On the cover:

The lichen species *Diplotomma cedricola* (Werner) Etayo was reported for the first time in the eastern Mediterranean by Aragón and Martínez, pp. 142–143.

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First record of the dinoflagellate *Tripos rotundatus* in the Adriatic Sea

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Abstract – This report presents the first record of *Tripos rotundatus* (Jørgensen) Gómez in the Adriatic Sea. The species was found in a net sample in the 50 – 100 m depth layer, taken on July 2021 off the southern coast of the Adriatic Sea. The species *T. rotundatus* was probably previously misidentified as the morphologically similar species *T. digitatus* (Schütt) Gómez.

Keywords: biodiversity, NE Mediterranean, phytoplankton, southern Adriatic, taxonomy

Introduction

Dinoflagellates are an important group of protists with a remarkable diversity of life forms (i.e. free-living, parasites, and mutualistic symbionts), habitats (plankton and benthos), and nutrition modes (heterotrophic, chloroplast-containing) (Gómez 2012).

In the Mediterranean Sea, 673 taxa have been identified, while 322 taxa have been reported for the Adriatic (Gómez 2003). In general, dinoflagellates are organisms dominantly found in oligotrophic waters (Gómez 2003).

Among dinoflagellates, the genus *Tripos* Bory includes the greatest number of taxa (~800) (Gómez 2021) and is globally widespread in marine waters. Previously, species of the genus *Tripos* were known as the marine section of the genus *Ceratium* Schrank, until morphological and molecular data supported the separation of marine and freshwater species of *Ceratium* at the genus level and restricted *Ceratium* to freshwater species (Gómez et al. 2010). Marine species are consistently large and robust, often with horns (Gómez 2021).

The aim of this study is to report the first record of *Tripos rotundatus* (Jørgensen) Gómez for the Adriatic Sea.

Materials and methods

Sampling was conducted on July 17, 2021 at the Lokrum coastal station, near Dubrovnik (southern Adriatic Sea, 42°37'21" N, 18°06'05"E) (Fig. 1). Vertical profiles of temperature and salinity were measured from the surface to the bottom (90 m depth) at each meter using a multiparametric conductivity-temperature-depth (CTD) probe, and density (sigma-*t*) was calculated from these data. Water samples were collected using 5-L Niskin bottles for dissolved oxygen, nutrients, chlorophyll *a* (Chl-*a*), and phytoplankton on the surface and at depths of 5, 10, 20, 50, 75, and 100 m. Net samples were collected using a Nansen net with 53 µm mesh and 200 µm mesh in two layers: 0–50 m and 50–100 m. Dissolved oxygen was determined by Winkler titration, and oxygen saturation (O_2/O_2') was calculated from the solubility of oxygen in seawater as a function of corresponding temperature and salinity (Weiss 1970, UNESCO 1987). Nutrient samples were analysed in the laboratory using a spectrophotometer according to Strickland and Parsons (1972). To estimate Chl-*a*, 1 L subsamples were filtered through Whatman GF/F glass microfiber filters and were analysed fluorimetrically (Holm-Hansen et al. 1965). The trophic index (TRIX) was calculated to classify the trophic status of the coastal marine area (Vollenweider et al. 1998). Phytoplankton samples were preserved in neutralized formaldehyde (2.5% final concentration) and observed with an Olympus IX-71 inverted microscope according to the Utermöhl method (Utermöhl 1958). For a detailed description of the method used to analyse nutrients, Chl-*a*, and phytoplankton (for details see Jasprica et al. 2022). The nomenclature of taxa follows Guiry and Guiry (2023).

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Fig. 1. Position of the coastal Lokrum station in the southern Adriatic Sea, where the dinoflagellate species *Tripos rotundatus* (Jørgensen) Gómez was found, 17th July 2021 (derived and adapted from Google earth).

Tab. 1. Physico-chemical parameters, chlorophyll *a* and total phytoplankton abundance in two layers (0–50, 50–100 m) of the water column on the Lokrum station in the southern Adriatic Sea, 17th July 2021 (n = 7).

Parameters	Layer (m)	
	0 – 50	50 – 100
Temperature (°C)	15.93 – 26.25	15.43 – 15.95
Salinity	36.56 – 38.93	38.93 – 38.96
Density (kg m^{-3})	24.36 – 28.78	28.79 – 28.93
Oxygen saturation (O_2/O_2')	0.95 – 1.06	0.83 – 0.86
Phosphate (µM)	0.01 – 0.06	0.04 – 0.06
Total inorganic nitrogen (µM)	0.23 – 0.33	0.29 – 0.60
Nitrate (µM)	0.01 – 0.08	0.06 – 0.09
Nitrite (µM)	0.008 – 0.03	0.008 – 0.32
Ammonium (µM)	0.21 – 0.23	0.19 – 0.22
Silicate (µM)	1.49 – 4.84	1.23 – 4.06
Chlorophyll *a* (mg m^{-3})	0.06 – 0.16	0.17 – 0.32
Total phytoplankton abundance (cells L^{-1})	9.1×10^4 – 1.4×10^5	7.7×10^4 – 2.1×10^5

Results

Vertical thermal stratification of the water column was found during sampling date. The water column in the upper layer (50 – 0 m) was stratified, with a temperature range (15.93 – 26.25 °C), salinity (36.56 – 38.93) and density (24.36 – 28.78) (Tab. 1). O_2/O_2' ranged from 0.83 to 1.06 and was lower (0.83 - 0.86) in the bottom layer.

TIN was calculated as the sum of nitrate (NO_3), nitrite (NO_2), and ammonium (NH_4). NH_4 accounted for the highest proportion of TIN in the entire water column, 59.5%, and NO_3 for the lowest, 16.3%. PO_4 ranged from 0.01 to 0.066 µM, with the highest value measured at 10 m depth. SiO_4 ranged from 1.23 to 4.84 µM, with higher values in the upper 50 m.

The highest Chl-*a* (0.32 mg m^{-3}) and phytoplankton abundance (2.1×10^5 cells L^{-1}) were found at a depth of 75 m. TRIX ranged from 1.1 to 2.8, classifying the station as oligotrophic.

Altogether, 48 phytoplankton taxa were identified in seven samples. Thirty-five taxa were dinoflagellates, 10 diatoms and three coccolithophorids. Among larger phytoplankton cells (> 20 µm cell long) *Thalassionema nitzschioides* (Grunow) Mereschkowsky, *Oxytoxum sphaeroideum* Stein, *Oxytoxum variabile* J. Schiller, *Oxytoxum caudatum* Schiller were the most abundant (> 945 cell L^{-1}). Nanophytoflagellates (2–20 µm cell long) dominated (96.3%) in total phytoplankton abundance.

Tripos rotundatus was found in the net sample in the 50–100 m depth layer (Fig. 2).

Discussion

Water column stratification is a common occurrence during summer, as is the occurrence of a thermocline and of water column stability (Ninčević Gladan et al. 2015).

The low nutrient and Chl-*a*, and in general the low trophic status, indicated a summer situation common in the oligotrophic coastal area. Moreover, the oligotrophy of the Lokrum station is confirmed by the abundance of phytoplankton dominated by nanophytoplankton, typical of the spring and summer period in the coastal southern Adriatic Sea (Caroppo et al. 1999), and by the highest abundance of dinoflagellates, typical organisms of oligotrophic waters (Gómez 2003).

As *T. rotundatus* was pooled as *T. digitatus* (Schütt) Gómez, and most of the records are not illustrated, we have

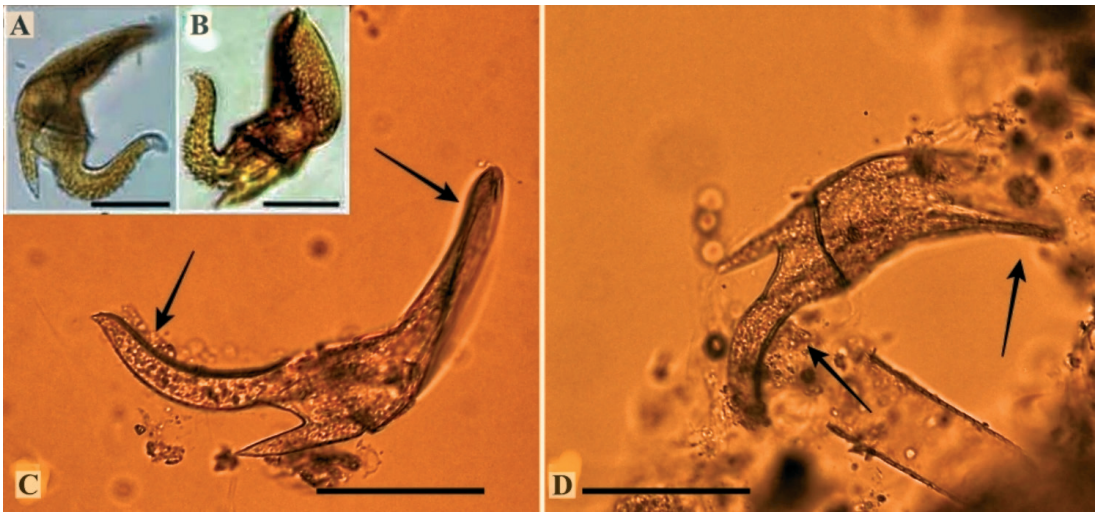

Fig. 2. Dinoflagellate species *Tripos digitatus* (Schütt) Gómez (A, B) (Gómez et al. 2021, with permission), *Tripos rotundatus* (Jørgensen) Gómez (C, D) found for the first time in the Adriatic Sea at the Lokrum station on 17th July 2021. Black arrows indicate the morphological differences between the species *T. rotundatus* and *T. digitatus*. Scale bars = 50 µm.

little information on the distribution of the species. *Tripos rotundatus* was previously reported as *T. tasmaniae* (E.J.F.Wood) F.Gómez in Australian Pacific waters (Wood 1963).

Tripos rotundatus also had a different taxonomic status in the literature (Guiry and Guiry 2023). Originally, *T. rotundatus* was named *Ceratium digitatum* Schütt var. *rotundatum* Jørgensen (Jørgensen 1920). The species was found west of the island of Rhodes in the Aegean Sea in August 1910. Our data coincided with Jørgensen's findings, regarding the sampling season (in summer) and the position of the station (coastal).

Tripos digitatus, a morphologically similar species, was found in the Mediterranean Sea (Gómez 2003) and in the northern Adriatic Sea (Revelante 1985). The differences in the morphological characteristics of these two taxa have been recently highlighted (Gómez 2021). There are differences in the epitheca and hypotheca. The epitheca of *T. digitatus* is strongly directed dorsally, the left antapical horn is directed anteriorly, and the apex has a short projection. In *T. rotundatus*, the epitheca is less bent towards the dorsal side, the short projection on the apex is missing, and the left antapical horn is directed laterally (Gómez 2021). The morphological description of the species *T. rotundatus* follows the morphology of the taxa recorded at the Lokrum station. In our case, the cell of *T. rotundatus* was 110 µm long and 30 µm wide.

This finding contributes to a better understanding of the diversity of dinoflagellates in the Adriatic Sea. However, further continuous studies of phytoplankton diversity in the coastal regions of all parts of the Adriatic Sea are required.

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Placoneis modaomensis sp. nov. (Bacillariophyta; Cymbellaceae), a new species from Guangdong Province, China

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Abstract – One new species, *Placoneis modaomensis*, found in a freshwater environment from a tributary of the Pearl River, which lies in Modaomen Channel, Zhuhai County, Guangdong Province, China, is described based on light and scanning electron microscope observations. *P. modaomensis* sp. nov. has the morphological features that are typical for the genus, including external terminal raphe fissures curved to the opposite sides and areolae with internal volate occlusions. The new species is similar to *P. amphibola* (Cleve) E.J. Cox, *P. amphiboliformis* (Metzeltin, Lange-Bertalot & Soninkhishig) Vishnyakov, *P. parvapolonica* Lange-Bertalot & Wojtal, *P. clementispronina* Lange-Bertalot & Wojtal and *P. nanoclementis* Lange-Bertalot & Wojtal in the shape of the valves and in having coarse striae but it can be easily distinguished by the two main morphological characteristics: external central raphe endings bent in the opposite directions, and areolae covered by volate occlusions externally. The latter feature appears to be new for taxa assigned to the genus *Placoneis*. Data on the associated diatom flora and its ecology are also given. These findings increase our understanding about the morphology of *Placoneis* in general and the distribution of the genus in China.

Keywords: diatoms, morphology, new species, *Placoneis*, taxonomy

Introduction

The genus *Placoneis* Mereschkowsky was erected by Mereschkowsky in 1903 for a group of species showing a single chloroplast with a central bridge and lateral lobes (Mereschkowsky 1903). With the shift to an emphasis on using frustular features to diagnose taxa (e.g., Hustedt 1930), species assigned to *Placoneis* were considered part of the large genus *Navicula* Bory (1822). Due to its structure of the chloroplast, Cox (1987) resurrected the genus *Placoneis* with *P. gastrum* (Ehrenberg) Mereschkowsky (Basionym: *Pinnularia gastrum* Ehrenberg) as the type species. Phylogenetic analysis of *Placoneis* based on morphological and molecular data showed that the genus was part of the Cymbellales, a group with valves that are asymmetrical to the apical and/or transapical axes, despite having symmetrical valves. Support for this phylogenetic placement includes the straight and expanded central raphe endings and more or less hooked distal ones, striae composed of rounded areolae which are internally closed by volae (tectulum), and a single chloroplast with a central bridge and lateral lobes extending under the valves (Cox 1987, 2003, 2004, Mann and Stickle 1995, Bruder and Medlin 2007). To date, more than 136 species are recognized to be part of this genus (Guiry and Guiry 2022).

The genus *Placoneis* has a relatively wide distribution range, including Europe (Cox 1987, Bruder and Medlin 2007, Levkov and Williams 2011, Kulikovskiy et al. 2016, Vishnyakov 2020), Asia (Mayama and Kawashima 1998, Metzeltin et al. 2009, Kulikovskiy et al. 2012, Pomazkina et al. 2019, Kezlya et al. 2020), North America (Johansen et al. 2004, Kociolek and Thomas 2010, Kociolek et al. 2014), South America (Metzeltin and Lange-Bertalot 1998, Metzeltin and Lange-Bertalot 2007, Straube et al. 2013, Maidana et al.

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2017), West Africa (Fofana et al. 2014) and Antarctica (Zidarova et al. 2009). In China, research on *Placoneis* has focused on the discovery of newly recorded species for the country, such as *P. prespanensis* Levkov, Krstic & Nakov (Li et al. 2010), *P. explanata* (Hustedt) S. Mamaya (Liu et al. 2012), *P. interglacialis* (Hustedt) E.J. Cox (Liu et al. 2012), *P. opportuna* (Hustedt) Chudaev & Gololobova (Lin et al. 2018), *P. anglophila* var. *signata* (Hustedt) Lange-Bertalot (Liu et al. 2020). New *Placoneis* species have been proposed from China by Gong et al. (2013) but otherwise only few new species of this genus have been reported. In Guangdong Province the diversity of diatoms is relatively rich (Qiu et al. 2016, Lin et al. 2018), but previous studies have mainly focused on *Navicula* Bory, *Nitzschia* Hassall, *Cymbella* C. Agardh and *Gomphonema* Ehrenberg (Wang et al. 2018, 2021). Our understanding of *Placoneis* from Guangdong Province is very limited (Wang et al. 2021).

The purposes of our study are: (i) to describe a *Placoneis* species new to science from the tributary of the Pearl River: Modaomen Channel, Zhuhai County, Guangdong Province, China based on detailed morphological observation using both light and scanning electron microscopy, (ii) to discuss its characteristics compared to related species, and (iii) to provide ecological information on this new species.

Materials and methods

Modaomen is located in Zhuhai County, Guangdong Province, China, one of the eight major gates at the mouth of the Pearl River. The length of the Modaomen Channel is about 45 km, and the water depth varies from 5 to 13 m. The Channel is relatively straight and about 2200 m wide (Chen et al. 2014, Tong et al. 2018).

In 2021, samples containing *Placoneis* were collected from the Modaomen Channel (22°24'20"N, 113°36'25"E). Channel water pH and specific conductance were measured using a YSI 650 multi-parameter display system (650 MDS, YSI Incorporated 1700/1725 Brannum Lane, Yellow Springs, OH 45387 USA) with a 600XL probe. The diatom samples were further processed as described by Battarbee (1986). After several rinses in distilled water, the partially cleaned diatom material was air-dried onto cover slips and mounted onto slides using Naphrax. The sample and slides were deposited in the Herbarium of the Institute for Ecological Research and Pollution Control of Plateau Lakes, Yunnan University, Kunming, P.R. China (YUK). The isotype slides were stored in the Key Laboratory of Biodiversity of Aquatic Organisms, Harbin Normal University.

Morphological observations of specimens were made under oil immersion at 1000× magnification with light microscopy (LM) using an OLYMPUS BX51-DIC research microscope and a C5060 Olympus digital camera. At least 500 valves were identified and counted in each surface sediment sample. Cleaned material for scanning electron microscope (SEM) analysis was air-dried onto cover glasses, mounted onto stubs, and coated with 20 nm of Au (EMSCOP SC 500 sputter coater). Resulting stubs were examined in the LEO 1530 scanning electron microscope (SEM). Description of the new species follows the terminology provided by Round et al. (1990), Cox (2003), Metzeltin et al. (2009) and Lange-Bertalot and Wojtal (2014).

Results

Taxonomy

Division Bacillariophyta Haeckel 1878: 95

Class Bacillariophyceae Haeckel 1878 emend D.G. Mann in Round et al. 1990: 651

Subclass Bacillariophycidae D.G. Mann in Round et al. 1990: 125

Order Cymbellales D.G Mann in Round et al. 1990: 653

Family Cymbellaceae Greville 1833: 263, 409

Genus *Placoneis* Mereschkowsky 1903: 3

Placoneis modaomenensis Y.-L. Li sp. nov. Fig. 1A–H; Fig. 1C is the holotype

LM (Fig. 1): Valves elliptical to broadly elliptical, nearly symmetrical about the apical axis, with rostrate or rostrate-rounded apices. Length 25.0–32.5 µm, width 14.0–16.5 µm, length/width ratio 1.62–2.16, median 1.89 (n = 30). Raphe filiform, almost straight with slightly expanded, but not clearly deflected to any side. External central raphe ends slightly straight, no significant expansion or bending. External terminal raphe fissures hooked to opposite sides. Axial area narrow, linear. Central area transverse, irregular to bow-tie-shaped, rarely asymmetrical, occupying nearly 1/2 of the valve width. Isolated pore is absent from the central area. Striae radiate throughout, 12–14 in 10 µm. Areolae visible, 11–14 in 10 µm.

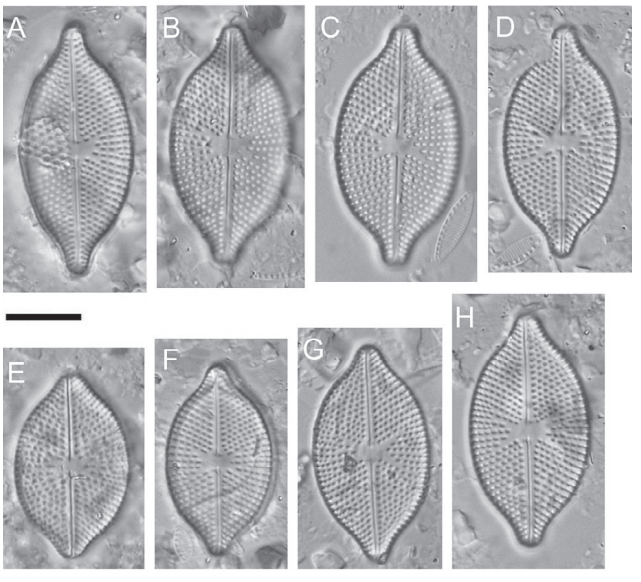

Fig. 1. *Placoneis modaomensis*, Light Microscopy (LM), Differential Interference Contrast (DIC). A–H – valve views, showing size range and variability of the holotype population. Scale bar = 10 µm.

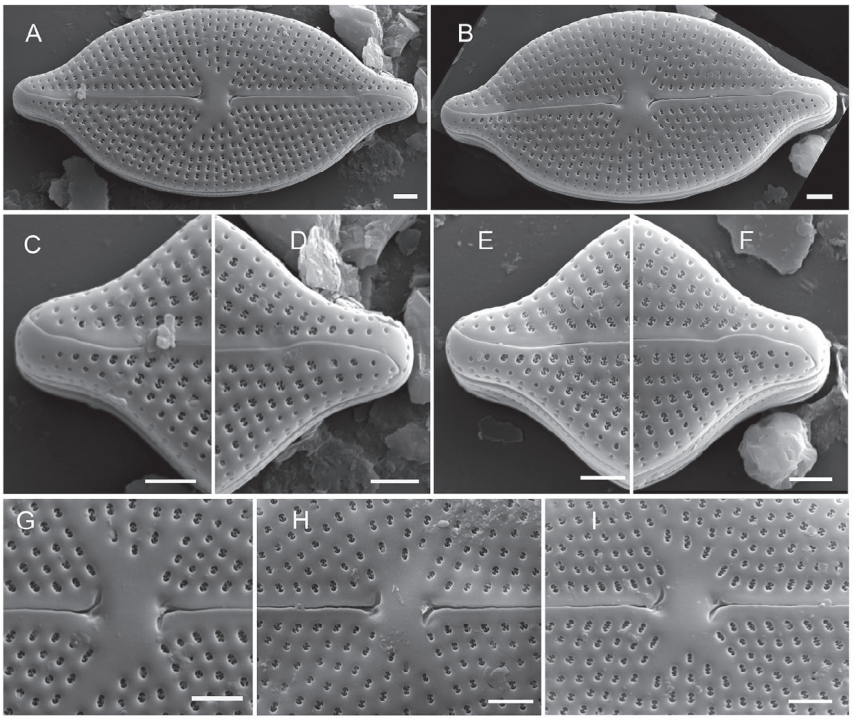

Fig. 2. *Placoneis modaomensis*, Scanning Electron Microscope (SEM), external views. A, B – external view of an entire valve. C, D, E, F – valve apices, striae with elliptic/rounded areolae and hook-shaped terminal raphe fissures bent onto the valve margin. G, H, I – external view of valve center, the central raphe endings hooked in the opposite directions from each other, note volate occlusions. Scale bar = 2 µm

In SEM (Fig. 2 and Fig. 3): External raphe narrow, central raphe endings hooked opposite to each other (Fig. 2A, B, G, H, I). The terminal fissures curved, deflected in the opposite directions, extend onto the valve margin (Fig. 2C, D, E, F). Striae uniseriate, composed of round or elliptical areolae, extending to valve margin (Fig. 2A–F). Areolae covered by

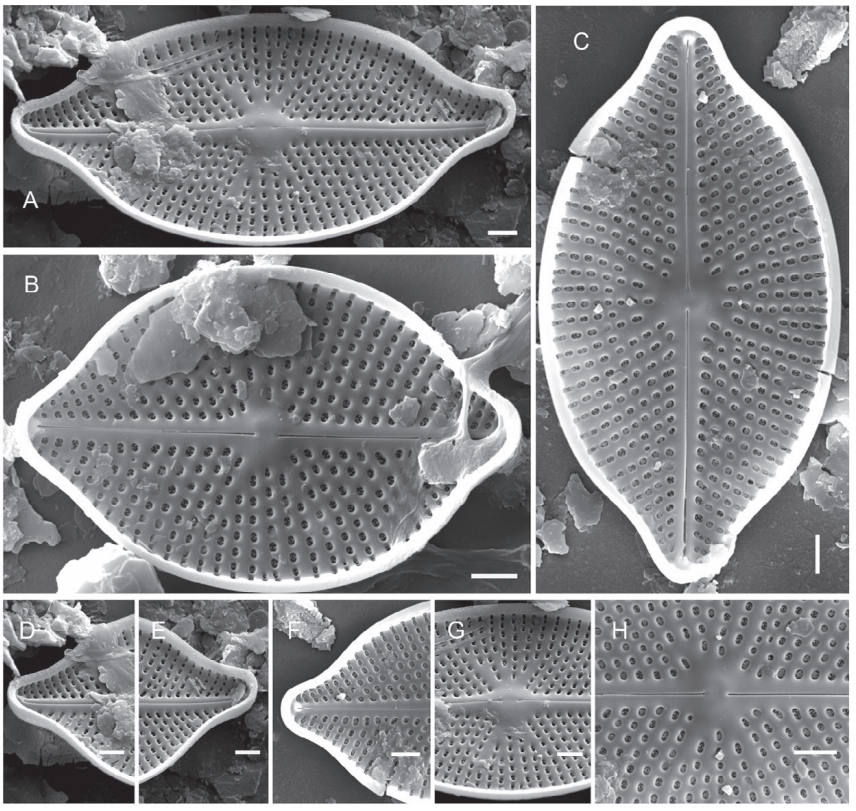

Fig. 3. *Placoneis modaomensis*, Scanning Electron Microscope (SEM), internal views. A, B, C – internal view of entire valve. D, E, F – internal view of valve apex with terminal raphe ends offset and bent slightly towards the margin. G, H – internal detail of central area showing raised central part and areolae covered by volate occlusions. Scale bar = 2 µm

volate occlusions (Fig. 2G, H, I). Each areola has almost 1–4 siliceous protrusions internally, forming a variety of different shapes (Fig. 2A–I). Internal striae uniseriate, formed by elliptical areolae, separated by robust silica ribs (Fig. 3A–H). The raphe sternum clearly raised above the valve plane and expanded in the central area. Internal raphe straight, discontinuous with intermissio, lying in a prominent and raised raphe sternum. Central raphe endings straight, not expanded. Terminal raphe ends terminate as helictoglossae, offset from the raphe branch, bent slightly towards the valve margin (Fig. 3D, E, F). Striae are uniseriate, distinctly radiate, formed by round to elliptical shaped areolae (11–14 in 10 μm) and covered by dentate occlusions (Fig. 3G, H).

Type: – CHINA. Guangdong Province: Zhuhai County, Modaomen Channel, GD1, 22°24'20" N, 113°36'25" E, elevation 0 m a.s.l., samples collected by Dr. Hong-Qu Tang, 26th July 2021. Holotype MDM202172601 in Coll. Yan-Ling Li, Yunnan University, Kunming, China. Fig. 1C is of the holotype; Isotype YUNGL20220218, Harbin Normal University, Harbin, China)

Etymology: – *modaomensis*, referring to the type locality from which the new species was obtained.

Associated diatom flora: *Placoneis modaomensis* is known from the Channel, situated at 0 m a.s.l. This species was associated with *Amphora linearis* F. Meister (1935: 97), *Seminavis strigosa* (Hustedt) Danielidis & Economou-Amilli (2003: 30), *Aulacoseira granulata* (Ehrenberg) Simonsen (1979: 58), *Gomphonema parvulum* (Kützing) Kützing (1849: 65), *Navicula schroeteri* F. Meister (1932: 38), *Navicula viridula* var. *rostellata* (Kützing) Cleve (1895: 15), *Nitzschia clausii* Hantzsch (1860: 40) and *Nitzschia frustulum* (Kützing) Grunow (1880: 98).

Ecology: The Modaomen Channel showed slightly alkaline conditions (pH 7.81), 29.8 °C of water temperature, 262 μS cm^{-1} of conductivity and 7.46 mg L^{-1} of dissolved oxygen (DO).

Discussion

Because *Placoneis modaomenensis* has symmetrical valve and radiate striae, it could easily be placed in one of four other genera: *Paraplaconeis* Kulikovskiy, Lange-Bertalot & Metzeltin (2012), *Geissleria* Lange-Bertalot & Metzeltin (1996), *Rexlowea* Kociolek & E.W.Thomas (2010) or *Navicula* Bory (1822). First, our new species resembles *Paraplaconeis* by symmetry of valve, but differs by the specific morphology of internal and external areolae patterns (Cox 1987, Cox 2003, Lange-Bertalot and Wojtal 2014). Second, *P. modaomenensis* is similar to the genus *Geissleria* on the basis of features observed with LM. These two genera are similar in terms of valve outline, but differ by the presence of the subpolar elongated areolae in the latter (Novais et al. 2013, Kulikovskiy et al. 2014). Third, *P. modaomenensis* is morphologically close to *Rexlowea* due to the valve outline, but these two genera are very distinct from one another by the arrangement, radiation and density of the striae (Kociolek and Thomas 2010). Fourth, *P. modaomenensis* can be confused with *Navicula* based on the valve symmetry. However, the former has exterior areolae covered by volate occlusions and internal areolae covered by dentate occlusions; while the latter areolae are all individually covered on the inside by a weakly convex hymen (Lange-Bertalot 2001, Li and Qi 2018). The characters found in *Placoneis modaomensis* are compared with those of morphologically most similar genera in Tab. 1.

