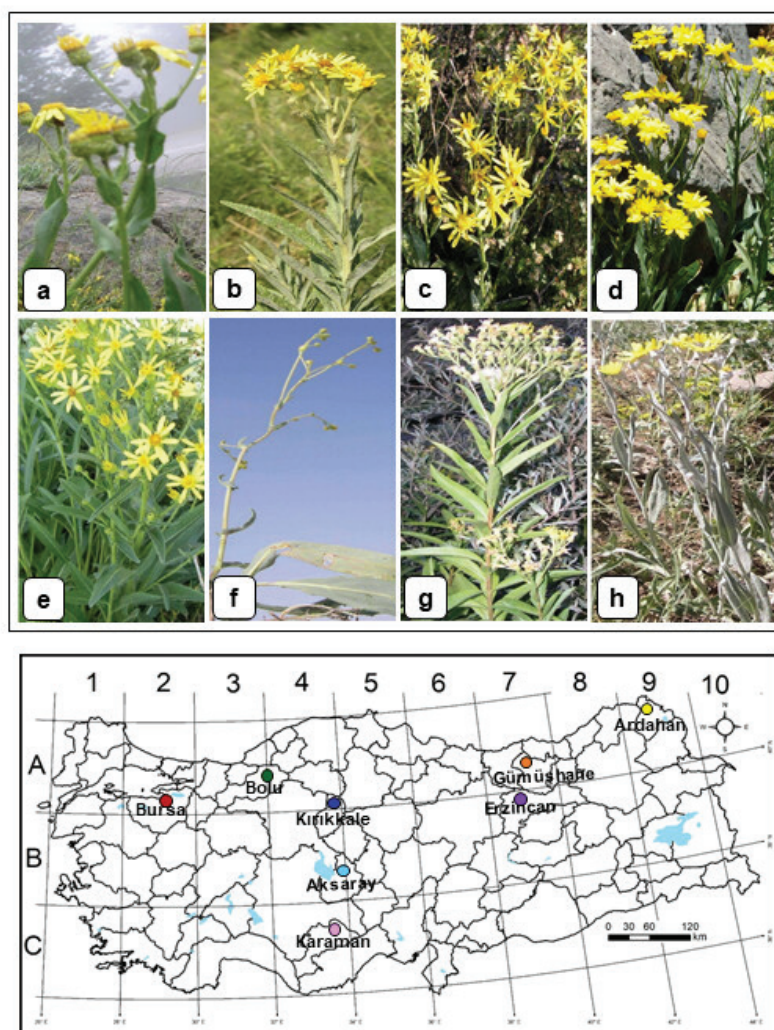


Fig. 1. Map of studied populations of *Senecio* taxa in Türkiye: a – *Senecio olympicus* (●), b – *Senecio paludosus* (●), c – *Senecio tauricolus* (●), d – *Senecio salsuginea* (●), e – *Senecio castagneanus* (●), f – *Senecio fluviatilis* (●), g – *Senecio pseudo-orientalis* (●), h – *Senecio racemosus* (●).

Material and methods

Plant materials belonging to *Senecio* species that were used in the study were collected from different regions of

Türkiye (Fig. 1). Regions, collectors, and collection data are listed in Tab. 1.

Mature cypselas of each taxon were pre-fixed with 3% glutaraldehyde for three hours. After the pre-fixation pro-

Tab. 1. Data on examined *Senecio* specimens.

Taxa	Collector and/or Locality Collection No.
<i>S. olympicus</i> Boiss.	Hamzaoglu 4399 Bursa: Uludağ, upper of Kırkpınar valley, 2100-2200 m, Hamzaoglu 4399, Aksoy & Budak (Bozok Hb.).
<i>S. paludosus</i> L.	Budak 1994 Bolu: The edges of the channels around the Abant lake, 1300 m, Budak 1994, Hamzaoglu & Aksoy (Bozok Hb.).
<i>S. tauricolus</i> V.A.Matthews	Budak 1735 Karaman: Between Ermenek-Karaman, 16 km, under the steep rocks to the left of the road, 1670 m, Budak 1735, Hamzaoglu & Aksoy (Bozok Hb.).
<i>S. salsuginea</i> H.Duman & Vural	Hamzaoglu 5377 Aksaray: Eskil, Tersakan location, 900 m, Hamzaoglu 5377, Aksoy & Budak (Bozok Hb.).
<i>S. castagneanus</i> DC.	Hamzaoglu 3774 Kırıkkale: Dinek Mountain is about 1600 m from the exit of Uzunlar village to Mamıkkaya hill, Hamzaoglu 3774, (Bozok Hb.).
<i>S. fluviatilis</i> Wallr.	Budak 2123 Erzincan: Between Aşkale-Tercan 20 km, 1550 m, Budak 2123, Hamzaoglu & Aksoy (Bozok Hb.).
<i>S. pseudo-orientalis</i> Schischk.	Budak 1900 Gümüşhane: Between Kelkit Akdağ village and Kuzuçimeni plateau, Kaz Mountain, 2340 m, Budak 1900, Hamzaoglu & Aksoy (Bozok Hb.).
<i>S. racemosus</i> (M.Bieb.) DC.	Budak 1956 Ardahan: Between Çamlıbel and Ardahan, 2000 m, Budak 1956, Hamzaoglu & Aksoy (Bozok Hb.).

Tab. 2. Anatomical features of the cypsela of the studied *Senecio* species.

Taxa	Papillae	Sclerenchyma tissue or /bundles	Secretory ducts	Crystals	Phytomelanin accumulation
<i>S. olympicus</i> Boiss.	+	+	+	-	+
<i>S. paludosus</i> L.	+	+	-	-	+
<i>S. tauricolus</i> V.A.Matthews	+	+	+	+	+
<i>S. salsuginea</i> H.Duman & Vural	-	+	-	-	-
<i>S. castagneanus</i> DC.	-	+	+	+	+
<i>S. fluviatilis</i> Wallr.	-	+	+	-	-
<i>S. pseudo-orientalis</i> Schischk.	-	+	-	+	+
<i>S. racemosus</i> (M.Bieb.) DC.	-	+	-	-	+

cess, the materials were washed with 0.1 M Na-P buffer (pH 7.2) and kept at 4 °C overnight, then post-fixed in 1% osmium tetroxide (Os₂O₄) for two hours. The materials were dehydrated through an ethanol series. After dehydration and saturation, the samples were placed in the embedding medium Epon 812 (Luft 1961). Semi-thin sections were taken from the prepared blocks using an ultramicrotome and these sections were stained with methylene and toluidine blue. Thin sections (ultrathin) were placed on copper grids and stained first with uranyl acetate (Stempak and Ward 1964), then with lead citrate (Sato 1967). They were exam-

ined with a JEOL CX II TEM and micrographs were taken. For LM, semi-thin sections of 1.5 µm or 2 µm were taken from the samples embedded in epoxy and stained with 1% toluidine blue. The sections were examined under a light microscope, and photographs were taken.

Results

Eight *Senecio* species were examined, cypsela anatomical characters and various diagnostic anatomical characters of these species were determined (Tab. 2). The cypsela

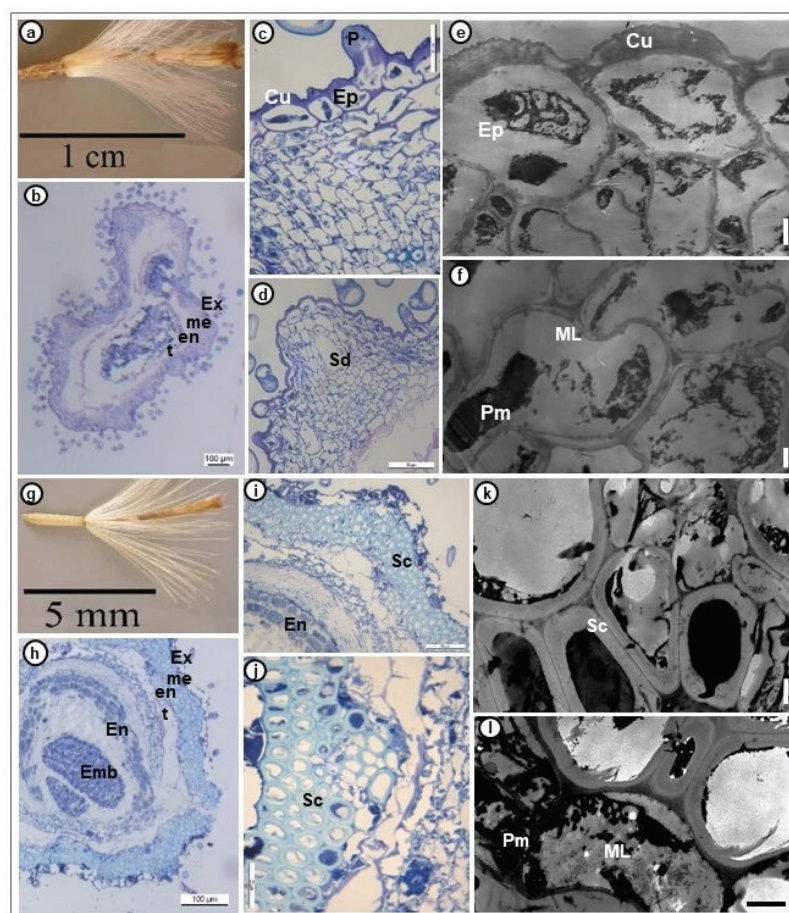


Fig. 2. *Senecio olympicus*: a – cypselum, b – general view of cypselum structure, scale bar = 100 µm, c – epidermal cells and papilla, scale bar = 20 µm, d – secretory duct in sclerenchyma bundle, scale bar = 50 µm, e – electron micrograph of epidermal cells, scale bar = 1 µm, f – electron micrograph of mid-layer cells, scale bar = 1 µm; *Senecio paludosus*: g – cypselum, h – general view of cypselum structure, scale bar = 100 µm, i – sclerenchyma cells, scale bar = 50 µm, j – sclerenchyma cells with large lumens, scale bar = 20 µm, k – electron micrograph of sclerenchyma cells, scale bar = 5 µm, l – electron micrograph of mid-layer cells, scale bar = 5 µm.

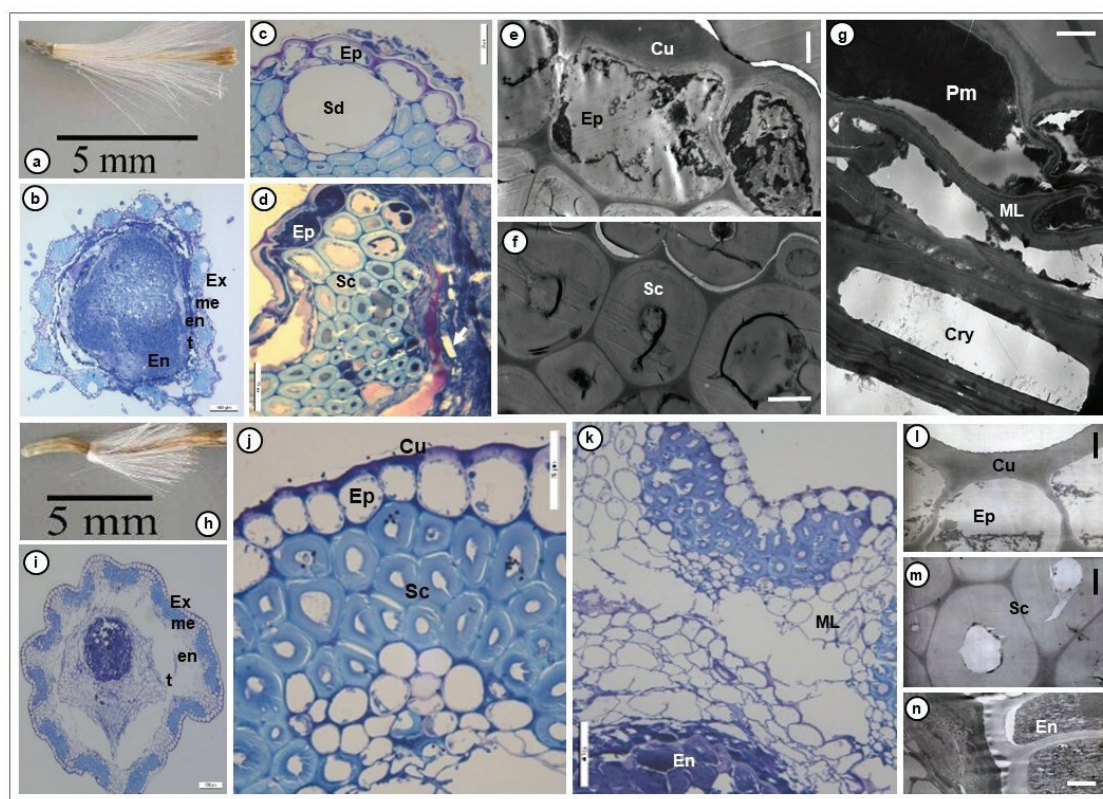


Fig. 3. *Senecio tauricolus*: a – cypselas, b – general view of cypselas structure, scale bar = 100 µm, c – epidermal cells and secretory duct, scale bar = 20 µm, d – sclerenchyma bundle, scale bar = 20 µm, e – electron micrograph of epidermal cells, scale bar = 1 µm, f – electron micrograph of sclerenchyma bundle, scale bar = 1 µm, g – electron micrograph of mid-layer cells, scale bar = 1 µm; *Senecio salsuginea*: h – cypselas, i – general view of cypselas structure, scale bar = 100 µm, j – epidermal cells and sclerenchyma bundle, scale bar = 20 µm, k – mid-layer cells, scale bar = 50 µm, l – electron micrograph of epidermal cells, scale bar = 5 µm, m – electron micrograph of sclerenchyma cells, scale bar = 5 µm, n – electron micrograph of endosperm cells, scale bar = 1 µm.

consists of the pericarp, testa, endosperm, and cotyledons, and the mature pericarp consists of three regions: the exocarp (outer epidermis), mesocarp, and endocarp (inner epidermis).

Senecio olympicus Boiss.

Cypselas wall is wavy (Fig. 2a) and the epidermis is papillary (Fig. 2b, c). The cuticle on the epidermal cells is darkly stained. There are 1-3 sclerenchyma cells among the parenchymatic cells under the epidermal cells (Fig. 2c). Below the exocarp, secretory ducts line the cypselas wall (Fig. 2d). Below the epidermis, the mesocarp contains 4-5 layers of mid-layer cells. In the innermost part, there are endosperm cells. Ultrastructural examinations revealed that epidermal cells are rectangular and that thick cuticle covers them (Fig. 2e). Mid-layer cells are wavy-walled and electron opaque. There are electron-dense phytomelanin deposits in some mid-layer cells (Fig. 2f).

Senecio paludosus L.

In the exocarp, the epidermis is papillary and thick-walled (Fig. 2g). The cuticle layer was darkly stained (Fig. 2h). Under the epidermal cells, there are 4-6 layers of sclerenchyma cells, which are located along the pericarp

wall (Fig. 2i). The lumens of sclerenchyma cells are wide. In some cells, the lumen is filled and the cell walls are electron-opaque (Fig. 2j, k). Beneath the sclerenchymatous cells, there are 3-5 layers of cells with large vacuoles and thin walls, which are parenchymatous cells. The schizogenous spaces of the mid-layer cells are filled with electron-dense phytomelanin aggregates (Fig. 2l).

Senecio tauricolus V.A. Matthews

The structure of the cypselas has a wavy wall and an oval shape (Fig. 3a, b). The cuticle layer is stained dark. Secretory ducts were found under the epidermal cells in the sclerenchyma bundles across the entire cypselas wall (Fig. 3c). Sclerenchyma cells are thick-walled. The interiors of some secretory ducts are empty. Under the sclerenchyma bunches, there are 2-3 layers of wavy-walled mid-layer cells. Prismatic and quadrangular crystals were observed in the mid-layer cells. In the innermost region, endosperm cells are present (Fig. 3d). Cypselas ultrathin sections have a thick cuticle layer on the epidermal cells (Fig. 3e). The walls of sclerenchyma cells are quite thick, and others are less electron-dense (Fig. 3f). The phytomelanin in the cypselas is observed in mid-layer cells with thick walls (Fig. 3g).

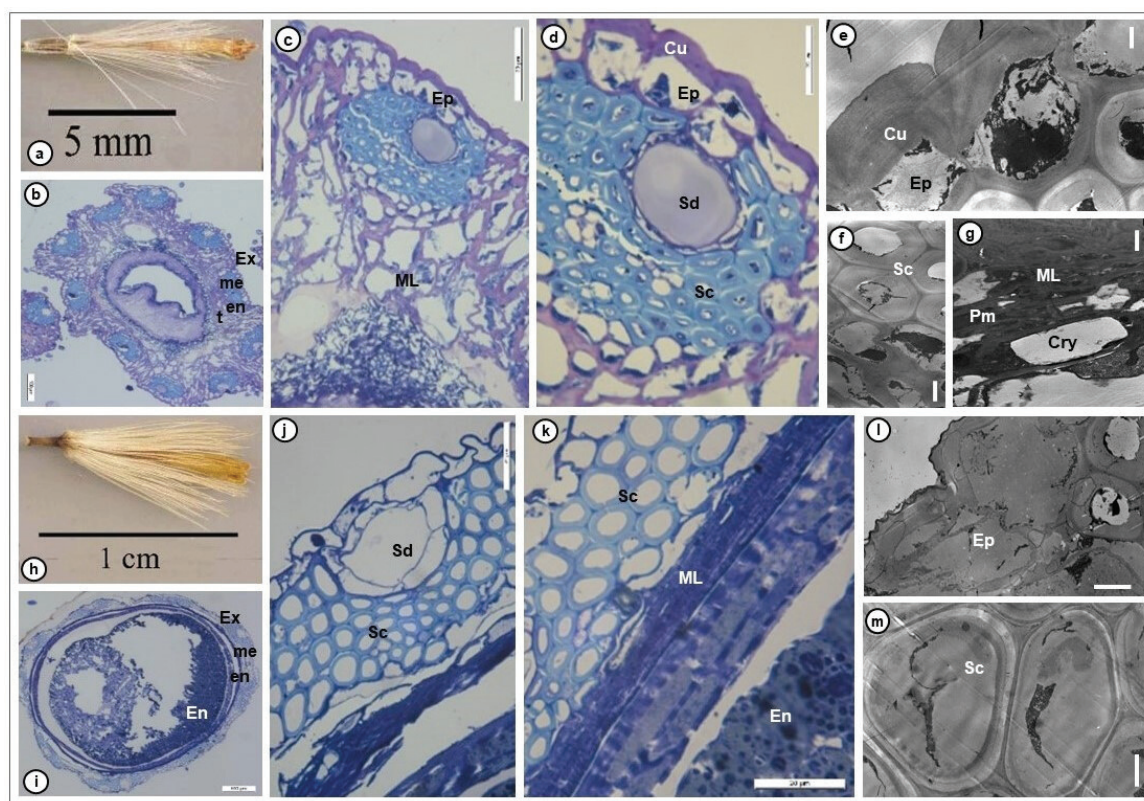


Fig. 4. *Senecio castagneanus*: a – cypselas, b – general view of cypselas structure, scale bar = 100 μ m, c – epidermal cells and mid-layer cells, scale bar = 50 μ m, d – secretory duct in sclerenchyma bundle, scale bar = 20 μ m, e – electron micrograph of epidermal cells, scale bar = 5 μ m, f – electron micrograph of sclerenchyma bundle, scale bar = 5 μ m, g – crystals in mid-layer cells, scale bar = 1 μ m; *Senecio fluviatilis*: h – cypselas, i – general view of cypselas structure, scale bar = 100 μ m, j – sclerenchyma bundle and secretory duct, scale bar = 20 μ m, k – mid-layer cells and endosperm, scale bar = 20 μ m, l – electron micrograph of epidermal cells, scale bar = 5 μ m, m – electron micrograph of sclerenchyma bundle, scale bar = 5 μ m.

Senecio salsuginea H. Duman & Vural

Cypselas has a wavy wall and oval shape (Fig. 3h, i). The cuticle layer on the epidermal cells was thick and dark-stained (Fig. 3j). Sclerenchyma bundles were observed in the wavy regions of the cypselas. The sclerenchyma cells are thick-walled, and their lumen is narrow. Under the sclerenchyma bundles are 2-3 layers of wavy-walled mid-layer cells. This layer consists of thin-walled, large parenchymatic cells (Fig. 3k). In the innermost part, there are endosperm cells. In the cypselas ultrathin sections, the epidermal cells are oval and have a thick layer of cuticle (Fig. 3l). Sclerenchyma cell walls are very thick and electron-opaque (Fig. 3m). Underneath these cells are mid-layer cells with wavy walls. Under the mid-layer are long rectangular endosperm cells with thickened walls (Fig. 3n).

Senecio castagneanus DC.

In this cypselas, sclerenchyma cells located in the form of bundles along the walls were observed (Fig. 4b, c). Under the epidermal cells, secretory ducts were observed along the pericarp wall. There are some thin parenchymal cells around the secretory ducts (Fig. 4d). Below the sclerenchyma bundles, there are 2-3 layers of wavy walled mid-layer cells. In electron microscopy images, epidermal cells were

observed to have dense cytoplasm (Fig. 4e). Sclerenchyma cell walls are very thick, and their lumens are filled in most cells. The walls of sclerenchyma cells are electron-opaque (Fig. 4f). The walls of the mid-layer cells are very thick, and crystals were observed in some cells. Additionally, these mid-layer cells have wavy walls beneath them. The crystals appear to be typically prismatic or rectangular and are electron-transparent. Phytomelanin accumulates inside the intercellular spaces in mid-layer cells (Fig. 4g).

Senecio fluviatilis Wallr.

Cypselas is circular (Fig. 4h, i). Under the epidermis, there are large secretory ducts surrounded by sclerenchymal cells (Fig. 4j). There are some thin-walled parenchymatic cells around the secretory ducts. Wavy wall mid-layer cells were observed under the sclerenchyma cells. Mid-layer cells are darkly stained. Inside, there are endosperm cells (Fig. 4k). Cypselas ultrathin sections have a thick cuticle layer on the epidermal cells (Fig. 4l). Sclerenchyma cell walls are thin and electron-transparent (Fig. 4m).

Senecio pseudo-orientalis Schischk.