While *Placoneis* seems easily placed as a member of Cymbellales, by virtue of its cytoplasmic features, its position in this lineage has been quite variable. *Placoneis* has been reported as outside the group of asymmetrical genera (Kociolek and Stoermer 1988, Nakov et al. 2014), deep within the cymbelloid lineage with some other genera naviculoid symmetry (Thomas et al. 2016, Kezlya et al. 2021) or in both positions depending upon the gene(s) used in the analysis (Bruder and Medlin 2007). Groupings within *Placoneis* are also enigmatic. Cox (1987) identified two groups within *Placoneis*, one with isolated pores, external terminal raphe fissures deflected in directions opposite one another and straight internal central raphe endings, while the other group has features of isolated pores absent, external terminal raphe fissures deflected in the same direction and recurved internal central raphe endings. Kociolek and Thomas (2010) noted some species from Colorado, USA, that did not conform to this organization of the genus. For example, *P. fourtanierii* Kociolek & Thomas (2010: 204) has external terminal raphe fissures that are deflected in opposite directions, but lack isolated pores and deflected internal raphe ends. *P. coloradoensis* Kociolek & Thomas (2010: 205) has external terminal raphe fissures that are deflected towards the same side, internal central raphe endings that are straight, but this species has isolated pores. In the case of *P. modaomenensis*, it has external terminal raphe fissures that curve in opposite sides, but has internal central raphe endings that are straight and no isolated pores. In addition, *P. modaomenensis* has areolae with volate occlusions positioned on the valve exterior which appears to be unique within *Placoneis*. Presence of central raphe endings turned opposite to one another in *P. modaomenensis* is shared with *P. uruguayensis* Metzeltin et al., a species without many features in common with other *Placoneis* species. The statement of Levkov et al. that "the full range of variation of morphology with the genus has been underestimated" (2007, p. 116) seems even more true now than when it was offered more than 15 years ago.

In terms of general valve shape and striae, *P. modaomenensis* shows some resemblance to *P. amphibola* (Cleve) E.J. Cox, *P. amphiboliformis* (Metzeltin, Lange-Bertalot and Soninkhishig) Vishnyakov, *P. parvapolonica* Lange-Bertalot & Wojtal, *P. clementispronina* Lange-Bertalot & Wojtal and *P. nanoclementis* Lange-Bertalot & Wojtal. However, *P. modaomenensis* differs strikingly from these species that are morphologically similar with respect to two features by the following: 1) external central raphe endings are bent in the

Tab. 1. Morphological comparison of *Placoneis* to the most similar genera.

	Placoneis modaomensis sp. nov.	***Placoneis***	***Paraplaconeis***	***Geissleria***	***Rexlowea***	***Navicula***
Reference	This study	Cox 1987, Cox 2003, Lange-Bertalot and Wojtal 2014	Kulikovskiy et al. 2012, Lange-Bertalot and Wojtal 2014	Novais et al. 2013, Kulikovskiy et al. 2014	Kociolek and Thomas 2010	Lange-Bertalot 2001, Li and Qi 2018
Areolae	Being covered by volate occlusions externally, being covered by dentate occlusions internally	Being closed internally by volae	Open internally and occluded externally	Straight slit-like pores openings without hymens, presence of the subpolar elongated areolae	Large and more coarsely arranged puncta on the marginal valve	Being all individually covered on the inside by a weakly convex hymen
Striae	Uniseriate, radiate throughout, extending to valve margin	Usually uniseriate but sometimes biseriate, radiate near the centre of the valve, more parallel or convergent, at the apices	Uniseriate only on the valve mantle, consistently biseriate on the valve face	Radiate throughout, the proximal three ones wider spaced than the others	Radiate, with the 1st and or 2nd striae at the apices parallel or, rarely, convergent	Uniseriate, rarely biseriate, parallel, radiate or convergent
Chloroplast	—	Single, asymmetrical chloroplast, central portion more or less along the apical axis of the cell, from which lobes extend under the valves	—	Single, large plastid is divided into two plates lying one against each valve, connected by a broad column	—	Two plastids, located on each side of the apical axis, and each plastid contains an elongated, slender protein nucleus
Raphe system	The external central raphe ends slightly straight, the terminal fissures deflected in the opposite directions; the internal central raphe endings straight, terminal raphe ends terminate as helictoglossae	The external central raphe endings are straight and slightly expanded; the internal endings usually deflected towards the secondary side, occasionally hooked in opposite directions	The external raphe slit together with terminal fissures often curving primarily to opposite sides but finally to the same side	The external raphe is almost straight; the internal raphe is not deflected between the central nodule and helictoglossa, internal rib has never been observed	The external proximal ends swollen, external distal ends hooked	The external terminal fissures are deflected to the secondary side; the internal central raphe ends are not deflected

Tab. 2. Morphological comparison of the currently described species in *Placoneis*.

	P. modaomensis sp. nov.	*P. parvapolonica*	*P. amphibola*	*P. amphiboliformis*	*P. clementispronina*	*P. nanoclementis*
Reference	This study	Lange-Bertalot and Wojtal 2014	Cox 2003	Metzeltin et al. 2009	Lange-Bertalot and Wojtal 2014	Lange-Bertalot and Wojtal 2014
Valve shape	Elliptical to broadly elliptical and the valve margin more convex	Broadly elliptical	Linear-elliptic to linear-lanceolate	Elliptical	Elliptical-lanceolate	Elliptical-lanceolate to elliptical
Central area	Bow-tie, transverse	Extended transapically	Bow-tie, transverse	Not given	Variable in size	Extended transapically, variable in shape
Isolated pores	Absent	Two	Not given	Not given	Two	Two
Valve length (µm)	25.0–32.5	16–20	37–75	34–63	20–40	14–23
Valve width (µm)	14.0–16.5	8.0–9.5	22–27	18–24	10–14	8.0–8.6
Number of striae in 10 µm	12–14	14–15	7–8	7–8	11–14	12–13
Number of areolae in 10 µm	11–14	40–44	Not given	10–12	38–41	40–44
External terminal raphe fissures	Undulate, deflected in the opposite directions, extend to valve margin	Turning first to opposite sides, finally turning to the same side as at the opposite pole	Deflected towards one side	Not given	Weakly sinuous subapically and deflected to opposite sides, at one of the poles curving back weakly to the same side	Shortly curved to apparently opposite sides
External central raphe end-ings	Hooked in the opposite directions from each other	Distinctly expanded central pores, not clearly deflected to any side	Expanded	Not given	Expanded but hardly deflected central pores	Distinctly expanded central pores, not clearly deflected to any side
Areolae	Uniseriate, exterior areolae covered by volate occlusions and formed a variety of different shapes, internal areolae are mostly round to elliptical covered by dentate occlusions	Uniseriate, circular to slightly elongate apically	Not given	Regularly elongated transapically	Small, circular to weakly elongated apically	Slightly elongated apically

opposite directions, and 2) both internal and external areolae are covered by volate occlusions. The characters found in *P. modaomenensis* are compared with other closely-related species of the genus in Tab. 2.

Based on valve outline, the new species is similar to *P. amphibola*, *P. amphiboliformis*, *P. parvapolonica*, *P. clementispronina* and *P. nanoclementis*, and *P. modaomenensis* sp. nov. most resembles *P. parvapolonica* based on its more convex valve margin and the structure of raphe and areola. However, they can be easily separated from one another. For example, in terms of the central raphe endings, the central raphe endings of the new species hooked in the opposite directions from each other while they do not clearly deflect to any side in *P. parvapolonica*. Comparing the shape of the areolae, the exterior areolae come in a variety of shapes and internal areolae are mostly round to elliptical in the new species, but *P. parvapolonica* differs in having areolae whose exterior and internal openings are nearly circular to slightly elongated in shape. Taking into consideration the occlusion of the areolae, the exterior and internal areolae are covered by volate and dentate occlusions respectively in the *P. modaomenensis* sp. nov., while there is an absence of occlusions in exterior and internal areolae in *P. parvapolonica*. Besides, the size range of *P. modaomensis* is larger than *P. parvapolonica* (16–20 × 8.0–9.5 μm). Finally, the stria and areola density are much lower compared to *P. parvapolonica* (stria: 14–15 μm, areola: 40–44 in 10 μm). In summary, these differences are sufficient to justify the description of *P. modaomensis* as an independent species.

Placoneis species are prevalent in freshwater bodies, including alkaline waters, mesotrophic and oligotrophic conditions (Cox 1987, Fujita and Ohtsuka 2005, Bruder and Medlin 2007, Kezlya et al. 2021). Of the five species similar to our new species, *P. amphibola* has a nordic-alpine distribution in Europe, and also occurs in freshwater fossil deposits (Cox 2003). Except for *P. amphibola*, the relevant information of nutrition, pH, and conductivity are not recorded, but other species have certain similarities or differences with our species. In terms of pH, *P. parvapolonica*, *P. clementispronina* and *P. nanoclementis* were found in alkaline waters, but *P. amphiboliformis* was known from acidic rivers (Metzeltin et al. 2009, Lange-Bertalot and Wojtal 2014). From the nutritional level, *P. clementispronina* and *P. nanoclementis* were distributed in eutrophic waters, and *P. parvapolonica* was found in mesotrophic waters (Metzeltin et al. 2009, Lange-Bertalot and Wojtal 2014). As for the conductivity, *P. parvapolonica* and *P. nanoclementis* are found predominantly in waters with moderate to high conductivity, whereas *P. amphiboliformis* was discovered rivers with low conductivity (Metzeltin et al. 2009, Lange-Bertalot and Wojtal 2014). Here, the new species of *Placoneis* was collected from alkaline waters with moderately high conductivity, which is the most similar to *P. parvapolonica* and *P. nanoclementis*.

In short, the discovery of this new species promotes the understanding of morphological features and ecological distribution about the genus *Placoneis,* and contributes to our understanding of diatom diversity, especially in Guangdong Province.

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A new subspecies of *Cephalaria pastricensis* Dörfl. & Hayek (Dipsacaceae) from North Macedonia

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Abstract – *Cephalaria pastricensis* subsp. *pologensis* Teofilovski (Dipsacaceae), from NW North Macedonia, is reported as a new subspecies to science. It is distinguished from *C. pastricensis* Dörfl. & Hayek subsp. *pastricensis* by its densely, ± patent-subvillous petioles and rachis of the leaves, and the midrib of the lower surface of the leaf lobes (vs. with sparse, short, ± appressed hairs), densely pubescent lower surface of the leaves (vs. with scattered hairs on the nerves), and densely pubescent to subglabrous upper surface of the leaves (vs. glabrous or with scattered hairs on the nerves). The new subspecies is a Macedonian endemic known from small areas in the Šar Mountains (near Brezno village) and Mt. Buković (near Gorna Đonovica village). Its distribution range is geographically distinct from that of *C. pastricensis* subsp. *pastricensis,* which is a Balkan endemic, distributed in Albania, Montenegro, Bosnia and Herzegovina, Kosovo, and Serbia.

Keywords: *Cephalaria pastricensis* subsp. *pologensis,* indumentum, new subspecies, North Macedonia

Introduction

The genus *Cephalaria* Schrader (Dipsacaceae) is distributed from N Africa and S & SE Europe to W China, with some species also occurring in parts of S Africa (Szabó 1940). This author quoted 65 species worldwide while since then, the number of known species has been raised to 94 (Göktürk and Sümbül 2014). With 11 native species, the Balkan Peninsula has a relatively low diversity [compared e.g. to 39 species representing the genus in Turkey (Göktürk and Sümbül 2014)]. In the flora of North Macedonia, the following six native species are known so far: *C. ambrosioides* (Sibth. & Sm.) Roem. & Schult., *C. flava* (Sibth. & Sm.) Szabó, *C. leucantha* (L.) Roem. & Schult., *C. pastricensis* Dörfl. & Hay., *C. transsylvanica* (L.) Roem. & Schult., and C. *uralensis* (Murray) Roem. & Schult. The only adventive species known in North Macedonia as well as in the Balkan Peninsula is the SW Asian *C. syriaca* (L.) Roem. & Schult.

Cephalaria pastricensis is a Balkan endemic, with a dispersed distribution in NE & SE Albania, Montenegro, C, E & S Bosnia and Herzegovina, W Kosovo, C & E Serbia, and NW North Macedonia (Hayek 1921, Szabó 1940, Millaku et al. 2013, Teofilovski 2014, Tomović et al. 2022). Babalonas (1983) reported this species also for NW Greece, however, according to Constantinidis and Phitos (2004) this report actually refers to a species belonging to another section.

In North Macedonia it is a very rare species, discovered relatively recently, in two localities near Brezno village in the Šar Mountains (Teofilovski 2014) [these data were erroneously referenced by Tomović et al. (2022) to "Vladimirov et al. 2014"]. Despite the identified differences in the indumentum between the collected specimens and typical *C. pastricensis*, due to the lack of additional information, they were not taken into consideration in the cited article. In the summer of 2022, during extensive fieldwork in the forest area south of Gostivar (NW North Macedonia), *C. pastricensis* was also recorded in Mt. Buković, with the indumentum of all the observed individuals matching those of the plants from Šar Mountains. The uniform indumentum of the plants from both mountain areas, obviously not matching that of the typical *C. pastricensis*, motivated the author to study the taxonomic position of the recorded populations. The comparative analysis showed that Macedonian populations should be classified as a separate subspecies of *C. pastricensis*.

Materials and methods

During fieldwork conducted in 2012, 2013, and 2022, appropriate parts of representative plants were collected and

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photographed. The collected material was herbarized and stored in the author's private herbarium, in the Herbarium of the Institute of Biology, Faculty of Natural Sciences and Mathematics in Skopje (MKNH), and the Herbarium of the Natural History Museum of the Republic of North Macedonia. Scans of herbarium specimens from the type collection and several other collections of *C. pastricensis* were used as comparative material (details listed in Discussion). Relevant literature related to the taxonomy of the genus *Cephalaria* was used during the morphological study of the collected material (Hayek 1921, Hayek 1928–1931, Szabó 1940, Matthews 1972, Diklić 1973, Ferguson 1976, Pignatti 1982, Kokkini 1991). Diagnosis of the newly described subspecies accompanied by a comparative table of the diagnostic characteristics and relevant photographs is presented. The distribution of the new subspecies is mapped.

Results

***Cephalaria pastricensis* subsp. *pologensis* Teofilovski, subsp. nov.**

Diagnosis: *Cephalaria pastricensis* subsp. *pologensis* Teofilovski, subsp. nov. differs from *C. pastricensis* Dörfl. & Hayek subsp. *pastricensis* by its densely, ± patent-subvillous petioles and rachis of the leaves, and the midrib of the lower surface of the leaf lobes (vs. with sparse, short, ± appressed hairs), densely pubescent lower surface of the leaves (vs. with scattered hairs on the nerves), and densely to sparsely pubescent upper surface of the leaves (or subglabrous in upper cauline ones) (vs. glabrous or with scattered hairs on the nerves) (Tab. 1, Fig. 1 and Fig. 2)

Holotype: North Macedonia, Mt. Bukovič, 2.2 km SW of Gorna Đonovica village, forest clearing in the zone of a mixed forest of *Ostrya carpinifolia* Scop. and *Fagua sylvatica* L., limestone, 1250 m, 41.690475 N, 20.892805 E, 10.8.2022, leg. & det. A. Teofilovski (MKNH).

Isotype: herb. A. Teofilovski.

Other examined collections: North Macedonia, Šar Mountains, 4.5 km NW of Brezno village, roadside in the zone of beech forest, siliceous substrate, 1460 m, 42.111214 N, 20.993406 E, 20.7.2012, leg. & det. A. Teofilovski (herb. A. Teofilovski) (Teofilovski 2014, sub *C. pastricensis*); North Macedonia, Šar Mountains, 1.8 km NW of Brezno village, pastures and shrubby places, siliceous substrate, 1570–1590 m a.s.l., 42.092039 N 21.017495 E, 5.8.2013, leg. & det. A.

Fig. 1. *Cephalaria pastricensis* subsp. *pologensis*: A – holotype (MKNH), B-D – details from the holotype. B – rachis of the basal cauline leaf, C – abaxial surface of the basal cauline leaf, D – abaxial surface of a middle cauline leaf, E – detail from the adaxial surface of a basal cauline leaf of a plant from the type locality. Scale bars = 3 mm (Photo: A. Teofilovski).

Tab. 1. Morphological differences between *Cephalaria pastricensis* subsp. *pologensis* Teofilovski, subsp. nov. and *C. pastricensis* Dörfl. & Hayek subsp. *pastricensis*.

	Cephalaria pastricensis subsp. *pologensis* (Fig. 1 and Fig. 3)	*C. pastricensis* subsp. *pastricensis* (Fig. 2)
Petioles, rachis of the leaves, and the midrib of the lower surface of the leaf lobes	densely, ± patently subvillous	with sparse, short, ± appressed hairs
Lower surface of the leaves	densely pubescent	with scattered hairs on the nerves
Upper surface of the leaves	densely to sparsely pubescent (or subglabrous in upper cauline ones)	glabrous or with scattered hairs on the nerves
Distribution	North Macedonia (Šar Mountains, Mt. Bukovič)	Albania, Montenegro, Kosovo, Serbia, Bosnia and Herzegovina

Teofilovski (herb. A. Teofilovski; Herbarium of the Natural History Museum of the Republic of North Macedonia) (Teofilovski 2014, sub *C. pastricensis*); North Macedonia, Bukovič Mt., 2.4 km SW of Gorna Đonovica village, mixed forest of *Acer obtusatum* Willd. and *Quercus cerris* L., limestone, 1285 m a.s.l., 41.688651 N, 20.896195 E, 10.8.2022, photo. A. Teofilovski; North Macedonia, Bukovič Mt., 1.3 km S of Gorna Đonovica village, forest margin, limestone, 1035 m a.s.l., 41.697198 N, 20.906883 E, 10.8.2022, leg. & det. A. Teofilovski (herb. A. Teofilovski).

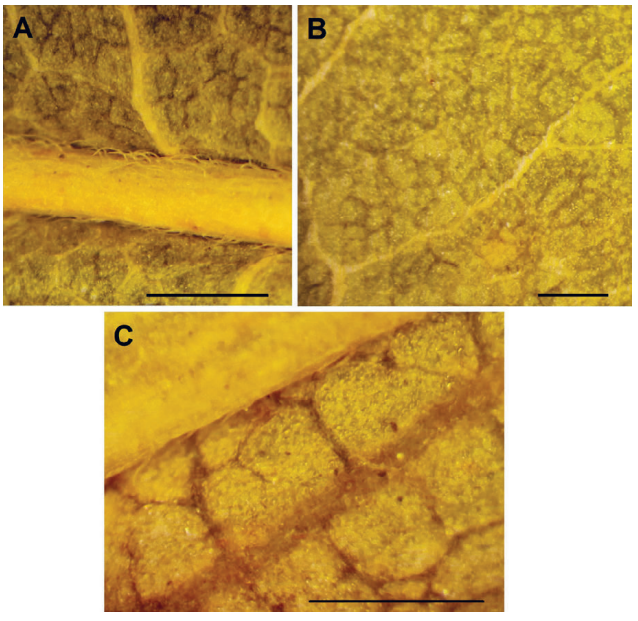

Fig. 2. *Cephalaria. pastricensis* subsp. *pastricensis*: A-B – details of the abaxial surface of a basal cauline leaf (Mt. Prokletije, Kosovo) (23367, BEO), C – detail of the abaxial surface of a middle cauline leaf (Mt. Suva Planina, Serbia). Scale bars = 1 mm (BEO) (Photo: M. Niketić).

Distribution: According to present knowledge, *C. pastricensis* subsp. *pologensis* is endemic to the valley of Polog (NW North Macedonia), occurring in two small areas – one in the Šar Mountains (near Brezno village) and the other on Mt. Bukovič (near Gorna Đonovica village) (Fig. 3). The distance between the two areas is ca. 47 km.

Habitats: The ecological preferences of *C. pastricensis* subsp. *pologensis* seem to be similar to those of *C. pastricensis* subsp. *pastricensis* reported in the literature and recorded in the available herbarium sheets. It grows in open and sparse forest habitats in the zone of mesic and meso-thermophytic forests, at an altitude of between 1036 and 1590 m a.s.l. It is indifferent as regards geological substrate, growing on both silicate (in the Šar Mountains) and limestone (on Mt. Bukovič).

Size of the population and threats: Despite the author's extensive fieldwork over many years in the valley of Polog, so far only 11 individuals were observed in Šar Mountains and ca. 60 individuals on Mt. Bukovič. The recorded populations are apparently highly endangered due to the frequent occurrence of wildfires and forest management activities in the areas.

Etymology: The epithet of the new subspecies refers to the valley of Polog, in which it is distributed.

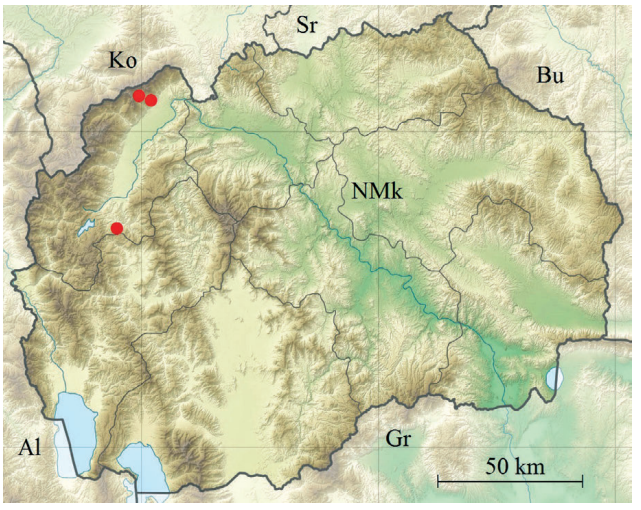

Fig. 3. Distribution of *Cephalaria pastricensis* subsp. *pologensis* in North Macedonia (red dots). Abbreviations: Al – Albania, Bu – Bulgaria, Gr – Greece, Ko – Kosovo, NMk – North Macedonia, Sr – Serbia.

Discussion

Cephalaria pastricensis is a robust, perennial species classified within Sect. *Atrocephala* Szabó and the monotypic Ser. *Rupestris* Szabó (Szabó 1940). It is described from the subalpine zone of the Albanian part of Mt. Paštrik (NE Albania) (Hayek 1921), with the following description provided in the protologue:

"*Perennis fere 2 cm alta. Caulis erectus glaber superne ramosus. Folia dilute viridia non nitentia, in pagina inferiore ad nervos minute puberula caeterum glabra*, 5-6-*jugo pinnatisecta segmentis oblongo-lanceolatis logitudine latitudinem* 6-7-*plo superante sensim acutatis anguste serratis, basi ad rhachim usque ad foliolum proximum anguste decurrentibus, summis late decurrentibus, terminali lateralibus subbreviore; infima longe caulina breviter petiolata, summa tantum* 3-*juga. Pedunculi elongati glaberrimi, lateralibus supra medium bracteolarum parvarum lanceolatarum pare, in quorum axilla capitula rudimentaria sessilia oriuntur, instructa. Capitula globosa plusquam 3 cm longa et lata. Bracteae nigricantes* 4–5 mm *longae triangulares breviter cuspidato-acuminatae hamato-recurvae extus dense sericeo-pilosae. Corolla* 10–12 mm *longa ochroleuca extus sericeo pilosa*."

The comparative morphological study showed that the newly described *C. pastricensis* subsp. *pologensis* differs from the typical *C. pastricensis* by the type and much higher density of the indumentum of the pedicels and leaves (Tab. 1, Fig. 1 and Fig. 2). These characteristics are rather uniform in the recorded populations, with none of the collected individuals or ca 30 examined in the field matching or approaching *C. pastricensis* subsp. *pastricensis*.

Besides the morphological differences, the recognition of the new subspecies is also justified by its apparently allopatric distribution in regard to *C. pastricensis* subsp. *pastricensis*. The closest known distance (c. 40 km) between localities of the two subspecies is the one between the type locality of *C. pastricensis* subsp. *pastricensis* (Mt. Paštrik, NW Albania) and the localities of *C. pastricensis* subsp. *pologensis* in the Šar Mountains. There are also no indications that plants approaching or matching *C. pastricensis* subsp. *pologensis* occur within the range of *C. pastricensis* subsp. *pastricensis*, as most of the chorological data for *C. pastricensis* s.l. (Hayek 1921, Hayek 1928–1931, Szabó 1940, Diklić 1973, Ferguson 1976) are accompanied by a morphological description strictly matching the typical *C. pastricensis*. In this context, of particular importance is the information provided by Szabó (1940) in his valuable monography of the genus *Cephalaria*, who quoted 11 checked by himself herbarium collections from Bosnia (Stolac, Višegrad, Silvolje, Čemerno), Herzegovina (Kokorje, sources of the Neretva River, Gacko), and Serbia (Ozren, Kopasnik, Biljanica, Ostra Čuka – Aleksinac). It should be mentioned in support of this author's opinion that the indumentum of all specimens in the following herbarium collections of *C. pastricensis* is typical for subsp. *pastricensis*:

– NE Albania, "District Hasi. Gerrolhalden an then südhängen des Paštrik, ca. 1800 m, nº 917," leg. I. Dörfler, 26.7.1918, 109004709, 109004710, 109004712, (B) (type collection);

– NE Albania, "Bergwiesen am westabhang des Paštrik", leg. H. Zerny, 13. July, 1918, 0047629 (GB);

– W Kosovo, Prokletije Mt., "*In rupestribus calcareis supra* pg. Lipa", leg. P. Černjavski, I. Rudski, V. Lindtner, det. P. Černjavski, 1.8.1933", 23367 (BEO) (Fig. 2A, B);

– W Kosovo, "m. Žljeb, u bukovoj šumi pored puta Stubice – Savine Vode" leg. P. Černjavski, I. Rudski, V. Lindtner, det. P. Černjavski, 3.8.1933, 23368, 23369, 23370, 23371 (BEO);

– SE Serbia, "Mt. Suva Planina, Golemo Stražište, Debalac", leg. M. Niketić, 17.8.2003. (BEO) (Fig. 2 C).

However, the infraspecific affiliation of the populations reported from SE Albania (Barina et al. 2017) needs to be checked. They represent the southernmost points in the distributional range of *C. pastricensis* s.l., situated significantly closer to the localities of *C. pastricensis* subsp. *pologensis* than those of *C. pastricensis* subsp. *pastricensis*.

Due to the indumentum of the leaves and pedicels *C. pastricensis* subsp. *pologensis* is reminiscent of *C. alpina* (L.) Roem. & Schult., distributed in SW & C Alps, Jura, and N Appennini. However, it differs at least by the following morphological characteristics that clearly classify it within *C. pastricensis*: intermediate setae of the involucels distinctly shorter than the longer ones (vs. slightly shorter), usually narrower shape of the lobes of the leaves (oblong-lanceolate to lanceolate vs. elliptic to oblong-lanceolate), and receptacular scales appressed sericeous (vs. patently villous).

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Effect of different substrates on *in vitro* symbiotic seed germination for soilless production of *Anacamptis laxiflora* orchid

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Abstract – In recent years, orchid species have become endangered due to excessive collection and habitat destruction. As with most flowering plants, seed production is the primary strategy for reproduction in orchids. Orchids produce tiny seeds consisting of a seed coat and a rudimentary embryo. However, it lacks the endosperm, which is generally required as the primary energy source during germination. The only way to germinate orchid seeds is to get nutrients from an external source. In nature, this is achieved by mycorrhizal symbiosis. This study used *Ceratobasidium sp.* inoculation of *Anacamptis laxiflora* (Lam.) seeds combined with media with various organic substrates to determine their effectiveness on *in vitro* germination and seedling development. The highest germination rate (35.78%) was obtained in the medium with the addition of young hazelnut leaves. Then, soilless *ex vitro* symbiotic germination was performed on young hazelnut leaves, the most effective organic substrate. Seed germination was determined to be 19.01% in this medium while 14.87% seedlings with developed leaves and roots were formed. For the first time, success was achieved by producing *A. laxiflora* from seed in *ex vitro* conditions without soil and adapting it to nature.