In semithin sections, the exocarp is wavy and oval (Fig. 5b). In dicotyledons, the embryo occupies a significant part

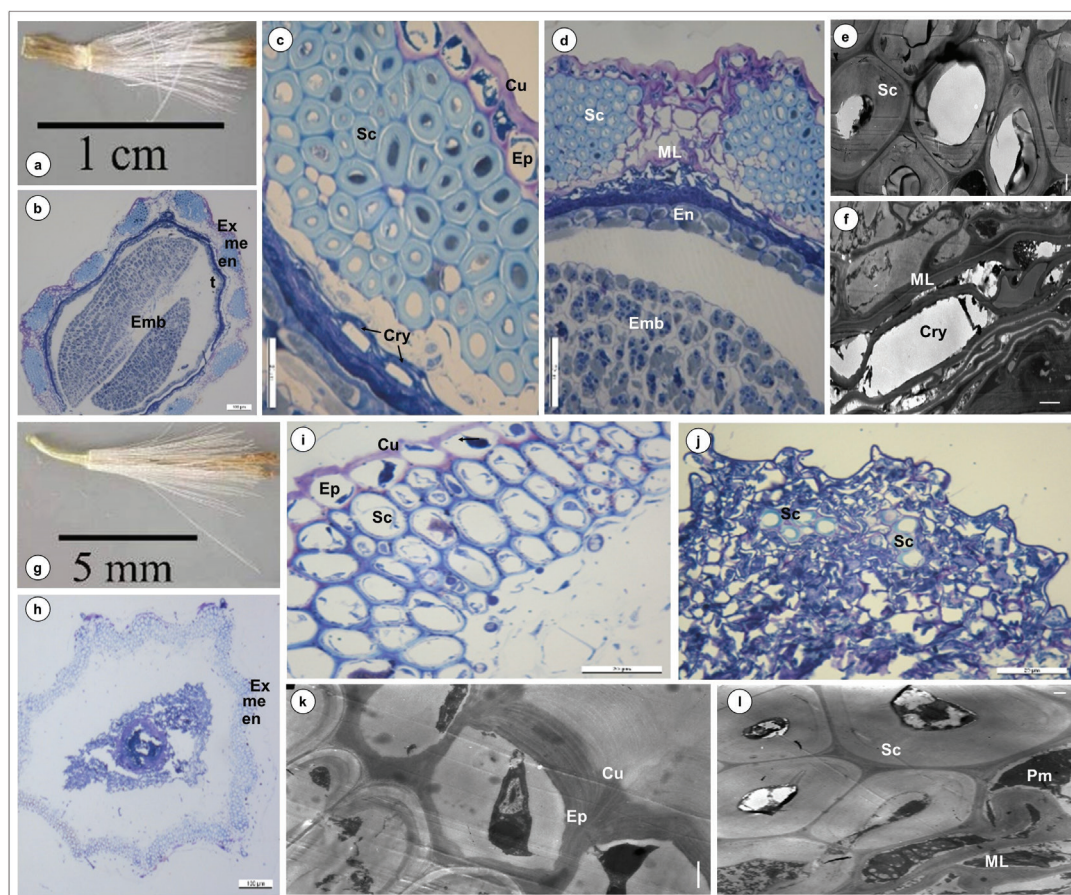


Fig. 5. *Senecio pseudo-orientalis*: a – cypsel, b – general view of cypsel structure, scale bar = 100 μ m, c – sclerenchyma cells, scale bar = 20 μ m, d – mid-layer cells, scale bar = 50 μ m, e – electron micrograph of sclerenchyma cells, scale bar = 5 μ m, f – electron micrograph of mid-layer cells and crystals, scale bar = 1 μ m; *Senecio racemosus*: g – cypsel, h – general view of cypsel structure, scale bar = 100 μ m, i – sclerenchyma cells, scale bar = 20 μ m, j – mid-layer cells, scale bar = 20 μ m, k – electron micrograph of epidermal cells, scale bar = 5 μ m, l – electron micrograph of sclerenchyma bundle, scale bar = 5 μ m.

of the seed. The cuticle of the epidermal cells is stained dark. Sclerenchyma bundles were observed in the wavy regions of the pericarp. Sclerenchyma cells have thick walls and narrow lumens. In some cells, the lumen is full. There are irregularly shaped mid-layer cells under the sclerenchyma bundles. Crystals in these cells are prismatic and rectangular, arranged in regular layers (Fig. 5c). Inside are polygonal endosperm cells (Fig. 5d). In ultrathin sections, the walls of sclerenchyma cells are electron opaque (Fig. 5e). Electron-dense phytomelanin was observed in mid-layer cells (Fig. 5f).

Senecio racemosus (M.Bieb.) DC

The pericarp and seed membrane are partially fused with each other, and the cuticle layer on the epidermal cells appears darkly stained (Fig. 5h, i). Below the epidermis, in the mesocarp, sclerenchyma cells are present with 3-4 layers of dark-stained and thickened walls. The sclerenchyma is uninterrupted, and the lumens of its cells are large. In some cells, the lumen is full. Cells stained dark in cypsel sections are mid-layer cells (Fig. 5j). Cytoplasm is dense in these cells. 2-4 sclerenchyma cells were observed at certain locations. In the cypsel ultrathin sections, the epidermal

cells are rectangular and have a thick layer of cuticle (Fig. 5k). It has been observed that sclerenchyma cell walls are thick, as in other species, and lumens are filled in some cells (Fig. 5l). The walls of sclerenchyma cells are electron-opaque. The cytoplasm was observed to be dense in the phytomelanin-containing mid-layer cells, which were located under the sclerenchyma cells.

Discussion

In this study, the cypsel structures of eight *Senecio* were described ultrastructurally and anatomically. Although ultrastructural studies can be used as a powerful tool in species delimitation, detailed studies on the cypselae of *Senecio* taxa have not been conducted. However, in our previous studies (Büyükkartal et al., 2017, 2018), 14 *Senecio* taxa were also examined.

Many studies have reported that the cypsel in the Asteraceae family is a single-chambered, dry fruit that does not open (Judd et al. 2002, Marzinek et al. 2008, Funk et al. 2009, Jeffrey 2009, Mandel et al. 2019). It has been stated that the characters of pericarp layer cells, the presence or absence of calcium oxalate crystals in these layer cells, phytomelanin

accumulation, secretory ducts, and different cell structures have taxonomic value (Roth 1977, Jana and Mukherjee 2014). In *Senecio*, the cypsela comprises the pericarp, testa, endosperm, and cotyledons. The mature pericarp is divided into three regions: the exocarp (outer epidermis), mesocarp, and endocarp (inner epidermis). There is a thick and dark cuticle layer on the epidermal cells of all species examined. Papillae were observed in the epidermal cells of *S. olympicus*, *S. paludosus*, and *S. tauricolus*. In our previous studies, trichomes were found in the epidermis of *S. grandidentatus* Ledeb., *S. trapezuntinus* Boiss., and *S. cilicius* Boiss. In *S. hypochionaeus* Boiss. subsp. *hypochionaeus*, papillae were observed in epidermal cells (Büyükkartal et al. 2017, 2018). Roth (1977) and Jana and Mukherjee (2014) reported that cypselae are generally variable in length, glabrous in structure, with or without pappus, and are ribbed or smooth. In this study, it was observed that sclerenchyma cells were less developed in the pericarp in *S. olympicus*, while they were highly developed in the other seven species. Others have reported that the presence of sclerenchyma tissue at the genus level in the Asteraceae family is taxonomically important (Wiklund 1983, Anderberg 1991, Zhu et al. 2006, Biswas et al. 2014).

Secretory ducts were observed along the pericarp wall in the mid-layer cells of *S. olympicus*, *S. tauricolus*, *S. castagneanus*, and *S. fluviatilis*. No secretory ducts were found in the other four species (*S. paludosus*, *S. salsuginea*, *S. pseudo-orientalis*, and *S. racemosus*). In our previous studies, secretory ducts along the pericarp wall were observed only in *S. grandidentatus* and *S. eriospermus* DC. subsp. *eriospermus* (Büyükkartal et al. 2017, 2018). Robinson (1981) reported that resin or secretory canals are commonly found in vegetative tissues and floral units in Asteraceae.

In this study, long prismatic calcium oxalate crystals were detected in the intermediate cells of *S. tauricolus*, *S. castagneanus*, and *S. pseudo-orientalis*. No crystals were found in the intermediate layer cells of the other five species. In our previous studies, calcium oxalate crystals were observed in the mid-layer cells of *S. aquaticus* Hill subsp. *erraticus* (Bertol.) VA. Mathews, *S. mollis* Willd., and *S. trapezuntinus* (Büyükkartal et al. 2017), *S. eriospermus* subsp. *eriospermus*, *S. eriospermus* subsp. *lorentii* (Hochst), and *S. hypochionaeus* subsp. *hypochionaeus* (Büyükkartal et al. 2018). Similarly, in a study conducted on the determination of fruit morphology and anatomy in Cardueae (Asteraceae), it was reported that the epidermal cell structure in the pericarp, the localization of calcium oxalate crystals, and the formation and location of secretion ducts in the mesocarp are important features for distinguishing taxa (Zarembo and Boyko 2008). Paul (2017) studied the external and internal characteristics of cypselae of *Emilia sonchifolia* (L.) DC. and *Calendula officinalis* L. species belonging to the tribes Senecioneae and Calenduleae. It was stated that the distribution and shape of the crystals are genetically controlled and thus represent taxonomically useful traits.

Electron-dense phytomelanin accumulation was observed in the schizogenous space between the outer and mid-layer cells in all studied species, except in *S. salsuginea* and *S. fluviatilis*. Phytomelanin is highly resistant to degradation and is secreted by fibers in the pericarp (De-Paula et al. 2013, Pandey et al. 2014). In addition, phytomelanin has been detected in many taxa belonging to the Asteraceae family (Freitas et al. 2015, Marques et al. 2021). It has been reported that phytomelanin accumulation in Asteraceae is of taxonomic importance (Freitas et al. 2015, Marques et al. 2022).

Previous studies have pointed out the need to consider cypsela anatomical and cytological features in resolving some taxonomic issues between species and genera. In this study, the anatomical and cytological differences among some *Senecio* species were revealed. The findings regarding the ultrastructural characteristics of cypsela in *Senecio* species appear to be consistent with the data obtained for other members of the Asteraceae family. Data based on anatomical and ultrastructural characteristics of cypselae, such as the organization of sclerenchyma tissue, the presence of papillae or trichomes in epidermal cells, the presence of secretory ducts along the cypsela wall, the localization of prismatic crystals in the middle layer cells, and phytomelanin accumulation in the intercellular spaces of the mesocarp and middle layer cells, can aid in classifying and identifying *Senecio* taxa. This study will further emphasize the value of the cypsela substructure for plant systematics and encourage future research on *Senecio* species. Gaps in our knowledge of *Senecio cypsela* substructure and differentiation make detailed studies essential.

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Author contribution statement

The plant materials were secured and collected by ÜB. Plants were photographed by ÜB and systematic revision work was carried out by the research team. HNB and HÇ conducted LM and TEM observations. EK and HNB wrote the first version of the manuscript. BK contributed to the sectioning and preparation processes. All authors have read and approved the final version of the manuscript.

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Research Article

Glucose-mixotrophic culture of the freshwater alga *Chlorella vulgaris* alleviates the phytotoxicity of silver nanoparticles

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Abstract – Silver nanoparticles (AgNPs) are widely used due to their specific physico-chemical properties, but their release in wastewater poses a threat to aquatic ecosystems. The microalga *Chlorella vulgaris* is often used as a model organism in toxicological research. Since this alga can switch from an autotrophic to a mixotrophic mode of life, we investigated whether glucose as an additional energy source can mitigate AgNP phytotoxic effects. Algae were cultivated in a nutrient medium with and without glucose and treated with a concentration of AgNPs allowing 60% cell survival. The effects were studied by monitoring oxidative stress and photosynthesis parameters. In the absence of glucose, AgNPs caused significant accumulation of reactive oxygen species, increased lipid peroxidation and carotenoid content with weak activation of antioxidant enzymes. In addition, reduced photosynthetic efficiency and ultrastructural damage were observed. The addition of glucose to AgNP treatments attenuated all negative effects by reducing oxidative stress and increasing the activity of antioxidant enzymes. Furthermore, *C. vulgaris* cells exposed in glucose-enriched media showed recovered photosynthesis by exhibiting the photosynthetic rate and chlorophyll fluorescence parameters of control algae. Ultrastructural analysis showed greater synthesis of lipid droplets and less structural damage. The study shows that glucose supplementation effectively mitigates the phytotoxic effects of AgNPs on *C. vulgaris*. By reducing oxidative stress, enhancing antioxidant enzyme activity and restoring photosynthetic function, glucose helps maintain cellular integrity and overall algal health in the presence of AgNPs, which suggests that mixotrophic cultivation may represent a protective strategy against nanoparticle-induced toxicity in aquatic microalgae.

Keywords: antioxidant enzymes, freshwater alga, mixotrophy, photosynthetic pigments, photosystem II, reactive oxygen species, ultrastructure

Introduction

Nanotechnology is a branch of science that modulates materials at the atomic and molecular level in the range of 0 to 100 nm. When the dimensions of a material are reduced to the nano level, there are significant changes in the physico-chemical properties, which consequently also change the properties of the material. These properties enable the use of nanomaterials in numerous areas, from medicine to the food industry (Anees Ahmad et al. 2020). Among metal nanoparticles, silver nanoparticles (AgNPs) are among the most widely used, mainly due to their unique properties such as their exceptional optical properties, electrical and thermal conductivity, but also because of their biological properties, which is why they have applications

in medicine, in commercially available products, in the food industry and, increasingly, in diagnostics, drug delivery and as anti-cancer agents (Zeng et al. 2024). Their primary biological activity is based on their biocidal effect, which is achieved through the gradual release of silver ions (Ag⁺) that can act on cells via various mechanisms by interfering with the thiol groups of proteins, inhibiting DNA replication and triggering oxidative stress (Anees Ahmad et al. 2020). With the increasing annual production and use of AgNPs, their release into the environment is also increasing, which means that they inevitably enter aquatic ecosystems via wastewater and rainwater, where microalgae are usually the dominant organisms (Zeng et al. 2024).

Microalgae are a diverse group of unicellular autotrophic organisms. Apart from the fact that microalgae, together

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with cyanobacteria, produce about 50% of the world's oxygen through the process of photosynthesis, they are also an excellent source of carbohydrates, proteins, lipids, nutrients, vitamins and minerals (Roth et al. 2019). For this reason, they are often used as food supplements, as well as in the pharmaceutical industry, in dye production, as animal feed, in aquaculture and in cosmetics (Castillo et al. 2021). *Chlorella vulgaris* is one of the best-known species of microalgae and is found in freshwater and in the sea. It is known for its high photosynthetic rate and rapid growth, especially under mixotrophic and heterotrophic conditions. Its ability to adapt to different conditions enables a wide range of applications in industry, but also makes it an excellent biosensor for environmental pollutants. In addition, *C. vulgaris* produces primary metabolites required for growth and maintenance of basic metabolic processes, while secondary metabolites are synthesized in response to stress conditions, further increasing its applicability in biotechnology (Ru et al. 2020).

Although microalgae are photosynthetic organisms, many species show the ability to switch to mixotrophic or heterotrophic conditions. Mixotrophic microalga growth is based on the addition of organic carbon sources (i. e. carbohydrates, organic acids, alcohols, amino acids) in the presence of light. Mixotrophic cultivation enables the production of valuable compounds such as fatty acids and polysaccharides and offers advantages over autotrophy such as faster growth and improved efficiency (Castillo et al. 2021). Glucose, the most commonly used carbon source in mixotrophic cultures, allows algae to utilize light energy and organic matter simultaneously. A key advantage of mixotrophic cultivation is the ability to adjust conditions to achieve an optimal balance between photosynthesis and the direct use of glucose as a carbon source (Ru et al. 2020). This not only accelerates algal growth but also allows precise control of the composition of metabolites, which is crucial for certain industrial applications such as food production or the synthesis of pharmaceutical compounds. Therefore, the use of glucose in mixotrophic cultures represents a promising approach to optimize microalgae cultivation, especially in situations where conditions for photosynthesis are suboptimal or when the goal is to achieve maximum biomass production in a shorter period of time (Castillo et al. 2021).

Previous studies have shown that the addition of glucose can have a significant effect on the photosynthetic apparatus of microalgae. Positive effects were observed in cultures of *Chlorella sorokiniana*, where the addition of glucose resulted in higher photosynthetic efficiency and a higher relative electron transport rate, which in turn led to biochemical changes in terms of protein and lipid content (Marchello et al. 2018). However, *Chromochloris zofingiensis* was shown to reversibly shut down photosynthesis in the presence of glucose (Roth et al. 2019), while in *Chlorella kessleri* the addition of glucose reduced chlorophyll content (Deng et al. 2019). Furthermore, higher glucose concentrations (> 3% w/v) were shown to inhibit chloroplast development in *Arabidopsis* seedlings by suppressing certain photosynthetic

genes (To et al. 2003) and reducing the photosynthetic rate and electron transport (Eckstein et al. 2012).

In addition to the effects on the photosynthetic apparatus, the addition of glucose as an additional carbon source can also mitigate the harmful effects of abiotic stress, as in the reduction of cadmium toxicity (Huang et al. 2024), and the reduction of nonylphenol toxicity in *Chlorella pyrenoidosa* (Yuan et al. 2025), the reduction of cadmium and copper toxicity in *Arabidopsis thaliana* (Shi et al. 2015) and *Cucumis sativus* (Yusuf et al. 2021) and the improvement of salt stress tolerance in *C. sativus* seedlings (Ma et al. 2020). In addition, studies have shown that glucose improves the tolerance of various microalgae species to high ammonia levels (Sutherland, 2022). However, the effects of glucose addition on AgNP toxicity have been very poorly investigated. A study on the phytotoxicity of AgNPs to human liver cells HepG2 has shown that glucose has a mitigating effect by reducing the cytotoxic effects of AgNPs mainly by increasing antioxidant defence and reducing the production of reactive oxygen species (ROS) (Zuberek et al. 2015). Moreover, another study showed that a high-carbohydrate diet can attenuate oxidative stress, inflammation and apoptosis induced by AgNPs in *Megalobrama amblycephala* (Chen et al. 2021). Although sparse, these data suggest that glucose may reduce AgNP-induced toxicity by mitigating oxidative stress and modulating various cellular processes.

Therefore, in this study we investigated whether the phytotoxic effects of AgNPs on the freshwater green alga *C. vulgaris* grown under autotrophic conditions can be mitigated by the addition of 0.5% glucose to the nutrient medium. To ensure comparability, the effective concentration of AgNPs allowing 60% cell survival (EC_{40}) was first determined in cultures grown without glucose (-Glu). This concentration was then applied to both groups of algae, cultivated in parallel: one in medium without glucose (-Glu) and the other in medium with glucose (+Glu), to evaluate whether glucose supplementation can attenuate AgNP-induced toxicity. During the study, we analysed the accumulation of silver, the occurrence and attenuation of oxidative stress, the effects on the photosynthetic apparatus and the ultrastructural changes in the cells.

Material and methods

AgNP synthesis and characterization

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade unless otherwise stated, while ultrapure water (Milli-Q, 18.2 MΩ-cm, Merck Millipore, Billerica, MA, USA) was used for all syntheses.

The synthesis of citrate-stabilised AgNPs (AgNP-citrate) was carried out according to the method of Peharec Štefanić et al. (2021). To 120 mL of boiling AgNO₃ solution (0.02 g), 5 mL of sodium citrate solution (1% w/v) was added. A colour change from clear to pale yellow signalled the formation of AgNPs, whereupon the suspension was rapidly

cooled to room temperature and stored at 4 °C until analysis. The physical and chemical properties of the AgNP-citrate stock suspension were analysed according to previously published procedures (Peharec Štefanić et al. 2021). The presence of AgNPs was confirmed by observing a characteristic surface plasmon resonance (SPR) peak using a UV-Vis spectrophotometer (Unicam, Cheshire, UK). The hydrodynamic diameter (d_H) and ζ -potential were measured by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using a NanoBrook 90Plus particle size analyser (Brookhaven Instruments, Holtsville, NY, USA), while the data were processed using Zeta Plus 5.71 software. The amount of Ag⁺ ions released by dissolving AgNPs in ultrapure water was determined by centrifugal ultrafiltration (Millipore Amicon Ultra-4 3K, with 3 kDa membrane) and by an inductively coupled plasma mass spectrometer (ICP-MS) (ELAN DRC-e, Perkin Elmer, Waltham, MA, USA) using a calibration curve with standards; the detection and quantification limits were 0.2 mg kg⁻¹ and 1 mg kg⁻¹, respectively. Finally, the synthesised and purified AgNP-citrate was visualised using a TEM (TF20, FEI Tecnai G2, equipped with an EDX detector, FEI, Hillsboro, OR, USA) according to the method described in Peharec Štefanić et al. (2021).

Chlorella vulgaris cell culture

C. vulgaris cultures (strain SAG 211-11b obtained from the Culture Collection of Algae at Göttingen University, Göttingen, Germany) were grown in liquid BBM medium (Nichols and Bold 1965) in autoclaved 200 mL Erlenmeyer flasks. Two versions of the medium were used for control purposes: BBM without glucose (-Glu) and BBM with 0.5% (w/v) glucose (+Glu). Cultivation was carried out in their corresponding media (-Glu and +Glu) throughout the experiment in a plant growth chamber at 24 °C under long-day conditions (16 h of light, 8 h of darkness) and a light intensity of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with constant orbital movement.

The growth of the culture was monitored for 30 days by daily cell counts using an automated cell counter (Luna II, Logos Biosystems, Anyang, Republic of Korea), which determined the exponential growth phase in the media -Glu and +Glu. To enable a direct comparison between treatments, cultures (-Glu and +Glu) were inoculated with different initial cell concentrations so that, after four days of cultivation, both control groups reached the same cell concentration of 1×10^6 cells mL⁻¹. At this point, both cultures were treated with 0.251 mg L⁻¹ AgNP-citrate, corresponding to the EC₄₀ value previously established in -Glu medium, i.e. the concentration that ensured 60% cell survival in -Glu medium.

Cell viability test using flow cytometry

To determine the EC₄₀ value, a culture with an initial concentration of 1×10^5 cells mL⁻¹ was prepared by inoculation in liquid -Glu medium in sterile Erlenmeyer flasks. The algae were then grown for four days under the indicated conditions with constant orbital mixing until a concentration of 1×10^6 cells mL⁻¹ was reached in the exponential

phase. According to OECD guidelines, these cultures were exposed to AgNP-citrate concentrations between 0.005 and 3.5 mg L⁻¹ for 72 h.

The EC₄₀ concentration after 72 h of treatment in -Glu medium was determined by cell survival analysis using flow cytometry in combination with the fluorescent dye propidium iodide (PI), as previously reported in Qi et al. (2022). Briefly, 1 mL of cell suspension was mixed with PI at a final concentration of 1 $\mu\text{g mL}^{-1}$, samples were incubated in the dark for 5 min, and then analysed using a flow cytometer (BD FACSVerse™, Piscataway, NJ, USA) with a 488-nm laser. In all, 100,000 cells per sample were analysed, and the concentration leading to EC₄₀ was determined using the four-parameter logistic (4PL) regression model (Dawson et al. 2012). The algal cultures without the addition of AgNP-citrate served as control samples for their corresponding media (Control (-Glu) and Control (+Glu)), and each experiment was performed in triplicate. Silver content, oxidative stress levels, antioxidant response parameters, as well as ultrastructural and photosynthetic activity analyses were determined in algal cells grown in -Glu and +Glu media. These cells were first cultivated for 4 days with initial cell concentrations adjusted to reach 1×10^6 cells mL⁻¹ and then exposed for 72 h to 0.251 mg L⁻¹ of AgNP-citrate. The results were compared with their corresponding control groups.

Measurements of the silver content

The silver content in algal cells was measured according to the previously described method (Komazec et al. 2023). The cell culture was centrifuged at $3500 \times g$ at room temperature for 5 min, and the resulting pellet was washed three times with ultrapure water. After each wash, the cells were centrifuged again under the same conditions, and the resulting pellet was freeze-dried for 24 h. The cells from the pellet were digested in a microwave oven (ETHOS SEL Milestone, Shelton, CT, USA) according to EPA method 3051a. In the first step, digestion was carried out in 10 mL of concentrated nitric acid (HNO₃) at 130 °C for 10 min and then at 180 °C for a further 15 min. In the next step, digestion was carried out in 1 mL of 30% hydrogen peroxide (H₂O₂) at 85 °C for 5 min and continued at 130 °C for 4 min. After cooling, the samples were diluted with 1% (v/v) HNO₃ to a final volume of 50 mL. The total silver content was determined with the ICP-MS instrument ELAN DRC-e, using a calibration curve from a series of standards with known concentrations for the calculation. The limits of detection and quantification (LOQ) were determined to be 0.05 mg kg⁻¹ and 0.1 mg kg⁻¹ and the percentage recoveries were 95.6%, 95.2% and 96.5%, respectively. The final results are expressed in micrograms of silver per 10⁶ cells.

Analyses of oxidative stress parameters

Determination of reactive oxygen species

ROS content was determined using the fluorescent markers dihydroethidium (DHE) and 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) according to the adapted

protocols described in Cvjetko et al. (2017) and Ng and Ooi (2021), respectively. DHE measures the superoxide anion ($O_2^{\cdot-}$), while H_2DCFDA measures the total ROS content. In a 96-well microplate, 80 μL of a 20 μM DHE or H_2DCFDA solution was added to each well containing 100 μL of cell suspension. Samples were then incubated in the dark for 30 min, after which fluorescence was measured using a GloMax microplate reader (Promega, Madison, WI, USA). An excitation wavelength of 520 nm and an emission wavelength of 600 nm were used for the DHE probe, while an excitation wavelength of 504 nm and an emission wavelength of 550 nm were used for H_2DCFDA . The results were normalized to the number of cells and expressed as a percentage of the ROS content in the control samples.

The H_2O_2 content was measured according to a modified protocol described in Mátaí and Hideg (2017). For this purpose, 200 ml of cell suspension was centrifuged at $3500 \times g$ for 5 min at 20 °C. After the supernatant was discarded, the cell pellet was washed three times with ultrapure water and centrifugation was repeated under the same conditions after each wash. The cell pellet was resuspended in cold 70% ethanol and homogenized in a Retsch homogenizer (MM200, Retsch, Haan, Germany) at a frequency of 30 Hz for 3 min at 4 °C with the addition of glass beads (425–600 μm). After homogenization, the samples were centrifuged at $15\,000 \times g$ for 10 min at 4 °C, and the supernatant was transferred to clean tubes and stored on ice. After extraction, 100 μL of the prepared sample was added to 1000 μL of a reaction mixture containing 124 μM xylenol orange (sodium salt), 99 mM sorbitol, and 0.248 mM ammonium iron (II) sulphate. After being briefly shaken, the mixture was incubated at room temperature for 15 min and the absorbance was measured at 560 nm. The total amount of H_2O_2 was determined by comparison with prepared standard solutions, and the results were expressed as $\mu mol\ H_2O_2$ per 10^6 cells.

Level of lipid peroxidation

The intensity of lipid peroxidation was determined by quantification of malondialdehyde (MDA) using an adapted protocol according to Heath and Packer (1968). First, 200 mL of the cell suspension was centrifuged at $3500 \times g$ for 5 min at 20 °C. The precipitate was then washed three times with ultrapure water and centrifuged again under the same conditions. Then, the cell pellet was homogenized in three cycles using a MM200 Retsch homogenizer at 30 Hz for 3 min, with the addition of glass beads (425–600 μm) to 700 μL of a solution containing 0.5% (w/v) 2-thiobarbituric acid (prepared in 20 % (w/v) trichloroacetic acid). The resulting extracts were incubated at 95 °C for 30 min and then centrifuged at $14\,000 \times g$ min at 4 °C for 30. Absorbance was measured at 532 nm, with the value measured at 600 nm subtracted to correct for non-specific turbidity. Final calculations of MDA content were normalized to the number of cells in each sample and expressed as a percentage of control cells.

Protein extraction

To prepare the protein extracts, 200 mL of cell suspension was centrifuged at $3500 \times g$ for 5 min at 20 °C. After the supernatant was discarded, the remaining pellet was washed three times with ultrapure water, with an additional centrifugation ($3500 \times g$, 20 °C, 3 min) after each wash. After the washing cycle, the cells were homogenized using a MM200 Retsch homogenizer at 30 Hz for 3 min at 4 °C in the presence of glass beads (425–600 μm) and 500 μL of potassium phosphate buffer (50 M, pH 7.0). The resulting homogenate was then centrifuged at $20\,000 \times g$ for 15 min at 4 °C, and the supernatant was transferred to clean tubes and centrifuged for another 45 min under the same conditions. Protein concentration was determined by the Bradford assay (Bradford 1976) using bovine serum albumin (BSA) as a standard. The extracts were then used for quantification of protein carbonyls and analysis of enzyme activity.

Activity assays of antioxidant enzymes

Enzyme kinetics were analysed at room temperature using a UV/Vis spectrophotometer (ATI UNICAM UV4, Cambridge, UK).

The activity of pyrogallol peroxidase (PPX, EC 1.11.1.7) was measured according to the protocol of Nakano and Asada (1981). The protein extract was added to the reaction mixture containing 50 mM potassium phosphate (pH 7.0), 20 mM pyrogallol and 5 mM H_2O_2 , at a final concentration of 2 % (v/v). The oxidation of pyrogallol, which led to an increase in absorbance at 430 nm (extinction coefficient $\epsilon = 2.6\ mM^{-1}\ cm^{-1}$), allowed the determination of the specific activity of PPX expressed as $\mu mol_{\text{purpurogalin}}\ min^{-1}\ mg^{-1}$ of protein.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was analysed using a modified version of the method of Nakano and Asada (1981). The protein extract was added to a reaction mixture of 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate and 10 mM H_2O_2 at a final concentration of 18 % (v/v). The decrease in absorbance at 290 nm (extinction coefficient $\epsilon = 2.8\ mM^{-1}\ cm^{-1}$) was used to calculate the specific APX activity, expressed as $\mu mol_{\text{oxidized ascorbate}}\ min^{-1}\ mg^{-1}$ protein.

Catalase activity (CAT, EC 1.11.1.6) was determined according to a modified procedure (Aebi 1984). The protein extract was added to a reaction mixture of 50 mM potassium phosphate (pH 7.0) and 20 mM H_2O_2 at a concentration of 5 % (v/v). The decrease in absorbance at 240 nm (extinction coefficient $\epsilon = 36\ mM^{-1}\ cm^{-1}$) was observed, and the results were expressed as $\mu mol_{\text{degraded } H_2O_2}\ min^{-1}\ mg^{-1}$ protein.

Analyses of photosynthesis parameters

Rate of photosynthesis

To determine the photosynthetic rate, the cell concentrations in all cultures were first measured using the Luna II device, while the photosynthetic rate was measured using

the Chlorolab 2 device (Hansatech, King's Lynn, UK). A 1.5 mL sample of the algal culture was added to the reaction vessel of the device, and the amount of oxygen produced was monitored under continuous stirring at 30 rpm at a temperature of 30 °C and a light intensity of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Between periods of illumination, the algal suspensions were incubated in the dark for 5 min. The data obtained showed the amount of oxygen released, and the photosynthetic rate values were calculated per second and expressed as nmol of oxygen per hour per 10^6 algal cells.

Parameters of chlorophyll *a* fluorescence

To determine the maximum quantum yield (F_v/F_m) of photosystem II (PSII) and the efficiency index (PI_{abs}) of PSII, the cell concentrations in the algal suspensions were measured after treatment and compared to the corresponding untreated controls using a Luna II cell counter. The algal suspensions were then incubated in the dark for 1 h to allow oxidation of PSII and deactivation of non-photochemical quenching mechanisms. Samples were prepared in cuvettes of the AquaPen instrument (Photon Systems Instruments, Drásov, Czech Republic) with 3 mL of the suspension added to each cuvette. The fluorescence of Chl *a* was monitored with the OJIP test using a superpulse of 53 % strength. The results were read with the program FluorPen ver. 1.1.1.3. (Photon System Instruments, Drásov, Czech Republic) and expressed as the ratio between variable and maximum fluorescence, adjusted to dark conditions (F_v/F_m), and as arbitrary PSII efficiency units (PI_{abs}).

Content of the photosynthetic pigments

To determine the concentrations of the photosynthetic pigments Chl *a* and *b* as well as of carotenoids, the cell concentrations in the suspensions were first measured using a Luna II cell counter. Based on these concentrations, the appropriate volume of the cell suspensions, containing 7.5×10^6 cells was transferred to clean 1.5 mL tubes. Pigment isolation was performed according to a previously modified protocol (Schumann et al. 2005). Samples were centrifuged at $3500 \times g$ for 5 min, then the supernatant was removed. The resulting pellet was washed 3 times with ultrapure water and centrifuged again under the same conditions after each wash. After the last wash, the pellet was resuspended in 800 μL of cold 90 % acetone and 0.083 g of silica beads (425–600 μm) were added. The cells were then homogenized in cold carriers on a MM200 Retsch homogenizer at 30 Hz for 4 min.

After homogenization, the samples were centrifuged again at $3500 \times g$ at +4 °C for 5 min. The supernatant was transferred to a dark 2.0 mL tube and stored at +4 °C. The pellet was resuspended in 500 μL of cold 90 % acetone, homogenized and centrifuged again under the same conditions. The resulting supernatants were combined, and the pellet was resuspended a third time in 500 μL of cold acetone, homogenized and centrifuged again under the same

conditions. All three supernatants were combined, and the volume was made up to 2 mL with cold 90 % acetone.

The absorbance of the samples was measured at the wavelengths of 647, 664 and 470 nm, and the concentrations of Chl *a* and *b* and carotenoids were calculated according to formulae described previously (Jeffrey and Humphrey 1975, Dere et al. 1998). The results obtained are expressed as μg of Chl *a* or *b* per 10^6 cells, and μg of carotenoids per 10^6 cells.

Analyses of cell ultrastructure

Ultrastructural changes were analysed by transmission electron microscopy (TEM). Algal cells were first centrifuged at $3500 \times g$ for 3 min at room temperature, after which the resulting pellet was fixed in 1 % (w/v) glutaraldehyde prepared in 50 mM cacodylate buffer (pH 7.2). Subsequently, 50 μL of the fixed cell suspension was transferred to clean tubes and mixed with liquid 1.5 % (w/v) agarose. The mixture was carefully resuspended, and after solidification, the separated agarose pieces with the cells were additionally fixed at 4 °C for 1.5 h. After fixation, the samples were washed twice with cold 50 mM cacodylate buffer (pH 7.2) and post-fixed with 1 % (w/v) osmium tetroxide in the same buffer at 4 °C for 1.5 h. This was followed by a double rinse with ultrapure water (10 min at 4 °C) before the samples were gradually dehydrated with ethanol. After the final dehydration step, the samples were embedded in Spurr resin. Ultrathin sections were stained with UranylLess (Em-Grade.com, Mauressac, France) and 3 % (w/v) Reynolds lead citrate (Em-Grade.com, Mauressac, France) and analysed using a monochromatic TF20 TEM (FEI Tecnai G2).

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA analysis followed by Newman-Keuls post-hoc analysis. The STATISTICA 14.0.0.15 software package (TIBCO Software Inc., Palo Alto, CA, USA) was used for the analysis, and statistically significant differences between the means values were determined at a significance level of $P \leq 0.05$.

Results

AgNPs physico-chemical properties

The AgNP stock suspension in ultrapure water was analysed by UV-Vis spectroscopy, TEM, DLS and ELS, and the results are presented in the Supplementary materials. Spectrophotometric analysis showed an SPR peak at 418 nm, indicating successful synthesis of AgNPs with a nominal diameter of 47 nm (On-line Suppl. Fig. 1A), while DLS analysis indicated an average d_H value of 41.4 ± 0.9 nm (On-line Suppl. Tab. 1). Furthermore, TEM micrographs revealed the spherical morphology of AgNPs with a diameter of 40–60 nm, with a small proportion of rod-like particles (On-line Suppl. Fig. 1B). The silver map (On-line Suppl. Fig. 1C) and energy dispersive X-ray microanalysis

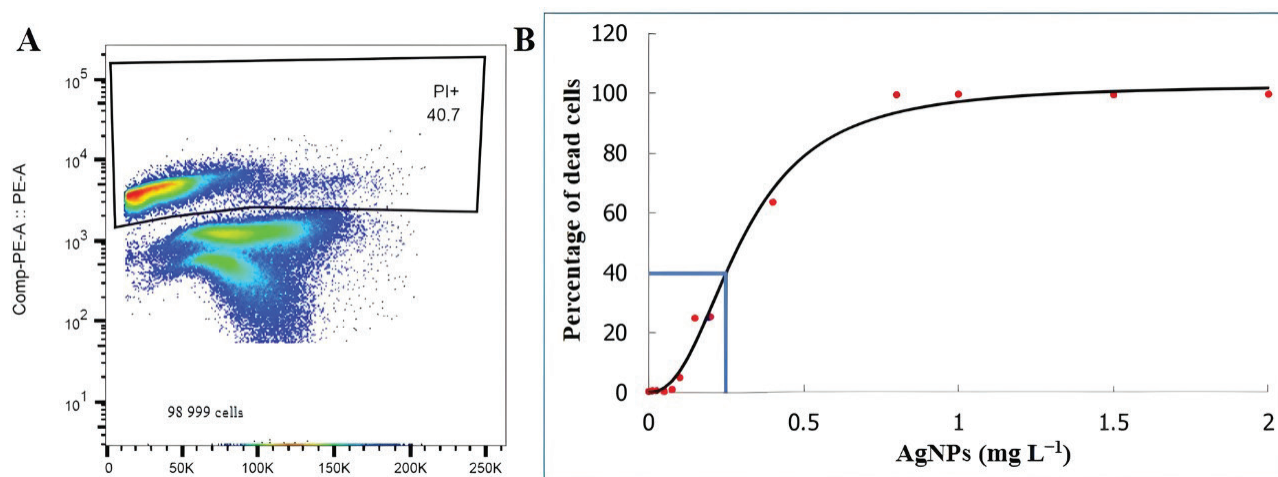


Fig. 1. The viability of *C. vulgaris* cells after 72 h exposure to increasing concentrations of AgNPs in BBM medium without glucose (-Glu) was determined by cell survival analysis using flow cytometry and the fluorescent dye propidium iodide. The results of flow cytometry (A) show approximately 40% dead cells. A four-parameter logistic (4PL) regression model was applied to determine the concentrations that caused EC_{40} (B), and the value obtained was used in all subsequent experiments. The values shown are the mean values of two independent experiments, each performed in triplicate ($N = 6$). The blue line indicates the EC_{40} value.

(On-line Suppl. Fig. 1D) confirmed the presence of silver in the particles. The AgNP ζ -potential was -40.50 ± 3.21 mV (On-line Suppl. Tab. 1). The silver concentration in the AgNP stock suspension measured by ICP-MS was 108.5 mg L^{-1} with 0.5% ionic silver (On-line Suppl. Tab. 1).

Cell viability response to AgNP exposure

Cell viability following 72 h exposure to varying concentrations of AgNPs in BBM medium is presented in Fig. 1. A clear concentration-dependent decrease in viability can be observed. The EC_{40} value, which indicates the concentration at which 60% of cells remained viable, was calculated using a four-parameter logistic (4PL) model. For the AgNP (-Glu) treatment, the EC_{40} was determined to be 0.251 mg L^{-1} , based on flow cytometry analysis with PI staining.

Silver uptake

Silver accumulation in algal cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium is summarized in Tab. 1. Detectable levels of silver were found in both treatment conditions, with significantly higher accumulation observed in the AgNP (-Glu) group ($1234.10 \pm 285.48 \text{ } \mu\text{g g}^{-1}$ dry mass) compared to AgNP (+Glu) ($404.31 \pm 60.46 \text{ } \mu\text{g g}^{-1}$

dry mass). In contrast, silver content in both control groups (-Glu and +Glu) remained below the detection limit. These results suggest that glucose reduces AgNP uptake in algal cells.

ROS formation

Use of the DHE probe revealed no significant difference in ROS content after either treatment with AgNPs than in the respective controls (Fig. 2A). However, treatment with AgNP (-Glu) resulted in a significant increase in ROS formation compared to the AgNP treatments with glucose (AgNP (+Glu)) (Fig. 2A). A similar pattern was observed with the H_2DCFDA probe, where exposure to AgNP (-Glu) resulted in a significantly increased value compared to all other treatments (Fig. 2B). The overall impression is that the addition of glucose reduces AgNP-induced ROS formation.

As for H_2O_2 content, exposure to AgNP (-Glu) also resulted in a significantly greater increase than all other treatments (Fig. 2C). An increase in H_2O_2 content was also observed with AgNP (+Glu) compared to the corresponding control, although it was not statistically significant (Fig. 2C). When comparing the two treatment groups, H_2O_2 formation increased after exposure to AgNP (+Glu) or AgNP (-Glu) compared to the corresponding controls, but

Tab. 1. Silver content ($\mu\text{g } 10^{-6}$ cells) in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). The silver content in the control cells was below the limit of quantification ($< 0.1 \text{ } \mu\text{g } 10^{-6}$ cells). Values are given as the mean $\pm$ SE of three biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

	Control (-Glu)	AgNPs (-Glu)	Control (+Glu)	AgNPs (+Glu)
Silver content ($\mu\text{g g}^{-1}$ dry mass)	0.00 ± 0.00^a	1234.10 ± 285.48^c	0.00 ± 0.00^a	404.31 ± 60.46^b

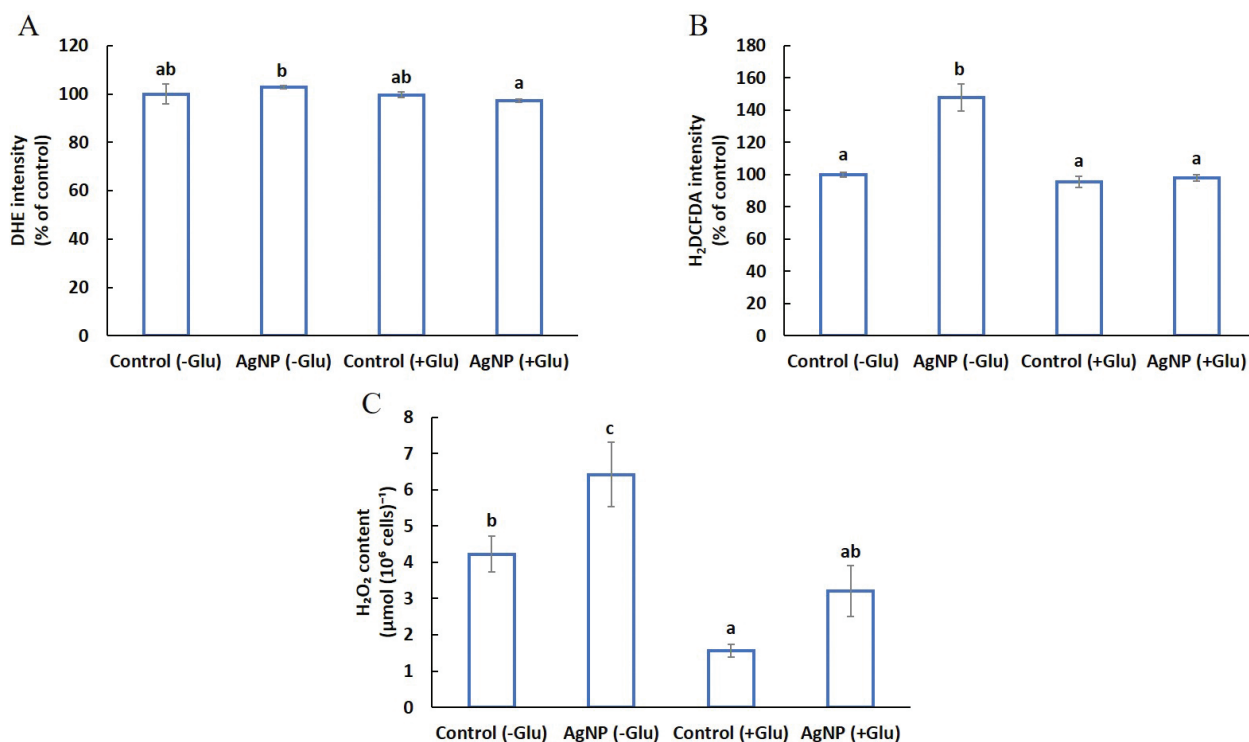


Fig. 2. The levels of reactive oxygen species (ROS) in *C. vulgaris* cells determined *in situ* after 72 h exposure to 0.251 mg L⁻¹ AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu), using dihydroethidium (DHE) (A) and 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA) (B), while hydrogen peroxide (H₂O₂) content (C) was analysed in cell extracts. Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

the amount of H₂O₂ increased significantly more after treatment with AgNP (-Glu), again indicating the alleviation effects of glucose.