Keywords: *Anacamptis laxiflora*, *Ceratobasidium*, Orchidaceae, reintroduction, symbiotic seed germination

Introduction

Orchids are one of the most diverse and widespread families of flowering plants, with approximately 28,500 species, and are classified among the most threatened plant groups worldwide (Indan et al. 2021, Štípková et al. 2021). Orchids are facing a rapid decline in their population worldwide; therefore, it is among the main species with respect to plant protection (Yi-Bo et al. 2003, Tikendra et al. 2021). Although a single orchid produces thousands of seeds during the breeding season, very few seeds survive as adult plants and the germination of seeds depends on the presence of compatible fungi in the soil and optimal microclimate and micro-edaphic conditions (Nicole et al. 2005, Jacquemyn et al, 2010, Cardoso et al. 2020, Phillips et al. 2020).

The density of fungi in the distribution area of the orchid affects the germination rate of seeds (Jacquemyn et al. 2012). Habitat destruction due to global climate change, urbanization, agricultural purposes, environmental pollution, and over-collection for food and medical purposes threaten the existence of orchids (Rasmussen 1995, Sezik 2002). Orchids with their very different life types, specific pollinators, and mycorrhizal associations are very interesting plants and the focus of interest for scientists from different research disciplines (Rasmussen 1995, Yi-Bo et al. 2003).

Terrestrial orchids (temperate) are mostly distributed in Eurasian-Mediterranean regions and most of them have tubers under the soil. Glucomannan, a starch-like polymer found in these tubers, is an important food additive (Sezik 2002, Hossain 2011). Especially in Greece, Iran, and Turkey, the use of orchids as a food additive is the most important factor threatening their extinction (Sezik 2002, Ghorbani et al. 2014). Against these threats, seedling production by *in vitro* and *ex vitro* symbiotic seed germination with compatible fungi seems to be a very favourable method for the propagation and reintroduction success of temperate orchids (Aewsakul et al. 2013, Mutlu and Kömpe 2021, Deniz et al. 2022). It has been determined that the adaptation success of healthy and strong seedlings to the natural environment is quite high (Deniz et al. 2022). It has also been shown that rich media and substrates for strong seedlings are effective in symbiotic reproduction (Aewsakul et al. 2013).

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Anacamptis laxiflora is an orchid distributed in wetlands and at altitudes up to 1600 m above sea level in the Mediterranean countries of Western Asia, and Central and Southern Europe (Wood and Ramsay 2004). *A. laxiflora*, attracts attention due to its specific wetland habitats, which are becoming gradually reduced, making the species more vulnerable to extinction threat (Kömpe et al. 2020). These orchids with showy flowers and large tubers are one of the most collected species for the well-sought-after hot beverage, salep (Sezik 2002). One individual with loose flowers and the pollination of all of these flowers produces hundreds of thousands of seeds (Wood and Ramsay 2004). Some research has been conducted on the propagation of this species through *in vitro*, symbiotic and asymbiotic germination (Özkoç and Dalci 1993, Kömpe et al. 2020). The most important goal in both commercial and conservation studies is to produce healthy and strong seedlings that are easily adapted to the natural environment. It has been determined that healthy seedlings obtained with compatible fungus under *in vitro* and *ex vitro* conditions can easily adapt to nature (Kömpe et al. 2020, Deniz et al. 2022). The success of symbiotic germination is strongly dependent on the compatible fungus as well as the additives added to the nutrient medium. This is because the symbiotic fungus transports carbon and other nutrients from the nutrient medium to the embryo. The presence of suitable substrates in the germination medium positively affects germination and seedling rate (Aewsakul et al. 2013). In addition, *ex vitro* symbiotic germination seems to be a practical and effective method by which to obtain strong seedlings in a short time and in simpler environments, as well as to ensure easier adaptation to nature (Aewsakul et al. 2013, Kömpe and Mutlu 2021, Deniz et al. 2022, Kömpe et al. 2022). In this context, the aim of this study is to determine the effect of different organic substrates added to a modified oat medium (Clements et al. 1986), which is used as an effective *in vitro* symbiotic culture medium, for the germination of *Anacamptis laxiflora* (Lam.) seeds and to demonstrate that the most effective substrate can be used as an *ex vitro* orchid propagation medium.

Materials and methods

Plant material

Seeds of *Anacamptis laxiflora*, which is commonly found in wet meadows in Samsun (Turkey), were collected in July 2017. The mature capsules were taken, opened in the laboratory, dried for a few days at room temperature, and then stored at 4 °C in brown glass bottles.

Fungal isolate

In this study, we used *Ceratobasidium* sp. (NCBI-accession number is MT605389) from the roots of *A. laxiflora* from previous research (Kömpe et al. 2020). The isolate is kept in the orchid-fungi collection of the Department of Biology at the University of Ondokuz Mayıs. A piece of the stock culture of this fungal isolate was taken and transferred to Petri dishes containing the fungus isolation medium (Clements et al. 1986) and the Petri dishes were wrapped with aluminium foil. They were incubated at 25 °C in the dark until the fungal hyphae completely covered the dishes. Fungus activation procedures were performed under sterile conditions and with sterile equipment throughout the entire study.

In vitro symbiotic seed germination

Modified oat medium (MOM) was used as a positive control in the symbiotic seed germination method (Clements et al. 1986). MOM contains $Ca(NO_3)_2 \times 2\ H_2O$ (0.2 g), KH_2PO_4 (0.2 g), KCl (0.1 g), $MgSO_4 \times 7\ H_2O$ (0.1 g), yeast extract (0.1 g), agar (10 g), ground oat (3.5 g) and sucrose (2 g) per litre and the pH is 5.8. Inorganic substances used for this medium were obtained from Merck (USA), and yeast extract and agar were obtained from Sigma.

Instead of ground oats, the same amounts (3.5 g L^{-1}) of ground old hazelnut leaves, young hazelnut leaves, straw, old oak leaves and young oak leaves, with sucrose (2 g L^{-1}) or without it, were added in the MOM. As young leaves, we used spring leaves and dried them in an oven at 40 °C. For old leaves we used dry brown leaves collected directly from the trees in autumn which were then dehumidified at 40 °C. These substrates were ground separately and added to each medium.

The medium prepared as above and adjusted to pH 5.8 was sterilized in an autoclave at 121 °C for 15 minutes and then poured evenly into Petri dishes in sterile laminar flow. After the MOM in the Petri dishes solidified, they were used for germination tests. In addition, MOM without sucrose and fungi were prepared as a negative control. All substrates added to MOM in this study and their modifications are given in Tab. 1.

Tab. 1. Characteristics of substrates tested for germination of *Anacamptis laxiflora* seeds.

Substrates	Abbreviations
Modified oat medium + fungus	MOM
Modified oat medium + fungus (no sucrose)	MOM (-S)
Modified oat medium (no fungus)	MOM (-F)
Modified oat medium (no sucrose and fungus)	MOM (-S and -F)
Old hazelnut leaf medium + sucrose+ fungus	OHL
Old hazelnut leaf medium + fungus (no sucrose)	OHL (-S)
Old oak leaf medium + sucrose + fungus	OOL
Old oak leaf medium + fungus (no sucrose)	OOL (-S)
Straw medium + sucrose + fungus	S
Straw medium + fungus (no sucrose)	S (-S)
Young hazelnut leaf medium + sucrose+ fungus	YHL
Young hazelnut leaf medium + fungus (no sucrose)	YHL (-S)
Young hazelnut leaf + fungus – *ex vitro*	YHL-EV
Young hazelnut leaf (no fungi) – *ex vitro*	YHL-EV (-F)
Young oak leaf medium +sucrose+ fungus	YOL
Young oak leaf medium + fungus (no sucrose)	YOL (-S)

The seeds were disinfected by immersion in 1% (v/v) solution of sodium hypochlorite (NaOCl) plus 0.1% (v/v) of Tween 20, followed by six rinses with distilled, autoclaved water. Seeds (approximately 100–150) were sown with a sterilized inoculation loop in Petri dishes (diameter 9 mm) containing the organic substrates tested in the study and incubated at 25 ± 2 °C in the dark for a week. Then, an agar block (approx. 0.5–7 mm^2) containing fungal hyphae was taken from a Petri plate containing the fungal isolate and put next to the seeds. Each application was repeated three times. All treatments were cultivated in a tissue culture chamber under 16:8 hours (light/dark) conditions at 25 ± 2 °C for 8 weeks.

Ex vitro symbiotic seed germination

After determining that the most effective substrate for total germination and seedling growth in *in vitro* conditions was young hazelnut leaves, this substrate was also used in *ex vitro* germination medium. Young hazelnut leaves were collected from a hazelnut orchard in Samsun/Turkey during spring (April-May), dried at 40 °C in an oven, ground, and then sterilized in an autoclave at 121 °C for 20 minutes before being placed into pots. Approximately 200–250 seeds were placed between sheets of water-resistant nylon mesh (45 μm pore size) and fixed between the dia frame (Rasmussen and Whigham 1993). Six seed packets were buried in each pot, and the pots were filled with equal amounts of young hazelnut leaves. In three pots, eight 1 cm^2 agar blocks containing fungal hyphae were placed around the seed packets, while the other pots were kept as a control group without any fungal inoculation. The pots were incubated under 16:8 hours (light/dark) conditions at 25 ± 2 °C in a climate chamber. Pots were irrigated once a week with a sterilized solution containing MOM's minerals and sucrose (1/2 strength).

Germination parameters

Three germination parameters were examined during 28 days to find out the potential effect of different substrates on seed germination of *A. laxiflora.* The observations were recorded daily for seeds.

Germination percentage was calculated according to Elezz et al. (2019), while germination index and mean germination time according to Marvin and Gonzales (2015):

$$\text{Germination percentage} = \frac{\text{seeds germinated}}{\text{total seeds tested}} \times 100$$

$$\text{Germination index} = \sum_{i=1}^{k} = \frac{\text{No. of germinated seed}}{\text{the count day}}$$

where: i = 1 is day one, k is the last day of observation.

$$\text{Mean germination time} = \frac{\sum_{i=1}^{k} ni * ti}{\sum_{i=1}^{k} ni}$$

where: ti is the time from day one to the last day of observation, ni is the observed number of germinated seeds every day, and k is the last day.

During seed germination, development was also monitored and described according to the following stages: S0 – no germination, S1 – swollen embryo with rupture of the seed coat, S2 – rupture of testa, globular embryo, rhizoids present (germination), S3 – protocorm formation, S4 – formation of first leaf and S5 – elongation of the first leaf and rooted (Zettler and Hofer 1998, Johnson et al. 2007). Stage 3 was considered the beginning of germination.

Data analysis

Data were subjected to analysis of variance (ANOVA) and mean values were compared by Duncan's multiple range test (DMTR), $\alpha = 0.05$) using IBM SPSS Statistics 24.

Results

Anacamptis laxiflora seeds germination

In order to evaluate the germination parameters, the germination status of the seeds in all culture media was checked daily from seed sowing. Germination started within 4 weeks of seed sowing and germination parameters were evaluated for the next 4 weeks (Tab. 2).

The effects of sucrose in combination with oat and other substrates on symbiotic germination and seedling development of *A. laxiflora* were firstly tested in *in vitro* conditions. MOM basic medium and its modifications, namely sucrose-free (MOM (-S)), fungus-free (MOM (-F)), and both sucrose- and fungus-free (MOM (-S and-F)), were used as control groups. No germination was observed in fungus-free substrates, including MOM (-F) and MOM (-S and-F), and consequently, data related to germination index (GI), germination rate (GR), and mean germination time (MGT) could not be obtained (Tab. 2). The highest GI values (Tab. 2) was achieved in YHL and OHL substrates (Tab. 1) and were significantly higher than that in MOM (Tab. 2). Other sucrose-containing substrates gave similar results to MOM. In the groups without sucrose, the lowest GI (Tab. 2) was observed in MOM (-S), OOL (-S), YOL (-S), and YHL (-S) substrates (Tab. 1).

The YHL substrate has been found to have the highest germination rate (GR) among all tested substrates *in vitro* (Tab. 2). It exhibited a germination rate three times as high as YHL (-S). Among other sucrose-containing media, including MOM, OHL had a significantly higher GR value. Among the sucrose-free substrates, MOM (-S), YHL (-S), and YOL (-S) were found to have lower GR rates. In contrast, OHL (-S) had the highest GR rate among the sucrose-free group, which was twice that of MOM (-S) (Tab. 2).

Regarding the mean germination time (MGT), sucrose-containing substrates *in vitro* exhibited the highest and lowest values in OOL and OHL media, respectively. However, there were no significant differences from MOM. Among the sucrose-free substrates, MOM (-S) had the highest MGT, and the lowest MGT (Tab. 2) was observed in YOL (-S) substrate, which was significantly different from the value observed in the same medium with sucrose.

Tab. 2. The effects of different organic substrates (see Tab. 1. for abbreviation explanation), with or without sucrose (-S) on symbiotic seed germination of *Anacamptis laxiflora*. The results are expressed as mean ± standard deviation, n = 3. One-way ANOVA and the post-hoc Duncan multiple range test was used to analyse differences between means. Statistical significance was set at $P < 0.05$. Means followed with the same letters denote that there was no statistically significant difference between groups.

Substrates	Germination index (GI)	Mean germination time (MGT)	Germination rate (GR) (%)
MOM	0.6 ± 0.4d	18.3 ± 4.7ab	15.60 ± 6.87cd
MOM (-S)	0.9 ± 0.1d	20.1 ± 2.7ab	14.42 ± 3.04d
MOM (-F)	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0e
MOM (-S and -F)	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0e
OHL	3.4 ± 1.5a	13.7 ± 3.6bc	31.16 ± 9.73ab
OHL (-S)	2.7 ± 1.4ab	13.6 ± 8.3bc	30.85 ± 5.67ab
OOL	1.3 ± 0.4bcd	22.1 ± 4.3a	24.95 ± 5.90bc
OOL (-S)	0.7 ± 0.4d	18.5 ± 1.1ab	15.87 ± 5.41cd
S	1.3 ± 0.9bcd	19.4 ± 4.1ab	24.13 ± 0.99bc
S (-S)	0.9 ± 0.2cd	19.4 ± 1.6ab	24.87 ± 6.12bc
YHL	2.6 ± 1.9abc	18.7 ± 2.3ab	35.78 ± 10.43a
YHL (-S)	0.7 ± 0.1d	18.4 ± 3.8ab	11.65 ± 1.11d
YOL	0.7 ± 0.1d	17.7 ± 1.9ab	14.40 ± 2.35d
YOL (-S)	0.5 ± 0.4d	7.9 ± 2.3c	9.54 ± 3.00d
YHL-EV	3.4 ± 1.21a	12.3 ± 0.5ab	19.01 ± 4.15cd
YHL-EV (-F)	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0e

Since under *in vitro* conditions, YHL had the highest GR and GI, it was used as the substrate for the *ex vitro* experiment. GI obtained in *ex vitro* conditions was slightly better than in *in vitro* conditions. However, GR was found to be approximately half of that in the *in vitro* and MGT was also slightly lower.

The developmental process of *Anacamptis laxiflora*

From the data collected for the germination of *A. laxiflora* seeds in the different treatments, the developmental stages were assigned to a qualitative scale, according to a modification from Zettler and Hofer (1998), and Johnson et al. (2007).

The effect of substrates added instead of oat in the modified medium on seedling development in the presence and absence of sucrose is shown in Tab. 3. Seedling growth was evaluated 8 weeks after seed sowing. According to the results, leafy seedlings (stage 5) were formed at different percentages in all *in vitro* substrate modifications as well as in *ex vitro* condition (FHL-EV). No growth was observed in

Tab. 3. Effects of substrates and mycorrhizal inoculation on the developmental stages of seeds of *Anacamptis laxiflora* at 8 weeks after seed sowing. Development of the seedlings was divided into stages: S0 – no germination, S1 – swollen embryo with rupture of the seed coat, S2 – rupture of testa, globular embryo, rhizoids present (germination), S3 – protocorm formation, S4 – formation of first leaf; S5 – rooted and leafy plantlet. The effects of substrates and their sucrose-free modifications on developmental stages were analyzed using one-way ANOVA. Results were compared using the standard deviation of the means (n = 3) and the post-hoc Duncan multiple range test. Statistical significance was set at $P < 0.05$. There is no statistically significant difference between groups with the same letters.

Substrates	Development stages %					
	S0	S1	S2	S3	S4	S5
MOM	77.64 ± 5.2abcd	6.77 ± 2.1ef	4.15 ± 1.2abc	3.14 ± 1.7bcde	4.15 ± 2.2abcd	4.15 ± 2.2cde
MOM (-S)	72.30 ± 3.8bcdef	13.28 ± 4.8bcd	3.34 ± 1.5bcd	2.91 ± 1.1bcde	1.66 ± 0.8de	6.50 ± 2.0bcd
MOM (-F)	82.10 ± 3.5ab	17.90 ± 2.42b	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e
MOM (-S and -F)	72.41 ± 5.9bcdef	27.59 ± 5.9a	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e
OHL	65.57 ± 9.3ef	3.27 ± 1.3f	5.21 ± 1.8ab	7.37 ± 4.9ab	3.84 ± 0.5abcd	14.73 ± 3.5a
OHL (-S)	63.27 ± 7.5fg	5.88 ± 2.3ef	4.52 ± 2.2abc	6.13 ± 3.8abcd	5.05 ± 2.4ab	15.16 ± 3.3a
OOL	67.76 ± 3.6def	7.30 ± 2.3def	3.42 ± 0.5bcd	7.28 ± 1.8ab	4.93 ± 2.1abc	9.32 ± 2.4b
OOL (-S)	78.52 ± 2.0abcd	5.62 ± 3.3ef	3.27 ± 1.2bcd	3.31 ± 1.3bcde	3.67 ± 1.8abcd	5.62 ± 1.9bcd
S	70.09 ± 2.8cdef	5.78 ± 2.4ef	3.14 ± 1.3bcd	6.99 ± 1.4abc	6.13 ± 2.6a	7.87 ± 2.6bc
S (-S)	67.72 ± 6.5def	7.41 ± 2.8def	4.83 ± 0.8ab	7.47 ± 4.6ab	3.34 ± 0.5bcd	9.23 ± 1.6b
YHL	55.47 ± 12.0g	8.75 ± 2.1def	6.81 ± 3.5a	9.04 ± 5.1a	4.88 ± 1.0abc	15.05 ± 4.3a
YHL (-S)	82.04 ± 3.5ab	6.31 ± 2.5ef	1.55 ± 0.3cde	2.21 ± 0.3cde	3.84 ± 0.5abcd	4.05 ± 0.9cde
YOL	74.50 ± 5.3abcde	11.11 ± 5.2cde	3.20 ± 1.9bcd	4.14 ± 1.8bcde	2.31 ± 0.8cde	4.74 ± 0.7cd
YOL (-S)	79.26 ± 3.abc	11.20 ± 3.8cde	1.72 ± 0.2cde	2.05 ± 0.7de	2.88 ± 1.1bcd	2.88 ± 1.1de
YHL-EV	78.07 ± 3.2abcd	2.93 ± 1.3f	0.98 ± 0.44de	1.60 ± 1.18de	1.56 ± 0.52de	14.87 ± 3.88a
YHL-EV (-F)	83.71 ± 4.8a	16.29 ± 4.8bc	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e

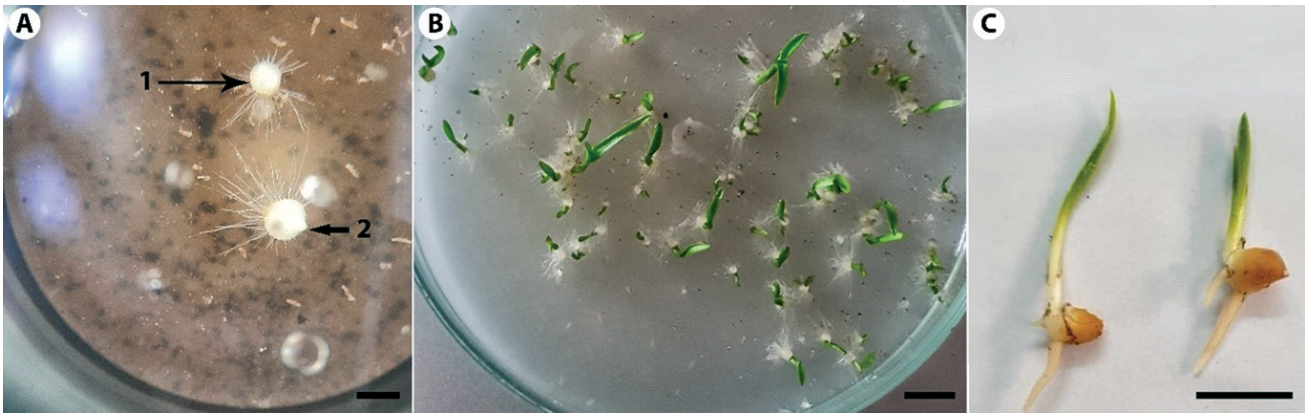

Fig. 1. Protocorm and progression of seedling development of *Anacamptis laxiflora* cultured on YHL medium. A) Stage 2 - protocorms and stage 3 - leaf primordium (arrows 1 and 2 show protocorm and leaf primordium, respectively), B) Stage 4 - leafy seedlings, C) Stage 5 – rooted and leafy seedlings. Scale bars: A - 1 mm, B and C - 1 cm.

the control groups without fungal inoculation (MOM (-F), MOM (-S and -F)).

The highest percentage of protocorm formation (S3) was observed in the YHL substrate (Fig. 1A) and it was approximately three times higher than in MOM. Results on other substrates showed no difference from those obtained with MOM. The protocorm formation in substrates that did not contain sucrose were similar to that obtained for substrates with sucrose except for YHL (-S) where significantly lower protocorm formation was observed (Tab. 3).

The highest percentage of first leaf formation stage (S4) was observed in the straw (S) substrate with sucrose, while the OHL (-S) had the highest value among the sucrose-free modifications. The lowest percentage was in MOM (-S) under *in vitro* conditions.

The highest percentage of seedlings with roots and leaves (S5) was observed in the YHL substrate (Fig. 1C), which was approximately 3.5 times higher than that of MOM. OHL and OOL media had also significantly higher values than MOM. The lowest percentage in sucrose-containing substrates was observed in the YOL, following MOM. In sucrose-free substrates, the best percentage was observed in OHL (-S) which was similar to the value observed for OHL and more than twice that of MOM (-S). The lowest percentage was in the YOL (-S) medium.

Regarding *ex vitro* conditions, although the germination rate in YHL medium was lower than that in the *in vitro* medium containing the same substrate, there was an interestingly high percentage of seedlings in the S5 stage (Fig. 2C), with a value similar to that for YHL *in vitro*. Moreover, the seedlings successfully survived transfer to nature (Fig. 2D).

Discussion

Orchids are in danger of extinction because of habitat destruction, over-harvesting, and the lack of a suitable fungus for seed germination. *In situ* and *ex situ* conservation methods are recommended for the protection of orchids. However, *ex situ* protection is also insufficient due to the rapid destruction and disappearance of habitats and the vulnerability of the specific habitats they are found in (Batty et al. 2006, Lemay et al. 2015, Štípková et al. 2021). Seed banks are also included in conservation programs, as it is known that orchid seeds can maintain their viability for many years (Kömpe et al. 2020). However, as this longevity is limited seed banks may not be a long-term solution. In recent years, it has been accepted that the most effective

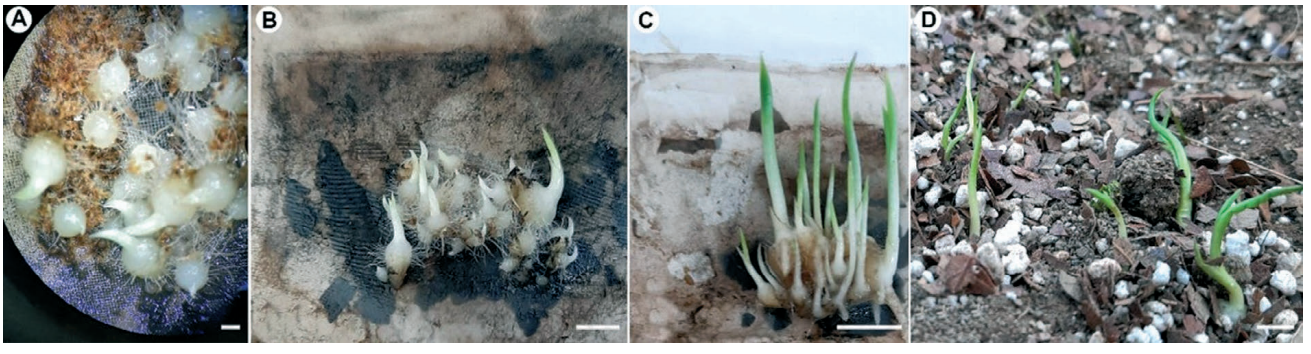

Fig. 2. *Anacamptis laxiflora.* A) Protocorms that developed 28 days after the seed packs were embedded in the fresh hazelnut leaf, B) the seedlings five weeks later, C) the seedlings with root that developed eight weeks later, D) transplanted seedlings in nature. Scale bar: A – 1 mm; B, C and D – 1 cm.

protection method is to transfer asymbiotic and/or symbiotic seedlings to the natural environment to establish a population (Smith et al. 2010, Duncan and Moloney 2018, Soares et al. 2020, Kömpe and Mutlu 2021, Wang et al. 2021, Zhao et al. 2021). The success of asymbiotic and symbiotic methods varies according to the orchid species, the nutrient media used, and the appropriate fungus (Rasmussen 1995). *A. laxiflora* is a wet meadow orchid found throughout Central Europe and the Mediterranean, including Turkey. There are some studies in which symbiotic and asymbiotic methods are applied to germinate seeds of this species (Mead and Bulard 1975, 1979, Özkoç and Dalcı 1993, Deconninck and Gerakis 2021, Kömpe et al. 2020). However, these studies mostly remained within the laboratory limits.

In this research, first, the efficiency of various organic substrates and sucrose on symbiotic seed germination and seedling growth was determined *in vitro*. Then *ex vitro* symbiotic germination was performed on the most effective substrate.

It is known that the basic functions of compatible fungi effective in the germination process of orchid seeds in nature are to carry carbon, nitrogen, and some other basic nutrients to the embryo (Jacquemyn et al. 2012). Detailed studies on the effect of carbon sources in *in vitro* replication studies were also conducted (Hadley and Perombel 1963, Smith 1966, Yam and Arditti 2009, Mehra et al. 2017). In addition to soluble carbon sources (such as monosaccharides, disaccharides, and polysaccharides), the effects of various organic substrates (milled oats, rotted leaves, cockroaches, peat moss, etc.) were also tested and different effects emerged (Mala et al. 2017). It was found that a compatible fungus and symbiotic method and substrates such as oat and peat moss were more effective in the germination of seeds of tropical exotic orchids (Mala et al. 2017). Orchid seeds do not contain endosperm, so they need all essential nutrients, especially carbon, to be transported by fungi until the photosynthetic stage. This occurs more abundantly and faster in the presence of rich substrates. Although the effect of hazelnut leaves has not been tested on the germination of any orchid seed before, Mala et al. (2017) suggested that natural substrates can be more effective in the development of symbiotic seedlings than oat in MOM.

In our study, it was revealed that different natural substrates significantly promoted germination and seedling development, and that sucrose increased this stimulation. This is not surprising since sucrose is a carbon source that plant cells use until the photosynthetic stage is established. The use of sucrose as a carbon source in the culture medium has been shown to be very important in orchid seed germination and protocorm development for both mature and immature seeds (Arditti 1967, Rasmussen et al. 2015, Gupta 2016).

Reintroduction studies have shown that if the transferred seedlings are well developed, the success and continuity of survival in nature are also higher (Aewsakul et al. 2013; Kömpe et al. 2022; Deniz et al. 2022). Therefore, in this study we performed an *ex vitro* experiment with the substrate that proved the most effective in order to obtain strong seedlings for reintroduction under *ex vitro* symbiotic conditions. *Ex vitro* symbiotic propagation studies conducted so far using different substrates have revealed very remarkable results. The *ex vitro* symbiotic method was first applied by Quay et al. (1995). These researchers inoculated fungi into a mulch obtained from *Allocasuarina fraseriana* trees. Although the germination rate was low, this was pioneering research for *ex vitro* germination (Quay et al. 1995). Aewsakul et al. (2013) inoculated two Epulorhiza isolates (isolated from *Dendrobium anosmum* and *Paphiopedilum sukhakulii*) onto different substrates (soil, coconut powder, peat moss) and planted *Spathoglottis plicata* seeds in these substrates. They achieved successful results with the best germination obtained in peat moss (Aewsakul et al. 2013).