Damage of membrane lipids

Changes in MDA content following AgNP treatment are presented in Fig. 3. Exposure to AgNP (-Glu) resulted in a significant 260% increase in MDA levels compared to the corresponding control (-Glu), indicating enhanced lipid

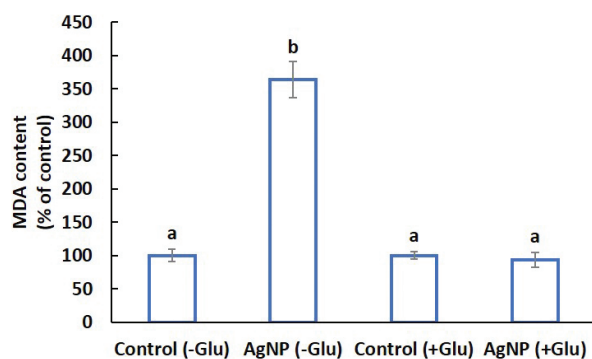


Fig. 3. Malondialdehyde (MDA) content in *C. vulgaris* cells determined after 72 h exposure to 0.251 mg L⁻¹ AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

peroxidation. In contrast, treatment with AgNP (+Glu) resulted in levels similar to those in the (+Glu) control, suggesting that glucose attenuates AgNP-induced damage of membrane lipids.

Regulation of antioxidant enzyme activity

The activity of all tested antioxidant enzymes was clearly and significantly the highest after exposure to AgNPs with the addition of glucose (AgNP (+Glu)) (Fig. 4). CAT and APX showed a similar response, as no significant changes in their activities were observed with any of the other treatments (Fig. 4A, B). The greatest differences between treatments were observed for the PPX enzyme, which also showed increased activity after exposure to AgNPs without the addition of glucose (AgNP (-Glu)) compared to the corresponding control, although this increase was significantly lower in comparison to the AgNP (+Glu) treatment (Fig. 4C). The results obtained indicate that the addition of glucose has a stimulatory effect on the enzymatic antioxidant system.

Photosynthesis rate

As shown in Fig. 5, none of the treatments caused statistically significant changes in the rate of photosynthesis compared to their respective controls. However, a slight decrease was observed in the AgNP (-Glu) treatment relative to both its control and the AgNP (+Glu) treatment.

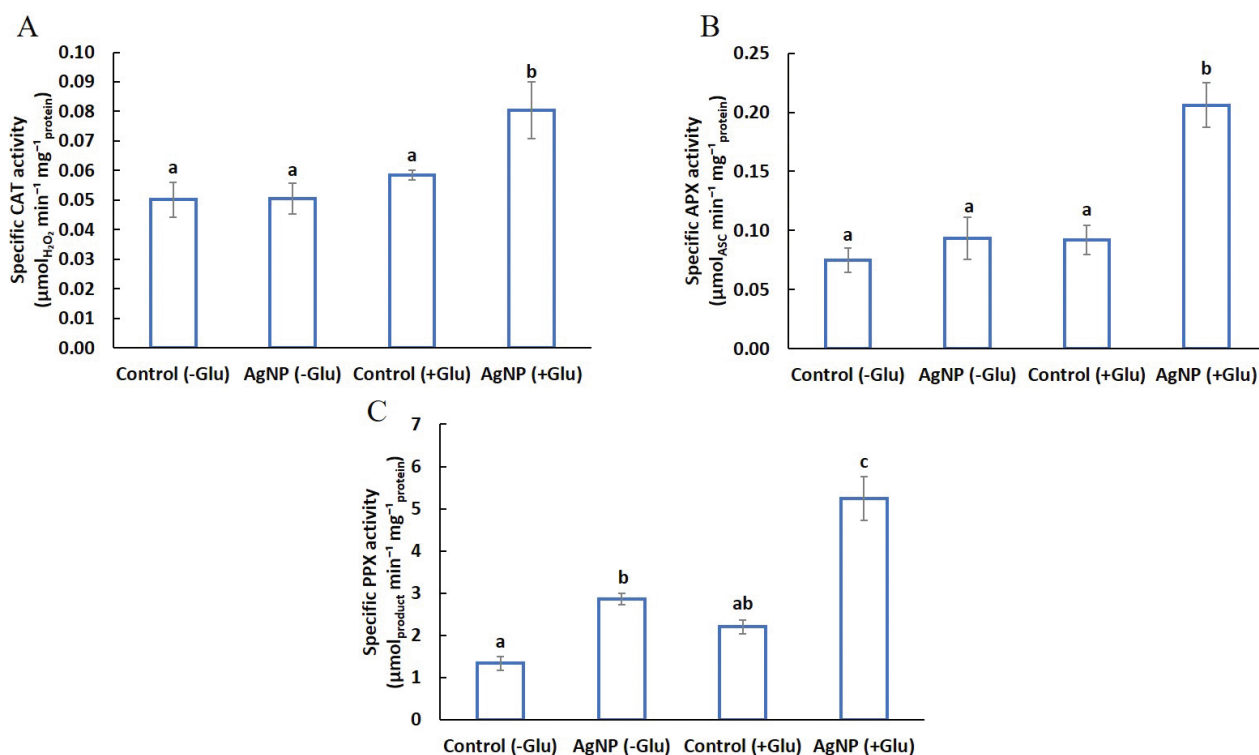


Fig. 4. Activities of the antioxidant enzymes catalase (CAT) (A), ascorbate peroxidase (APX) (B) and pyrogallol peroxidase (PPX) (C) in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean $\pm$ SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

Chlorophyll *a* fluorescence parameters

The F_v/F_m decreased significantly after treatment with AgNP (-Glu) compared to the control (-Glu) as well as to AgNP (+Glu) and the control (+Glu) (Fig. 6A). A similar trend was observed in the PI_{abs} , which also showed a significant decrease after exposure to AgNP (-Glu) compared to all other treatments (Fig. 6B). No significant differences in F_v/F_m or PI_{abs} were observed between AgNP (+Glu) and its control, while the PI_{abs} value was higher in the control (-Glu)

than in the control (+Glu). Interestingly, however, both F_v/F_m or PI_{abs} were significantly higher in glucose treatments compared to AgNP (-Glu), suggesting that the addition of glucose restores the effectiveness of photosynthesis.

Photosynthetic pigment content

The analysis of the photosynthetic pigments showed treatment-specific responses (Fig. 7). A significant increase in Chl *a* content was observed after exposure to AgNP

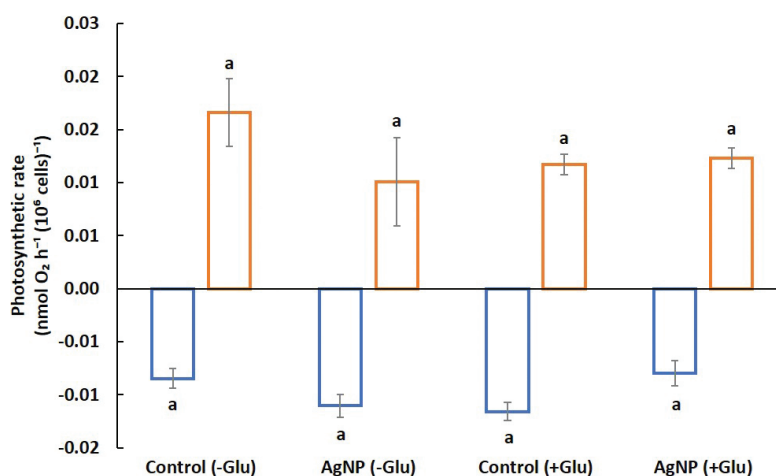


Fig. 5. Photosynthetic rate in *C. vulgaris* cells after 72 h exposure to 0.251 mg L^{-1} AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu) in the dark (blue) and at a light intensity of $80 \mu\text{mol}_{\text{photons}} \text{m}^{-2} \text{s}^{-1}$ (orange). Values are given as the mean $\pm$ SE of three biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

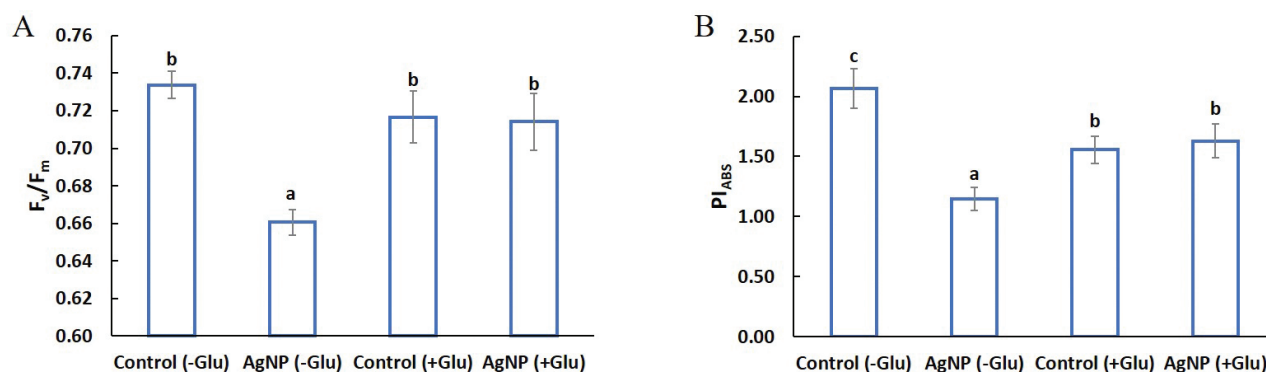


Fig. 6. Maximum quantum yield of PSII (F_v/F_m) (A) and photosynthetic efficiency index (PI_{abs}) (B) in *C. vulgaris* cells after 72 h exposure to 0.251 mg L⁻¹ AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

(-Glu) compared to the corresponding control (Fig. 7A), while no significant changes in Chl *b* or carotenoid content were observed (Fig. 7B, C). The treatment with AgNP (+Glu) had no significant effect on the content of photosynthetic pigments compared to its control, although the addition of glucose significantly reduced the content of Chl *a* compared to AgNP (-Glu). It is also interesting to note that the treatments without glucose resulted in a significantly higher content of carotenoids than treatments with glucose addition.

Ultrastructural changes

Ultrastructural changes in *C. vulgaris* cells following AgNP exposure were observed by TEM and are presented in Fig. 8. Both AgNP treatments caused visible alterations compared to their respective controls. In cells treated with AgNP (-Glu), plasmolysis, cell wall instability, and increased starch granule accumulation were evident (Fig. 8B). Addition of glucose led to enhanced formation of lipid droplets and starch granules even in the control group (Control (+Glu)) (Fig. 8C), while the most notable accumulation of

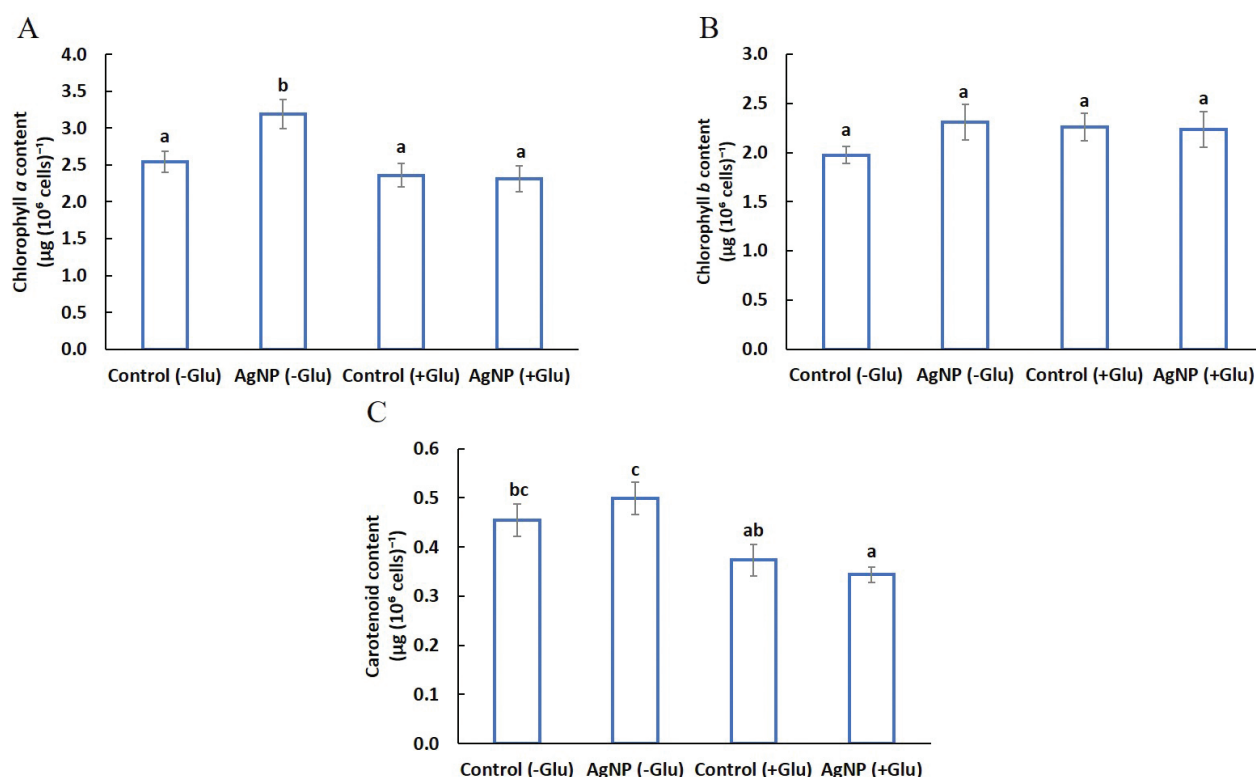


Fig. 7. Chlorophyll *a* (A), chlorophyll *b* (B) and carotenoid (C) content in *C. vulgaris* cells after 72 h exposure to 0.251 mg L⁻¹ AgNPs in BBM medium without glucose (-Glu) and with 0.5% glucose (+Glu). Values are given as the mean ± SE of six biological replicates. Treatments significantly different at $P \leq 0.05$ (one-way ANOVA followed by a Newman-Keuls post hoc test) are indicated with different letters.

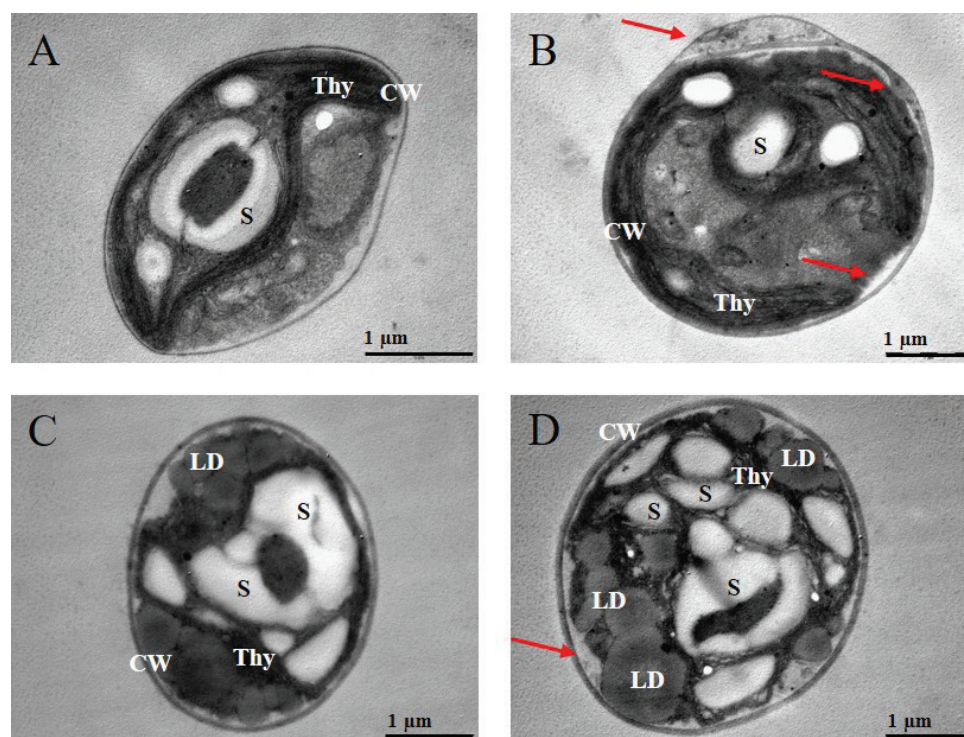


Fig. 8. Ultrastructure of control *C. vulgaris* cells in BBM medium without glucose (-Glu) (A) and with 0.5% glucose (+Glu) (C), and cells after 72 h exposure to 0.251 mg L⁻¹ AgNPs in BBM medium without glucose (-Glu) (B) and with 0.5% glucose (+Glu) (D), imaged by transmission electron microscopy. Thy - thylakoids; S - starch; CW - cell wall; red arrows indicate changes in the cell wall. Scale bar = 1 µm.

starch granules was observed in cells treated with AgNP (+Glu) (Fig. 8D). These results clearly indicate that glucose addition stimulates starch production.

Discussion

The increasing use of AgNPs increases their concentration in aquatic ecosystems and consequently has a negative impact on the organisms living there. According to studies, the concentration of AgNPs in wastewater treatment plant effluents worldwide ranges between 16.4 and 17 000 ng L⁻¹, while concentrations in surface waters are between 0.088 and 10 000 ng L⁻¹ (Maurer-Jones et al. 2013). In our study, we chose the AgNP concentration that allows the survival of 60% of algal cells (EC₄₀) after 72 h of treatment. Given the measured and estimated concentrations of AgNPs in the environment, this concentration is environmentally relevant, as it is likely that the AgNP concentrations at micro-locations will increase to the indicated level. Furthermore, AgNPs in the environment have chronic, longer-term effects on organisms, whereas our experiment aimed to assess the acute effects following a 72 h exposure of algae to AgNPs and thus focus on the immediate toxic responses of the algae. Although microalgae are naturally photosynthetic, many species can adopt mixotrophic or heterotrophic growth in the presence of organic compounds (Castillo et al. 2021). In this study, we used glucose to induce mixotrophic growth of *C. vulgaris* and to investigate its potential mitigating effect on the toxicity of AgNPs.

Most studies that have investigated the phytotoxicity of AgNPs to freshwater algae have focused on changes in cell morphology and photosynthesis as well as silver accumulation in algal cells (Afkar 2010, Dash et al. 2012, Mariano et al. 2020, Romero et al. 2020). In recent years, however, research has increasingly focused on oxidative stress caused by ROS and thus on the antioxidant response (He et al. 2012, Lekamge et al. 2020). In algae, ROS are produced in chloroplasts, peroxisomes, cytosol and mitochondria as by-products of photosynthesis and cellular respiration, but their accumulation is enhanced under various environmental stress conditions, including exposure to AgNPs (Rezayian et al. 2019, Romero et al. 2020). To determine the effect of glucose on ROS formation and oxidative stress after exposure of *C. vulgaris* to AgNPs, we designed an experiment in which the algal cells were exposed to the same concentration of AgNPs in a medium without glucose and with glucose, and the results obtained were compared with each other and with the respective controls. Two indicators were used for ROS determination (Ng and Ooi 2021), one for the total ROS content and the other for the content of O₂⁻, and increased values were recorded after exposure to AgNPs in a medium without glucose (-Glu). These results are consistent with the findings obtained in previous studies when different microalgae species were exposed to AgNPs (Hazeem et al. 2019, Shanab et al. 2019, Lekamge et al. 2020, Zhao et al. 2021). However, the addition of glucose in the treatment with AgNPs in our study resulted in a significant decrease in ROS content with both indicators compared to AgNP (-Glu),

suggesting that algae with an additional food source can successfully maintain the antioxidant system in the fight against ROS. This attenuating effect of glucose on the formation of ROS molecules was even more pronounced in the H_2O_2 content, which was significantly lower in the treatments with glucose, both in the control (+Glu) and AgNP (+Glu), compared to the control and AgNP treatment without glucose. This is also consistent with the results of our previous study (Komazec et al. 2023) and shows once again that algae can use an additional energy source for an effective defence against oxidative stress.

One of the harmful effects of ROS is the peroxidation of cell membrane lipids, which usually leads to the formation of MDA, a cytotoxic product that can cause irreversible damage to proteins and DNA molecules (Vavilin et al. 1998, Rezayian et al. 2019). Previous studies on different algal species have shown that increased ROS content in algal cells can lead to higher levels of lipid peroxidation upon exposure to AgNPs (Oukarroum et al. 2012, Shanab et al. 2019, Zhao et al. 2021). This was confirmed in this study, as exposure of *C. vulgaris* to AgNP (-Glu) led to a significantly increased MDA content compared to the control (-Glu). On the other hand, the addition of glucose to the culture medium in exposure to AgNPs reduced MDA production to the control level, again indicating a possible mitigating effect of glucose.