The results obtained in this study with natural substrates different from those used by Aewsakul et al. (2013) are promising for both conservation and economic purposes. We observed that hazelnut leaves (young and old) had a remarkably positive effect on germination and seedling development compared to other substrates. This situation may arise from the structural or functional molecule content of the substrates. Various secondary metabolites present in straw and oak leaves may have negatively affected fungal activity and/or germination process. It is well known that many phenolic compounds have antifungal or allelopathic activity (Wang et al. 2017), so they may have negatively affected fungal activity under symbiotic germination conditions. The reason why specifically young hazelnut leaves are more effective than other substrates is most likely because during the active photosynthetic phase leaves contain more carbon (including soluble carbon), nitrogen, and other essential elements. Since most of the nutrient elements are transferred to younger leaves during the aging process, old leaves are poorer in terms of nutrient content (Taiz and Zeiger 2010). However, old leaves also contain structural polysaccharides, cellulose, which can be degraded into glucose monomers and transported to the embryo during orchid seed germination (Gong et al. 2023). This can explain satisfactory germination results obtained in our study with substrate from old hazelnut leaves, even without the presence of sucrose. The chemical profiles of these substrates should be evaluated in detail in future studies to determine their potential effects on symbiotic germination.

Conclusions

The addition of organic materials as a supplementary substrate to the nutrient medium was found to enhance the germination and seedling development of *A. laxiflora* in *in vitro* conditions. The highest germination and seedling growth was obtained using fresh hazelnut leaves. Sucrose, together with natural organic additives, strongly supported germination and seedling growth. Fresh hazelnut leaves also proved successful in *ex vitro* conditions providing high

percentage of seedlings with developed leaves and roots. Given the importance of orchids and their status as a threatened species, it is critical to develop cheap, easy, and fast methods for their mass production. The use of these substrates for this purpose is promising, as it would not only reduce the cost but also decrease the laboratory dependency, thereby providing new avenues for future research. Further detailed studies are needed to optimize these methods and fully understand their potential for application in orchid production.

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In vitro cultivation and biocontrol potential of *Botryosphaeria visci* against European mistletoe (*Viscum album* L.)

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Abstract – To improve the biological control of white mistletoe (European mistletoe) additional detailed information on *Botryosphaeria visci* infection, its basic nutritional requirements, growth, and *in vitro* growth characteristics is needed. The objectives of this study were to isolate and identify the fungus *B. visci* associated with *Viscum album* from *Sorbus aucuparia*, to provide information on its *in vitro* introduction and growth characteristics on different media, and to test the pathogenicity of the isolated fungus. To achieve these goals, the morphology of conidiophores from infected parts of mistletoe was evaluated by microscopy. The isolate from fresh collections of *V. album* was identified as *Sphaeropsis visci* anamorph of ascomycete *Botryosphaeria visci*. The morphology of the vegetative mycelium and growth of *B. visci* varied depending on the media used. The best medium supporting growth and sporulation was oatmeal. Re-infection of European mistletoe in laboratory conditions showed positive results on liquid media, and in field conditions but only after mechanical damage to the mistletoe leaves. Our results expand the knowledge regarding the optimal cultivation of this fungus. This may facilitate further mycological and pathological studies involving *B. visci* isolates, and the results have a theoretical basis for the implementation of measures for the prevention and control of mistletoe.

Keywords: biological control, laboratory tests, pathogenic fungus, *Sphaeropsis visci*

Introduction

Viscum album L. (white mistletoe or European mistletoe) is a semi-parasitic plant that is common in South-West Asia and most European countries, including Ukraine. It inhabits numerous decorative and economically important tree species. *V. album* like many other mistletoes is believed to cause growth retardation, premature defoliation, reduction of photosynthetic potential and subtle changes in the water-carbon balance of host trees, which inevitably leads to a decrease in the resistance of woody plants to other damaging factors (Barbu 2012, Sanguesa-Barreda et al. 2013, Alvarado--Rosales and Saavedra-Romero 2021, Thomas et al. 2022). This can be a serious threat to host plants.

There are various methods of controlling mistletoe (pruning of infected branches, removal of infected trees, use of systemic herbicides, etc.), but all of them significantly affect the host plants (Varga et al. 2012a, Watson 2019, Mudgal et al. 2022). There are some promising methods of biological control, but they require further elaboration and development and/or the use of special equipment (Krasylenko et al. 2023). The methods that target pollinators and dispersers are unacceptable because they lead to significant negative environmental consequences. Hyper parasitism (parasitism of one mistletoe on another) causes overloading of the host plant and can lead to its death. However, the use of natural antagonists, such as bacteria and fungi, is considered a more promising option (Karadžić et al. 2004, Kotan et al. 2013, Chen et al. 2018).

There are several genera of pathogenic fungi associated with mistletoe diseases, among which *Botryosphaeria visci* is relatively more aggressive as it causes mistletoe dieback (Kotan et al. 2013, Karadžić et al. 2004, Kahle-Zuber 2008). Traditional research on *B. visci* to date has focused on

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taxonomic profiling, particularly based on molecular, macro- and microscopic studies (Phillips et al. 2008, Poczai et al. 2015, Varga et al. 2014). It should be noted that studies of the growth of this pathogenic fungus *in vitro* can reveal some important physiological features. However, research on *B. visci* is fragmentary and almost none has been conducted on the factors influencing its *in vitro* growth. Studies of *B. visci* and the disease it causes have included culturing the fungus on various media for growth and sporulation (Varga et al. 2012b, Varga et al. 2013) and examining temperature parameters (Zlatković et al. 2016, Tkaczyk and Sikora 2020). Due to the high pathogenicity of this fungus, several studies have focused on evaluating it as a promising candidate for the biological control of mistletoe (Karadžić et al. 2004, Varga et al. 2012a, Kotan et al. 2013, Tkaczyk and Sikora 2020). At the same time, it must be remembered that mistletoe is part of ecosystems, and its complete removal reduces biodiversity. Thus, the greatest potential for the use of *B. visci* as a biocontrol agent for *Viscum album* should be expected in the protection of commercial plantations and orchards.

The purpose of this work was to isolate and identify the fungus *Botryosphaeria visci* associated with *Viscum album* L. from *Sorbus aucuparia*, to elaborate its introduction and growth *in vitro*, as well as to test the pathogenicity of the isolated fungus.

Materials and methods

Sampling

Diseased parts of common mistletoe (stems and leaves) were collected in Ukraine (Sumy region) in August 2021, where a mass appearance of white mistletoe was observed. Samples of mistletoe plants were taken from host-tree *Sorbus aucuparia* L., usually moderately damaged due to infection with the above-mentioned plant pathogen (from 10 to 18 bushes). From these trees, ten shoots were taken with visible black pycnidia and preserved as dry herbarium samples (stored in paper bags, with the sampling information).

Identification

The fungus was removed with a sterile needle and transferred to a small drop of twice-distilled water on a clean slide and covered with a cover glass. Identification of samples was based on morphological characteristics of conidia from infected host materials under an Axiostar microscope, (Zeiss, Germany), photographs were taken using the AxioCam MRc5 digital camera (Zeiss, Germany) and the AxioVision 4.7 software (Zeiss, Germany) and microscope Nicon SMZ18 SHR Plan Apo with camera Nikon DS-FI3 and software NIS-Elements D5.03.02 (Build 1545). A total of 20 conidiomata and 50 conidia were measured to calculate the mean size and standard deviation (SD). The taxonomic affiliation of the fungus was determined by a combination of morphological and cultural features (Phillips et al. 2013).

Isolation

Isolation was carried out by transferring conidia, after surface sterilization with 3% hydrogen peroxide for 1–2 minutes on potato-dextrose agar (PDA) (Varga et al. 2012a). Single germinating spores were transferred to fresh PDA plates and incubated at 25 °C in the dark. Maternal cultures of the introduced fungi strains were kept on PDA slants at 4 °C before use. A total of 8 fungal isolates were obtained in pure culture. However, 5 isolates were identified as *Botryosphaeria visci*. These pure fungal cultures were deposited in the collection of microorganism strains and plant lines at the Institute of Food Biotechnology and Genomics at the National Academy of Sciences of Ukraine. The *B. visci* strain IFBG 96 with the best growth characteristics was selected for further research in this study.

Cultivated media

The choice of media for cultivation was based on the information available in the literature about the growth of the fungus; for a more effective comparison and reproduction of the results, the synthetic media that are most often used were chosen (Varga et al. 2012b, 2013, 2014).

Morphological characteristics of vegetative mycelia and evaluation of the isolate's growth were provided using the tested media: PDA, Difco, USA; Sabouraud dextrose agar (SDA), Difco, USA; glucose-peptone-yeast agar (GPYA), containing, in g L^{-1}: 25.0 glucose, 3.0 yeast extract, 2.0 peptone, 1.0 K_2HPO_4, 1.0 KH_2PO_4, 0.25 $MgSO_4 \times 7H_2O$, and 15.0 agar (pH 6.0); oatmeal agar (OMA), Difco, USA.

The liquid media used (PDB, GPYB, SDB, OMB) had the same composition as above except for agar. Media pH was adjusted before autoclaving. The media were distributed to 9 cm sterile Petri dishes. Flat plates were inoculated with a mycelial block of the fungus cut from GPYA Petri dishes using a sterile 8 mm diameter drill bit at the stage of active mycelial growth. The morphology of the cultures and pigment production were assessed visually and noted after 1 week of growth on the test media. Colony colors (surface and reverse) were rated according to Rayner's color chart (1970).

For liquid suspension culture, 100 mL media was inoculated (in 250 mL vol. cap. flasks) with three mycelial wells cut from GPYA Petri dishes as above. Cultivation on solid and liquid media was carried out at 25 °C in the dark at static conditions.

Biomass dry weight

On the 14th day of growth, the mycelium was separated from the liquid medium by filtering through Whatman filter paper No. 4, washed with distilled water, and dried to constant weight at 105 °C. Fungi biomass was estimated by absolutely dry weight (a.d.w.).

Pathogenicity tests

Reinfections in laboratory conditions were performed with four fresh sterilized (in 3% H_2O_2, 2 minutes) healthy

mistletoe leaves which were placed into Petri dishes with a colony of *B. visci* grown on solid media (OMA and SDA) and in a vial (culture mattress) with the mycelial matt of *B. visci* grown in SDB. The fungal isolate was grown on these suitable growth media at 25 °C under light during the day for spore formation and in the dark at night. Repeated infections in laboratory conditions were carried out in three replicates.

Tests for pathogenicity in field conditions were carried out by spraying with culture liquid. A spray bottle was used with a suspension collected after 21 days of growth of *B. visci* in SDB medium. A sterile wire loop was used to scrape the conidia and bring them to a suspension. The suspension was filtered through a muslin cloth, and the collected filtrate was serially diluted to 1×10^7. A haemocytometer was used to adjust the spore concentration. About 30 live mistletoe bushes were sprayed with the resulting liquids using manual sprayers. These bushes grew on different host trees. The distance between the treated *V. album* plants was between 2 and 15 m. All mistletoe bushes were located at a height below 2 m. Sterilized distilled water was used as a negative control. Additionally, some mistletoe leaves were mechanically damaged with a blade to allow liquid contact with parenchymal cells. The processing was carried out in autumn (September). The conditions were quite favourable for the growth of *B. visci*, since the species is able to grow actively until the onset of cold weather (Tkaczyk and Sikora 2020). The average daily temperature was +13.5 °C and the humidity was 63%. Over the following two months, the impact on mistletoe was recorded. According to the formation of pycnidia or of their absence on leaves, shoots or berries of mistletoe, pathogenicity was evaluated as positive or negative, respectively. Re-isolations were performed from infected leaves and shoots (Varga et al. 2014), and the resulting isolates were identified as previously described.

Statistical analysis

The experimental values were expressed as mean ± SD (standard deviation) of at least three replicates. Statistical significance analysis was performed by Fisher Least Significant Difference (LSD) test at 0.05 probability level using computer Software SPSS 24.0 (SPSS Inc, Chicago, IL, USA).

Results

The fungus was isolated from fresh collections of the European mistletoe, *Viscum album* from the host-tree *Sorbus aucuparia*. Based on the morphological characteristics of the conidia from the infected host material, it was established that this is an anamorph *Sphaeropsis visci* of the ascomycete *Botryosphaeria visci* (Fig. 1). Below is a taxonomic description of the fungus according to Ukrainian samples. Its morphological features are described in detail and illustrated with photomicrographs obtained using light microscopy. Taxonomic placement and author citations are provided according to Index Fungorum Database (2023).

Botryosphaeria visci (Kalchbr.) Arx & E. Müll. Beitr. Kryptfl. Schweiz **11**(1): 41, 1954. Syn: *Phaeobotryon visci* (Kalchbr.) Höhn., Sber. Akad. Wiss. Wien, Math.- naturw. Kl., Abt. 1 **128**(7–8): 591. 1919.

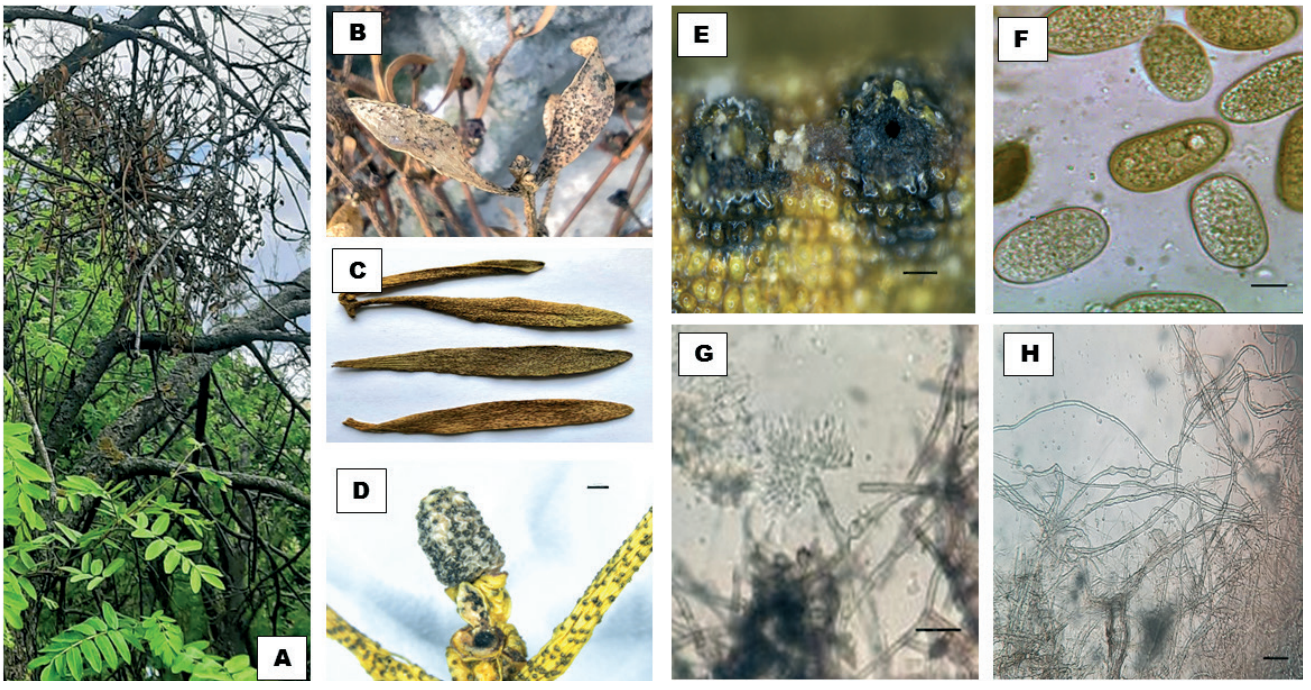

Fig. 1. *Botryosphaeria visci* and infected diseased mistletoe: A – infected European mistletoe (*Viscum album* L.) on *Sorbus aucuparia* L.; B-E – pycnidia forming on leaves and berries of mistletoe; F – conidia of the pathogenic fungus; G – conidiogenous cells and conidia of pycnidia isolated from a colony on Sabouraud dextrose agar medium; H – mycelium of *B. visci*. Scale bars: D = 1 mm, E = 0.1 mm, F = 10 µm, G = 200 µm, H = 100 µm.

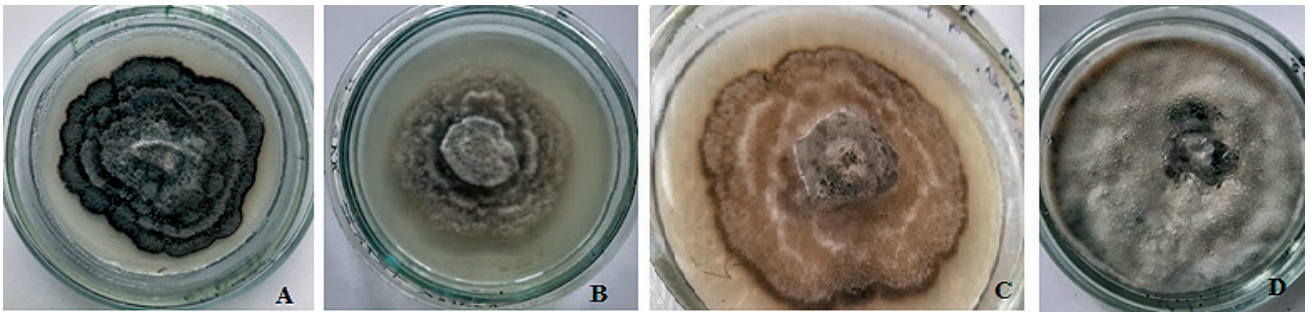

Fig. 2. Morphological differences of *Botryosphaeria visci* colonies grown on different media: A – potato dextrose agar (PDA), B – glucose-peptone-yeast agar (GPYA), C – Sabouraud dextrose agar (SDA), D – oatmeal agar (OMA).

Anamorph: ***Sphaeropsis visci*** (Alb. & Schwein.) Sacc., Michelia **2**(6): 105, 1880.

Descriptions: Conidiomata pycnidia, initially submerged in leaf tissue, erupt and become superficial at maturity, scattered to gregarious, globose, dark brown to black, approximately 300 µm in diameter. Pycnidia unilocular single-nested, with dark layered walls, the inner wall is formed of dark brown angular cells, *textura angularis*. Pseudoparaphyses 4–5 µm wide, hyaline, cylindrical, aseptic, unbranched. Conidiogenous cells are located between pseudoparaphyses, hyaline or yellowish-brown, 8–11 × 5.5–6 µm, with periclinal thickenings. Pycnoconidia are brown, oval or ovoid, 28–42 × 17–22 µm, with truncate or obtuse base and obtuse apex, thick-walled with a fine warty surface texture inside.

Sexual morph: not observed.

Culture characteristics: *B. visci* isolate IFBG 96 was found to grow on all semi-solid media but with variations in mycelial growth rate and colony morphology. Colonies on PDA, GPYA, and SDA had appressed moderately dense matted mycelium, with irregular sparse zonal aerial mycelium or rosette-like appearance, wavy edge not reaching the edge of the Petri dish for 4 weeks (Fig. 2A-C). The surface of the colony on PDA was smoke-grey to black, reverse side was dark black (Fig. 2A). Colony surface on GPYA was pale mouse-grey, reverse side mouse-grey to dark mouse-grey (Fig. 2B). The surface of the colony on SDA was apricot to chestnut; reverse side umber to chestnut (Fig. 2C). Colonies on OMA were off-white with dense aerial mycelium to cottony, covering the dish after 7 days, reverse side black (Fig. 2D). Pycnidia were unevenly distributed on the surface of the SDA medium. The first pycnidia appeared beside the inoculation block, then they sporadically covered the media surface.

Colony morphology of *B. visci* on liquid media was similar to that on agar media. The surface mycelia of *B. visci* started to grow on the third day and during 14 days of cultivation completely covered the surface of all media in the flasks. The mycelium was downy, uniform in texture and consistency, white to greyish-green in colour, with airy hyphae on the surface of the liquid media (Fig. 3A). Liquid media also had a significant effect on mycelial growth of *B. visci* isolate IFBG 96 (Fig. 3B). The results showed that OMB was the most suitable liquid culture medium for the growth of *B. visci*. Moderate mycelial growth was observed in SDB medium followed by GPYB medium. Limited growth of

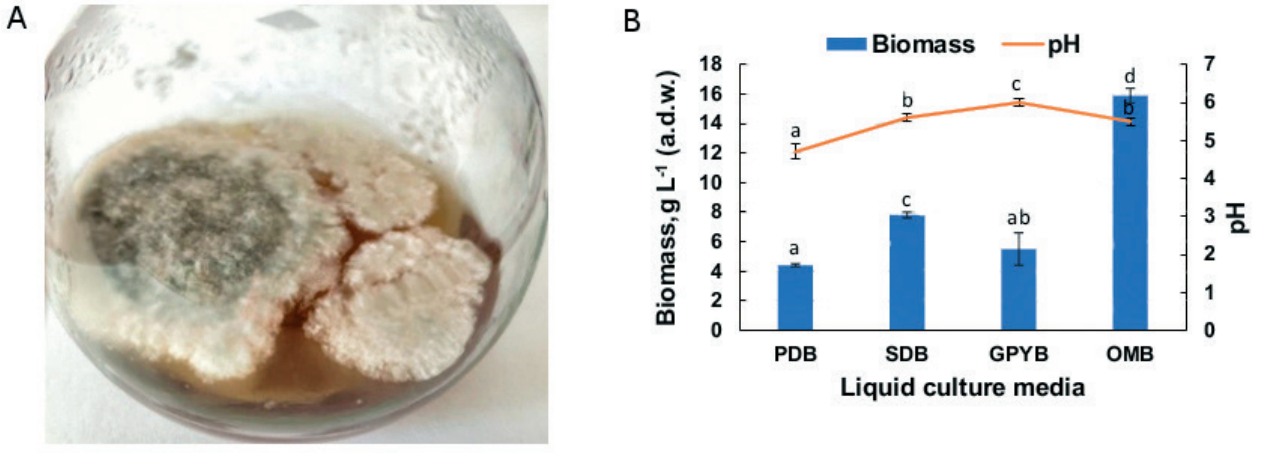

Fig. 3. The morphology of *Botryosphaeria visci* colony on the 10th day of growth in GPYB medium (A), and biomass production of *B. visci* after 14 days of inoculation in liquid culture media (B): PDB – potato dextrose broth, SDB – Sabouraud dextrose broth, GPYB – glucose-peptone-yeast broth, OMB – oatmeal broth. Data are mean ± standard deviation, n = 3. Different letters indicate significant differences at $P < 0.05$ by Fisher`s LSD test.

Fig. 4. Possibility of mistletoe infection in the laboratory on 21 days on oatmeal agar (A), on Sabouraud dextrose agar (B), in Sabouraud dextrose broth (C, D), and in the field 60 days after treatment with *B. visci* liquid culture, obtained after growth in Sabouraud dextrose broth (E–I).

mycelium was established in PDB. The final pH of the culture media varied from 4.7 to 6.0.

Possibility of infection: Laboratory experiments of mistletoe pathogenicity were modelled on different nutrient media. A negative result of mistletoe leaf infection was obtained on solid agar media; no infection of mistletoe leaves placed on fungal colonies in Petri dishes on OMA (Fig. 4A) and SDA (Fig. 4B) media was detected. Neither did mechanical damage to *V. album* leaves before they were placed on fungal colonies in Petri dishes contribute to infection in our experiments. A positive result of infection of *V. album* leaves was obtained with *B. visci* grown in liquid medium. The formation of characteristic pycnidia on the mycelium of *B. visci* was visually observed starting from the 21st day of cultivation in Sabouraud dextrose broth. It should be noted that the highest concentration of pycnidia was found in the immediate vicinity of the leaves (Fig. 4C-D), followed by infection and development of infection on 45–50% of the leaf area of *V. album*.

The experiment in the field showed that only a few plants had initial signs of infection after spraying with liquid culture of *B. visci* (Fig. 4E). Only previously damaged plants were infected. Their number was about 6.5% of all processed mistletoe bushes. Undamaged plants had no signs of infection.

The weakening of the host plant contributed to the development of the infection. After processing the samples, we noted mechanical damage to one of the host trees, which had a negative impact on the overall condition of the mistletoe. As a result, pycnidia formation was observed on 65% of inoculated leaves and 28% of inoculated shoots (Fig. 4F-H). At the same time, no damage was detected on the berries.

The results of the pathogenicity test in field conditions indicated that the isolated pathogen can infect *V. album* only at minimal effectiveness under the condition of mechanical pre-treatment and in already weakened mistletoe. Conidiomata isolated from the laboratory experiments under liquid conditions and from re-infested field conditions were re-isolated by implanting on PDA medium, thus fulfilling Koch's postulates. All isolates were confirmed to belong to *Botryosphaeria visci*.

Discussion

The rapid spread of semi-parasitic mistletoe, which has become a serious pest of trees, requires the search for and development of simple, effective means of control (Mudgal et al. 2022). The literature supports the possibility of biotechnological interventions such as *Sphaeropsis visci* fungi as a biological control agent for mistletoe (Varga et al. 2012b, 2013, Tkaczyk and Sikora, 2020). These studies concern *B. visci*, obtained from *Viscum album* on host trees such as *Acer saccharinum* L. (Varga et al. 2012b, 2013), *A. pseudoplatanus* L. and *Populus nigra* L. (Varga et al. 2012a), as well as from pine plantations (Tkaczyk and Sikora 2020). To the best of our knowledge, this is the first report of an *in vitro* study of

B. visci, isolated from *V. album* on the host tree *Sorbus aucuparia* L. which belongs to the category of secondary hosts (Krasylenko et al. 2020). The morphological characteristics of conidiomata (pycnidia) from collected samples of European mistletoe clearly identify the anamorph *Sphaeropsis visci* of ascomycete *Botryosphaeria visci*. Identification was based solely on morphological characteristics of conidia from infected host material, but molecular analysis of the fungus is also required. This fungus is an aggressive pathogen on mistletoe in various countries, for example in Romania (Sutton 1980), Croatia (Idžojtić et al. 2008), Ukraine (Phillips et al. 2008), Austria, the Czech Republic, Egypt (Phillips et al. 2013), Hungary (Varga et al. 2014, Poczai et al. 2015), Serbia, Luxemburg (Zlatković et al. 2016, Zlatković et al. 2015), China (Chen et al. 2018), and Poland (Tkaczyk and Sikora 2020).

Introduction of the fungus into the *in vitro* culture and investigation of its growth and physiology are necessary for a comprehensive study of the fungus. Despite the ecological and often physiological diversity, the specific adaptation to different woody hosts, species from the *Botryosphaeriaceae* family can be grown in culture conditions (Luque et al. 2005, Abdollahzadeh et al. 2009, Yang et al. 2017, Zhang et al. 2021, Wu et al. 2021). In this study, *S. visci* isolates belonging to *Botryosphaeriaceae* could be cultured on PDA, which is consistent with previous reports (Varga et al. 2012a, Varga et al. 2013, Varga et al. 2014). No ascomata were detected either on the host or in culture, which is consistent with previous studies (Varga et al. 2014, Chen et al. 2018, Tkaczyk and Sikora 2020).

Vegetative mycelial growth and colony morphology are important criteria for culture characterization. Growth and morphology of *B. visci* were strongly affected by the nutrient media probably due to the use of test media that differed in carbon sources. This result of our study confirms the observations of similar studies on the effect of the medium on fungus growth (Varga et al. 2012b, Varga et al. 2013). The isolated fungus grew on all solid and liquid media at different growth rates. To our knowledge, this is the first time that SDA and SDB media have been used for the isolation and cultivation of *B. visci*, and that the effect of different liquid media on *B. visci* growth has been evaluated by mycelial dry weight. Although PDA as well as PDB were commonly used for culturing the fungus in previous studies, this medium proved ineffective for our research because of the slow growth of *B. visci*. Fungal strains and isolates may differ in the degree of cultivation in different media compositions. At the same time, the use of natural environments can enhance the growth and/or sporulation of fungal pathogens. Rather, PDA was the first medium tested, but oatmeal medium is the most common and effective medium for *B. visci* cultivation. The OMA as well as OMB media in this study effectively supported the growth and sporulation of *B. visci*, which could be a low cost alternative for use in routine mycological and pathological studies involving *B. visci* isolates. Our results are consistent with previous studies that found good growth and sporulation of this fungus on OMA (Varga et al. 2012b, Varga et al. 2013). This finding indicates that *B. visci* prefers the oatmeal medium regardless of the fungal strain, probably due to the presence of a plant component in the medium.

Isolation and functional testing of pathogenic fungal effectors has an important role to play in developing new approaches to disease control, particularly the effective control of pathogen damage. The conducted phytopathogenic test of *B. visci* was positive in the case of liquid culture conditions and negative when tested on solid agar media. This can be explained by the fact that under the conditions of liquid cultivation, the isolate had a significantly larger mycelial surface and a larger volume of nutrient medium, which contributed to the intensive production of conidia and, accordingly, the formation of pycnidia. The formation of pycnidia by the fungus *B. visci* in the immediate vicinity of the host plant, followed by infestation, indicates a direct relationship with mistletoe. Some studies have also shown that the use of pathogenic fungi such as *B. visci* can be considered as a potential method for the biological control of European mistletoe pathogen (Varga et al. 2012a, Kotan et al. 2013, Tkaczyk and Sikora 2020). As a rule, fungal isolates in the form of fungal suspensions were tested for pathogenicity on the young leaves of white mistletoe by using polyethylene bags (Varga et al. 2012a, Tkaczyk and Sikora 2020) or by using a bottle sprayer in field conditions. According to our results, placing host samples on the surface of the fungal mycelium should be considered as an alternative, easy-to-perform test for phytopathogenicity only during product development phase that allows visual observation of the relationship between the pathogen and the host plant. The phytopathogenicity test of *B. visci* conducted in the field was positive only after mechanical pre-treatment, which is consistent with the findings of Karadžić et al. (2004). A low intensity of infection in our field conditions can be related both to deteriorating climatic conditions (a drop in temperature to negative values in autumn) and to a change in the physiological state of the white mistletoe, which, together with the host plant, suppresses its physiological activity for the winter (Agne et al., 2014, Mutlu et al., 2016). Abiotic factors (temperature, humidity, etc.) have also been identified as key factors for successful infection in studies by Varga et al. (2012a). However, the strongest effect after treating mistletoe plants with a mycopesticide was observed when the mistletoe was weakened or suppressed by previous exposure to chemicals (Baltazár 2016). Pre-treatment with herbicides is also proposed as a way to reduce the vital activity of mistletoe (Baltazár 2016), but this, in our opinion, contradicts the very idea of biocontrol. An alternative approach to suppress mistletoe growth by using bacterial or fungal isolates, such as *Alternaria alternata* and *A. kiliense*, as pathogenic for mistletoe, has also been proposed (Kotan et al. 2013).

Conclusion

The obtained information expands our knowledge on the cultivation of the fungus and may further contribute to

both mycological and pathological studies involving *B. visci* isolates and the diseases they cause. Fungus collected from fresh European mistletoe (*V. album*) growing on *Sorbus aucuparia* L. was identified as an anamorph *Sphaeropsis visci* of the ascomycete *Botryosphaeria visci*. Our results show that it can be easily introduced into culture and that the growth and morphology of *B. visci* colonies can vary greatly depending on the media used. The highest mycelium growth and sporulation was found on liquid oatmeal media. The possibility of European mistletoe re-infection in laboratory and field conditions has been evaluated. Our results indicate the biocontrol potential of *B. visci* against *Viscum album* infecting various trees.

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Aluminum accumulation and tolerance in four *Amaranthus* species

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Abstract – About one-third of the earth's land area consists of acidic soils. The rhizotoxic Al^{3+} is one of the primary constraints associated with low soil pH. Various *Amaranthus* species are important components of the weed flora in tea plantations on acid soils in north Iran. In this study, four *Amaranthus* species (*A. blitoides*, *A. retroflexus*, *A. cruentus*, and *A. tricolor*) were grown under hydroponic conditions with total Al concentrations of 0, 20, 50, 200, and 400 μM corresponding to free Al^{3+} activity of 0, 3.75, 11.97, 60.34, and 125 μM, respectively. Low Al concentrations (20, 50, or 200 μM) stimulated plant growth; *A. tricolor* demonstrated the highest improvement in shoot growth (93%), whereas *A. retroflexus* exhibited the greatest improvement in root biomass (367%), total root length (173%), and taproot length (32%). Although the response of shoot biomass to 400 μM Al varied among species, all species were able to accumulate Al in the leaves above the critical level for Al hyperaccumulation (1 mg g^{-1} DW). Our findings revealed Al accumulation in *Amaranthus* species for the first time at the genus and family levels, suggesting that these species are suitable for the restoration and revegetation of acid-eroded soils.

Keywords: Aluminum hyperaccumulation, *Amaranthus*, tea gardens, weed flora

Introduction

Soil acidity (pH < 5.5) significantly limits plant growth and yield. Approximately 30% of the world's land area comprises acidic soils, which are found in both hemispheres (Von Uexküll and Mutert 1995). Low soil pH considerably impacts the availability of nutrients and other soil elements for plant uptake. A primary effect of low pH is the dissociation and release of the soluble Al ion (Al^{3+}), which is readily absorbed by plants, causing toxicity (Kochian et al. 2005).

The root is the first organ contacting the toxic Al^{3+}. Rhizotoxic Al^{3+} concentrations inhibit cell division in the tips of main and lateral roots, increase cell wall rigidity by cross-linking pectins, and inhibit root elongation (Kopittke et al. 2016). These effects interfere with water and nutrient uptake. Consequently, the degree to which rhizotoxic concentrations of Al^{3+} suppress root growth is a primary determinant of the plant's overall response to Al. Staining intensity of root tips with hematoxylin, a dye that localizes Al, correlates with the Al-sensitivity of plants (Barceló and Poschenrieder 2002) and is used to rank species and genotypes according to Al susceptibility (Cançado et al. 1999).

Plants growing in acidic soils have evolved mechanisms to withstand low pH and Al toxicity (Poschenrieder et al. 2008, Chandra and Keshavkant 2021). Among these plants, certain species have a high capacity to accumulate Al in their leaves, i.e., "Al accumulators". The Al concentration in the aerial parts of accumulator species exceeds the critical value of 1 mg g^{-1} DW and the plants exhibit efficient internal detoxification mechanisms, including Al^{3+} chelation with low molecular weight compounds, especially organic acids and phenolic compounds (Poschenrieder et

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al. 2015). Al accumulator species have primarily been found in the acidic soils of the humid tropics, which are covered by savannahs and tropical rain forests. These species are predominantly shrubs and trees of Melastomataceae, Polygonaceae, Theaceae, Caesalpinaceae, Rubiaceae, and Hydrangeaceae (Jansen et al. 2002, Poschenrieder et al. 2015). Currently, there is limited knowledge regarding the status of Al accumulation in the flora of acid soils from outside the tropics.

In Iran, tea cultivation is restricted to regions with acidic soils in the south of the Caspian Sea, north Iran. In these tea plantations, leaching through rainfall and the use of ammonium fertilizers have drastically decreased the soil pH to values between 4.0 and 4.5. Numerous plant species are growing as weeds in these tea gardens, favored by the moderate temperatures, high humidity, and abundant rainfall. However, there is no information on Al accumulation and tolerance in this weed flora.

Amaranthaceae is a prevalent cosmopolitan family found from the tropics to cool temperate regions. This botanical family consists of annual or perennial plants, primarily herbs, but also occasionally shrubs, small trees, and vines (Simpson 2010). After the combination of Amaranthaceae and Chenopodiaceae into a single family (Judd et al. 1999, Stevens 2001), the extended Amaranthaceae family comprises approximately 175 genera and 2,000 species and is considered the most species-diverse lineage within the Caryophyllales (Müller and Borsch 2005). Important economic crop plants, forage crops, fodder plants, vegetables, and euhalophytes can be found in the Amaranthaceae family, which thrives in various environmental conditions (Simpson 2010). Amaranthaceae contain approximately 50% of all known C_4 species among eudicots (Kadereit et al. 2003) and are distinguished by their diverse chemistry, which includes betalains, flavonoids, and phenolic acids (Mroczek 2015).

Amaranthus L. is a genus of about 70 annual monoecious and dioecious species with C_4 photosynthesis and global distribution (Kigel 1994, Kadereit et al. 2003). Several *Amaranthus* species are used as ornamentals (*A. caudatus*, *A. cruentus*, *A. hypochondriacus*, *A. tricolor*), as medicinal plants (*A. blitum*, *A. caudatus*, *A. cruentus*, *A. dubius*, *A. hypochondriacus*, *A. spinosus*, *A. thunbergh*, *A. tricolor*), vegetable leaves (*A. blitum, A. caudatus, A. cruentus, A. dubius, A. graecizans, A. hybridus, A. hypochondriacus, A. lividus, A. thunbergh, A. tricolor*) or for food grains (*A. caudatus, A. cruentus, A. hypochondriacus*) (Adegbola et al. 2020, Manyelo et al., 2020). Many are found as weeds in agricultural fields (Sellers et al. 2003). Despite their cosmopolitan behavior and the widespread human use, information is lacking on the adaptation to acid soils and Al accumulation capacity in the species of this genus, its family (Amaranthaceae), and order (Caryophyllales) (as per Jansen et al. 2002 and subsequently published works).

In northern Iran's tea gardens, *Amaranthus* species are found as a weed (Hajiboland, unpublished data). The low pH and high Al^{3+} availability in the soils of these tea plantations imply a high Al tolerance and likely Al-accumulating capacity. However, no studies have so far examined these features in these species. This study aimed to investigate Al accumulation and tolerance in four *Amaranthus* species to determine their soil acidity tolerance, Al toxicity, and Al accumulation capacity. Low and high Al treatment levels were applied in both short- and long-term experiments with a special emphasis on the root growth and morphology parameters as indicators of plant Al response. This study may extend our knowledge on Al tolerance and accumulation in Amaranthaceae, a family which has not yet been studied for Al response or accumulation.

Materials and methods

Collection of seeds and cultivation of plants

The seeds of *A. blitoides* and *A. retroflexus* were collected from acid soils of a tea garden (pH 4.5), N Iran, while the seeds of *A. cruentus* and *A. tricolor* were collected from calcareous soil, Khosrow-Shahr (pH 7.5), NW Iran.

The seeds were surface-sterilized with 10% (w/v) sodium hypochlorite and were germinated in perlite under dark conditions at 25 °C. Young seedlings were transferred to light and irrigated with 25% Hoagland nutrient solution (pH 6.0) (Johnson et al. 1957) for another week.

Two-week old plants of similar sizes were transferred to aerated hydroponic pots filled with 100% Hoagland nutrient solution (pH 6.0) and precultured for two weeks.

Plants treatments

A short-term experiment was undertaken for evaluation of the effect of increasing Al concentrations on the shoot growth, various root growth parameters and some biochemical attributes in four *Amaranthus* species. Two-weeks after preculture, the nutrient solution was replaced with a low--strength nutrient solution (pH 4.0) containing macronutrients (mM): 1.2 KNO_3, 0.8 $Ca(NO_3)_2{\times}4H_2O$, 0.1 $NH_4H_2PO_4$, 0.1 $MgSO_4{\times}7H_2O$, 0.3 NH_4Cl, 0.1 $MgCl_2$ and micronutrients (μM): 10 KCl, 5 H_3BO_3, 0.4 $MnSO_4{\times}H_2O$, 0.4 $ZnSO_4{\times}7H_2O$, 0.1 $CuSO_4{\times}5H_2O$, 0.1 H_2MoO_4, 4 Fe-EDTA with four Al levels (0, 20, 50, 200 and 400 μM as $AlCl_3$).

The activity of free Al^{3+} was 0, 3.75, 11.97, 60.34 and 125 μM respectively calculated by GEOCHEM-PC (Parker et al. 1995).

Three weeks after starting the treatments, plants were harvested and the fresh weight (FW) and dry weight (DW) of the shoots and roots were determined. The tap root length was measured with a ruler and the total length of roots was determined by the line intercept method (Tennant 1975).

In addition, some physiological and biochemical parameters including leaf chlorophyll content, chlorophyll fluorescence, photosynthetic rate, anthocyanin concentration

and activity of nitrate reductase were determined in the young leaves (the second and third youngest, fully-expanded leaves) of the experimental plants treated with 0, 50 and 400 μM Al as described below.

Root staining was undertaken in plants treated with 0, 50, 200 and 400 μM Al according to the method described below.

A long-term experiment was undertaken for evaluation of the long-term effect of Al treatment on the biomass of and Al accumulation capacity in four *Amaranthus* species. Plants were grown with addition of 0, 50, 200 and 400 μM of $AlCl_3$ in the hydroponics as described above. Six weeks after start of the treatments, plants were harvested.

In addition to measurement of the biomass, Al concentrations in the young (the second youngest) leaves and old (the second oldest) leaves were analyzed in the experimental plants as described below.

In both experiments, the plants were grown in a growth chamber with 16/8 h of light/dark photoperiod at 25/17 °C, relative humidity of 50–60%, and at a photon flux density of about 400 μmol m^{-2} s^{-1} provided by fluorescent lamps. The nutrient solutions were replaced weekly with fresh solutions.

Root staining

Haematoxylin staining of root apices was performed according to Polle et al. (1978). Roots were excised at 20-mm distance from the tip, rinsed with distilled water for 15 min, and subsequently placed in micro-tubes filled with 0.2% haematoxylin and 0.02% (w/v) potassium iodide (KI) for 20 min. Thereafter, the root tips were washed three times with distilled water, mounted on the glass slides and photographed.

Determination of leaf chlorophyll content, chlorophyll fluorescence and gas exchange parameters

The leaf chlorophyll content was measured as SPAD value with soil plant analysis development (SPAD) chlorophyll meter (SPAD-502, Minolta). The maximum quantum efficiency of PSII (F_v/F_m) was recorded in the attached leaves using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK).

The net photosynthetic rate (A) was determined using a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) during the light period between 9:00 and 13:00 h under a photosynthetic photon flux density of approximately 300 μmol m^{-2} s^{-1} provided by incandescent lamps.

Determination of leaf anthocyanins

The leaf anthocyanin concentration was analyzed using a pH differential method (Tonutare et al. 2014). The leaves were extracted in methanol/HCl (98:2, v/v) and the homogenate was centrifuged at 5000 *g*. The supernatant was diluted (1:2) with two different buffers including 25 mM KCl (pH 1.0) and 400 mM Na acetate (pH 4.5). The absorbance of both solutions was determined at 510 and 700 nm and the anthocyanin content was expressed as the cyanidin-3-O-glucoside equivalents using its extinction coefficient of 26900 M^{-1} cm^{-1} (Tonutare et al. 2014).

Assay of nitrate reductase activity

Activity of NR (E.C. 1.6.6.1) was determined using the method described by Jaworski (1971). Leaf samples were cut into 5-10 mm sections and placed in incubation buffer (100 mg tissue for 10 mL of buffer) containing 25 mM potassium phosphate buffer (pH 7.2), 25 mM KNO_3 and 1% Triton X-100. The samples were infiltrated using a vacuum (80 kPa). After 3 h, the vacuum was released and the samples were incubated at 30 °C in darkness for 1 h, then placed in a boiling water bath to stop the NR activity. The resulting nitrite was determined spectrophotometrically at 540 nm in a reaction mixture containing sulfanilamide and naphthylethylenediamine dihydrochloride (N-NEDA).

Elemental analyses

Oven-dried samples of the young and old leaves were ashed at 500 °C in a muffle furnace then were resolved in 10% HCl and made to volume by double-distilled water. The aluminum concentration was determined by an atomic absorption spectrophotometer (AA-6300, Shimadzu, Japan).

Experimental design and statistical analyses

The experiment was undertaken using a completely randomized design with four independent pots as four replicates. Pairwise comparison of means was performed by Tukey's test ($P < 0.05$).

Results

Plant biomass and root morphology after three weeks of growth in different Al concentrations

The shoot biomass of two species whose seeds were collected from acidic soils (*A. retroflexus* and *A. blitoides*) was significantly improved with 20 and 50 μM Al, but was constricted under 200 and 400 μM Al (Fig. 1A, C).

Like the shoot biomass, the root biomass was increased in plants exposed to 20 and 50 μM Al in both species. The highest Al concentration (400 μM) significantly decreased root biomass in both species, whereas 200 μM Al had no effect (Fig. 1B, D).

In the two species whose seeds were collected from calcareous soils (*A. cruentus* and *A. tricolor*), low concentrations of Al (20 and 50 μM) also improved the shoot DW (Fig. 2A, C). This parameter was repressed by 400 μM Al in *A. cruentus,* but showed no effect in *A. tricolor* (Fig. 2A, C).

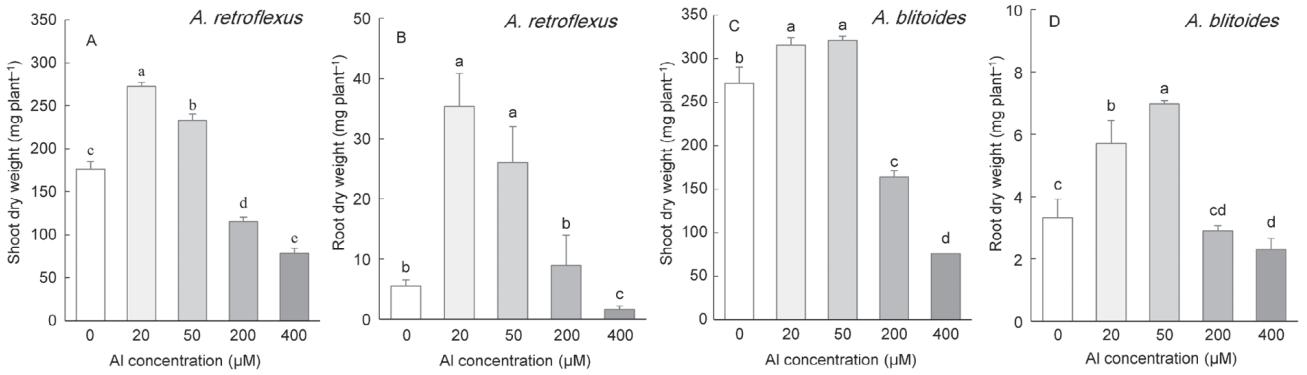

Fig. 1. Shoot and root biomass in *Amaranthus retroflexus* (A, B) and *A. blitoides* (C, D) grown for three weeks under different Al concentrations in the hydroponic medium. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$).

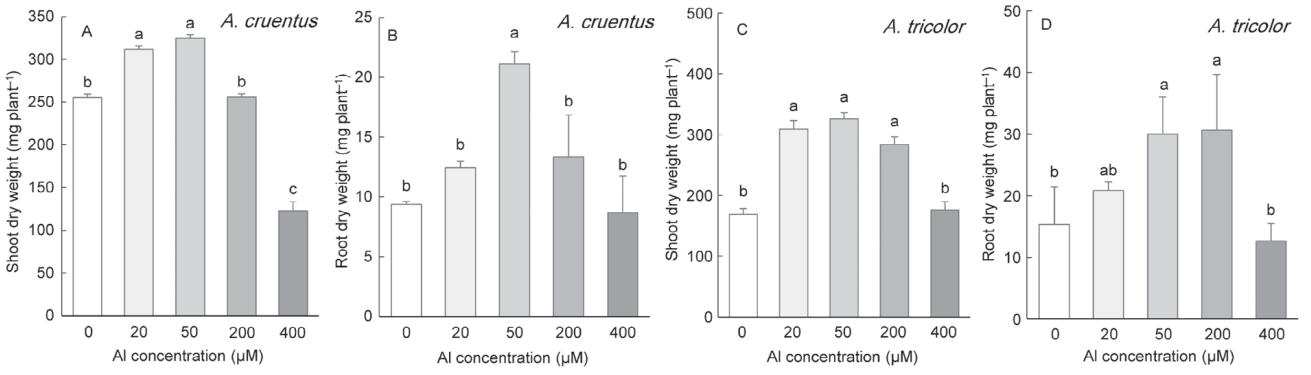

Fig. 2. Shoot and root biomass in *Amaranthus cruentus* (A, B) and *A. tricolor* (C, D) grown for three weeks under different Al concentrations in the hydroponic medium. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$).

Even at the highest Al concentration applied to the medium (400 μM Al), root biomass was not inhibited. The root DW increased at 50 μM Al in both species and at 200 μM Al in *A. tricolor* (Fig. 2B, D).

Al treatment significantly influenced the root morphology of *A. retroflexus* and *A. blitoides* (Fig. 3). In *A. retroflexus*, both the taproot length and the total root length were stimulated by 20 to 200 μM Al, but inhibited by 400 μM Al. (Fig. 3A-C). The application of 20 μM Al to *A. blitoides* substantially increased the taproot length. However, total root length was increased with Al concentrations up to 50 μM. These parameters were not affected by 200 μM Al in this species but were significantly inhibited by 400 μM Al (Fig. 3D-F).

A. cruentus and *A. tricolor* exhibited a similar effect of Al on root morphology (Fig. 4). Taproot length was significantly greater in plants exposed to 20 μM Al (in *A. cruentus*) or 20–50 μM Al (in *A. tricolor*), remained unchanged at 200 μM Al, but decreased significantly under 400 μM Al in both species (Fig. 4A, D). In response to a range of 20–200 μM Al concentrations, however, the total root length of both species was stimulated. Both species exposed to 400 μM Al exhibited a significant reduction in root length (Fig. 4B, E).

According to relative increase (% over control) in shoot growth under 50 μM Al, *A. tricolor* was the most responsive species with a 93% increase, followed by *A. retroflexus* with a 32% increase. *A. retroflexus* exhibited the greatest stimulation of root biomass (367%), total root length (173%), and taproot length (32%) (On-line Suppl. Fig. 1A).

Al susceptibility based on the relative inhibition of shoot biomass by 400 μM Al followed the order *A. blitoides* > *A. retroflexus* > *A. cruentus* > *A. tricolor* (On-line Suppl. Fig. 2B).

The inhibitory effect of Al on root growth was, however, dependent on the growth parameters, *A. retroflexus* was most sensitive to 400 μM Al as measured by root biomass and total root length, while it was the second most susceptible species in terms of taproot length. Similarly, *A. cruentus* was the most resistant to Al concerning root biomass, but the most sensitive for taproot length. In addition, the response of shoot growth was independent of that of root

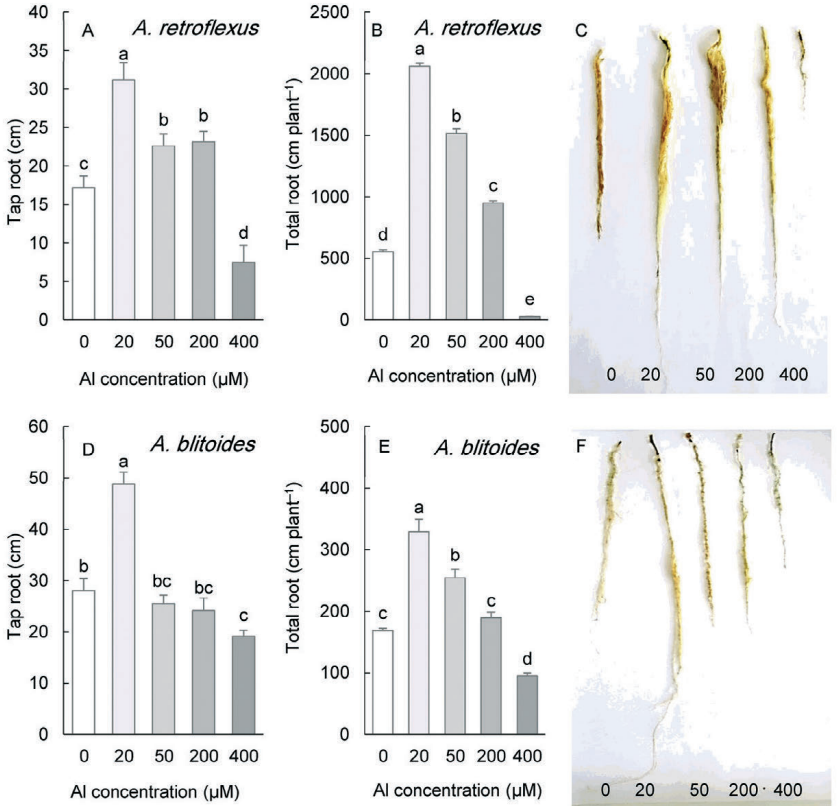

Fig. 3. Length of the tap roots and total length of the roots in *Amaranthus retroflexus* (A, B) and *A. blitoides* (D, E) grown for three weeks under different Al concentrations in the hydroponic medium. The right panels (C, F) represent the roots of plants under different Al concentrations. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$).

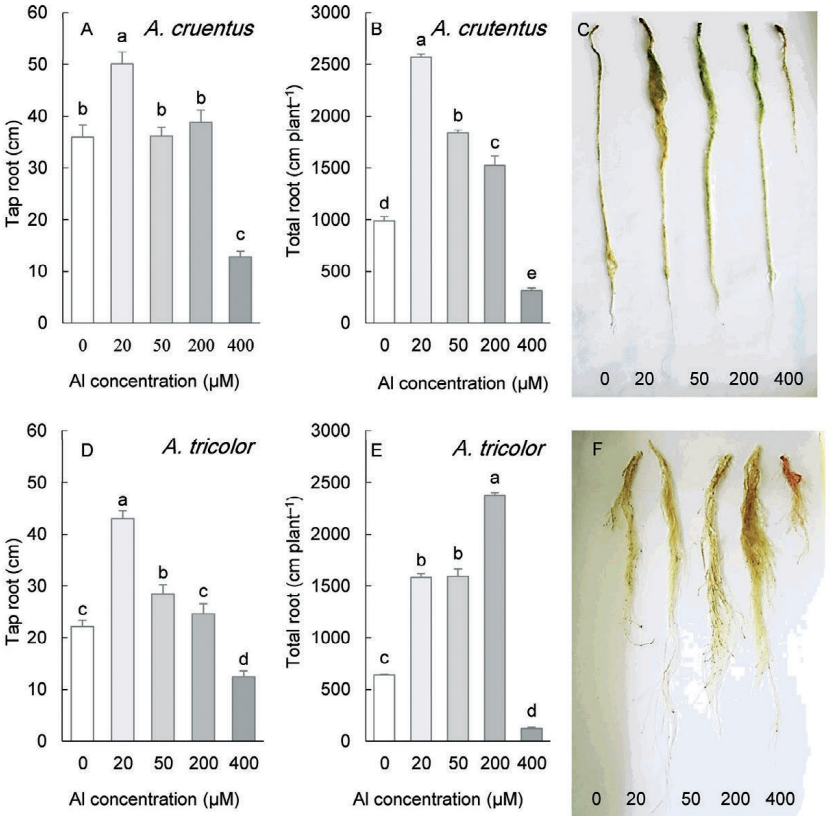

Fig. 4. Length of the tap roots and total length of the roots in *Amaranthus cruentus* (A, B) and *A. tricolor* (D, E) grown for three weeks under different Al concentrations in the hydroponic medium. The right panels (C, F) represent the roots of plants under different Al concentrations. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$).

growth. *A. blitoides* was the most susceptible regarding the response of shoot biomass to 400 μM Al, whereas it was the most tolerant species regarding the response of taproot and total root length (On-line Suppl. Fig. 1B).

The principal component analysis (PCA) of growth parameters revealed a close relationship between the tap root length and total root length under low Al concentration (50 μM Al), while the root biomass was clustered separately. The shoot biomass response was also weakly correlated with root growth parameters under low Al concentration (On-line Suppl. Fig. 1C). Under higher Al concentration (400 μM), a poor correlation was observed between the shoot and root Al responses (On-line Suppl. Fig. 1D).

Root staining with hematoxylin following three weeks of growth at varying Al concentrations

The violet-blue color of the Al-haematoxylin complex was not observed in the roots of *A. retroflexus* plants treated with 50 μM Al. In *A. blitoides* and *A. tricolor*, only the 0–2 mm root tips were stained with hematoxylin, while in *A. cruentus*, both the root tip and basal parts (0–15 mm) were stained (On-line Suppl. Fig. 2). Except for *A. cruentus*, which displayed a uniform hematoxylin staining at 200 μM Al, the root tips of the other three species displayed a localized hematoxylin staining between 0 and 2 mm. Under the highest Al concentration applied to the plants (400 μM), the basal root parts of all four studied species stained visibly. However, the intensity of staining was significantly greater in *A. blitoides* and *A. cruentus* than in *A. retroflexus* and *A. tricolor* (On-line Suppl. Fig. 2).

Physiological and biochemical parameters after three weeks of growth at various Al concentrations

Low (50 μM) and high (400 μM) Al treatments decreased leaf SPAD values in *A. retroflexus*, whereas only high Al concentration affected this parameter in *A. blitoides* and *A. cruentus* (Tab. 1). In *A. tricolor*, however, leaf SPAD values remained unaffected under either of the Al treatments (Tab. 1).