In response to the induction of oxidative stress by AgNPs, algae have a wide range of defence strategies, including the activation of enzymatic antioxidant system, to eliminate excess ROS (Romero et al. 2020). In this study, we investigated the activities of three antioxidant enzymes, all of which degrade H_2O_2 and thus protect cells from oxidative stress (Kalehjahi et al. 2023). Treatment with AgNP (-Glu) had no effect on the specific activity of CAT and APX compared to the control (-Glu). A similar result was reported by Lekamge et al. (2020), who found increased CAT activity only when the alga *Raphidocelis subcapitata* was exposed to higher AgNP concentrations (0.8 mg L^{-1}), while no significant change occurred at lower concentrations ($< 0.2 \text{ mg L}^{-1}$), which was similar to that used in our study. In addition, studies on the effects of AgNPs on the algae *Nannochloropsis oculata* (Fazelian et al. 2020) and *Chlamydomonas reinhardtii* (Zhao et al. 2021) showed that APX activity also increased only at higher AgNP concentrations ($< 5 \text{ mg L}^{-1}$). However, the lack of increased CAT or APX activity could also result from the altered gene regulation of these enzymes (Gupta et al. 2018), which could be a consequence of DNA damage (Cameron et al. 2018), or from binding of the enzyme to the AgNP surface, resulting in damage to the heme unit within the enzyme and consequently inhibition of its activity (Liu et al. 2020b). The latter is the more likely scenario, as exposure to AgNPs significantly increased ROS production, especially H_2O_2 , and lipid peroxidation, which is undoubtedly associated with oxidative stress. The addition of glucose in the control treatment had no significant effect on CAT and APX activity, but exposure to AgNP (+Glu) resulted in significantly increased levels, suggesting that the antioxidant defence of *C. vulgaris* is more effective in the

presence of glucose, probably due to the greater availability of energy. As for the PPX, its activity increased significantly after treatment with AgNP (-Glu) compared to the control (-Glu), which is due to increased ROS and lipid peroxidation levels, as previously reported for *Chlamydomonas reinhardtii* (Zhao et al. 2021) and *Dunaliella salina* (Bahador et al. 2019). This result also suggests that PPX may be more resistant to the toxic effects of AgNPs than CAT and APX. Furthermore, the addition of glucose increased the activity of the PPX enzyme in general as a slightly, although not significantly, increased activity was also measured in the control (+Glu) compared to the control (-Glu). However, the most significant increase was recorded after exposure to AgNP (+Glu). Since all enzymes use H_2O_2 as a substrate, the increase in their activities upon treatment to AgNP (+Glu) can be related to the significantly reduced H_2O_2 content measured in the presence of glucose in the culture medium, which enables a more efficient detoxification of peroxide. From the results obtained, it can be concluded that the addition of glucose to the culture medium increases the antioxidant capacity of the algal cells, as evidenced by the increased activity of the enzymes CAT, APX and PPX, which ultimately leads to a more effective reduction in oxidative stress, e.g. by reducing ROS and lipid peroxidation levels, suggesting that protection against oxidative stress is more stable and effective in the presence of glucose due to the increased energy status of the cell.

Aquatic ecosystems are vitally important for photosynthesis, as microalgae and cyanobacteria together produce about half of the Earth's atmospheric oxygen. Consequently, any reduction in their photosynthetic activity could pose a serious ecological threat. In this study, treatment with AgNP (-Glu) resulted in a slight decrease in photosynthetic rate and a significant reduction in chlorophyll fluorescence parameters (F_v/F_m and PI_{abs}) compared to the control (-Glu), which is consistent with the results of previous studies in *C. vulgaris* (Romero et al. 2020) as well as in *Euglena gracilis* (Li et al. 2015) and *Chlamydomonas reinhardtii* (Navarro et al. 2015, Salas et al. 2019, Zhan et al. 2024). The reduction in photosynthetic rate, and consequently carbon fixation capacity and chlorophyll fluorescence parameters, has been linked to the AgNP-induced negative effects, as the decrease in chlorophyll fluorescence indicates a disruption of the electron transport chain (ETC) within PSII, which consequently translates into a decrease in photosynthetic rate (Liu et al. 2020a). In a study on *Vicia faba* leaves, chlorophyll fluorescence was reduced after AgNP treatment, which is likely due to the transfer of excited energy from chlorophyll molecules to AgNP (Queiroz et al. 2016). Moreover, the structure and function of PSII can be damaged by AgNP-induced ROS, which can disrupt the normal course of electron transport (Jiang et al. 2017); the results on chlorophyll fluorescence and photosynthetic rate obtained in this study are consistent with this finding. Indeed, elevated ROS levels can trigger lipid peroxidation, which affects all cell membranes, including thylakoids (Dash et al. 2012, Mariano et al. 2020, Kalehjahi et al. 2023). On the other hand, the

increased content of photosynthetic pigments, particularly Chl *a*, recorded after treatment with AgNP (-Glu) probably indicates a compensatory response of the algal cells to the reduced photochemical efficiency. This interpretation is further supported by ultrastructural observations, which revealed clear alterations in chloroplast structure, including disrupted and disorganized thylakoid membranes as well as evident plasmolysis suggesting stress-induced damage to cellular structure. These structural damages are consistent with impaired photosynthetic performance and are likely caused by AgNP-induced oxidative stress. Previous studies have also shown that AgNP can stimulate pigment synthesis in the algae *Porphyridium purpureum* (Rudi et al. 2024) and *C. vulgaris* (Hazeem et al. 2019) as well as in *Lactuca sativa* L. seedlings (Jurkow et al. 2020), *Nicotiana tabacum* and *Trigonella foenum-graecum* (Sadak 2019). The increased content of Chl *a* and *b* can improve the absorption of light energy to compensate for the reduced energy conversion efficiency at the thylakoid membranes (Matorin et al. 2013, Liu et al. 2021). Additionally, carotenoids also function as antioxidants in algal cells, and their increased levels likely reflect an increased oxidative stress response due to AgNP exposure, along with enhanced light energy uptake (Zuluaga et al. 2017).

In contrast, the presence of glucose in the control medium resulted in slightly reduced photosynthesis compared to the control (-Glu) in terms of F_v/F_m and PI_{abs} , which was somewhat expected as the algal cells are more dependent on photosynthesis for food production in the absence of glucose. Obviously, there is a sufficient carbon source in the medium with glucose, which is also confirmed by the increase in the number of lipid droplets and starch granules, suggesting a metabolic shift towards heterotrophy. This is consistent with findings obtained on *Chlorella* sp. and *Nannochloropsis* sp. (Cheirsilp and Torpee 2012), where an external organic carbon source promoted intracellular storage compound synthesis. Moreover, AgNP exposure did not result in significant changes in chlorophyll fluorescence parameters, photosynthetic rate or pigment content compared to the control (+Glu), supporting the hypothesis that the addition of 0.5% glucose provides an additional carbon and energy source that helps the algal cells to mitigate the oxidative stress caused by AgNP exposure. In fact, in the presence of glucose, the algae may be less inclined to increase pigment production as they partially meet their energy and carbon requirements heterotrophically. Ultrastructural analysis further supports this interpretation, as TEM images of algae cells after AgNP (+Glu) treatment showed an even more pronounced accumulation of starch granules and lipid droplets compared to the control (+Glu), which is likely both a stress response and a consequence of extra energy and carbon availability. Furthermore, cells after AgNP (+Glu) treatment exhibited a preserved chloroplast structure and visible thylakoid membranes, in contrast to the disorganization observed in cells after AgNP (-Glu). The maintained cellular integrity and absence of pronounced plasmolysis in the AgNP (+Glu) treatment further indicate

that glucose helps stabilize internal structures and alleviate AgNP toxicity by providing sufficient energy for adequate activation of antioxidant defence mechanisms, as indicated in the oxidative stress results.

Finally, if we compare the results of treatment with AgNP (-Glu) and AgNP (+Glu), a significant increase in Chl *a* fluorescence parameters is observed, accompanied by a significant decrease in the content of photosynthetic pigments, again suggesting that in the absence of glucose, the compensatory increase in chlorophyll content is likely to be a more pronounced response to compensate for the reduction in lack of photosynthetic efficiency caused by AgNPs, and that the addition of glucose may mitigate the phytotoxic effects of AgNPs on photosynthesis in algae.

Conclusion

The acute toxicity of AgNPs to *C. vulgaris* at ecologically relevant concentrations was confirmed in this study by an increase in oxidative stress and disruption of photosynthetic processes. In the absence of glucose, AgNPs caused significant accumulation of ROS, resulting in increased lipid peroxidation, ultrastructural damage in the form of plasmolysis and cell wall degradation, and decreased photosynthetic efficiency. These effects were accompanied by little or no activation of antioxidant enzymes. In contrast, glucose supplementation attenuated the negative effects of AgNPs on *C. vulgaris* by bringing ROS accumulation and lipid peroxidation to control levels through the significant activation of all analysed antioxidant enzymes. This was accompanied by a preserved photosynthetic performance and a low degree of ultrastructural damage as well as increased lipid droplet synthesis. These results suggest that an additional carbon source such as glucose likely improves the energy status of *C. vulgaris* cells and facilitates the management of oxidative stress by supporting the activation of antioxidant defence, which in turn enables modulation of physiological and biochemical responses to AgNPs. This study demonstrates that nutrient and energy availability significantly modulates the toxicity of nanoparticles to primary producers, emphasizing the importance of the environmental context in assessing the ecological risk of nanomaterials.

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Author contribution statement

B.K., P.P.Š. – conceptualization and design of the experiments; B.K. – nanoparticle synthesis; B.K., P.P.Š. – nanoparticle characterization; B.K., K.G. – algal exposure; B.K., S.Š. – silver content analyses; B.K., K.G. – ROS, MDA and antioxidants analyses; P.P.Š. – TEM analyses; B.K. – statistical analysis; B.K., P.P.Š. – writing – original draft preparation; B.B. – writing – review and editing; P.P.Š. – supervision. All authors have read and agreed to the published version of the manuscript.

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Research Article

Heat stress responses in common bean genotypes: membrane damage, proline accumulation, and heat shock protein expression

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Abstract – This study investigated the physiological and molecular responses of two common bean (*Phaseolus vulgaris* L.) genotypes, “Balkız” and “Local Genotype”, which differ in their relative heat tolerance, with the “Local Genotype” being more tolerant than “Balkız”, to high-temperature stress during the seedling stage, aiming to identify genotype-specific physiological and biochemical responses and to determine reliable indicators of thermotolerance. Leaf relative water content (RWC), turgor loss, membrane damage, proline levels, total soluble protein (TSP), and the expression of heat shock proteins HSP23 and HSP60 were examined. The “Local Genotype” exhibited greater thermotolerance, with lower membrane damage while the temperature at which 50% injury occurs (LT_{50}) was higher, despite the accumulation of less proline than “Balkız”. Genotype-specific variations detected in protein expression indicate distinct biochemical responses to heat stress. HSP23 showed a rapid, transient increase at moderate temperature (40 °C) in both genotypes, suggesting it has a role in early heat stress response. HSP60 levels were maintained near LT_{50} temperatures, supporting sustained stress adaptation, but declined at 48 °C, reflecting extreme stress rather than genotype-specific differences. These findings emphasize LT_{50} and HSP dynamics as key indicators of thermotolerance and provide insights for understanding heat stress responses in common bean seedlings.

Keywords: High temperature stress, HSP23, HSP60, LT_{50} , *Phaseolus vulgaris* L., soluble protein, thermotolerance, water balance

Introduction

Abiotic stressors are major contributors to crop losses worldwide. Among these, high temperatures disrupt plant growth and development, limit productivity, and, in extreme situations, cause plant death (Raza et al. 2023). Rising global temperatures are expected to drastically affect agricultural productivity, particularly in moderate zones, where many major crop production areas are located. Heat stress, changing precipitation patterns, and more frequent extreme weather events are predicted to reduce global yields of major staple crops, including wheat by 6.0%, rice by 3.2%, maize by 7.4%, and soybean by 3.1%, posing a significant threat to global food security (Zhao et al. 2017). Although some high-latitude locations may benefit modestly, evidence suggests that climate change has slowed the growth of agricultural productivity worldwide, and the net effect in many regions is unfavourable (IPCC 2023).

Temperature elevation triggers a range of biochemical and physiological disruptions in plants. High temperatures

promote protein denaturation and reduce membrane fluidity, leading to oxidative stress (Pant et al. 2025). The increase in the kinetic energy and mobility of biomolecules under heat stress weakens chemical bonds, resulting in membrane injury (Zhao et al. 2021). Leaf relative water content (RWC) is a widely used physiological indicator for assessing plant water balance and stress adaptation (Soltys-Kalina et al. 2016). This parameter provides valuable insight into the balance between water uptake and transpiration, which directly influences growth, gas exchange, and metabolic activity. Likewise, the maintenance of turgor has been shown to be critical for normal cell function and for supporting growth (Kumari et al. 2021). Previous studies have reported that high temperatures reduce leaf RWC and increase turgidity loss by enhancing transpiration (Kumar et al. 2011, Tokyol and Turhan 2019).

Proline is an essential molecule that acts as an osmo-protectant and molecular chaperone (Meena et al. 2019). It aids in osmotic adjustment and protects cellular structures

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under stress (Kavi Kishor et al. 2022). However, its integrative role in legumes under heat stress and its precise physiological functions remain unclear. Although increased proline accumulation is generally associated with improved thermotolerance in crops like common bean and moth bean (Chavez-Arias et al. 2018, Tiwari et al. 2018), this relationship is not universal; in *Vigna aconitifolia*, proline accumulation under heat stress was not associated with thermotolerance (Harsh et al. 2016). Heat stress also induces the accumulation of heat shock proteins (HSPs), which play a critical role in maintaining protein structure and cellular homeostasis under stressful conditions (Scharf et al. 2012). While their general function as molecular chaperones is well established, recent studies have revealed genotype-specific variations in HSP expression and their contributions to thermotolerance (Ergin et al. 2016, Haq et al. 2019). Comparative analyses across crop species indicate that genotypes with higher and sustained HSP levels generally exhibit enhanced membrane stability, improved water retention, and better growth recovery following heat exposure (Xu et al. 2011, Kumar et al. 2024).

Common bean (*Phaseolus vulgaris* L.) is an important legume crop valued for its protein, vitamin, and mineral content. It also contributes to soil fertility through biological nitrogen fixation (Blair 2013). However, high temperatures are a major limiting factor for common bean production and quality. Heat stress reduced yield by 26–37%, primarily due to impaired pollen viability, defective seed set, and poor seed filling (Vargas et al. 2021). Most studies on abiotic stress in common bean have addressed drought, salinity, and cold (Jha et al. 2024), whereas research on heat stress has largely focused on reproductive-stage responses, such as pollen viability (Soltani et al. 2019, da Silva et al. 2020, Vargas et al. 2021). Relatively few studies (Chavez-Arias et al. 2018, Tokyol and Turhan 2019) have examined the seedling stage, which is critical for successful plant establishment under stress conditions. Moreover, integrating physiological and molecular investigations provides a more comprehensive understanding of the mechanisms underlying plant thermotolerance (Tiwari et al. 2018). In this study, we examined proline accumulation and the expression patterns of HSP23 and HSP60, alongside physiological parameters such as RWC, turgor maintenance, and membrane integrity in two *Phaseolus vulgaris* genotypes which differ in their relative heat tolerance, to elucidate the genotype-specific molecular mechanisms underlying heat tolerance in common bean seedlings. Our findings provide new insights into early-stage thermotolerance mechanisms in common bean.

Material and methods

Plant material and growth conditions

In this experiment, the genotypes “Balkız” and “Local Genotype” of common bean (*Phaseolus vulgaris* L.) were used. In our previous study, the “Local Genotype” was shown to be relatively more heat-tolerant than “Balkız” (Tokyol and Turhan 2019), which guided the selection of

these genotypes for comparative analysis. Seeds of each genotype were obtained from our laboratory common bean germplasm collection and sown in pots filled with a 1:1:1 mixture of peat, perlite, and soil. The plants were kept for four weeks in a controlled greenhouse environment with 65% relative humidity (RH) and temperatures ranging from 15 to 30 °C (night/day). After emergence, seedlings were irrigated using a pot tensiometer (Irrometer Co. Riverside, Calif, USA), ensuring uniform water availability across all pots. The greenhouse phase of the experiment was conducted from 15 October to 30 November 2019, followed by heat-stress treatments completed during the first two weeks of December 2019.

Heat stress treatments

When the seedlings reached the 5–6 leaf stage, they were transferred to a growth chamber (DAIHAN WGC-1000, South Korea) set at 65% humidity and a light intensity of 450 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (Tokyol and Turhan 2019). The temperature was gradually increased from 30 °C by 2 °C per hour until it reached 40 °C. Seedlings were maintained at 40 °C for 2 hours, after which samples were collected. The temperature was then gradually increased by 2 °C per hour to 44 °C, maintained for 2 hours, and sampled again. Finally, the temperature was gradually increased by 2 °C per hour to 48 °C, maintained for 2 hours, and sampled. For each genotype and temperature treatment, five pots (each containing one seedling) were used per replicate. The experiment was conducted with three independent biological replicates, each performed at a different time. Control seedlings were maintained under standard greenhouse conditions (30 °C, 65% RH) without heat stress.

Sampling for analyses

After each treatment, leaf samples were collected and divided into two groups. One group was used to measure membrane injury, leaf RWC, and turgor loss, while the second group was immediately frozen in liquid nitrogen and stored at –80 °C for proline and protein analyses. Fully expanded young leaves were collected for all measurements, with approximately 2.5–3 g of tissue per replication. For each genotype and temperature treatment, leaf samples were collected from five plants per replicate, with three biological replicates in total.

Cell membrane injury (CMI)

The method of Arora et al. (1998) was used with some modifications to assess CMI according to the percentage of total electrolyte leakage (EL). Leaf discs (1.5 cm in diameter) were collected from both control and high temperature-treated plants of each bean genotype. The discs were placed in test tubes containing 15 mL of double-distilled water (ddH₂O). The samples were incubated on an orbital shaker (Thermo SCIENTIFIC, 4334, USA) for 4 h. The electrical conductivity (EC) of the solution was measured using a conductivity meter (Mettler-Toledo, Seven Compact Conductivity S230,

Switzerland) to determine initial ion leakage (EC1). The samples were then autoclaved (ALP, CLG-32L, Japan) at 121 °C for 15 min, and incubated again on the orbital shaker for 4 h before the final conductivity reading (EC2) was made. Electrolyte leakage (EL, %) was calculated using the following equation:

$$EL (\%) = (EC1/EC2) \times 100$$

The percentage of cell membrane injury (CMI, %) was determined according to Arora et al. (1998) using the formula:

$$CMI (\%) = [EL(t) - EL(c)] / [100 - EL(c)] \times 100$$

where EL(t) and EL(c) refer to the EL values of the treatment and control samples, respectively.

Additionally, LT₅₀ (semi lethal temperature), defined as the temperature at which 50% CMI occurs, was calculated by nonlinear regression analysis of CMI values derived from EL measurements.

Leaf relative water content (RWC) and turgor loss

Leaf RWC (%) and turgor loss were determined using the method described by Barr and Weatherley (1962). Leaf discs (1.5 cm in diameter) were obtained from each sample. The fresh weight (FW) of the discs was recorded immediately. The discs were then soaked in distilled water for 4 h to reach full turgor, after which their turgid weight (TW) was measured. Finally, the discs were dried in an oven at 70 °C for 24 h, and the dry weight (DW) was recorded.

Leaf RWC and turgor loss were calculated using the following formulas:

$$RWC (\%) = [(FW - DW) / (TW - DW)] \times 100$$

$$Turgor\ loss (\%) = [(TW - FW) / TW] \times 100$$

Proline content

Proline content was determined using the method described by Bates et al. (1973). For the analysis, 200 mg of plant material previously stored at -80 °C was homogenized in 1 mL of 3% sulfosalicylic acid and centrifuged at 5000 g for 15 min at 4 °C. To 200 µL of the resulting supernatant, 400 µL ninhydrin reagent, 400 µL glacial acetic acid, and 200 µL of 3% sulfosalicylic acid were added and mixed thoroughly. The mixture was incubated in a water bath at 100 °C for 1 h. The samples were then placed on ice, 2 mL of toluene was added, and the mixture was centrifuged for 20 s at 4000 g. Proline content was quantified spectrophotometrically at 520 nm (Perkin Elmer Lambda 25, USA) using a standard curve prepared with L-proline (0, 50, 100, 200, 400, 800 µM).

Protein content and expression

The extraction method for total soluble protein (TSP) followed the procedure described by Shen et al. (2003) with some modifications. The extraction solution was prepared by

adjusting the pH to 7.8 using 2-morpholinoethanesulfonic acid monohydrate (MES) and contained 25 mM Tris-base, 275 mM sucrose, 2 mM EDTA, 10 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). For the extraction, 250 mg of plant material was homogenized with 1 mL of the extraction solution. The samples were then centrifuged at 10000 rpm for 10 min at 4 °C. TSP concentration was determined using the Bradford method (1976), with absorbance measured at 595 nm.

Protein profiles in leaf tissues were evaluated using a Mini PROTEAN Tetra electrophoresis device (Bio-Rad, Hercules, USA). An electrical current of 150 V was applied to the gel using a Bio-Rad PowerPac Basic power supply. For protein separation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared manually. A 4% stacking gel and a 12.5% separating gel were used. The gels were cast immediately before use. The samples were run on the gel for 1 h and 20 min at ambient temperature. Prior to electrophoresis, samples were mixed with 6× sample buffer containing 1 M Tris (pH 6.8), glycerol, SDS, DTT and bromophenol blue and denatured at 100 °C for 3 min. The samples were then cooled on ice for 5 min, and 10 µg of protein was loaded for each sample. Coomassie Brilliant Blue G-250 dye was used to visualize the protein bands in the gel, which were then imaged using Vilber's Quantum ST4 Gel Imaging System (France). Molecular weights were determined using the SDS-PAGE molecular weight standard (Bio-Rad, Precision Plus Protein Unstained Standard).

Western blotting was performed to detect HSPs according to the method of Arora and Wisniewski (1994). Proteins were transferred from the gel onto nitrocellulose membranes (Nitrocellulose membranes BioRad) using a semi-dry transfer system (Bio-Rad Trans-Blot Turbo). The proteins were probed with a 1:1500 dilution of the antibody directed against HSP23 [Monoclonal Anti-Heat Shock Protein 23 (rabbit, Sigma)] and HSP60 [Monoclonal Anti-Heat Shock Protein 60 (mouse, Sigma)]. The alkaline phosphatase assay was performed with a ProtoBlot Western Blot AP Kit (Promega) to detect immunoreactive bands. The band images that appeared on the membranes were captured using a scanner and transferred to digital media. The experiments were repeated three times, and densitometric analysis of the bands was performed in triplicate using the Public Domain NIH Image program (available at <http://rsb.info.nih.gov/nih-image/>), also known as ImageJ Software. Band intensities were normalized relative to the control sample of the “Balkız” genotype, which was set to 100%, and all other treatments were expressed relative to this.