The maximum quantum yield of PSII (F_v/F_m) decreased by the highest Al treatment (400 μM) in *A. retroflexus* and *A. blitoides*. This parameter was unaffected by Al levels in *A. tricolor* but increased in *A. cruentus* when a low Al concentration (50 μM) was applied (Tab. 1).

The photosynthesis rate was reduced by both Al concentrations (50 and 400 μM) in *A. retroflexus* and *A. tricolor*, it decreased at the highest Al level (400 μM) in *A. blitoides*, but was unaffected by any of the applied Al concentrations in *A. cruentus* (Tab. 1).

The concentrations of anthocyanins in the leaves of *A. retroflexus* were unaffected by Al, while a simulation was observed with increasing Al levels in the other three species. The constitutive concentrations of anthocyanin were highest in *A. tricolor*, followed by *A. cruentus* (Tab. 1) and lower in *A. retroflexus* and *A. blitoides*.

The leaf activity of NR increased under 50 μM Al in all four studied species (Tab. 1).

The response of NR activity to higher Al concentration (400 μM) was species-dependent, it decreased in *A. cruentus*, remained unchanged in *A. retroflexus* and *A. blitoides*, and increased in *A. tricolor* (Tab. 1).

Tab. 1. Leaf chlorophyll content as SPAD (soil plant analysis development) value, maximum quantum yield of PSII (F_v/F_m), photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}), concentrations of anthocyanins (μg cyaniding-3-glucoside g^{-1} FW) and activity of nitrate reductase (NR, nmol NO_2^- mg^{-1} prot. min^{-1}) in the young leaves of four *Amaranthus* species grown hydroponically and treated with three different Al (0, 50 and 400 μM) concentrations for three weeks. Data of each column within each species indicated by the same letters are not significantly different ($P < 0.05$). Prot. – proteins, FW – fresh weight.

Plant species	Al treatments	SPAD	F_v/F_m	A	Anthocyanins	NR activity
A. retroflexus	0 μM	30.9 ± 1.7 [a]	0.71 ± 0.00 [a]	7.39 ± 0.47 [a]	45 ± 11 [a]	1.10 ± 0.15 [b]
	50 μM	24.4 ± 1.1 [b]	0.69 ± 0.02 [a]	5.97 ± 0.06 [b]	47 ± 5 [a]	1.99 ± 0.14 [a]
	400 μM	25.0 ± 0.1 [b]	0.62 ± 0.02 [b]	5.15 ± 0.57 [b]	48 ± 8 [a]	1.10 ± 0.06 [b]
A. blitoides	0 μM	43.7 ± 0.7 [a]	0.71 ± 0.03 [a]	6.41 ± 0.17 [a]	54 ± 2.3 [c]	0.56 ± 0.17 [b]
	50 μM	43.6 ± 0.9 [a]	0.71 ± 0.01 [a]	5.85 ± 0.13 [a]	87 ± 6.2 [b]	1.79 ± 0.06 [a]
	400 μM	41.1 ± 0.5 [b]	0.61 ± 0.01 [b]	4.22 ± 0.46 [b]	107 ± 13 [a]	0.56 ± 0.07 [b]
A. cruentus	0 μM	24.8 ± 1.4 [ab]	0.70 ± 0.02 [b]	8.20 ± 1.50 [a]	102 ± 10 [c]	1.86 ± 0.11 [b]
	50 μM	26.6 ± 1.3 [a]	0.76 ± 0.00 [a]	7.87 ± 0.33 [a]	159 ± 19 [b]	2.41 ± 0.13 [a]
	400 μM	22.3 ± 1.7 [b]	0.68 ± 0.02 [b]	6.58 ± 0.31 [a]	232 ± 26 [a]	1.09 ± 0.18 [c]
A. tricolor	0 μM	35.8 ± 1.7 [a]	0.74 ± 0.03 [a]	9.25 ± 0.23 [a]	159 ± 19 [c]	0.82 ± 0.03 [c]
	50 μM	35.1 ± 2.0 [a]	0.74 ± 0.01 [a]	7.75 ± 0.05 [b]	240 ± 14 [b]	1.86 ± 0.15 [a]
	400 μM	32.7 ± 2.1 [a]	0.74 ± 0.00 [a]	5.78 ± 0.06 [c]	486 ± 37 [a]	1.58 ± 0.11 [b]

Biomass and Al accumulation following six weeks of growth at various Al concentrations

Under long-term cultivation (six weeks), Al concentrations below 400 μM did not significantly affect shoot growth in any *Amaranthus* species (Fig. 5 and Fig. 6).

In contrast, the root biomass increased under 50 or 200 μM Al, depending on the species, except for *A. tricolor*, whose root biomass did not change in this Al concentration range.

The highest Al treatment level (400 μM) significantly reduced the shoot and root biomass of *A. blitoides* and *A. retroflexus* but did not affect the shoot biomass of *A. cruentus* and *A. tricolor* (Fig. 5 and Fig. 6).

Al accumulation in the leaves increased as Al concentration in the medium increased (Fig. 7).

The accumulation of Al was greater in older leaves than in younger leaves ($P < 0.05$). Al accumulation in the old leaves of plants treated with 400 μM Al exceeded the critical level established for Al accumulators in all studied species (Fig. 7). In the old leaves of *A. blitoides* and *A. cruentus*, Al accumulation of approximately 1 mg g^{-1} DW was also caused by 200 μM Al treatment (Fig. 7B, C).

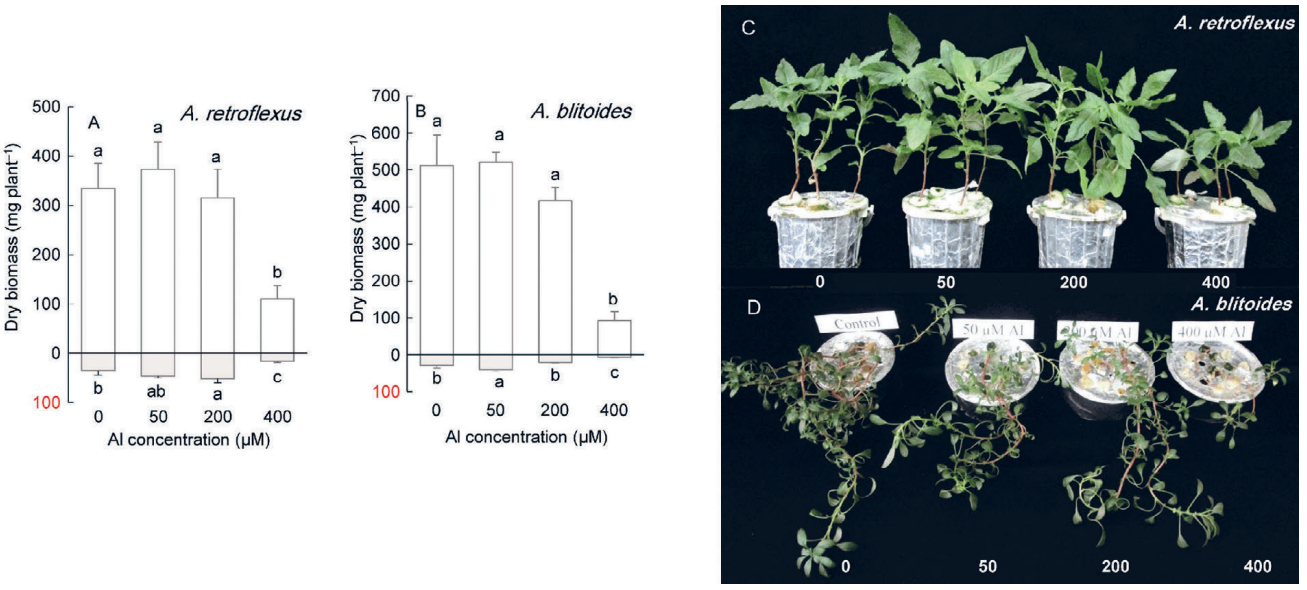

Fig. 5. Shoot (above the x-axis) and root (below the x-axis) biomass in *Amaranthus retroflexus* (A) and *A. blitoides* (B) grown for six weeks under different Al concentrations in the hydroponic medium. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$). Pictures (C, D) show the plants at harvest.

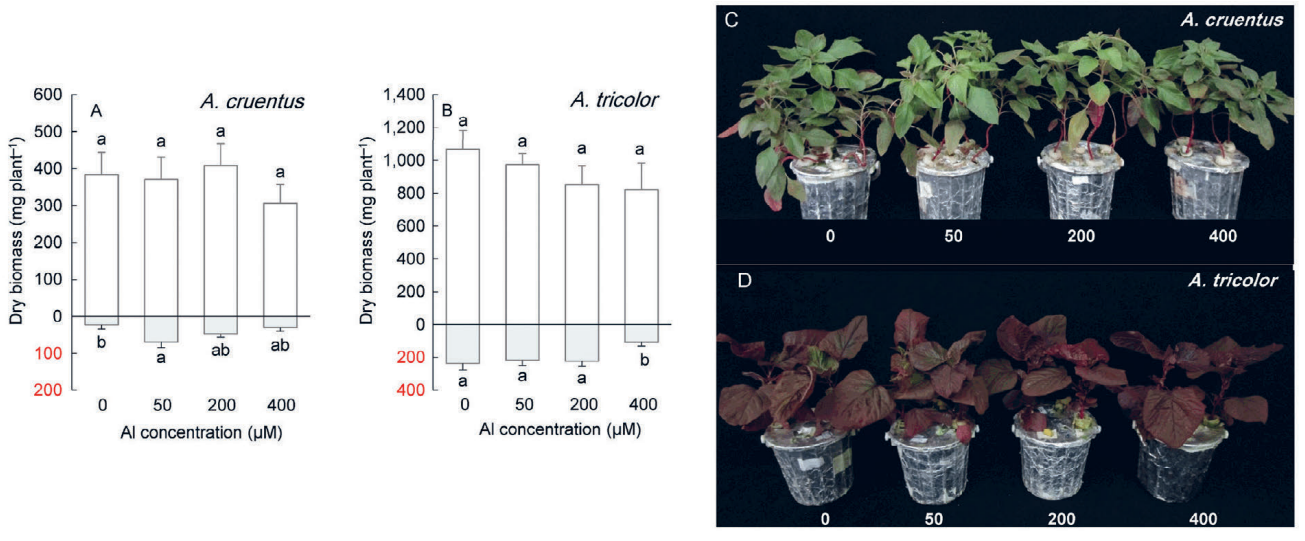

Fig. 6. Shoot (above the x-axis) and root (below the x-axis) biomass in *Amaranthus cruentus* (A) and *A. tricolor* (B) grown for six weeks under different Al concentrations in the hydroponic medium. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$). Pictures (C, D) show the plants at harvest.

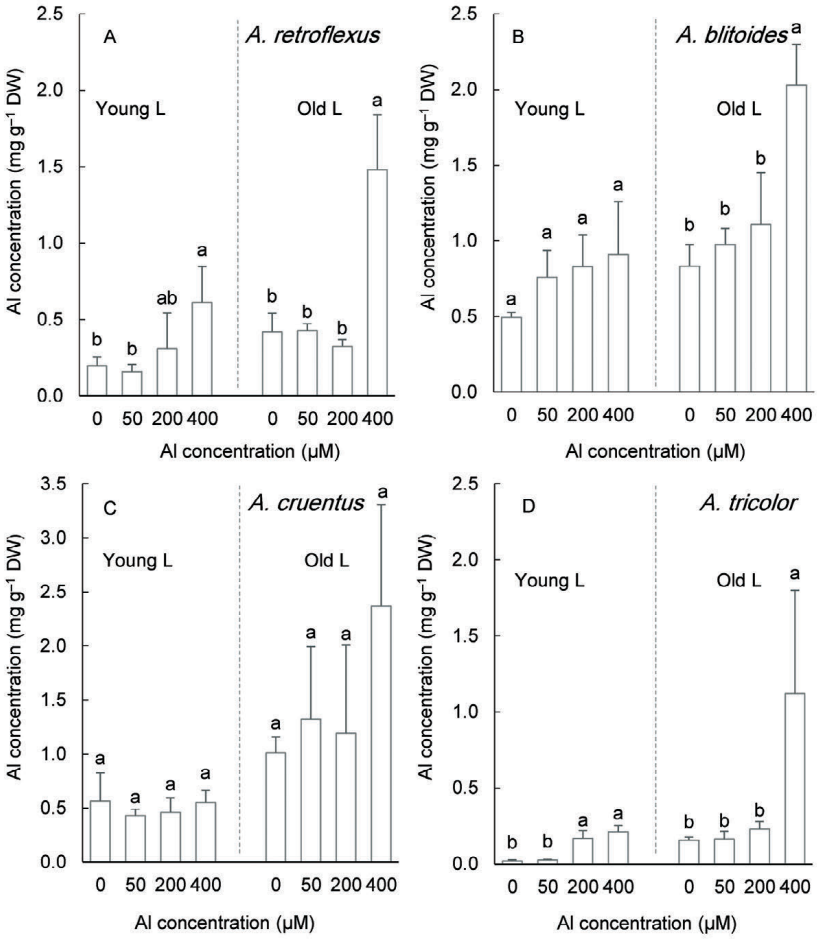

Fig. 7. Aluminum concentration in the young and old leaves (L) in four *Amaranthus* species (A – *A. retroflexus*, B – *A. blitoides*, C – *A. cruentus*, D – *A. tricolor*) grown for six weeks under different Al concentrations in the hydroponic medium. Data are mean values of 4 replicates ± standard deviation. Bars indicated by the same letters are not significantly different ($P < 0.05$). DW – dry weight.

Discussion

After three weeks of treatment with low Al concentrations (20–50 μM), the shoot biomass of all four *Amaranthus* species was significantly higher than that in control plants (Fig. 1 and Fig. 2). Even 200 μM Al increased the shoot biomass by 68% in *A. tricolor*. Growth stimulation by low concentrations of Al is relatively common in plant species adapted to acid soils and has been attributed to activation of photosynthesis and nitrogen assimilation, enhancement of nutrient uptake, alleviation of the toxic effects of H^+, Fe^{2+} and Mn^{2+}, activation of antioxidant defense and reduction of lignin (Bojórquez-Quintal et al. 2017, Hajiboland et al. 2022). In our work, only the activity of NR was improved among the physiological and biochemical parameters determined for the *Amaranthus* species. Nonetheless, higher nitrogen assimilation was not associated with a higher photosynthetic rate under these conditions, thus, its role in the growth promotion of these species remains unclear. Enhanced capacity for nutrient acquisition resulting from root growth stimulation observed in the experiment, could also be an additional mechanism of higher shoot biomass production in soil-grown plants.

In contrast to the root biomass, the improvement of shoot growth mediated by low Al concentrations disappeared under longer periods of time in all four studied species (Fig. 5 and Fig. 6). Such transient growth responses may be the consequence of the attenuation of the factors responsible for growth improvement under short-term Al exposure, but the underlying causes remain obscure. Regardless of the mechanisms, a significant growth improvement by Al in *Amaranthus* species during the early growth stage may be of great importance for the establishment of young plants in acidic soil conditions and is likely an advantage over species lacking such stimulating responses to Al.

The elongation rate of roots in response to Al exhibits three distinct patterns: growth stimulation by low Al level or short-term Al exposure due to alleviation of proton toxicity, consistent reduction of elongation due to Al^{3+} toxicity, and induction of tolerance mechanisms and compensation of growth inhibition following a transient reduction (Barceló and Poschenrieder 2002). In this study, the taproot length, which may directly reflect the Al effect on the root elongation rate, was seen to be significantly stimulated in all *Amaranthus* species within a lower Al concentration range, the most prominent effect being observed at 20 μM Al, with

a 39–95% increase depending on the species. However, a higher Al concentration (200 μM) only stimulated the taproot length in *A. retroflexus*.

At 400 μM Al concentration, a significant decrease in the tap-root length was observed in all studied species. Rhizotoxic Al^{3+} inhibits root cell multiplication and expansion, thereby preventing its elongation (Doncheva et al. 2005). Recently, the involvement of the plant DNA damage pathway in the root response to Al has been demonstrated. Al-mediated root growth inhibition results from DNA damage and induction of repair mechanisms by the components of this pathway, which ultimately stop cell cycle progression (Pedroza-Garcia et al. 2022).

The effect of Al on root growth was more pronounced when biomass data and total length were considered. Al concentrations (50–200 μM, depending on species) improved these parameters without causing an increase in tap root length (Fig. 3 and Fig. 4). Under these circumstances, more lateral root initials developed, resulting in an increase in total root length and root biomass. In susceptible species, this range of Al concentrations (12–60 μM free Al^{3+} activity) is primarily toxic causing strong root growth inhibition (Poschenrieder et al. 2008). The Al-induced increase in ethylene and auxin, both of which are closely related to root growth, elicits significant adaptive responses to Al. These phytohormones are responsible for initiating more lateral roots in Al-tolerant species (Kopittke 2016), modifying the cell wall to reduce Al binding, and regulating the release of organic acids (Gao et al. 2022). Al-mediated improvement of root growth may be a crucial adaptive strategy for species native to acidic soils. A greater spatial availability may be vital for nutrient-poor, acidic soils typical of some regions, such as the Cerrado biome (Haridasan 2008). In addition, higher lateral root initiation primarily located in the upper portions of the roots in *Amaranthus* species may be more advantageous for plants in capturing the available nutrients in the topsoil. This soil profile is highly susceptible to leaching and nutrient loss, particularly of phosphorus (Withers et al. 2003).

The most Al^{3+}-sensitive portion of the root apex is the transition zone, which is the zone between the active cell division zone and the fast cell elongation zone (Sivaguru and Horst 1998, Poschenrieder et al. 2008). In the present study, the absence of hematoxylin staining in the 1.0–5.0 mm tip region (coincident with the transition and elongation zones) under 20–200 μM Al concentrations in *Amaranthus* species (except *A. cruentus*) suggests that these species are able to inhibit the binding of Al^{3+} to the cell wall fractions in this zone, thereby preventing Al-induced inhibition of root elongation. As in other Al-tolerant species (Barceló and Poschenrieder 2002), excluding Al^{3+} from the vulnerable root zones, i.e., apoplastic detoxification, was most likely accomplished via the exudation of chelating molecules, such as phenolics and organic acids. Excretion of oxalate and citrate in *A. hypochondriacus* (Fan et al. 2016) and phenolics in *A. blitoides* (Hajiboland, unpublished data) has been found in Al-exposed plants. Greater staining of the root axis under 50 μM Al in *A. cruentus* was associated with the least stimulation of tap root length of all the four species. Nevertheless, lower staining under 400 μM Al in *A. retroflexus* and *A. tricolor* was not correlated with less growth inhibition of total root length or taproot length in these species. Under these conditions, Al was likely entered into the root-tip symplast, resulting in less cell wall localization. The transporter mediating uptake of Al in a chelated form with organic acids (NIP1;2) has been identified in *Arabidopsis*, which is responsible for removing Al^{3+} from the root cell wall (Wang et al. 2017).

In four *Amaranthus* species, higher growth stimulation at low Al concentrations (20–50 μM) was not necessarily associated with greater tolerance to the toxic Al concentration (400 μM). This is likely due to the involvement of two distinct mechanisms in the plant's response to these varying Al concentrations. The growth improvement by low concentrations of metal ions (including Al^{3+}) is primarily attributable to enhanced tolerance to certain latent stress factors (the hormesis effect) (Poschenrieder et al. 2013). In contrast, tolerance to higher Al concentrations is due to specific mechanisms for sequestration and internal detoxification of Al^{3+} (Bojórquez-Quintal et al. 2017, Hajiboland et al. 2022).

The root growth response has been considered a major criterion for the evaluation of the response of whole plants to Al (Barceló and Poschenrieder 2002). However, in our hydroponic study, root and shoot growth responded differently to Al (On-line Suppl. Fig. 2). In addition, although the beneficial effect of Al on root growth was observed in both short- and long-term treatments, no improvement in shoot biomass growth was detected in the long term Al treatments (Fig. 5 and Fig. 6). The absence of a correlation between shoot and root growth under both beneficial and toxic Al^{3+} concentrations was likely the result of sufficient nutrient availability in the hydroponic medium, independent of the root surface area. However, root function may be crucial for the shoot response to Al for soil-grown plants due to its important role in nutrient acquisition from acid soil that tends to be deficient in essential macronutrients such as P, Ca, Mg, and K. The availability and concentration of these nutrients are major limiting factors for plant performance under acidic soil conditions (Kochian et al. 2004).

The biosynthesis of anthocyanins, a large group of water-soluble secondary plant metabolites, is stimulated by environmental stresses (Sakihama et al. 2002), indicating a functional relationship between their accumulation and stress adaptation. The formation of a complex between Al and anthocyanins has been shown under *in vitro* conditions (Moncada et al. 2003). The hydroxyl substituents in the B-ring of anthocyanins are bound to metal ions such as Al and form supramolecular complexes with colorless molecules (Quina et al. 2009), resulting in the flower's color in Al-accumulator species such as hydrangea (Schreiber et al. 2011). However, the function of anthocyanins as chelating molecules for internal Al detoxification has not been addressed directly. *Amaranthus* species are abundant in anthocyanins (Paśko et al. 2009), however, their significance for Al chela-

tion and detoxification has not yet been studied. In the present study, Al accumulation in the leaves of four *Amaranthus* species was not associated with correspondingly higher anthocyanin concentration. However, in the long-term experiment (Fig. 6), the red colored species (*A. cruentus* and *A. tricolor*) exhibited much less inhibition of shoot growth under 400 μM Al compared to the green colored species (*A. retroflexus* and *A. blitoides*) (Fig. 5). This may be indirect evidence for a role of anthocyanin in Al detoxification in the leaves.

After six weeks of growth under 400 μM Al, the concentration of Al in the old leaves of four *Amaranthus* species reached the critical Al accumulation level (1 mg g^{-1} DW). The Al concentrations in the old leaves of *A. blitoides* and *A. cruentus* were greater than this critical level even under 50 and 200 μM Al treatments, which suggests that these species have a higher accumulation capacity than others.

Conclusions

There is currently a lack of information regarding the tolerance of *Amaranthus* species to acid soils, particularly their Al accumulation capacity, despite their global distribution and adaptation to various soil and climate conditions. According to this study, all the four *Amaranthus* species grown in a hydroponic medium exhibited a high Al tolerance and Al accumulation capacity. Although the seeds of these species were collected from acid (*A. blitoides* and *A. retroflexus*) or calcareous (*A. cruentus* and A. *tricolor*) soils, there was no effect of habitat on their tolerance level to Al. This indicates that these species have the capacity to adapt to a broad range of soil conditions which is consistent with their cosmopolitan nature. However, for exploring an intraspecific variation driven by different soil pH, the comparison of plant populations from different habitats with contrasting soil pH is necessary.

According to our findings, these *Amaranthus* species could be used for the restoration and revegetation of acid-eroded soils, as model species for the physiological and molecular study of internal Al detoxification, and for phylogenetic studies by botanists interested in the origin of the Al accumulation trait in angiosperms. Considering the edible nature of some *Amaranthus* species, the Al concentration in the old leaves should be regarded as a potential human health risk.

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Sugar beet cells' cellular and extracellular events taking place in response to drought and salinity

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Abstract – Salt and drought stress are important abiotic factors that negatively affect plant growth and yield. To understand how these stress factors affect metabolism at the cellular level, we analyzed cation concentrations and expression of cellular and extracellular proteins, as well as their functions and types. Cells of the industrially important halophyte sugar beet were exposed to 300 mM NaCl and 600 mM mannitol as stressors in modified Gamborg B_5 liquid nutrient medium (PG0). Severe stress altered the intracellular concentrations of most of the measured cations. The cellular proteome revealed that both stressors provoked significant differential regulation of 110 cellular proteins. About 80% of the identified proteins were classified in metabolism, energy, or cell rescue, defense and virulence categories. We identified several novel proteins that respond to stress, including a member of the bZIP family of transcription factors, a member of the glycine-rich RNA-binding proteins, and the K^+ channel beta subunit. Among extracellular proteins we found previously unreported stress-responsive proteins, a beta-xylosidase and an isoform of chitinase. The obtained results indicate that salt and drought stress disturbed the concentrations of cellular cations and affected the expression of cellular and extracellular proteins in sugar beet cells.

Keywords: extracellular proteins, mannitol, osmotic stress, proteome, salt stress

Introduction

Salt stress and drought are major abiotic stressors that significantly affect all aspects of plant physiology, resulting in yield losses of more than 50% and a loss of more than $10.3 billion per year (Ma et al. 2020). Future global scenarios, envisioned by the Intergovernmental Panel on Climate Change indicate a decrease in precipitation and an increase in evapotranspiration rates (Pörtner et al. 2022). Initially, both stressors cause water deficit in plants, but under prolonged salinity, plants respond to hyper-ionic and hyper-osmotic stress in addition to dehydration (Chaves et al. 2009). Plant physiological responses to these stressors aim to minimize water deficit and restore ion homeostasis (Ma et al. 2020). Osmotic adjustments are achieved through the synthesis and accumulation of osmoprotective compounds (Chen and Murata 2002), while disturbances caused by excess sodium ions are remedied by changes in the activity and abundance of sodium/proton exchangers (Deinlein et al. 2014). In addition, both salinity and drought can induce the production of reactive oxygen species (ROS) that can cause lipid, protein, and DNA damage, which, however, can be counteracted by complex nonenzymatic antioxidants (glutathione, ascorbate, carotenoids) and antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) (Apel and Hirt 2004).

Transcriptomics studies in Arabidopsis revealed that 1008 and 1123 mRNAs are regulated in response to water deficit and salt stress, respectively (Apel and Hirt 2004), implying that both stresses involve complex processes. In sugar beet, an experiment with salinization and alkalinization identified 4773 and 2251 differentially expressed genes in leaves and roots, respectively (Geng et al. 2020). This and other studies identified ROS-scavenging enzymes, ion transporters and channels, proteins involved in signal transduction, and regulatory proteins, kinases, phosphatases, and transcription factors responsible for triggering the

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stress response (Zhu 2002, Yoshida et al. 2014). However, changes in mRNA levels do not correlate well with protein levels, and many gene products undergo posttranslational modifications that can alter protein activity (Varshavsky 1996, Deyholos 2010). Therefore, protein levels must be determined directly rather than extrapolated from transcript abundance. A complementary approach is to use proteomic analyses such as two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) to quantify protein abundance and identify it on a large scale (Hajheidari et al. 2005). To date, several papers have been published analysing the response to salt and drought stress using proteomic approaches (Hajheidari et al. 2005, Singh et al. 2022). Although there are common proteins that are regulated during salt and drought stress, each plant species has been shown to respond differently to these stressors. The differences between salt-tolerant (halophytes) and salt-sensitive (glycophytes) plants are particularly pronounced (Askari et al. 2006, Zhang et al. 2012). Most proteomic research has focused on changes in the abundance of cellular proteins, while knowledge of stress-induced expression of extracellular proteins is limited. Only recently, it has been shown that the extracellular matrix and its constituent proteins are involved in the response to various stressors in rice, poplar and sweet potato (Zhang et al. 2009, Kim et al. 2013).

In this study we aimed to gain insight into the cellular and extracellular processes involved in responses to drought and salinity. To this end, we decided to use cells grown *in vitro*, since they represent a homogeneous system in which all cells are of similar origin and type, and the conditions of plant tissue culture allow the control of stress homogeneity and the characterization of cell behavior under stress conditions independently of the regulatory systems acting at the whole plant level (Errabii et al. 2007). On the other hand, plants are composed of a number of cell types that exhibit different cellular characteristics leading to different responses to stimuli. The N, HO, and HNO sugar beet cell lines have proven useful as *in vitro* models for studying epigenetic mechanisms, cell differentiation, and metabolism in plants (Le Dily et al. 1990, Causevic et al. 2006). The N line is a normal callus dependent on plant growth regulators, in contrast to the autonomous habituated HNO line and the tumorous T line, which is the result of cell transformation with the *Agrobacterium tumefaciens* Ti plasmid B6S3 (Pavoković et al. 2012b). In this study, we used the differentiated N line, which contains mainly parenchyma cells. It is photosynthetic and grows in response to 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. It exhibits normal nuclear morphology and cell wall cellulose deposition (Pavoković et al. 2012b). To investigate the effects of salt- and mannitol-induced stress at the cellular level, we sought to identify stress-related proteins that are differentially expressed in non-stressed and stressed cells. We also determined possible changes in the concentrations of cellular macro- and microelements. In addition, this study was extended by analyzing the expression of stress-related extracellular proteins. We report the disruption of macro- and microelement homeostasis as a consequence of stress and the identification of novel proteins in sugar beet as stress-related proteins.

Materials and methods

Plant material

Sugar beet N cell line (*Beta vulgaris* L. subsp. *vulgaris* var. *altissima* Döll) was grown *in vitro* in modified Gamborg B_5 liquid nutrient medium (PG0) (Negrutiu et al. 1975, Pavoković et al. 2007). The growth chamber was maintained at 22 °C and a 16-h photoperiod (80 μmol photons m^{-2} s^{-1}). Cells were subcultured every two weeks by transferring 10 mL of the old cells into 40 mL of fresh PG0 medium. The suspensions were shaken on a reciprocal shaker at 125 rpm.

Experimental conditions and harvesting

Salt stress was generated by growing cells in liquid PG0 medium containing 300 mM NaCl, while physiological drought was provoked by growing cells in the same medium containing 600 mM mannitol. Cells were harvested after 72 h of incubation, washed thoroughly with distilled H_2O, dried, and rapidly frozen in liquid nitrogen until use.

Macro- and microelements analysis

Five samples were used to determine macro- and microelement concentrations. Samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using the Prodigy High Dispersion ICP instrument (Teledyne Leeman Labs, Hudson, NH). ICP multi-element standard solution IV (Merck, Darmstadt, Germany) was used to control plasma positioning and to prepare standard solutions for calibration. All calibration standards were prepared by appropriate dilution of standard stock solutions (1 g L^{-1}) in a concentration range from 0.1 to 5.0 mg L^{-1}. Lyophilized samples were dried at a constant temperature of 70 °C for 1 h and then pulverized in a porcelain mortar. An amount of 0.15 g of each dried sample was weighed with analytical accuracy and placed in Teflon vials, except for the solutions of the nutrient medium. 4 mL of concentrated nitric acid, HNO_3, 1.0 mL of hydrogen peroxide, (w = 30%) and 1.0 mL of ultrapure deionized water (R ≈ 18 MΩ) were added and the vials were left open for 30 min. The vials were sealed and placed in a rack holder of a microwave-assisted high-pressure digestion system (Berghof, Germany). Digestion was performed in several steps for 40 minutes. After cooling to room temperature, the solutions were filtered, transferred to 10 mL volumetric flasks, and filled to the mark with ultrapure deionized water. All samples were digested and analyzed as duplicates; blanks were also prepared in the same manner as the samples. To verify the accuracy of the digestion procedure, the same digestion scheme was applied to the certified reference material (SRM 1571 – Orchard leaves). The results are presented as mg macroelement per g of dry weight (DW) or μg microelement per g of DW together with the standard deviation of measurements.

Analysis of cellular proteins

The frozen cells were ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. For 2-DE, the phenol extraction protocol was performed according to a published procedure (Faurobert et al. 2007). Protein concentration was determined by the modified Bradford method using a UV/Vis spectrophotometer UV-4 (Unicam, UK) and bovine serum albumin (BSA) as a standard (Faurobert et al. 2007).

The first dimension, isoelectric focusing (IEF), was performed using 18 cm long nonlinear, immobilized pH gradient (IPG) strips, pH 3–10, in the IPGphor system (GE Healthcare, USA), according to Pavoković et al. (2012). The IPG strips were stored at -80 °C until use. IPG strips were thawed and incubated for 15 min in a buffer composed of 0.05 M Tris-HCl pH 8.8, 6 M urea, 2% SDS (w/v) containing 130 mM dithiothreitol (DTT) and then for 15 min in a buffer of the same composition, but with 135 mM iodoacetamide instead of DTT. The second dimension was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Pavoković et al. (2012a), using the PROTEAN II xi system (BioRad, USA).

Analysis of extracellular proteins

Extracellular proteins were harvested from the liquid medium after cells were removed. To remove debris, the medium was filtered through No 1. Whatman filter papers (Whatman, UK) and again through 0.45 μm Millipore filters (Millipore, USA). Proteins were concentrated on Amicon ultrafiltration devices (Millipore, USA) with the cut-off of 3 kDa. Protein concentration was determined by the Bradford method using a spectrophotometer and a BSA as standard (Bradford 1976). Proteins were mixed with Laemmli buffer (Laemmli 1970) and loaded onto a large vertical electrophoresis system. Electrophoresis was performed for 30 min at 100 V in stacking gel containing 4% T and 2.67% C and then at 220 V in running gel (12% T, 2.67% C) until the bromophenol blue ran off the gel.

Gel staining, image acquisition and analysis

Protein spots were visualized using Coomassie Brilliant Blue R-250 (CBB) or by silver staining (Blum et al. 1987). After protein visualization, gels were scanned with a flatbed scanner (HP, USA) at a resolution of 600 dpi and analyzed with Proteomeweaver 2.2 (Definiens, Germany) using the proposed working pipeline. Protein spots were detected using the following parameters: intensity limit, 10000; contrast limit, 50; and radius limit, 10. Statistical analyzes were performed using Proteomeweaver software. At least five gels, each from a biological replicate, were used to create the master gel for the control and for salt and mannitol treatments.

In-gel digestion, peptide sample preparation, and peptide mass spectrometry

Spots and bands of differentially expressed proteins were excised from the gels with a scalpel and the gel pieces were washed thoroughly using destaining buffer (30% methanol, 10% glacial acetic acid in H_2O) and prepared for matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis as described in Pavoković et al. (2012b). Mass spectra were obtained using a MALDI-TOF/TOF MS (4800 Plus MALDI TOF/TOF analyzer, Applied Biosystems Inc., Foster City, CA, USA) equipped with a 200 Hz, 355 nm Nd:YAG laser, operated in the positive ion reflector mode.

For protein identification, we applied the global protein server explorer software (version 3.6, Applied Biosystems, USA) for Mascot (Matrix Science version 2.1, UK) search against the National Center for Biotechnology Information protein database (NCBIprot, http:// www.ncbi.nlm.nih.gov/protein).

Bioinformatics

The identified proteins were assigned to functional groups according to the MIPS Functional Catalogue FunCat database (Ruepp et al. 2004) and involved mechanisms reported previously in the literature. The target organelle of proteins was predicted using the TargetP web tool (Emanuelsson et al. 2007), which predicts whether protein sequences contain a mitochondrial target peptide, a chloroplast transit peptide or a signal peptide for secretion.

At the time of the initial experiment, mass spectrometry was used to identify protein sequences derived from organisms other than just sugar beet. To expand the disorder analysis to a set of proteins exclusive to this plant, we obtained sequences from the recently published sugar beet genome. We performed a similarity search of the initially identified protein sequences against the sugar beet genome assembly RefBeet-1.2 using the built-in BLAST within the Beta vulgaris Resource (http://bvseq.molgen.mpg.de/blast/) (Dohm et al. 2014).

The Universal Protein Resource (UniProt) was used for Gene Ontology analysis (GO, http://www.geneontology.org) of all identified proteins.

Statistical analysis

For the macro- and microelements analysis the results were analyzed with one-way ANOVA using the STATISTICA 13.0 (Stat Soft Inc., USA) software package. Differences between means were considered statistically significant at $P < 0.05$ (Tukey post hoc test for unequal sample sizes).

Statistical significance for protein expression in 2-D gels was calculated when the protein spot was present on at least three gels. Univariate mean differences between the abundance of protein spots in the control gel and in the gel obtained under salt or mannitol stress were examined using the non-parametric Mann-Whitney-Wilcoxon and Kolmogorov-Smirnov tests.

Results

Disruption of homeostasis of macro- and microelements

Salt- and mannitol-induced stress resulted in disruption of cellular cation homeostasis (Tab. 1 and Tab. 2). Depend-

Tab. 1. The concentration of macroelements in control cells of the sugar beet N cell line, in cells treated with 300 mM NaCl or 600 mM mannitol, and in modified Gamborg B_5 liquid nutrient medium (PG0) measured by inductively coupled plasma atomic emission spectroscopy. Results are mean values ± standard errors (n = 5). DW - dry weight.

Sample	mg g^{-1} DW			
	Na	K	Ca	Mg
cell – control	15.00 ± 0.08^c	54.58 ± 0.90^b	4.465 ± 0.03^b	2.568 ± 0.01^b
cell – 300 mM NaCl	116.8 ± 2.56^a	39.7 ± 0.87^c	1.941 ± 0.12^c	2.536 ± 0.12^b
cell – 600 mM mannitol	1.738 ± 0.02^d	21.65 ± 0.48^d	1.599 ± 0.03^c	2.528 ± 0.08^b
PG0 medium	72.47 ± 2.01^b	222.0 ± 3.6^a	76.79 ± 2.20^a	56.37 ± 1.5^a

Tab. 2. The concentration of selected microelements in control cells of the sugar beet N cell line, in cells treated with 300 mM NaCl or 600 mM mannitol, and in modified Gamborg B_5 liquid nutrient medium (PG0) measured by inductively coupled plasma atomic emission spectroscopy. Results are mean values ± standard errors (n = 5). DW - dry weight.

Sample	μg g^{-1} DW			
	Fe	Cu	Mn	Zn
cell – control	330.9 ± 2.12^a	4.382 ± 0.13^a	17.53 ± 0.13^a	46.15 ± 0.13^b
cell – 300 mM NaCl	258.3 ± 14.04^b	2.928 ± 0.33^c	9.382 ± 0.53^b	46.98 ± 0.66^a
cell – 600 mM mannitol	164.8 ± 3.64^c	11.86 ± 0.33^b	14.93 ± 0.26^c	35.25 ± 0.59^c
PG0 medium	330.9 ± 2.12^a	4.382 ± 0.13^a	17.53 ± 0.13^a	46.15 ± 0.13^b

ing on the stress and element, different accumulation patterns were evident. The concentration of Na^+ ions was 7.7-fold higher in salt-treated cells than in the control but 8.3-fold lower with the mannitol treatment (Tab. 1). The concentrations of K^+ and Ca^{2+} ions were decreased in both types of stress compared with the control, but this was particularly pronounced for K^+ ions in mannitol treatment (Tab. 1). In contrast, the stress did not affect the concentration of Mg^{2+} ions. Both types of stress altered the concentrations of cellular microelements in different ways: NaCl decreased the content of Fe^{2+}, Cu^{2+}, and especially Mn^{2+} ions and increased the concentration of Zn^{2+} ions, whereas mannitol decreased the concentration of Fe^{2+}, Mn^{2+}, and Zn^{2+} ions and increased that of Cu^{2+} ions (Tab. 2).

Salt- and mannitol-responsive cellular proteins

We applied a proteomic approach to analyze and compare the effect of salt and mannitol on the expression profile of sugar beet proteins. Salt stress resulted in statistically different regulation of 43 proteins, of which 22 were up-regulated and 21 were down-regulated (Fig. 1). Mannitol-induced stress affected the expression of 67 proteins, of which

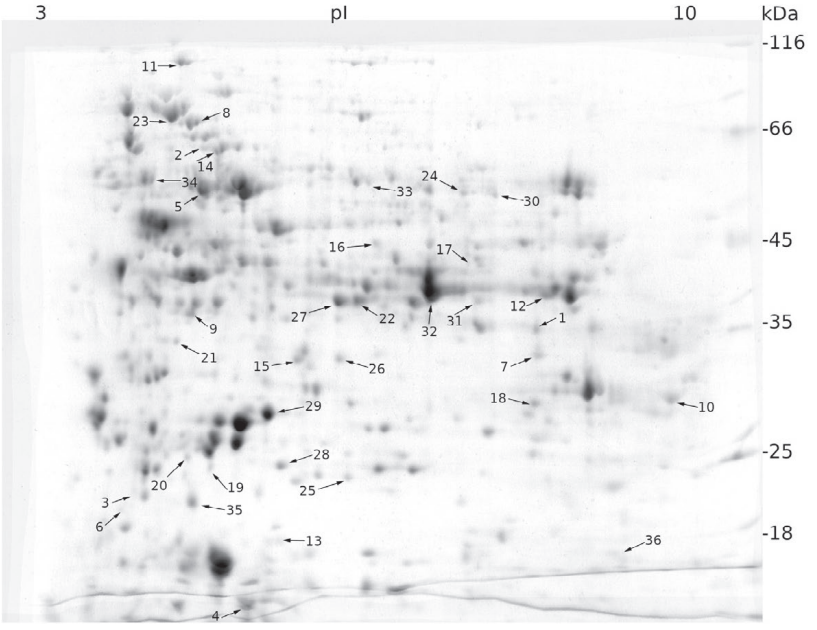

Fig. 1. Expression patterns of cellular proteins from a sugar beet N cell line exposed to salt stress (300 mM NaCl) for 72 h obtained by two-dimensional electrophoresis (2-DE). In the first dimension (IEF), 300 μg of proteins were resolved in 18 cm IPG strips with a non-linear pH gradient of 3-10. In the second dimension, proteins were separated on a 12% SDS polyacrylamide gel and then stained with Coomassie Brilliant Blue. Black arrows indicate proteins whose expression was statistically different to that of the control sample (see Materials and methods) and were identified using MALDI-TOF/TOF mass spectrometry.

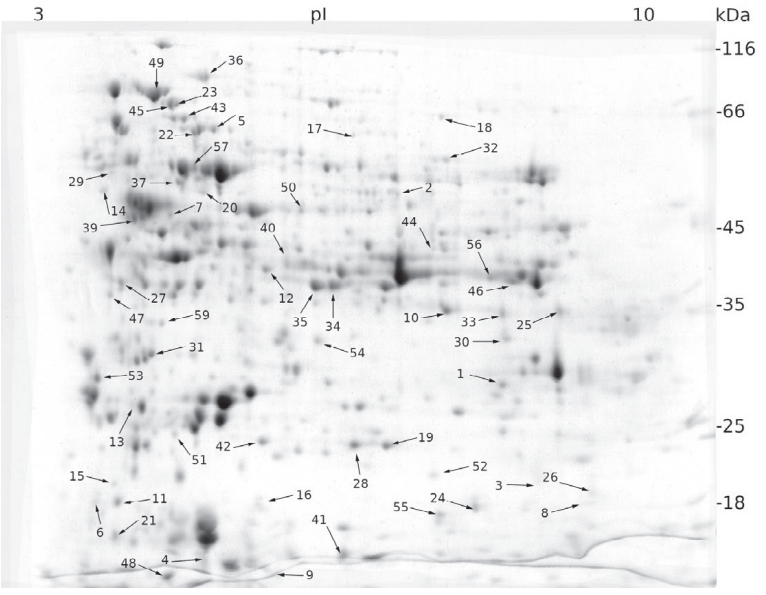

Fig. 2. Expression patterns of cellular proteins from a sugar beet N cell line exposed to mannitol stress (600 mM mannitol) for 72 h obtained by two-dimensional electrophoresis (2-DE) (for details see Fig. 1). Black arrows indicate proteins whose expression was statistically different to that of the control sample (see Materials and methods) and were identified using MALDI-TOF/TOF mass spectrometry.

41 were up-regulated and 26 were down-regulated (Fig. 2). Protein spots were excised and subjected to protein identification by MALDI-TOF/TOF MS. Of the proteins that were differentially expressed under salt stress, 25 were successfully identified, of which 15 were up-regulated and 10 were down-regulated (On-line Suppl. Tab. 1). Identification of proteins that showed differential expression after mannitol stress was successful for 29 of them, of which 18 were up-regulated and 11 were down-regulated (On-line Suppl. Tab. 2). Some salt- and mannitol-responsive spots were not excised due to their low abundance on the gel.

To obtain more information about unresolved proteins or those with low scores, we searched for protein homologs using the BLAST tool (Altschul et al. 1990). Functional classification of proteins was performed using the FunCat database (http://mips.helmholtz-muenchen.de/funcatDB/) (Ruepp et al. 2004). According to this classification, most of the identified proteins were involved in metabolism, energy and cell rescue, defense, and virulence, accounting for approximately 80% of the proteins (Fig. 3).

GO analyses revealed that of the proteins that were up-regulated by salt stress, most were associated with the oxidative stress response (4 proteins) and the malate metabolic process (2 proteins), whereas other biological processes were represented by only one protein (Fig. 4a). Molecular function analysis revealed that most of the up-regulated proteins were related to hem-, ribosome-, and ATP-binding and to L-malate dehydrogenase activity (Fig. 4b) and exerted their function in the cytoplasm and chloroplast (7 and 4 proteins, respectively) (Fig. 4c). As for the down-regulated proteins, most of them are involved in the response to unfolded proteins (Fig. 4a) and have functions in ATP binding (Fig. 4b) in the cytoplasm (Fig. 4c).

For most of the proteins that were up-regulated on mannitol, it was not possible to obtain information on the biological processes in which they are involved, but for those

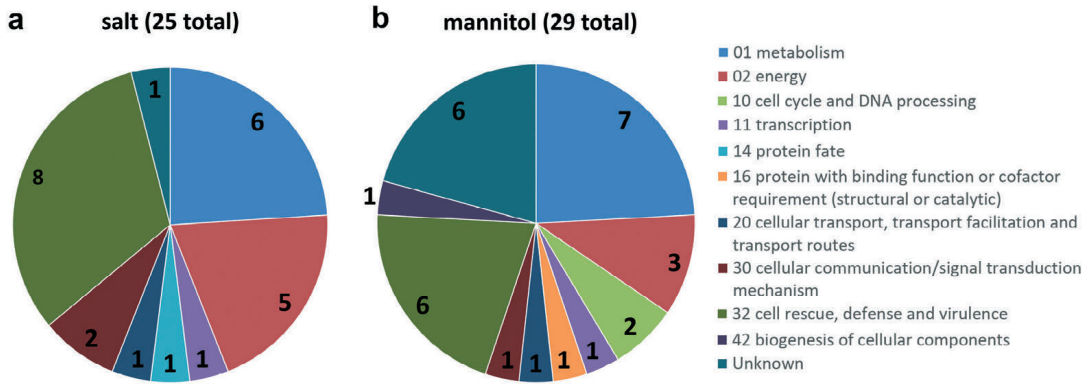

Fig. 3. Functional classification of the identified proteins: salt-induced stress proteins (a), mannitol-induced stress proteins (b). The functional grouping of the different proteins was based on FunCat web tool. The numbers on the pie-chart indicate the number of identified proteins belonging to the respective section.

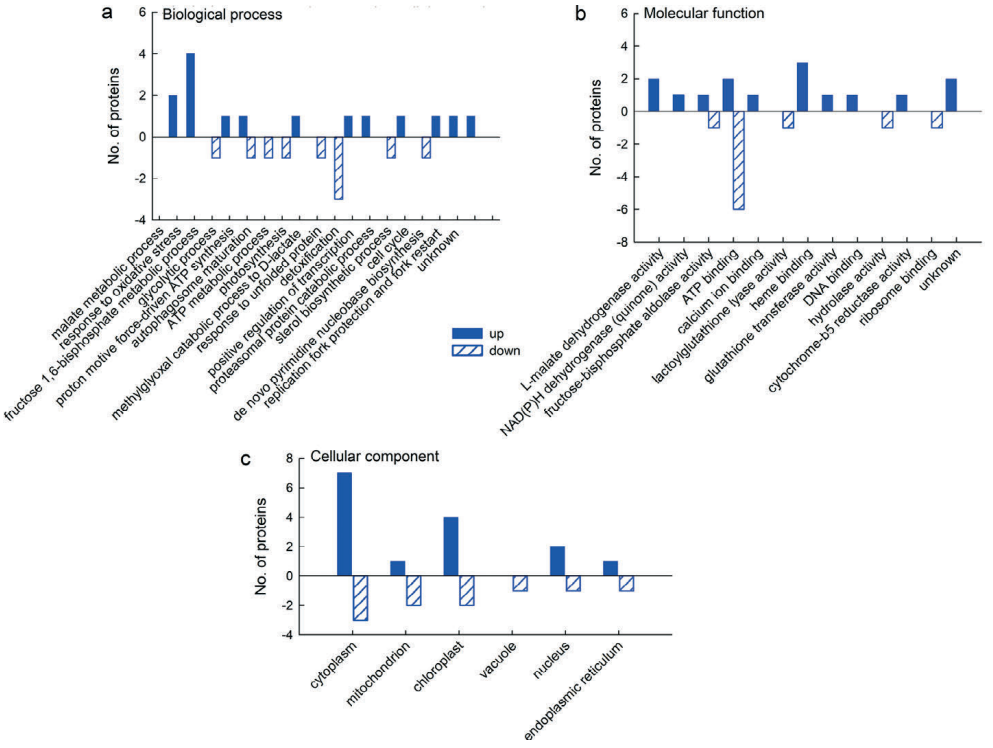

Fig. 4. Gene ontology (GO) analysis of cellular proteins from a sugar beet N cell line exposed to salt stress (300 mM NaCl) for 72 h: biological process (a), molecular function (b), and cellular compartment (c). GO analysis was derived through Uniprot hit accessions. Differently abundant proteins were identified by MALDI-TOF/TOF MS according to the NCBIprot database.

for which it was possible, GO analysis revealed that they are involved in malate metabolism and photosynthesis, as well as in the response to fungi and oxidative stress (2 proteins in each category) (Fig. 5a). Analysis of molecular function showed that two proteins had L-malate dehydrogenase activity, whereas other categories were represented by only one

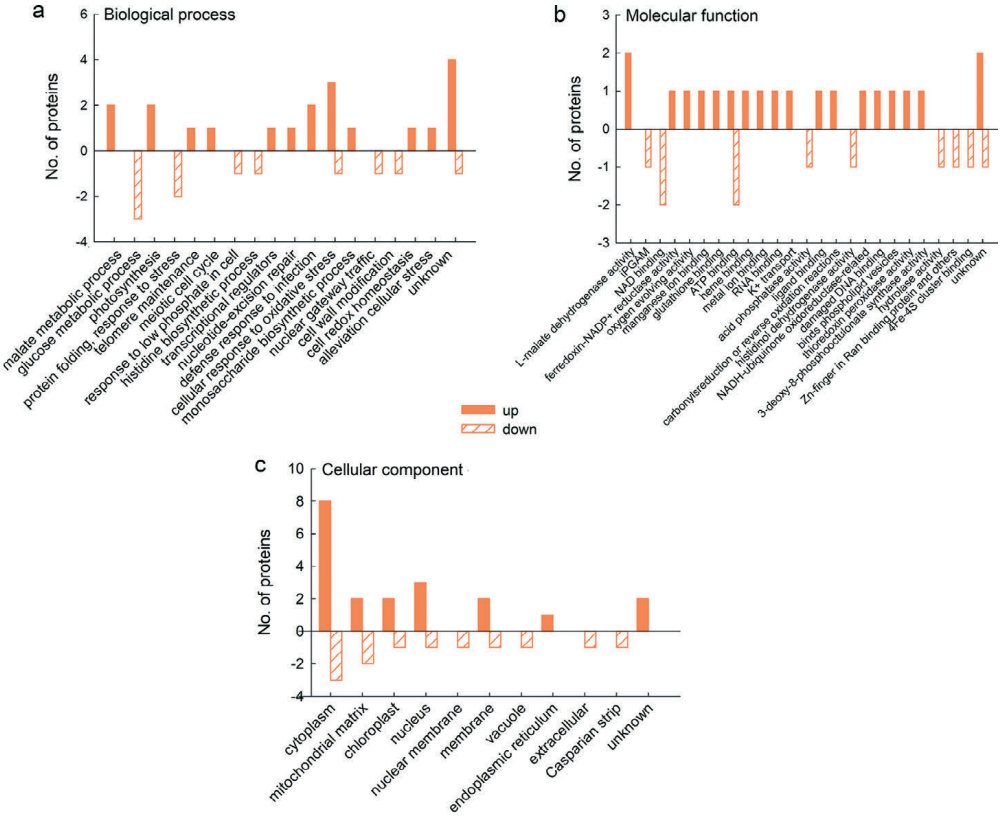

Fig. 5. Gene ontology (GO) analysis of cellular proteins from a sugar beet N cell line exposed to mannitol stress (600 mM mannitol) for 72 h: biological process (a), molecular function (b), and cellular compartment (c). GO analysis was derived through Uniprot hit accessions. Differently abundant proteins were identified by MALDI-TOF/TOF MS according to the NCBIprot database.

protein (Fig. 5b); the majority of the up-regulated proteins exerted their function in the cytoplasm (Fig. 5c). Of the down-regulated proteins, three are involved in glucose metabolism and two in protein folding and response to stress (Fig. 5a); their function is mainly in NAD and ATP binding (2 proteins per category) (Fig. 5b) in the cytoplasm and mitochondrial matrix (Fig. 5c), whereas other categories are represented by only one protein.

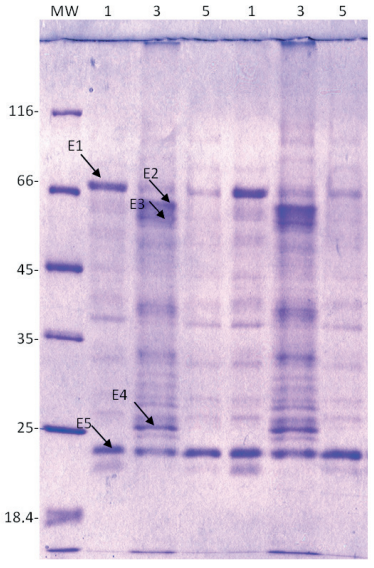

Fig. 6. Expression patterns of extracellular proteins from a sugar beet N cell line cultured for 72 h in modified Gamborg B_5 liquid nutrient medium (PG0) with or without the addition of 300 mM NaCl or 600 mM mannitol. Proteins were resolved on a 12% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue. MW – molecular weight markers, 1 – control, 3 – 300 mM NaCl, 5 – 600 mM mannitol. E1-E5 represent proteins submitted to MALDI-TOF/TOF MS for identification.

Salt- and mannitol-responsive extracellular proteins

Using experiments in cell culture, we investigated whether extracellular proteins are involved in the response to salt and mannitol stress. We found five protein spots (labelled E1-E5 in Fig. 6) whose expression changed significantly after salt and mannitol treatment compared with the control. They were excised from the gel and identified using MALDI-TOF/TOF MS.

Two of the five proteins with altered expression (labelled E1 and E4 in Fig. 6) were successfully identified. The other three proteins labelled E2, E3, and E5 are unknown (Fig. 6). Salt stress increased the expression of chitinase precursor (E4) and unknown proteins E2 and E3, whereas it down-regulated the expression of β-D-xylosidase 4 (E1) and unknown protein E5. The expression of β-D-xylosidase was also down-regulated by mannitol (On-line Suppl. Tab. 3).

GO analyses revealed that three proteins up-regulated by salt stress are involved in cell tip and root growth and carbohydrate metabolism and have a molecular function in binding copper ions and chitin. The proteins down-regulated by NaCl belong to arabinan catabolism, with alpha-L-arabinofuranosidase activity. The single mannitol-responsive protein that was down-regulated also belongs to arabinan metabolism, with alpha-L-arabinofuranosidase activity (Fig. 7).

Discussion

In this study, we investigated the cellular processes in sugar beet cells after a brief but severe exposure to salt and mannitol stress. Although sugar beet is an industrially important crop, there is little proteomic and bioinformatic information on the response of this plant to abiotic stress. By using cell lines instead of whole plants, our intention was to eliminate differences in protein expression between different tissues and focus on the effects of stress on a small number of cell types. We chose to use high concentrations of salt and mannitol to elicit a stronger cellular response. This approach allowed us to identify a number of previously unknown cellular and extracellular proteins involved in the response to salt and drought stress in sugar beet. A dose-dependent response to salt was also observed in the halo-

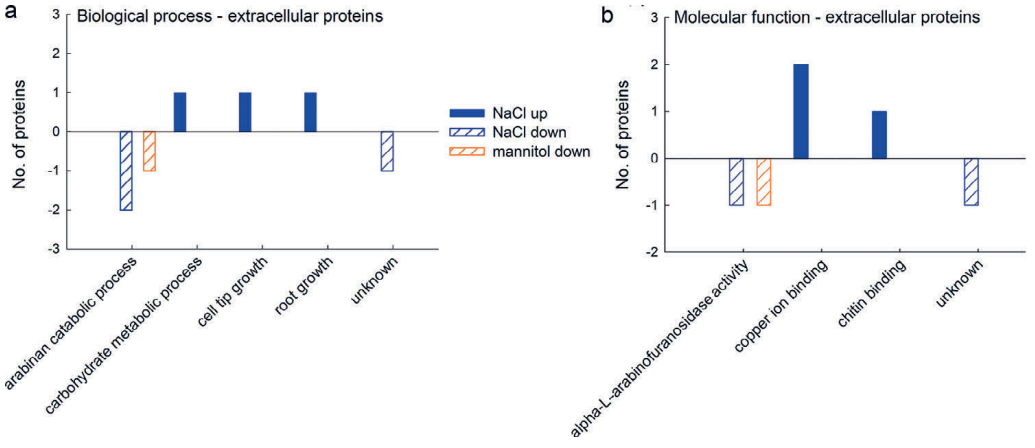

Fig. 7. Gene ontology (GO) analysis of extracellular proteins from a sugar beet N cell line exposed to salt (300 mM NaCl) and mannitol stress (600 mM mannitol) for 72 h: biological process (a) and molecular function (b). Differently abundant proteins were identified by MALDI-TOF/TOF MS according to the NCBIprot database. GO analysis was derived through Uniprot hit accessions.

phyte *Suaeda aegyptica*, where the number of regulated proteins was positively correlated with salt concentration (Askari et al. 2006).