Statistical analysis

The experiment was conducted as a completely randomized design with three independent biological replicates, each consisting of five pots (one plant per pot). Percentage-based traits (CMI, RWC and turgor loss) were arcsine square root transformed to satisfy the assumptions of normality and homoscedasticity. Normality and homoscedasticity of

residuals were checked using standardized residual plots and Shapiro-Wilk tests ($P > 0.05$). Data were analyzed using SPSS software (version 22, Chicago, IL, USA) using two-way ANOVA followed by Tukey HSD post hoc test. Data are presented as mean $\pm$ standard error (SE) of three biological replicates.

Results

Cell membrane injury (CMI) and high temperature tolerance (LT_{50})

The leaf CMI percentages and LT_{50} values for both genotypes are presented in Fig. 1. The “Balkız” genotype exhibited a higher CMI percentage (42.09%) than the “Local Genotype” when averaged across all temperature treatments. CMI percentage increased proportionally with temperature, with the most pronounced rise observed at 48 °C. From 40 to 44 °C, CMI increased approximately 3.3-fold in the “Balkız” genotype and 3.8-fold in the “Local Genotype”. A further increase from 44 to 48 °C resulted in an additional 2.9-fold rise in “Balkız” and a 7.9-fold rise in the “Local Genotype”. The higher relative increase observed in the “Local Genotype” is attributable to its considerably lower initial CMI value at 40 °C. Nevertheless, absolute CMI values at 48 °C were comparable between the genotypes. LT_{50} values further indicated that the “Local Genotype” (46.2 °C) exhibited relatively greater heat tolerance than “Balkız” (45.3 °C) (Fig. 1).

A two-way ANOVA revealed that temperature, genotype, and their interaction had significant effects on membrane injury (Tab. 1).

Leaf RWC and turgor loss

In both genotypes, leaf RWC began to decline noticeably from 40 °C onward and continued to decrease in parallel with rising temperatures (Fig. 2A). At 48 °C, the RWC of the “Local Genotype” was still higher (40.32%) than that of the “Balkız” genotype (28.97%) (Fig. 2A). In the “Balkız” geno-

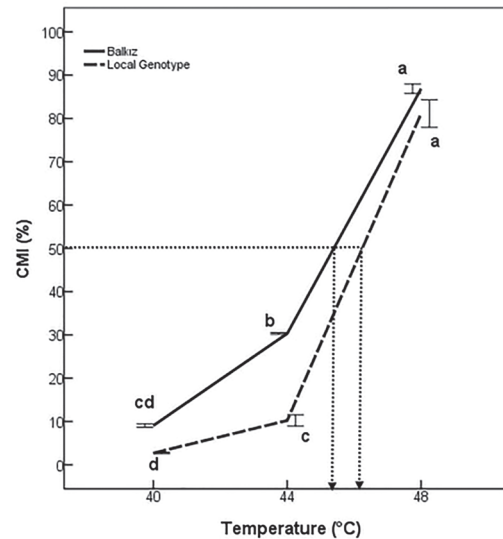


Fig. 1. Cell membrane injury (CMI, %) of “Balkız” and “Local Genotype”, two genotypes differing in heat tolerance, measured at 40, 44, and 48 °C. For each temperature treatment, leaves were collected from all five seedlings within each pot representing one replicate, and the experiment was conducted with three independent biological replicates. The horizontal dotted line shows the 50% CMI threshold, and vertical arrows indicate the LT_{50} (semi lethal temperature) of each genotype, calculated from electrolyte leakage (EL; %) using nonlinear regression. Different lowercase letters indicate significant differences among temperature treatments within each genotype ($P < 0.05$). Vertical bars represent $\pm$ standard errors (SE) of three replicates.

type, RWC decreased by approximately 38% from control to 40 °C, followed by additional decreases of 18% (40–44 °C) and 32% (44–48 °C). In the “Local Genotype”, the corresponding decreases were approximately 38%, 13%, and 10%, respectively. Although, the “Local Genotype” maintained higher RWC than the “Balkız” genotype at 44 °C and 48 °C, the differences between genotypes were not statistically significant ($P < 0.05$). A two-way ANOVA revealed a significant effect of temperature on RWC ($P < 0.05$), while the genotype $\times$ temperature interaction was not significant (Tab. 1).

Tab. 1. Results of analysis of variance (ANOVA) showing the effects of genotype, temperature, and their interactions on cell membrane injury, relative water content (RWC), turgor loss, proline content, total soluble protein (TSP) content and expression of heat stress proteins (HSP) in leaf tissues. Seedlings of two common bean genotypes differing in heat tolerance, “Balkız” and “Local Genotype”, were exposed to different temperature treatments (30, 40, 44, and 48 °C). For each temperature, measurements were performed on leaves from three biological replicates. df1 and df2 denote degrees of freedom for the factor and error, respectively. F and P values were obtained from two-way ANOVA.

Dependent variable	Independent variable								
	Genotype			Treatment			Genotype x Treatment		
	(df, df ₂)	F	P	(df, df ₂)	F	P	(df, df ₂)	F	P
Injury	(1, 12)	90.620	<0.000	(2, 12)	1129.511	<0.000	(2, 12)	10.265	<0.003
RWC	(1, 14)	2.072	=0.172	(3, 14)	82.219	<0.000	(3, 14)	1.055	=0.399
Turgor loss	(1, 15)	3.598	=0.077	(3, 15)	40.124	<0.000	(3, 15)	2.435	=0.105
Proline	(1, 16)	21.493	<0.000	(3, 16)	96.838	<0.000	(3, 16)	12.340	<0.000
TSP	(1, 16)	2.023	=0.193	(3, 16)	218.516	<0.000	(3, 16)	37.829	<0.000
HSP23	(1, 16)	0.969	=0.339	(3, 16)	186.243	<0.000	(3, 16)	32.736	<0.000
HSP60	(1, 16)	88.932	<0.000	(3, 16)	199.696	<0.000	(3, 16)	20.970	<0.000

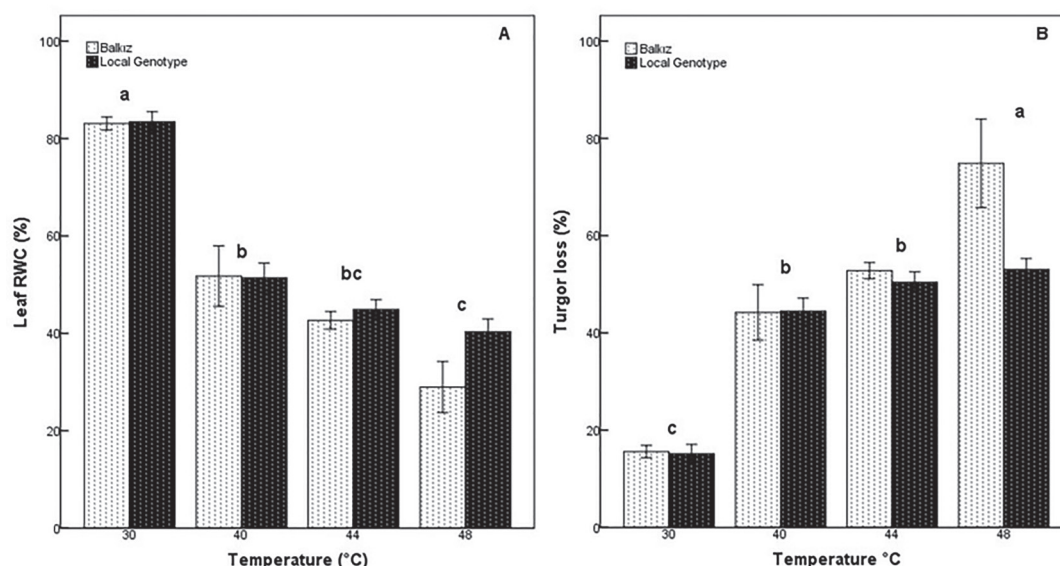


Fig. 2. Relative water content (RWC) (A) and turgor loss (B) of two common bean genotypes differing in heat tolerance, “Balkız” and “Local Genotype”, under different temperature treatments. Control conditions were 30 °C and 65% relative humidity. Two-way ANOVA revealed a significant effect of temperature ($P < 0.05$), while genotype and genotype $\times$ temperature interaction were not significant. Different small letters above the bars indicate statistically significant differences among temperature treatments (Tukey’s test, $P < 0.05$). Vertical bars represent $\pm$ standard errors (SE) of three replicates.

Additionally, turgor loss increased progressively with rising temperature in both genotypes (Fig. 2B). In the “Balkız” genotype, turgor loss increased by approximately 184% from 30 to 40 °C, followed by further increases of 20% (40–44 °C) and 42% (44–48 °C). In the “Local Genotype”, the corresponding increases were approximately 193%, 14%, and 5%, respectively. A two-way ANOVA revealed a significant effect of temperature on turgor loss (30 °C < 40 °C < 44 °C < 48 °C; $P < 0.05$), whereas neither the genotype effect nor the genotype $\times$ temperature interaction were significant (Tab. 1).

Proline content

Proline content increased progressively with rising temperature in both genotypes (Fig. 3A). In the “Balkız” genotype, proline increased by approximately 39% at 40 °C, 155% at 44 °C, and 308% at 48 °C relative to the control. In the “Local Genotype”, increases were about 18%, 42%, and 127% at the same temperatures, respectively. Across all treatments, the “Balkız” genotype had a higher average proline content than the “Local Genotype”. At elevated temperatures (44 and 48 °C), “Balkız” accumulated significantly more proline than the “Local Genotype” (Tukey,

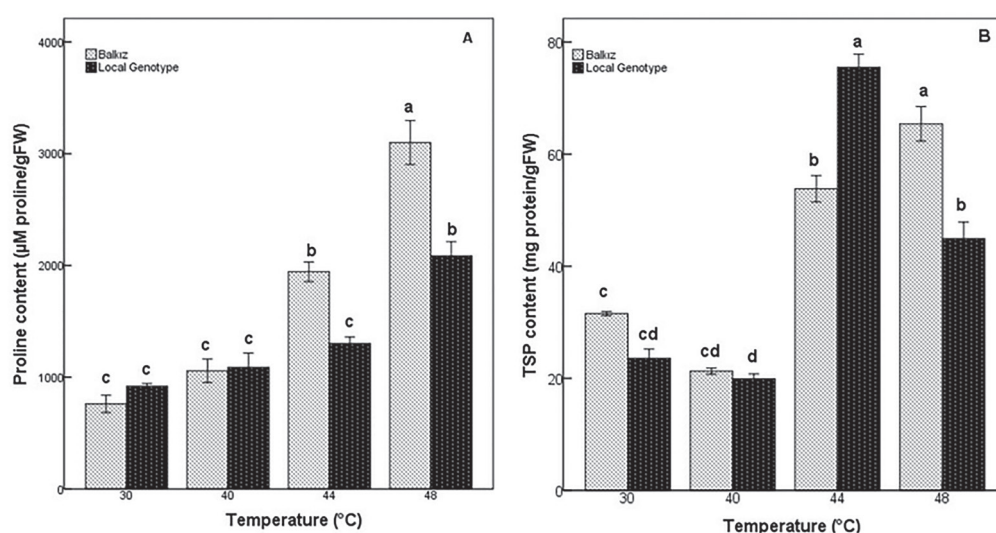


Fig. 3. The content of proline (A) and TSP (B) in common bean genotypes “Balkız” and “Local Genotype” differing in heat tolerance, under heat-stress treatments. Control conditions were 30 °C and 65% relative humidity. Two-way ANOVA revealed significant effects of temperature, genotype, and genotype $\times$ temperature interaction ($P < 0.05$). Different small letters above the bars indicate statistically significant differences among temperature treatments within each genotype (Tukey’s test, $P < 0.05$). Vertical bars represent $\pm$ standard errors (SE) of three replicates.

$P < 0.05$), and within each genotype, all temperature points differed significantly from each other. Two-way ANOVA showed significant effects of temperature, genotype, and their interaction ($P < 0.05$), indicating a strong, genotype-dependent, temperature response (Tab. 1).

Protein content and expression pattern

Total soluble protein (TSP) content changed with increasing temperature in both genotypes (Fig. 3B). Relative to control, TSP in “Balkız” changed by approximately -33% at 40 °C, +71% at 44 °C, and +107% at 48 °C, whereas in the “Local Genotype”, the corresponding changes were about -16%, +220%, and +91%. Overall, both genotypes showed a temperature-dependent TSP pattern, with significantly higher values at 44 and 48 °C than in control (30 °C) and at 40 °C (Tukey, $P < 0.05$). Two-way ANOVA indicated a significant effect of temperature and a significant genotype $\times$ temperature interaction ($P < 0.05$), whereas the main effect of genotype was not significant.

In the SDS-PAGE analysis, protein expression patterns differed between the two genotypes (Fig. 4A). In the “Balkız” genotype, the strongest overall band intensities were observed at 40 °C, followed by a clear reduction at 44 °C and an even weaker expression at 48 °C. In contrast, the “Local Genotype” displayed its highest protein expression at both 40 and 44 °C, with a decrease at 48 °C. In the “Balkız” genotype, the 43, 64, and 120 kDa bands increased at 40 °C compared with the control (30 °C), decreased slightly at 44 °C, and were strongly reduced or absent at 48 °C. In the “Local Genotype”, the 43 kDa band increased at 40 °C, decreased at 44 °C, and disappeared at 48 °C, while the 64 and 120 kDa bands, not detected at control, appeared at 40 and 44 °C but were absent at 48 °C. In the “Balkız” genotype, the 13 and 14 kDa protein bands increased at 40 and 44 °C, a pattern not observed in the “Local Genotype”. Proteins of 22, 25, and 38 kDa were more prominent at higher temperatures in both genotypes compared to the control except for the 22 and 25 kDa bands at 48 °C. Notably,

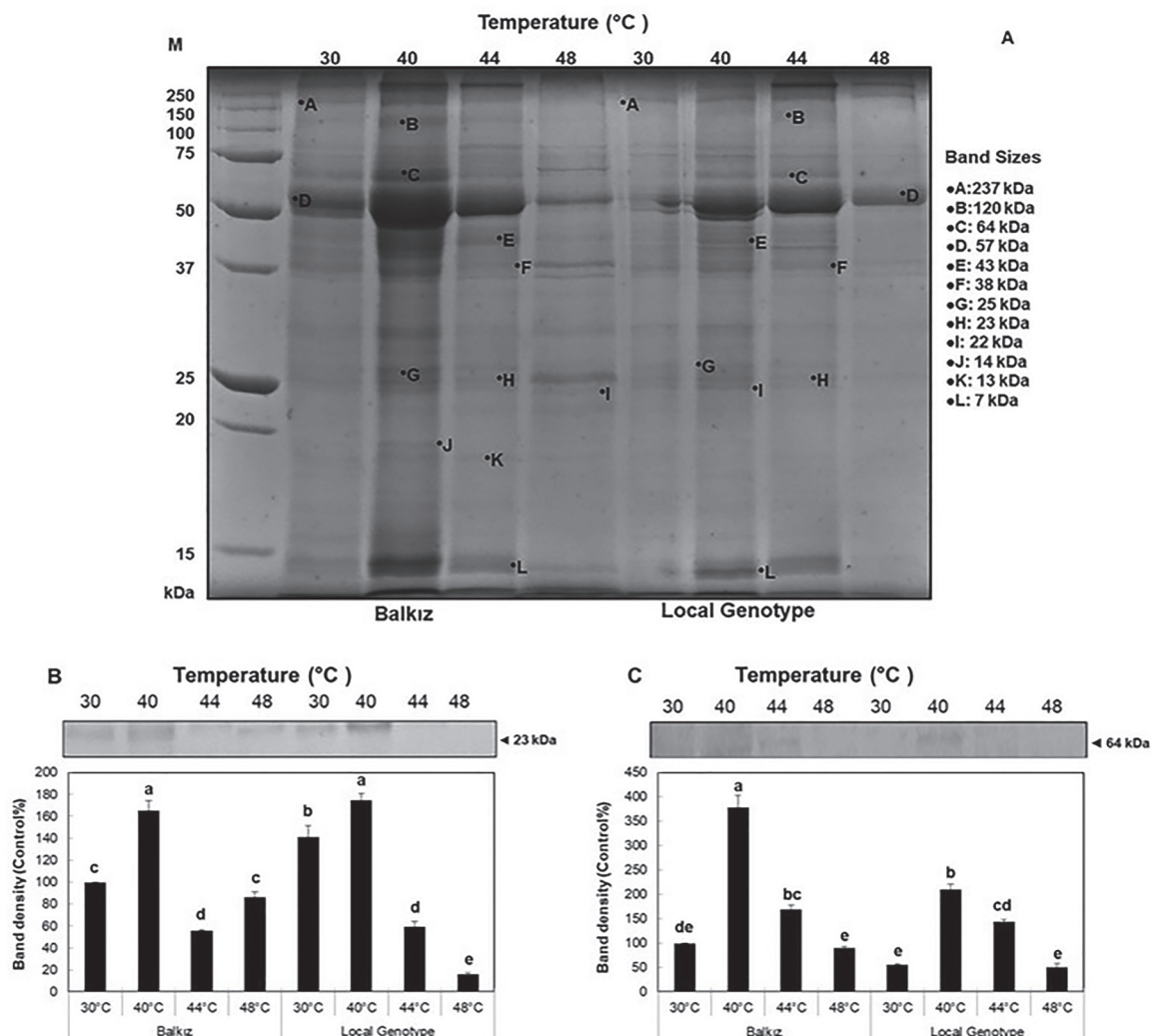


Fig. 4. Total protein profiles (A), along with the expression of heat shock proteins HSP23 (B) and HSP60 (C) in two common bean genotypes “Balkız” and “Local Genotype” differing in heat tolerance, under heat-stress treatments. Control conditions were 30 °C and 65% relative humidity. M: MW standard. For each sample, 10 µg of protein was loaded. Different letters indicate significant differences among temperature treatments within each genotype according to Tukey’s test ($P < 0.05$). Vertical bars represent $\pm$ standard errors (SE) of three replicates.

the 23 kDa band increased at 48 °C in the “Balkız” genotype. A 57 kDa protein band was clearly visible in both genotypes and is presumed to be a structural protein. The overall intensity of protein bands declined with rising temperature, with the most pronounced reduction observed at 48 °C in both genotypes.

Fig. 4B and 4C presents the HSP profiles identified through immunoblot analysis following high temperature treatments. A 23 kDa band corresponding to HSP23 was detected in both genotypes (Fig. 4B). In the “Balkız” genotype, HSP23 expression increased significantly at 40 °C, dropped sharply below control value at 44 °C, and returned to control levels at 48 °C. HSP23 expression in the “Local Genotype”, which was higher than in the “Balkız” genotype at 30 °C (basal expression), increased markedly at 40 °C, fell below control levels at 44 °C, and reached its minimum at 48 °C. These patterns indicate clear genotype-specific differences in HSP23 response, with the “Balkız” genotype maintaining expression at 48 °C, unlike the “Local Genotype”. A 64 kDa protein corresponding to HSP60 was detected, and its accumulation varied depending on genotype and temperature (Fig. 4C). In the “Balkız” genotype, HSP60 expression rose sharply at 40 °C, and although it declined at 44 °C, it remained above control levels, and returned to control levels at 48 °C. A similar pattern was observed in the “Local Genotype”, i.e. HSP60 accumulation increased significantly at 40 °C, remained elevated above control at 44 °C, and returned to control levels at 48 °C. The findings reveal genotype-specific differences in HSP60 dynamics, with the “Balkız” genotype showing higher accumulation at 40 °C (response to moderate heat stress), while the “Local Genotype” maintains HSP60 more steadily at 44 °C.

Discussion

Measuring ion leakage is one of the most effective methods for detecting cell membrane damage caused by high-temperature stress. In this study, CMI increased with rising temperature in both genotypes, and was consistently higher in the “Balkız” genotype, resulting in an LT₅₀ difference of 0.86 °C, which supports the superior thermotolerance of the “Local Genotype”. Similar results have also been reported in other species, including strawberry (Ergin et al. 2016) and cucumber (Ali et al. 2019), as well as in common bean (Tokyol and Turhan 2019). Mechanistically, high temperature perturbs lipid-protein interactions and increases membrane fluidity, which, together with protein denaturation, compromises barrier function and promotes ion efflux. Elevated temperatures also enhance reactive oxygen species (ROS) production, driving lipid peroxidation, which further disrupts membrane architecture and permeability (Bitá and Gerats 2013). Plants counter these effects by accumulating protective molecules and chaperones: for example, heat-tolerant accessions have been shown to increase membrane stabilizers including proline, HSPs, and saturated lipids to maintain membrane integrity under heat stress (Niu and Xiang 2018). Together, these processes explain

how differences in membrane stabilization and cellular protection, rather than baseline water status alone, may underlie the genotypic variation in thermotolerance observed here.

Relative water content (RWC) reflects the balance between leaf water status and transpiration rate and is a key indicator for evaluating plant resistance to stress (Mullan and Pietragalla 2012). High-temperature exposure has been shown to negatively affect water retention in legumes. Kumar et al. (2011) reported a reduction in leaf RWC in mung bean, while Tokyol and Turhan (2019) observed both a decline in leaf RWC and increased turgor loss with rising temperature in common bean. Besides, it has been found that the increase in RWC and the decrease in turgor loss are associated with heat tolerance in moth bean (Tiwari et al. 2018). Lower leaf RWC and higher turgor loss observed in both genotypes in the present study suggest that high-temperature stress disrupts normal physiological processes. However, these values did not differ significantly between genotypes, indicating that these physiological traits are not the primary determinants of thermotolerance in the common bean genotypes examined. This finding underscores the importance of integrating molecular and biochemical markers to better characterize genotype-specific responses to heat stress.

Proline accumulation is a common response in plants to various abiotic stresses. While moderate proline levels help stabilize membranes, protect enzymes, and scavenge ROS (Kavi Kishor et al. 2022), excessive amounts can disrupt cellular homeostasis or increase oxidative damage via the proline/P5C cycle (Spormann et al. 2023). In the present study, the “Balkız” genotype accumulated more proline than the “Local Genotype” but experienced greater membrane injury, suggesting that proline accumulation alone does not confer thermotolerance. These results, which are consistent with observations under heat, drought, and salinity stress (Raza et al. 2023), indicate that proline’s protective role is context-dependent and relies on coordination with other antioxidant and protective mechanisms. To enhance stress adaptation, breeding strategies should target genotypes that maintain optimal proline levels while ensuring efficient redox regulation.

High-temperature stress induces complex changes in protein metabolism, including the degradation of existing proteins and the synthesis of stress-related proteins, which can vary depending on genotype-specific tolerance mechanisms and the severity of the stress (Kosová et al. 2011). In many species, high temperatures lead to a reduction in TSP levels, due to protein denaturation and proteolysis, as reported in strawberry (Ergin et al. 2016) and moth bean (Tiwari et al. 2018). However, Tiwari et al. (2018) also noted that heat-tolerant moth bean genotypes exhibited only a slight reduction in protein content compared with sensitive genotypes. In contrast, in the present study, although TSP levels showed a slight decrease at 40 °C in both genotypes, this change was not considered significant. However, at 44 °C and 48 °C, TSP content increased markedly and significantly in both genotypes, indicating that, beyond a moderate

threshold, high-temperature exposure predominantly triggers the accumulation of temperature-responsive proteins rather than their degradation. Moreover, the results indicate that while both genotypes adjust their protein metabolism under heat stress, the “Balkız” genotype maintains a stronger TSP response at higher temperatures. Rani et al. (2016) reported the induction of two protein bands (25.8 and 30.7 kDa) in mustard subjected to 45 °C. Similarly, dehydrin proteins of 21, 23, and 27 kDa were detected in sugarcane leaves under high-temperature stress (Wahid and Close 2007). In line with these findings, the SDS-PAGE results in the present study showed both the disappearance of some protein bands and the appearance of new bands at higher temperatures, indicating active proteome reconfiguration. However, in the present study, SDS-PAGE profiles displayed the strongest banding at 40 °C and progressively weaker, less-defined bands at higher temperatures. This pattern, which appears inconsistent with the TSP results, can be explained by heat-induced changes in protein solubility and stability; severe heat promotes protein unfolding, aggregation and chaperone association, shifting a portion of the proteome into aggregated or chaperone-bound forms that are less resolved by SDS-PAGE, even as newly synthesized, highly soluble stress proteins such as small HSPs and dehydrins elevated TSP levels (Kosová et al. 2011, McLoughlin et al. 2019).

Heat shock proteins (HSPs) are evolutionarily conserved molecular chaperones that assist in the refolding of stress-damaged proteins (McLoughlin et al. 2019). HSP23, HSP36, and HSP66 have been linked to thermotolerance in turf grasses exposed to both gradual and sudden heat stress (Xu et al. 2011). According to Ergin et al. (2016), greater accumulation of a 23 kDa HSP was associated with improved heat tolerance in strawberry cultivars. Silencing of CaHSP60-6 in pepper reduced antioxidant enzyme activities under heat stress, indicating that HSP60 not only induces antioxidant enzyme synthesis but also preserves their functional structure as a molecular chaperone (Haq et al. 2019). In the present study, HSP23 accumulated transiently at 40 °C in both genotypes, but the expression was higher in the “Local Genotype”, than in the “Balkız” genotype at 30 °C (basal expression). Although HSP23 expression in the “Local Genotype” declined at higher temperatures, the genotype exhibited better thermotolerance than “Balkız”, likely due to its higher basal HSP23 expression at 30 °C and the combined effect of other protective mechanisms. This also indicates that HSP23 expression alone does not reflect heat tolerance. Accumulation of HSP60 also increased in both genotypes, showing a greater absolute band intensity in “Balkız” but a larger relative increase from control (30 °C) in the “Local Genotype”. Relatively higher HSP60 levels persisted at higher temperatures in the “Local Genotype,” suggesting genotype-specific regulatory mechanisms that enhance protein stability and organellar integrity. HSP60, a chaperonin located in mitochondria and plastids, is essential for maintaining protein homeostasis under stress. It facilitates protein import across the mitochondrial membrane and proper folding within organelles, thereby supporting

chloroplast protein maturation and Rubisco assembly (Roy et al. 2019). HSP60 functions in an ATP-dependent manner and exhibits stress- and species-specific expression, with minimal responsiveness to low-temperature or osmotic stressors (Kumar et al. 2024). These findings suggest that prolonged HSP60 accumulation contributes to thermotolerance by stabilizing organellar proteins and maintaining cellular homeostasis.

Conclusions

High temperature is a major environmental factor negatively affecting plant growth and development. In this study, thermotolerance was primarily associated with LT₅₀ and HSP expression patterns. According to LT₅₀ values, the “Balkız” genotype showed lower heat tolerance than the “Local Genotype”. HSP23 contributed to the early phase of heat stress response at moderate temperatures (around 40 °C), while HSP60 provided a sustained response at temperatures below or around LT₅₀, contributing to stress adaptation. Notably, extreme temperature (e.g. 48 °C) overwhelmed both genotypes, emphasizing the physiological limits of seedlings under heat stress. Together, LT₅₀ measurements and HSP dynamics emerge as robust indicators for screening thermotolerant genotypes, providing useful information for breeding heat-resilient common beans. However, to fully understand genotype-specific responses, future studies should also investigate additional physiological, biochemical, and molecular mechanisms that may contribute to thermotolerance, alongside regulatory mechanisms controlling HSP expression.

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Author contribution statement

Tugba Bicer: Methodology, Investigation, Data curation, Formal analysis, Validation, Writing- Original draft preparation. Ece Turhan: Conceptualization, Methodology, Writing- Reviewing and Editing, and Supervision.

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Lathyrus palustris – a newly discovered species in Slovenia

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Abstract – A conspicuous wetland species, *Lathyrus palustris* L. with Holarctic distribution has been discovered as new for the flora of Slovenia, although the wetland flora and vegetation of the country is relatively well studied. We recorded the species in the area of the intermittent Lake Cerknica, growing in different herbaceous wetland plant communities. The ecology fits the data from the surrounding regions. Our finding fills an important gap in the distribution area between Southeastern and Central Europe, where the species' rarity has resulted in it being declared endangered and accordingly protected.

Keywords: flora, Lake Cerknica, new records, wetlands

Introduction

The species *Lathyrus palustris* L. is included in the *Orobis* section of the genus *Lathyrus* (Kenicer et al. 2005). It is widespread with a Holarctic distribution (Kenicer et al. 2005) and was reported from every country neighboring Slovenia (Fischer et al. 2008, Barthä et al. 2015, Bartolucci et al. 2018, Nikolić 2020). The species is not mentioned in any of the identification keys for the Slovenian flora (Piskernik 1951, Martinčič et al. 2007 and previous versions of Mala flora Slovenije) but it was, however, included in the list of vascular plants of the Slovenian ethnic territory (which is larger than defined by the current political borders) in Mayer (1952). The only mention of the species for Slovenia was in a local vegetation paper by Gaži and Dubravec (1978), which seems to have been overlooked subsequently. The species was reported from Vojnik near Celje without a specific location, occurring in one vegetation relevé in a community dominated by *Scirpus sylvaticus* L. No further information or evidence is available to confirm the record.

The species is characterized by a narrowly winged stem, leaves with usually (two to) three pairs of leaflets, a tendril, and 2–6 pink flowers in the inflorescence (Fig. 1, On-line Suppl. Fig. 1, On-line Suppl. Fig. 2) (Fischer et al. 2008, Nikolić 2020). These characters distinguish it from other similar pink-flowered species.

L. palustris L. is a wetland species. According to Mucina et al. (2016), it is characteristic of three phytosociological classes: *Alnetea glutinosae* Br.-Bl. et Tx. ex Westhoff et al. 1946 (European mesotrophic regularly flooded alder carr and birch wooded mires), *Phragmito-Magnocaricetea* Klika in Klika et Novák 1941 (Reed swamp, sedge bed and herbland vegetation of freshwater or brackish water bodies and streams of Eurasia), and *Molinio-Arrhenatheretea* Tx. 1937 (Anthropogenic managed pastures, meadows and tall-herb meadow fringes on fertile deep soils at low and mid-altitudes (rarely also high altitudes) of Europe).

In the paper, we report on the first documented findings of the species for the territory of Slovenia, report on its ecology, and discuss its distribution and conservation status in the wider region.

Material and methods

Lake Cerknica (SW Slovenia) is one of the largest intermittent lakes in Europe, occurring on a typical karst polje (plain), 26 km² of the area being inundated at high flooding levels. Different parts of the plain experience different depths and periods of flooding, resulting in a variety of wetland plant communities.

The study is a result of i) intensive field vegetation recording (over 150 relevés in 2022–2023) for the purpose

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Fig. 1. A specimen of *Lathyrus palustris* from Lake Cerknica collected on 7th June, 2023, stored in the LJS herbarium collection (ID 13137).

of a monitoring survey of donor and recipient plots for the LIFE for Seeds project (LIFE20 NAT/SI/000253), and ii) *Allium suaveolens* Jacq. population monitoring (2024) in the Lake Cerknica area. The vegetation relevés were made according to the Braun-Blanquet approach using the modified abundance scale (+ – 5) (Westhoff and van der Maarel 1978), in plot sizes of 5 × 5 m. All occurrences of the species were found by chance.

We identified the unknown species as *L. palustris* using the identification manuals Fischer et al. (2008) and Nikolić (2020). To ascertain whether it has been reported for Slovenia, we checked Google Scholar, Hladnikia journal, and the basic floristic publications for the territory of Slovenia (Mayer 1952, Trpin and Vreš 1995, Jogan et al. 2001, Bakan 2006, Martinčič et al. 2007, Anderle 2023). We

also checked herbarium specimens in the Herbarium of the University of Ljubljana (LJU) and the Herbarium of the Jovan Hadži Institute of Biology ZRC SAZU (LJS) herbaria (Thiers 2025) for any misidentifications, stored under the following names: *L. hirsutus* L., *L. latifolius* L., *L. linifolius* (Reichard) Bässler, *L. membranaceus* C. Presl., *L. sylvestris* L., and *L. tuberosus* L.

We compared the specimens we collected in the field with photos of herbarium specimens from Virtual herbaria (JACQ Consortium) and with two specimens from the Herbarium of the University of Zagreb (ZA).

Results and discussion

We found the individuals of *Lathyrus palustris* in eight sites in the area of the intermittent Lake Cerknica, that can

be spatially grouped into three clusters (On-line Suppl. Fig. 3, On-line Suppl. Tab. 2). The habitats are different wetland communities, i.e. tall sedge-dominated beds, *Molinia caerulea* meadows, and *Schoenus ferrugineus*-dominated fens. We made two vegetation relevés with present *L. palustris* (On-line Suppl. Tab. 1).

No specimens examined in the herbaria LJU and LJS corresponded to *L. palustris*. Specimens collected at the two sites where the two vegetation relevés were made are stored in the LJS herbarium under IDs 12621 and 13137 (Fig. 1, On-line Suppl. Tab. 2).

The vegetation and flora of wetland habitats in Slovenia have been relatively well studied, including the area of the intermittent Lake Cerknica; however, the species *L. palustris* is not mentioned or listed in the publications. In the Balkans and the wider neighborhood, the species is rare, corresponding to the edge of its area of distribution. It has recently been (re)discovered for Bulgaria (Hájek et al. 2006), and confirmed for North Macedonia (Teofilovski 2018) in a basophilic fen and a sedge bed. In accordance with its rarity, it is often assessed as threatened: in Austria as Endangered with decline (Schratt-Ehrendorfer et al. 2022), in Croatia as Data Deficient (Nikolić and Topić 2005), in Italy as Endangered (Orsenigo et al. 2021), and in Hungary it is Near Threatened (Barina et al. 2007). Globally, the species is considered Least Concern (Rowe and Kavak 2019).

The species is protected in the following countries: Croatia (strictly protected) (Anonymous 2016), Hungary (Anonymous 2012), Austrian Carinthia (Anonymous 2007a) but not in Austrian Styria (Anonymous 2007b) or Italy (Anonymous 2013). As the species has not been recorded in Slovenian territory, it is not protected. Still, due to several locations found in a span of only a couple of years since the discovery, we can expect the species to be present in even more localities in the area of Lake Cerknica, possibly also in other regions. Nevertheless, it is unlikely to be widespread and common in the country, and additional attention to this conspicuous species in the future years will enable us to assess its conservation status.

Here, we also propose that the species be named “močvirski grahor” in Slovenian, following the established Slovenian name for the majority of species in the genus *Lathyrus* “grahor” with the adjective “močvirski” being the translation from Latin “palustris” and from names in other languages (Croatian “močvarna” graholika (Nikolić 2020), and English “Marsh” Pea (Rowe and Kavak 2019)).

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Author contribution statement

F.K. conceived and wrote the manuscript; all authors recorded and identified the species in the field; all authors critically revised and approved the manuscript.

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Short Communication

Two *Potamogeton* species (Potamogetonaceae) new to the Caucasus

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Abstract – This study presents the first records of *Potamogeton compressus* L. and *P. friesii* Rupr. in the Caucasus, where both are confined to Lake Tabatskuri, Georgia. *Potamogeton compressus* was identified from 1965 herbarium specimens found in TGM, while *P. friesii* was documented in 2023. These findings extend their known southern ranges, highlighting the importance of high-altitude lakes in preserving boreal aquatic flora in the South Caucasus.

Keywords: aquatic plants, boreal species, high-altitude lakes, Lake Tabatskuri, Samtskhe-Javakheti region

Introduction

The genus *Potamogeton* L. s.s. (Potamogetonaceae) is represented in the Caucasus by 13 species and two hybrids: *P. acutifolius* Link, *P. alpinus* Balb., *P. berchtoldii* Fieber, *P. coloratus* Hornem., *P. crispus* L., *P. gramineus* L., *P. lucens* L., *P. natans* L., *P. × nitens* Weber, *P. nododus* Poir., *P. perfoliatus* L., *P. praelongus* Wulf., *P. pusillus* L., *P. × salicifolius* Wulf., and *P. trichoides* Cham. & Schldtl. (Grossheim 1939, Tzvelev 2006, Tikhomirov et al. 2024). However, the individual regions of the Caucasus remain unevenly studied regarding the diversity and distribution of pondweeds, with only a few recent records.

In this report, two pondweeds, *P. compressus* L. and *P. friesii* Rupr., are first recorded for the Caucasus. Both species are common in northern temperate regions, but in the Caucasus, they are known only from single localities restricted to the Georgian part of the Lesser Caucasus, north of the Armenian Highlands (Fig. 1A).

Material and methods

Material of *P. compressus* was found during herbarium studies at TGM (herbarium acronyms follow Thiers 2025) and originates from the Samtskhe–Javakheti region. It was collected in the vicinity of Lake Tabatskuri in 1965 and initially identified as *P. gramineus*, one of the commonest pondweeds in the region. A few leaves were isolated for detailed microscopic studies. *Potamogeton friesii* was observed by the author during field studies in 2023 in the same area. A few plants of *P. friesii* were found only once, in

a small shallow bay in the western part of the lake dominated by communities of *P. gramineus*, *P. crispus*, and *Ceratophyllum demersum* L. Specimens were collected by hand, pressed for herbarium storage and deposited at IBIW. Collection of TBI was also checked for possible specimens. The specimens were studied under an NSZ818 Nexcope stereomicroscope (Ningbo Yongxin Optics Co., Ltd., China).

Results and discussion

Potamogeton compressus L. 1753, Sp. Pl.: 127 (Fig. 1B, C). – [Georgia, Samtskhe–Javakheti region, Borjomi municipality] Djavachethi. Ad ripam lac. Tabatzkuri [41.65° N, 43.63° E]. 2000 m.s.m., coll.: K. Kimeridze, Aug 24, 1965, TGM 24145!, TGM 67357! The studied specimens are characterized by strongly flattened stems, approximately 2–4 mm wide, and long (up to 200 mm), linear leaves that are 5-veined and 2.5–4.5 mm wide, with numerous sclerenchymatous strands. These specimens lack fruits, with apical and axillary turions present.

Potamogeton friesii Rupr. 1845, Beitr. Pflanzenk. Russ. Reiches 4: 43. (Fig. 1D, E) – Samtskhe–Javakheti, Borjomi municipality, Lake Tabatskuri, 1991 m a.s.l., Moliti, 41.63875° N, 43.58911° E, water depth 1 m, water conductivity 130 µS cm⁻¹, coll., det.: V.S. Vishnyakov, Aug 17, 2023, IBIW. The specimens are characterized by compressed stems and linear leaves 45–55 × 1.5–2.5 mm, which are 3- or 5-veined. Nodal glands are well developed. These specimens are sterile and fragile, with apical turions.

Both *P. compressus* and *P. friesii* are widely distributed in boreal and temperate zones but become rare in southern

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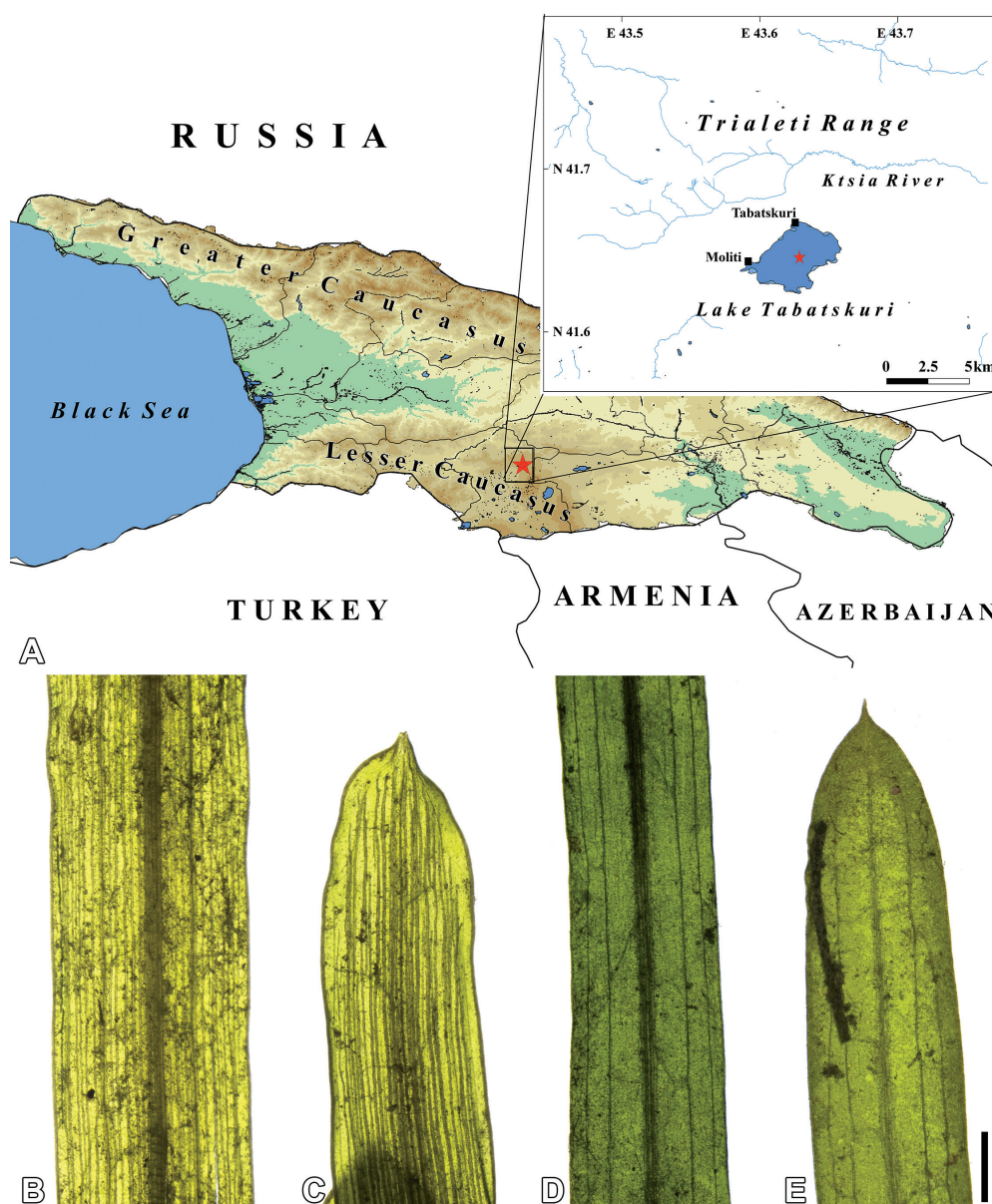


Fig. 1. *Potamogeton* species from Georgia new to the Caucasus: A – map of the Lake Tabatskuri area; B, C – *P. compressus*, 5-veined leaves with numerous sclerenchymatous strands, TGM 67357; D, E – *P. friesii*, 5-veined leaves without sclerenchymatous strands, IBIW. Scale bar = 1 mm (photo: Vasily S. Vishnyakov).

regions (Casper and Krausch 1980, Wiegleb and Kaplan 1998, GBIF 2025, POWO 2025). The nearest known *P. compressus* records are approximately 900 km to the north (e.g., Seregin and Stepanova 2025a). The only similar species in the region, *P. acutifolius*, differs in having narrower, 3-veined leaves. In Europe, both species exhibit broad areas of overlap (Kaplan and Marhold 2012); however, their distributions within the Caucasus are distinct: *P. acutifolius* is confined to steppe plains near the Black and Azov seas, barely entering western Ciscaucasia, whereas *P. compressus* appears to be restricted to the mountainous region of southwestern Transcaucasia. The nearest *P. friesii* records are ca. 820 km to the north (Seregin and Stepanova 2025b) and 590 km to the southeast (Abbasi et al. 2015). This species can be distinguished from the similar *P. pusillus* and *P. berchtoldii*, previously recorded throughout the Caucasus,

by its broader leaves with rounded to apiculate tips, more numerous veins (not always 3-veined), and white stipules connate at the base with coarse veins.

The new records extend the southern range of these two pondweed species, filling a large gap in their distribution within the Caucasus. A review of herbarium specimens, combined with field surveys, confirms that both species are rare in this region, being restricted to a single locality – Lake Tabatskuri, one of the largest of Georgia's lakes. Situated on the volcanic Javakheti Plateau at 1991 m a.s.l., Lake Tabatskuri (83.1 km²) has a maximum depth of 40 m. Its hydrographic network is poorly developed, lacking permanent tributaries, with an outlet draining underground into the Ktsia River (Caspian basin). The lake is primarily fed by low-mineral groundwater, supplemented by minimal precipitation and temporary streams. This groundwater

influx helps maintain cool summer temperatures, with an August maximum of 15 °C. Severe climatic conditions lead to complete ice cover from late December for about three months (Apkhazava 1975). Previously classified as oligotrophic, the lake has recently shown signs of macrophyte overgrowth, as reported by local residents in 2023. Despite these changes, its environmental conditions remain similar to those of remote northern regions, characterized by cool temperatures, prolonged ice cover, and low ion content.

It is likely that the high-altitude positioning of waterbodies in the Armenian Highlands and adjacent regions provides favorable conditions for pondweed species typically found in more northern latitudes, such as *P. alpinus* and *P. praelongus* (İkinci and Bayındır 2021). The Javakheti region, in particular, has long been recognized as a refuge for remnants of boreal aquatic flora that have persisted since cooler and wetter climatic periods, such as the Last Glacial Maximum, and which now occur only rarely and in low abundances (Zedelmeyer 1933). The occurrence of *P. compressus* and *P. friesii* in this area may similarly reflect the long-term survival of relict populations in isolated habitats. However, their presence could also be the result of recent introductions linked to human activity, as Lake Tabatskuri was stocked with whitefish originating from Lake Ladoga in northern European Russia during the 1930s (Kuljanishvili et al. 2021).

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Secondary metabolites of the culturable endophytic fungus *Penicillium chrysogenum* and their plant growth regulator activity

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Abstract – This study reports the isolation of two natural compounds, zearalenone and hyrtiosulawesine, from the endophytic fungus *Penicillium chrysogenum* isolate SR192 which is associated with the invasive plant species *Solanum rostratum*. In general, both isolated compounds exhibited a dose-dependent stimulatory effect on the growth of four weed species when concentrations ranging from 5 to 100 $\mu\text{g mL}^{-1}$ were tested. However, suppressive effects were observed when the concentration was increased to 500 $\mu\text{g mL}^{-1}$, suggesting a biphasic response. Our findings indicate that these compounds have the potential to be further investigated as environment-friendly plant growth regulatory agents.

Keywords: Hyrtiosulawesine, *Penicillium chrysogenum*, *Solanum rostratum*, zearalenone

Introduction

Fungal endophytes are microbes that symbiotically inhabit the inner tissues of their hosts for part or all of their life cycles without causing visible pathogenic symptoms and potentially modulating plant reproductive capacity, growth dynamics, and resilience to abiotic and biotic stressors (Rho et al. 2018, Ripa et al. 2019). They have been identified as important sources of novel biologically active compounds like steroids, alkaloids, terpenoids, phenols, quinones, isocoumarins, lignans, and lactones (Dwibedi and Saxena 2019, Kuchkarova et al. 2020). These compounds have significant potential for further research in medical applications or as eco-friendly agrochemicals, owing to their diverse range of biological properties, including antiviral, anticancer, immunosuppressive, antidiabetic, antioxidant,

antimicrobial, phytotoxic, and plant growth regulatory activities (Koul et al. 2016, Kuchkarova et al. 2024a).

The increasing global population and land scarcity underscore the importance of plant growth regulators (PGRs) in sustainable agriculture. Despite significant advancements in the PGR industry between 2003 and 2022, existing research overlooks industry trends, challenges, and innovation opportunities. PGRs demonstrate considerable market potential, bolstering global crop production and contributing to sustainable development (Rademacher, 2015).

Numerous endophytic fungal species can be found in *Solanum rostratum*, and these species produce bioactive secondary metabolites (Kuchkarova et al. 2024b). An endophytic fungus called *Penicillium chrysogenum* SR192 isolated from the invasive plant *S. rostratum* produces secondary metabolites with significant plant growth regulatory activity

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(Kuchkarova et al. 2020). The study aimed to isolate and identify biologically active compounds with potential plant growth regulatory effects. Further, this study provides a theoretical basis for the responsible use of secondary metabolites from *S. rostratum* endophytic fungi.

Material and methods

The endophytic fungal isolate SR192 was obtained from the leaf of the invasive *Solanum rostratum* growing in suburban Urumqi, Xinjiang Province, China. The fungal isolate was identified as *P. chrysogenum* (Accession No. OM698376), based on sequence analysis of the rDNA ITS. The sequence fragment was identified using the basic local alignment search tool BLAST (<http://www.ncbi.nlm.nih.gov/>) of the NCBI and Mycobank (<https://www.mycobank.org>) databases. The isolate SR192 is preserved at the State Key Laboratory of Desert and Oasis Ecology, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi, China (Kuchkarova et al. 2024a).

The endophytic fungal isolate SR192 was cultivated for 21 days at 28 °C and 120 r/min on a rotary shaker in a 14.4-liter potato dextrose broth (PDB) medium. Then, the broth was fully extracted with ethyl acetate four times, and the ethyl acetate extracts were evaporated by using a rotary evaporator (IKA RV8, Germany) under reduced pressure at 40 °C to harvest a brown oily gum (2.5 g). For fractionation, the dried extract was processed by silica gel (200–300 mesh, Qingdao Haiyang Chemical Factory, Qingdao, China) column chromatography (CC) with a n-hexane/ethyl acetate gradient (100:0, 90:1, 70:1, 50:1, 25:1, 12:1, 6:1, 4:1, 2:1 and 1:1 v/v) to afford ten fractions (F.1–10). All the fractions underwent preliminary screening for plant growth regulatory activity on the test plant *Amaranthus retroflexus* for promotive activity at 20 µg mL⁻¹ and inhibitory activity at 500 µg mL⁻¹. Fractions F.8 and F.9 with significant growth regulatory activity were combined (0.5 g, brown gum) and subjected to column chromatography on silica gel (300–400 mesh) using a gradient elution with ethyl acetate followed by ethyl acetate/methanol mixture (9:1, 6:1, 4:1, 2:1, 1:1, and 0:1 v/v), resulting in separation into 12 subfractions (F.9.1–F.9.12). Subfraction F.9.10 was further purified by column chromatography on Sephadex LH-20 (H&E Co. Ltd., Beijing, China) using chloroform/methanol (1:1) as an elution solvent, yielding 12 subfractions. Then, following the same method, subfraction F.9.10.5 was purified again by using CC on Sephadex LH-20 to obtain compound 1 (4.7 mg) and compound 2 (3.9 mg). Thin-layer chromatography (TLC) was applied to determine the purity of natural compounds (Toshmatov et al. 2024). Sulfuric acid/ethanol (5:95 v/v) mixture was sprayed onto fraction spots on the TLC plates, and then heated at 105 °C for visualization. ¹³C NMR, ¹H NMR, and 2D NMR spectra were recorded on Varian MR-400 and VNMR-600 NMR spectrometers with tetramethylsilane (TMS) as an internal standard (Harris et al. 2001).

The plant growth regulatory activity of isolated compounds was screened using two monocot plants, *Lolium*

perenne, and *Setaria viridis*, and two dicot plants, *A. retroflexus* and *Medicago sativa*. Before use, the seeds of the test plants were surface-sterilized with 75% ethanol. The two compounds were diluted with methanol to obtain 5, 20, 100, and 500 µg mL⁻¹ concentrations. Subsequently, Petri dishes containing a layer of filter paper were filled with 0.5 mL of the corresponding methanol solution. The test seeds (10 seeds/dish) and 0.5 mL of distilled water were added to the corresponding Petri dishes once the methanol had evaporated entirely. Afterwards, Petri dishes were kept at 25 °C in the dark and sealed with parafilm. After seven days of seedling growth, the lengths of the roots and shoots were measured. Each bioassay was done in three replicates. Ultimately, thirty plant seedlings per concentration were selected for measurement (N = 30). Data were analyzed using Fisher's LSD test at a significance level of P < 0.05 to compare differences among treatments, and results were expressed as mean ± standard error (SE) (Kuchkarova et al. 2024b).

Results and discussion

Penicillium chrysogenum is a valuable source of new and known natural products, and numerous studies have investigated its biological activities (Chen et al. 2022, Lu et al. 2024). Furthermore, Anh et al. (2024) isolated and identified one new and nine known compounds from the methanol extract of the marine fungus *P. chrysogenum* VH17, and they were discovered to possess antimicrobial and cytotoxic activities. In this study, two compounds were isolated from the subfraction (F.9.10) of the SR192 fungal extract. The spectral data of the isolated compounds (PMR, ¹³C NMR, HSQC, and HMBC) were compared with published literature (Yang et al. 2008, Sauleau et al. 2006), leading to their identification as zearalenone and hyrtiosulawesine.

Zearalenone (1), light-orange powder (MeOH), C₁₈H₂₂O₅. ¹H NMR spectrum (600 MHz, CD₃OD, δ, ppm, J/Hz): 1.37 (3H, d, J = 6.2, H-11'), 1.56 (2H, m, H-4', 8'), 1.63 (1H, m, H-5'), 1.77 (2H, m, H-4', 5'), 2.06 (1H, m, H-8'), 2.11 (1H, m, H-3'), 2.16 (1H, m, H-9'), 2.29 (2H, m, H-7', 9'), 2.65 (1H, m, H-3'), 5.71 (1H, dd, J = 15.0, 10.3, 3.9, H-2'), 6.22 (1H, d, J = 2.3, H-3), 6.38 (1H, d, J = 2.3, H-3), 6.98 (1H, dd, J = 15.7, 1.8, H-1'). ¹³C NMR spectrum (150 MHz, CD₃OD, δ, ppm): 21.0 (C-11'), 22.0 (C-8'), 23.3 (C-4'), 32.1 (C-9'), 35.9 (C-5'), 37.5 (C-7'), 43.8 (C-3'), 74.4 (C-10'), 102.9 (C-3), 104.0 (C-1), 109.5 (C-5), 133.0 (C-2'), 134.5 (C-1'), 144.8 (C-6), 164.0 (C-4), 166.3 (C-2), 172.7 (C-12'), 213.8 (C-6').

Hyrtiosulawesine (2), a yellow powder (MeOH), C₂₀H₁₃N₃O₃. ¹H NMR (600 MHz, CD₃OD, δ, ppm): 6.82 (1H, d, J = 8.8 Hz, H-6'), 7.14 (1H, dd, J = 8.8, 1.5 Hz, H-7), 7.32 (1H, d, J = 8.3 Hz, H-7'), 7.55 (1H, d, J = 8.8 Hz, H-8), 7.57 (1H, s, H-5), 8.02 (1H, s, H-4'), 8.16 (1H, d, J = 4.9 Hz, H-4), 8.43 (1H, d, J = 4.9 Hz, H-3), 8.89 (1H, s, H-2'); ¹³C NMR (150 MHz, CD₃OD, δ, ppm): 106.76 (C-5), 108.01 (C-4'), 113.27 (C-7'), 113.83 (C-6'), 113.90 (C-8), 116.05 (C-3'), 118.47 (C-4), 119.82 (C-7), 122.61 (C-5a), 129.87 (C-3'a),

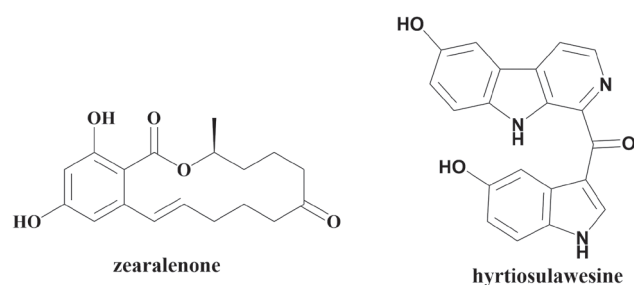


Fig. 1. Structures of compounds isolated from *Penicillium chrysogenum* extract.

132.35 (C-7'a), 132.48 (C-4a), 137.31 (C-8a), 137.31 (C-9a), 137.39 (C-3), 139.09 (C-2'), 140.36 (C-1), 152.65 (C-6), 154.45 (C-5'), 189.86 (C-8'), (Fig. 1).

Compound 1, zearalenone, is a mycotoxin primarily synthesized by the fungal genera *Aspergillus* and *Fusarium*. It has also been isolated from a marine strain of *Penicillium* sp., obtained from the surface of drifting cotton clothing collected on Namhae Island, Gyeongnam, Korea (Yang et

al. 2008). Compound 2, hyrtiosulawesine, was originally isolated from the *Hyrtios erectus* Red Sea marine sponge and studied for its antiphospholipase A₂ activity (Sauleau et al., 2006). More recently, Toshmatov et al. (2024) reported its isolation from the seeds of the invasive plant *S. rostratum* and evaluated its phytotoxic activity on *A. retroflexus* and *L. perenne* seedlings. Our current research expands the scope by evaluating the biological activity of hyrtiosulawesine on four weed species. Two dicot plants, *A. retroflexus*, and *M. sativa*, as well as two monocot plants, *L. perenne*, and *S. viridis*, were used to screen the plant growth regulatory effects of the two compounds at concentrations of 5, 20, 100, and 500 $\mu\text{g mL}^{-1}$ (Figs. 2 and 3).

The growth of the seedlings generally exhibited a dose-dependent pattern as the concentration of the compound increased. Compounds 1 and 2 significantly accelerated the root growth of *A. retroflexus* by 51.53% and 40.49%, and shoot growth by 29.54% and 27.27%, respectively, at a concentration of 5 $\mu\text{g mL}^{-1}$. At 20 $\mu\text{g mL}^{-1}$ and higher concentrations of both compounds, the promotion of growth was

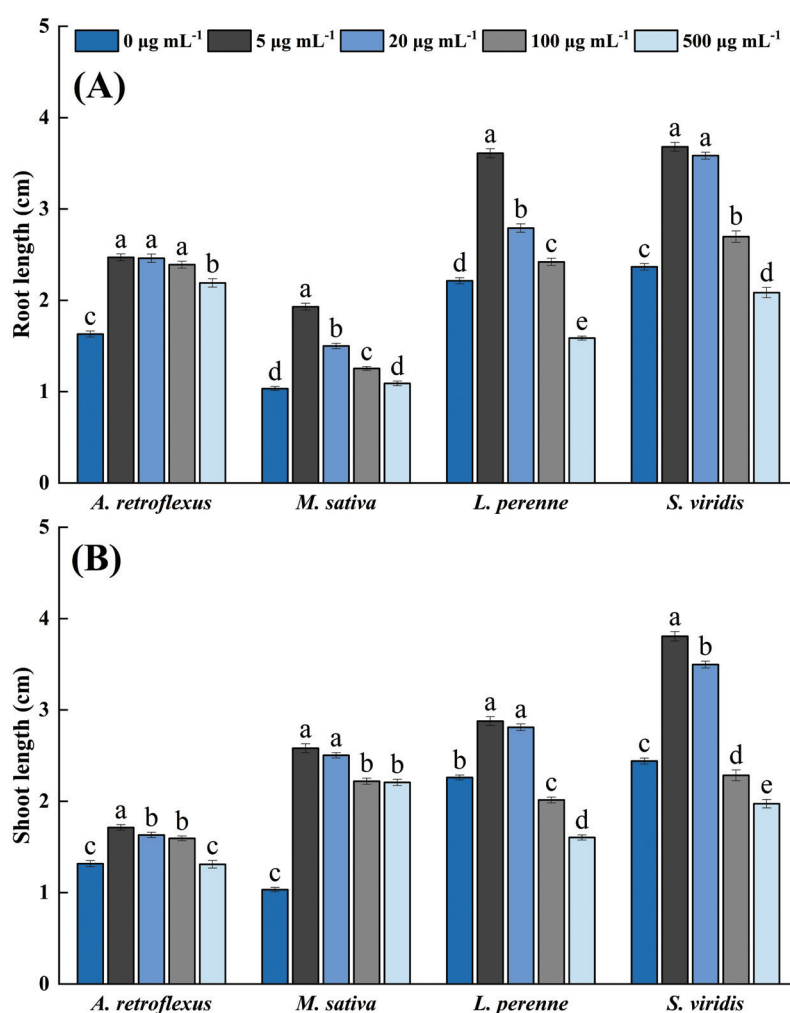


Fig. 2. Plant growth regulatory effects of zearalenone (compound 1) isolated from *Penicillium chrysogenum* extract on the root length (A) and shoot length (B) of *Amaranthus retroflexus*, *Medicago sativa*, *Lolium perenne*, and *Setaria viridis* plant seedlings. Each value is the mean of three replicates $\pm$ standard error. Different letters indicate significant differences among different concentrations at $P < 0.05$ level according to Fisher's LSD test.

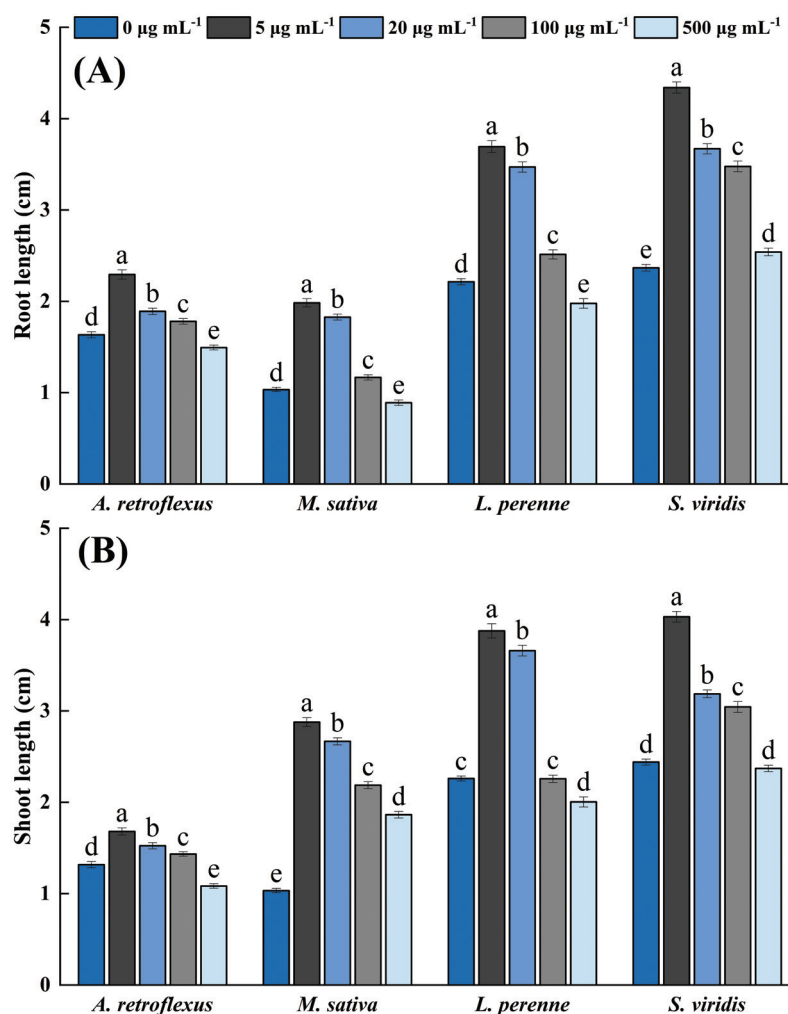


Fig. 3. Plant growth regulatory effects of hyrtiosulawesine (compound 2) isolated from *Penicillium chrysogenum* extract on the root length (A) and shoot length (B) of *Amaranthus retroflexus*, *Medicago sativa*, *Lolium perenne*, and *Setaria viridis* plant seedlings. Each value is the mean of three replicates $\pm$ standard error. Different letters indicate significant differences among different concentrations at $P < 0.05$ level according to Fisher's LSD test.

reduced, and even suppressed by compound 2 at 500 $\mu\text{g mL}^{-1}$. Both compounds observably promoted root elongation of *M. sativa* by 87.38% and 92.23%, respectively, at 5 $\mu\text{g mL}^{-1}$. At 20 $\mu\text{g mL}^{-1}$, the promotion was reduced to 45.63% and 77.67%, respectively. In contrast, the opposite trend was found in the case of shoot lengths. These results showed that compound 1 inhibited the shoot lengths of *M. sativa* by 0.77%, 3.85%, 14.62%, and 15.00% at 5 – 500 $\mu\text{g mL}^{-1}$ concentrations, respectively. Moreover, the plant growth regulatory effect of compound 2 showed different results at all concentrations. For instance, at 5 and 20 $\mu\text{g mL}^{-1}$, shoot lengths of *M. sativa* slightly increased by 10.77% and 2.69%, respectively, while they were suppressed by 15.77% and 28.46% at higher concentrations of 100 and 500 $\mu\text{g mL}^{-1}$, respectively.

At 5 $\mu\text{g mL}^{-1}$, compounds 1 and 2 significantly increased *L. perenne* and *S. viridis* root growth by 63.35%, 66.97%, 55.27%, and 83.12% respectively, demonstrating significant stimulant effects. Compound 2 greatly promoted *L. perenne* and *S. viridis* shoot growth at 5 $\mu\text{g mL}^{-1}$ by 71.68% and

65.16%, and at 20 $\mu\text{g mL}^{-1}$ by 61.94% and 30.74%, respectively. However, as the compound concentrations increased, the promoting effect gradually decreased, and the inhibitory effect gradually increased (Figs. 2 and 3).

Compound 2, hyrtiosulawesine, exhibited stronger stimulatory effects on root and shoot growth of *M. sativa* and *S. viridis* at 5 and 20 $\mu\text{g mL}^{-1}$ than zearalenone, suggesting its potential as an environmentally friendly plant growth regulator. The compounds isolated from *S. rostratum*, which included hyrtiosulawesine, showed a moderate phytotoxic effect on the dicot *A. retroflexus*, but exhibited a strong promotive effect on the monocot *L. perenne* (Toshmatov et al. 2024).

In conclusion, this study demonstrates that the endophytic fungus *Penicillium chrysogenum* SR192, isolated from the invasive plant *Solanum rostratum*, produces two bioactive secondary metabolites, zearalenone and hyrtiosulawesine, with significant plant growth regulatory activity. Both compounds exhibited dose-dependent effects, stimulating root and shoot growth at lower concentrations and

inhibiting growth at higher concentrations. This is the first report of the isolation and characterization of these compounds from *P. chrysogenum* SR192, underscoring the potential of endophytic fungi from invasive plants as valuable sources of novel plant growth regulators. Further research on the mechanisms and field applications of these compounds could contribute to sustainable agricultural practices.

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Author contribution statement

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