Cation homeostasis

Salt and mannitol stress caused profound disruption of cation homeostasis in sugar beet cells. Salt-stressed cells increased the concentration of Na^+ ions, decreased the concentration of K^+ and Ca^{2+}, whereas the concentration of Mg^{2+} ions did not change. Accumulation of Na^+ ions and loss of K^+ ions were observed in sugar beet treated with salt and sorbitol (Wu et al. 2014) and in *Mammilaria gracilis* callus and tumor exposed to NaCl (Balen et al. 2013). The loss of K^+ during salt stress appears to be related to antagonism of Na^+ and K^+ ions at uptake sites or inhibition of K^+ uptake at the plasma membrane (Hu and Schmidhalter 2005). This cellular uptake inhibition could lead to the measured lower Na^+ and K^+ levels and contribute to osmotic adjustment, in whole sugar beet seedlings during drought, for instance (Wu et al. 2014). Lower Ca^{2+} levels can be explained by the displacement of extracellularly bound Ca^{2+} ions by Na^+ ions and precipitation of Ca^{2+} under saline conditions. Such Ca^{2+} deficiency also allows passive uptake of Na^+ ions into cells, resulting in growth arrest and changes in morphology (Cramer et al. 1988). Loss of Ca^{2+} has been observed upon salt stress in tomato plants and can be alleviated by small heat shock proteins (HSP) (Fu et al. 2016). Salinity affects micronutrients and nutrient solubility differently depending on the plant species (for a detailed discussion, see Hu and Schmidhalter 2005). It appears that high salinity reduces concentrations of these ions, as has been reported for shoots and roots of marigold (Koksal et al. 2016).

Mannitol decreased the concentration of all measured macroelements except Mg^{2+}. A strong decrease in the concentrations of Na^+, K^+, and Ca^{2+} ions was also observed upon mannitol stress in *M. gracilis* callus (Balen et al. 2013). In addition, a small reduction in the concentration of K^+ and Na^+ ions and a large reduction in Ca^{2+} ions were observed in various organs of sugar beet cv. Janus during long-term drought (Choluj et al. 2008). Ca^{2+} concentration was reduced in shoots of two drought-sensitive cultivars during both mild and severe short-term drought (Wu et al. 2014). In addition, mannitol decreased Fe, Mn, and Zn microelement content but increased Cu content. The decrease in Fe, Mn, and Zn during drought is thought to be due to their lower solubility (Hu and Schmidhalter 2005). However, the decrease in accumulated ions under mannitol stress may be due to decreased ion uptake rather than the decreased ion mobility that occurs in soil-grown plants during drought (Choluj et al. 2008).

Proteome analysis

We compared the protein expression profile of control cells with that of cells under salt or mannitol stress. An approximately 1.3-fold difference in abundance compared with control was chosen as the threshold for protein significance, consistent with other similar proteomics studies (Parker et al. 2006, Yan et al. 2006, Jiang et al. 2007). We have identified proteins regulated by salt and drought stress such as H^+ATPases (Kirsch et al. 1996), malate dehydrogenases (Song et al. 2001), heat shock proteins, oxidoreductases, and SOD, but also some new proteins that add to the available information on stress-related proteins in sugar beet. Classification using the FunCat tool showed that most of the identified proteins could be classified into three classes: (1) cell rescue, defense and virulence, (2) metabolism, and (3) energy.

Cell rescue, defense and virulence

ROS, such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), are formed in small amounts under normal growth conditions. Under abiotic stress, the production of ROS increases, which can cause damage to biological molecules (Hajheidari et al. 2005). To counteract ROS, plants employ families of enzymes that are part of their antioxidant system (Deyholos 2010). In the present study, a salt-dependent increase in protein abundance was observed for several APX isoforms and glutathione S-transferase (GST) 6, whereas mannitol increased protein abundance of the stromal APX isoform, GST F3 and Mn-SOD II. SODs catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2, and form the first line of defense against the toxic effects induced by ROS (Salekdeh et al. 2002). H_2O_2 is then detoxified to H_2O by the APX family, which consists of several isoforms localized in thylakoid and glyoxisome membranes as well as in the chloroplast stroma and cytosol (Gill and Tuteja 2010). In addition, H_2O_2 is degraded by peroxiredoxins, and the isoform up-regulated in this study is targeted to chloroplasts, suggesting that ROS production was increased in this organelle (Dietz 2007). Endogenous and xenobiotic toxic compounds can be detoxified by GSTs, which form a large family consisting of forty-seven members identified in the Arabidopsis genome (Gill and Tuteja 2010). The GSTs up-regulated in this study appear to belong to the plant-specific Type I (phi-class), whose function is to counteract oxidative damage induced by herbicides and abiotic stress (Wagner et al. 2002, Gill and Tuteja 2010).

Salt- and mannitol-induced stress regulates another large group of proteins: the family of heat shock proteins (HSPs). In plants, HSPs are classified into five families according to their molecular weight, amino acid sequence homology, and functions: HSP100, HSP90, HSP70, HSP60, and the small HSP. They can be up-regulated under abiotic stress to support proper folding, unfolding and translocation of proteins to target organelles (Gupta et al. 2010). In this study, the chloroplast and mitochondrial HSP isoforms, together with a chloroplast 60 kDa chaperonin subunit, were significantly down-regulated after salt- and mannitol-induced stress, which may be due to the lower protein synthesis rate as reported in barley roots after salt shock (Hurkman and Tanaka 1987). Down-regulation of chloroplast HSP70 and HSP90 isoforms has also been reported

during salt stress in *Camellia sinensis* (Wang et al. 2015, Chen et al. 2018). Moreover, HSP70 was down-regulated in callus and tumor tissues of *M. gracillis* treated with either NaCl or mannitol, while HSP60, which is critical for achieving native forms of newly synthesized proteins, was also down-regulated in tumor exposed to NaCl (Rogić et al. 2015). HSP70 can inhibit the transcription of other HSPs by binding to the heat shock transcription factor (HSF) (Kim and Schöffl 2002, Oliver et al. 2011). Reducing HSP70 levels allows for increased production of small HSPs, which play an important role in protection against drought and salt stress (Zou et al. 2012).

Glyoxalase I (lactoylglutathione lyase) was down-regulated in sugar beet cells under salt stress. It is an enzyme involved in the detoxification of the cytotoxic compound methylglyoxal (MG), which is a byproduct of glycolysis. This enzyme was also down-regulated in tobacco cells under salt stress (Hoque et al. 2008), suggesting that detoxification of MG via the glyoxalase system was insufficient in cells under salt stress. Another possible explanation could be that the metabolism of triose phosphates and thus the production of MG was reduced in cells under salt stress.

Metabolism

At the physiological level, salt stress and drought decrease stomatal conductance and photosynthesis, and increase photorespiration (Atkin and Macherel 2009). During detoxification of photorespiration products, there is an increased requirement for the oxidation of NADH and regeneration of NAD^+, which occurs in mitochondria using the mitochondrial NADH dehydrogenase type II or the malate/oxaloacetate shuttle across the inner mitochondrial membrane (Atkin and Macherel 2009). A deficiency of NAD^+ in the cell is detrimental because an adequate amount of ATP cannot be provided during stress. Therefore, the increased abundance of cytoplasmic and mitochondrial malate dehydrogenases detected in our study during salt- and mannitol-induced stress serves to ensure ATP and support normal chloroplast function (Liu et al. 2012). An increase in malate dehydrogenase levels was also observed under 150 mM NaCl stress in Arabidopsis, under treatment with 300 mM NaCl in *Eremochloa ophiuroides*, and during drought in *Elymus elongatum* (Jiang et al. 2007, Salekdeh et al. 2007, Liu et al. 2012).

We observed up-regulation of one isoform of fructose-biphosphate aldolase and down-regulation of another isoform, aldolase superfamily protein, after salt stress. In plants, there is a cytosolic and a chloroplast isoform of this enzyme (Konishi et al. 2004). We performed protein localization estimation based on the target peptide sequence using the TargetP algorithm (Emanuelsson et al. 2007), and the cytosolic isoform was up-regulated, whereas the chloroplast isoform was down-regulated. Up-regulation of the cytoplasmic isoform of the enzyme appears to help plants to cope with anaerobic conditions by promoting the function of the glycolytic pathway for ATP synthesis (Konishi et al. 2004). The down-regulation of the chloroplast isoform is likely a consequence of lower photosynthetic rates and the establishment of tolerance following stress induction (Yamada et al. 2000).

Salt stress resulted in up-regulation of putative quinone reductase, an enzyme belonging to the large family of oxidoreductases that acts on NADH or NADPH with a quinone or similar compound as acceptor. It appears to be involved in the detoxification of reactive carbonyls in plants (Yamauchi et al. 2010). Quinone reductase was also found to be up-regulated after salt stress in tomato (Zhou et al. 2009). We also observed mannitol-induced down-regulation of histidinol dehydrogenase, which is part of amino acid metabolism and catalyzes the final step in the production of histidine from histidinol. Down-regulation of this enzyme has also been reported in *Chlamydomonas reinhardtii* exposed to heat shock, suggesting that a reduction in protein biosynthesis occurs during abiotic stress, perhaps as a means to reduce problems with *de novo* protein folding under adverse conditions (Mühlhaus et al. 2011).

Energy

Four different proteins belonging to ATPases were differentially regulated after salt stress: proteins belonging to the endoplasmic reticulum (ER), mitochondria, and V-type ATPases were down-regulated, whereas a subunit of the chloroplast ATPase was up-regulated. Transitional ER ATPase provides energy for vesicle budding, which is responsible for transport between ER and Golgi (Zhang et al. 1994). Down-regulation of this enzyme after salt stress could be a consequence of decreased protein synthesis, which then leads to decreased vesicle production and protein transport. V-type and mitochondrial ATPases are important in salt stress to stimulate the transport of Na^+ ions in the vacuole and to restore ion balance, respectively (Lehr et al. 1999, Ndimba et al. 2005). However, the beta-subunit of mitochondrial ATP synthase was down-regulated in rice roots under severe salt stress (500 mM for 2 h), which ultimately triggered programmed cell death in salt-sensitive rice (Chen et al. 2009).

Salt stress up-regulated two proteins involved in photosynthesis: a β-subunit of chloroplast ATP-synthase (AtpB) and a 23 kDa polypeptide of the oxygen-evolving complex (OEC) (PsbP) of the photosystem II (PSII), whereas mannitol up-regulated the 33 kDa OEC protein (PsbO). PsbP and PsbO are extrinsic proteins located on the luminal side of the PSII complex in chloroplasts, where they serve to maintain oxygen evolution at physiological rates and ensure thylakoid stability (De Las Rivas et al. 2007). Their increase under salt stress could help maintain sufficient water-splitting activity or stabilize PSII under these conditions (Yamauchi and Sugimoto 2010). Up-regulation of PsbP protein has also been observed in Norway spruce needles under mild drought conditions (Blödner et al. 2007).

Mannitol up-regulated ferredoxin-$NADP^+$ reductase (FNR), an enzyme that catalyzes the final step of electron

transfer in PSI and is responsible for NADPH production. A general increase in FNR transcripts was observed in Arabidopsis plants exposed to drought stress (Lehtimäki et al. 2010), and it was concluded that FNR is important in redox regulation and in antioxidant mechanisms in chloroplasts during drought stress.

Mannitol also up-regulated mitochondrial NADH-ubiquinone oxidoreductase, also known as Complex I of the respiratory chain. The complex transfers electrons from NADH to coenzyme Q and translocates protons across the inner mitochondrial membrane, contributing to the build-up of the electrochemical potential needed to produce ATP (Brandt 2006). The up-regulation of the enzyme during drought could help mitochondria to restore ROS imbalance after stress (Nwugo and Huerta 2011, Sharma et al. 2011).

Mannitol stress resulted in down-regulation of the glycolysis enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGAM). GAPDH, which catalyzes the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, requires NAD(P)H for its activity and is normally inhibited when cells are exposed to oxidative stress (Chernyad'ev and Monakhova 2006, Ralser et al. 2007). IPGAM was found to be down-regulated in *Thellungiella halophila* upon salt stress, likely due to decreased energy metabolism and subsequent formation of ROS (Gao et al. 2008). Moreover, expression of both enzymes was also reduced in *M. gracilis* tumor exposed to salt and mannitol (Rogić et al. 2015). Taken together, these results suggest that primary metabolism in exposed tissues was significantly affected by salt- and mannitol-induced stress and that, in response to salinity and osmotic stress, energy metabolism is decreased to reduce the excessive production of ROS that could trigger oxidative stress (Gao et al. 2008).

Other categories of proteins

The proteasome is a large protein complex that eliminates damaged or misfolded proteins in cells. Here, we found down-regulation of the α-subunit of the proteasome 20S complex in response to salt stress. Considering that stress increases the number of misfolded and damaged proteins, one would expect an increase in 20S abundance. However, in wheat roots, 20S proteasome subunit abundance decreased under salt stress, although activity increased (Shi et al. 2011), suggesting that regulation of proteasome activity is not always well correlated with the stress severity. In addition, the 26S proteasome subunit α was found to be down-regulated in *M. gracillis* tumor tissue treated with NaCl (Rogić et al. 2015), and the authors speculated that under stress, plants process misfolded proteins mainly by refolding. Proteasome abundance may also be down-regulated during programmed cell death (Kurepa and Smalle 2008).

The down-regulation of receptors for activated C kinase 1 (RACK1) by salt stress appears to be related to its regulation by the stress hormone abscisic acid (ABA). RACK1, considered a versatile scaffold protein, is a negative regulator of ABA responses. Induction of ABA during salt and drought stress down-regulated the expression of three RackK1 isoforms in Arabidopsis (Guo et al. 2009a). ABA can modulate gene expression depending on whether or not new protein synthesis is required (Zhang et al. 2006). Group A of the bZIP family of transcription factors, the ABRE-BINDING PROTEIN/FACTOR (AREB/ABF) family, is responsible for gene expression that has a ABA-responsive cis-element (ABRE) in its promoter domain (Kim et al. 2011). The bZIP transcription factor up-regulated in this study may be a member of the AREB family in sugar beet responsible for mediating ABA responses.

Mannitol up-regulated a glycine-rich RNA-binding protein. The superfamily of glycine-rich proteins is involved in post-transcriptional processes such as mRNA and rRNA processing, RNA export, and stability (Hu et al. 2011). A β-subunit of a probable voltage-gated potassium channel localized in the plasma membrane was strongly up-regulated by mannitol. In mammals, β-subunits are not directly involved in potassium transport but in modulating channel activity and are stoichiometrically related to α-subunits transporting ions according to the formula α4β4 (Capera et al. 2019). Ardie et. al. (Ardie et al. 2011) used a β-subunit of a K^+ channel from the halophyte *Puccinellia tenuiflora* and induced it transiently in yeast and Arabidopsis. The activity of the protein can alter the levels of K^+ and Na^+ in plant parts. During drought, cells suffer from a deficiency of K^+ ions (Hu and Schmidhalter 2005) and plants try to maintain a high K^+:Na^+ ratio to ensure the integrity of cell membranes. These results suggest that in sugar beet cells, as in mammals, β-subunits may be involved in modulating K^+ channel activity during stress.

Extracellular proteins

In this study, we observed only five extracellular proteins with altered expression. Although the number is lower than reported in other studies (Zhang et al. 2009, Pechanova et al. 2010), this is the first analysis of this type in sugar beet. A β-xylosidase was down-regulated during salt- and mannitol-induced stress. This enzyme belongs to a family of exo-hydrolases but also has significant transglycosylase activity (Franková and Fry 2011). It is involved in the degradation/reconstruction of xylose-containing polysaccharides in the plant cell wall during various physiological events (Franková and Fry 2011). The down-regulation of the enzyme during elevated salinity and drought appears to be part of a defense mechanism in which no cell wall modification serves to alleviate cell damage.

AT1G76160 [*Arabidopsis thaliana*] L-ascorbate oxidase (SKU5 similar protein 5, SKS5), hypothetical protein At1g41830 (SKU5 similar proteins 6, SKS6), and a chitinase were up-regulated only during salt stress. SKS5 and SKS6 belong to a family of multicopper oxidase-like proteins related to ferroxidases, ascorbate oxidases, and laccases

(Jacobs and Roe 2005). SKS6 is involved in the formation of vascular patterns in cotyledons during Arabidopsis development, and is positively influenced by plant hormones such as ABA, 1-aminocyclopropane-1-carboxylic acid (ACC, the direct precursor of ethylene), indole-3-acetic acid (IAA, the most common naturally occurring plant hormone of the auxin class), and 2,4-dichlorophenoxyacetic acid (2,4-D, a synthetic auxin) (Jacobs and Roe 2005). The role of SKS5 in the cell wall is not known but may be related to hyperhydric stress (Sen and Alikamanoglu 2013). The chitinase protein has a domain of glycoside hydrolase 19 (GH19) and belongs to Class IV chitinase by size. While the primary role of this class of chitinases is in defense against pathogens, up-regulation at the transcriptional level has been observed in Arabidopsis under salt and drought stress (Takenaka et al. 2009, Vaghela et al. 2022).

In conclusion, we showed that both stressors altered ion status in cells. Most of the regulated proteins were responsible for alleviating the increased ROS production and cellular damage and restoring homeostasis. In addition to previously known cellular proteins commonly identified as stress-responsive, we have identified several new proteins that appear to be involved in the stress response. Their role in abiotic stress in sugar beet requires further investigation. By extending our study to the extracellular space, we also identified several extracellular proteins involved in the stress response.

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Short communication

First record of *Diplotomma cedricola* in the eastern Mediterranean

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Abstract – The present paper reports the first record of *Diplotomma cedricola* (Werner) Etayo in the eastern Mediterranean (Creta Island, Greece), the nearest record being rom Corsica (France). This lichen species was found near Iérapreta on decorticated *Cupressus sempervirens* L. and *Pinus brutia* Ten. trees. This finding constitutes a great expansion of the distribution range of this species.

Keywords: Greece, lichen, lignicolous species, new record

Introduction

Diplotomma cedricola (Werner) Etayo (=*Buellia cedricola* Werner) is a lignicolous specialist species growing on decorticated parts of conifer trunks in semi-open woodlands at elevations of 700 to 2400 m (Burgaz and Sarrión 1995, Bungartz et al. 2007). The known range of the species is extended to a few localities in the southwestern United States (Nordin 1999, Bungartz et al. 2007), Canary Islands (Nordin 2000, Giralt and van den Boom 2011) and western Mediterranean countries including the north of Morocco (Werner 1970, 1974), central-southern areas of Spain (Burgaz and Sarrión 1995, Aragón et al. 2004) and Corsica (France) (Werner and Deschatres 1974).

In this contribution, we present the first record of *Diplotomma cedricola* in Greece (Crete Island), significantly expanding the range of this species to southeastern Europe.

Materials and methods

Specimens were collected on 5th December 2022, in Selakano Forest, near the village of Selakano in Crete. The samples were deposited in the MACB Herbarium (Faculty of Biology, Complutense University of Madrid). To verify the identification, the collected specimens were compared with Spanish material (MA and MACB herbaria). The nomenclature follows Index Fungorum (www.indexfungorum.org). Habitat description is based on personal observations.

Localities of sampling: Greece: Crete Island, Iérapetra, Selakano, 35°05'06''N, 25°31'10''E, 1174 m a.s.l., on decorticated *Cupressus sempervirens* L., G. Aragón nº 1009 et al., December 5, 2022, MACB. Iérapetra, Selakano, 35°04'52''N, 25°31'51''E, 1080 m a.s.l., on decorticated *Pinus brutia* Ten., G. Aragón nº 1010 et al., December 5, 2022, MACB.

Results and discussion

Diplotomma cedricola is distinctive by its yellowish tinged thallus (usnic acid), K+ red (norstictic acid), black apothecia cryptolecanorine to adnate, and its submuriform to muriform spores (21–26 × 10–13 µm). A complete and detailed description of the species is available in Burgaz and Sarrión (1995) and Bungartz et al. (2007).

The species is found on decorticated and hardened parts of old *Pinus brutia* and *Cupressus sempervirens* trees, in mountain forests, at 1000–1200 m altitude (Fig. 1). The species appears only together with *Lecanora varia* (Hoffm.) Ach. Host tree species were similar to those previously reported for *Diplotomma cedricola*. In general, the species appears on a variety of mostly decorticated coniferous trees such as *Cedrus atlantica* (Endl.) Carrière in Morocco (Werner 1970), *Juniperus* L. (*J. oxycedrus* L., *J. thurifera* L.) and *Pinus* spp. in southern Europe and the Canary Islands (Werner and Deschatres 1974, Nordin 2000, Aragón et al. 2004, Martínez et al. 2002) and *Juniperus deppeana* Steud. in North America but it has also been observed on *Cupressus arizonica* Greene and several species of *Pinus* (Nordin 1999).

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Fig. 1. A – Decorticated trunk of *Cupressus sempervirens* L., 1174 m a.s.l., Crete, is a typical habitat of *Diplotomma cedricola* (Werner) Etayo. Photo: L. Jiménez-Eguizábal. **B** – *Diplotomma cedricola*. Scale bar: 1.0 cm. Photo: G. Aragón.

The new locality situated in the east of Crete is composed of abrupt and unequal mountain landscape, where open forests are developed on limestone with intensive livestock management (goats). The dominant tree species are *Pinus brutia* and *Cupressus sempervirens* at medium altitudes, and *Quercus coccifera* L. at lower altitudes, while the vegetation of the ravines is composed mainly of *Platanus orientalis* L. and *Nerium oleander* L.

Diplotomma cedricola is reported here from SE Europe for the first time and its distribution area is now enlarged to Crete (Greece). This species was not reported in the recent checklist of Greek lichens (Arcadia 2022), the nearest known locality being situated in Corsica (France) (Burgaz and Sarrión 1995).

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Short communication

The new association *Pimpinello lithophilae-Centaureetum lovricii* (*Crithmo-Staticetea*) from the island of Vis (southern Croatia)

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Abstract – This paper presents the new association *Pimpinello lithophilae-Centaureetum lovricii*, described for the halotolerant vegetation of the order *Helichrysetalia italici* (*Crithmo-Staticetea*) on the island of Vis (southern Croatia). The new association replaces the *Pimpinello lithophilae-Centaureetum issaeae*, originally published invalidly because its name was formed from the invalid taxon name '*Centaurea issaea* Lovrić'.

Keywords: Adriatic, *Capparo-Aurinion*, halotolerant vegetation, ICPN, phytosociological nomenclature, plant association, syntaxonomy

Introduction

The Vis Archipelago is located off the Eastern Adriatic (central Dalmatian) coast, in Croatia. The archipelago shows an exceptional concentration of endemic and rare plant species (e.g., *Centaurea* spp. and *Limonium* spp.) (Nikolić et al. 2015). Many of them inhabit halophytic and chasmophytic coastal and subcoastal habitats within rare plant communities (Terzi et al. 2020).

In Croatia, according to the European syntaxonomic framework of the EuroVegChecklist (EVC: Mucina et al. 2016), the halophilous coastal vegetation is included in the order *Crithmo-Staticetalia* whereas the halotolerant belt is classified in the order *Helichrysetalia italici* (Škvorc et al. 2017). Both orders belong to the *Crithmo-Staticetea* class. Although Škvorc et al. (2017) were the first to recognize the occurrence of the *Helichrysetalia italici* in Croatia, this order was widely described and documented for the first time for the eastern Adriatic by Terzi et al. (2020).

The order *Helichrysetalia italici* occurs between coastal vegetation under the direct influence of sea-borne salt spray and a vegetation belt notably less influenced by salt spray. In the island of Vis, within the order *Helichrysetalia italici* Terzi et al. (2020) reported two alliances (1) *Anthyllidion barbae-jovis*, with a Central Mediterranean distribution, and (2) *Capparo orientalis-Aurinion leucadeae* – an endemic alliance of the central Adriatic islands (Croatia) and the Tremiti (Italy).

The alliance *Capparo-Aurinion*, alongside four associations described from neighbouring islets in the Vis Archipelago and the Tremiti as well, includes the stenoendemic association *Pimpinello lithophilae-Centaureetum issaeae* Terzi, Bogdanović, D'Amico et Jasprica 2020 from the island of Vis. This association was, however, invalidly described because one of the name-giving taxon, *Centaurea issaea* Lovrić, had not been validly published at that time (Art. 3 l of the ICPN, International Code of Phytosociological Nomenclature, Theurillat et al. 2021). In fact, according to Bogdanović et al. (2022), *Centaurea issaea* is a *nomen nudum*. Therefore, these authors described the new species *C. lovricii*. Consequently, the new association *Pimpinello lithophilae-Centaureetum lovricii* that is described here.

Materials and methods

This study was carried out according to the Braun-Blanquet approach (Westhoff and van der Maarel 1980). In the type relevé of the new association, the extended 9-point Braun-Blanquet cover-abundance scale was used, with a subdivision of symbol 2 into 2m, 2a and 2b (see Westhoff and van der Maarel 1980 and references therein). Nomenclatural decisions follow the fourth edition of the ICPN (Theurillat et al. 2021). Taxonomic nomenclature follows

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Flora Croatica Database (Nikolić 2023) except for *Limonium issaeum* and *Centaurea lovricii*, the nomenclature of which follows the revisions carried out by Bogdanović and Brullo (2015), and Bogdanović et al. (2022), respectively. Syntaxonomic nomenclature follows the Euro VegChecklist (EVC: Mucina et al. 2016). For complete description of the alliance *Capparo-Aurinion* and belonging tables, see Terzi et al. (2020).

Results and discussion

Syntaxonomic scheme:

Class: *Crithmo-Staticetea* Br.-Bl. in Br.-Bl. et al. 1952

Order: *Helichrysetalia italici* Biondi et Géhu in Géhu et Biondi 1994

Alliance: *Capparo orientalis-Aurinion leucadeae* Lovrić ex Terzi, Bogdanović, D'Amico et Jasprica 2020

Association: *Pimpinello lithophilae-Centaureetum lovricii* Jasprica et Terzi *ass. nov.*

Holotypus: Relevé no. 50 of Supplement S3 in Terzi et al. (2020), from the island of Vis. Plot size: 20 m^2; coordinates (WGS84): latitude 43°04'28", longitude 16°06'33"; altitude 30 m a.s.l., aspect: 360°; slope 60°; vegetation cover 40%; date: 30 May 2018. List of taxa: *Brachypodium retusum*, 2m; *Brassica incana*, 2m; *Centaurea lovricii*, 2a; *Coronilla valentina* subsp. *valentina*, +; *Crithmum maritimum*, +; *Desmazeria rigida*, +; *Dorycnium hirsutum*, r; *Erica manipuliflora*, +; *Helichrysum italicum* subsp. *italicum*, 2m; *Inula verbascifolia*, 2a; *Juniperus phoenicea*, +; *Limonium issaeum*, 1; *Pimpinella tragium* subsp. *lithophila*, 2m; *Reichardia picroides*, +; *Silene vulgaris* subsp. *angustifolia*, +; *Valantia muralis*, 1. Name-giving taxa: *Pimpinella tragium* Vill. subsp. *lithophila* (Schischk.) Tutin and *Centaurea lovricii* Bogdanović, Boršić, Ljubičić, Brullo et Giusso.

Distribution records: The stands occur along the northern coast between Dragodid Bay and Oključina Bay, the island of Vis (Fig. 1). The association is localized in a narrow coastal belt of ca. 0.5 km^2.

Fig. 1. *Pimpinello lithophilae-Centaureetum lovricii*, *ass. nova*. A – the habitat type, B – *Pimpinella tragium* subsp. *lithophila*, C – *Centaurea lovricii*, D – detail of the association with *C. lovricii* and *Brassica incana*. (Photo: N. Jasprica; the island of Vis, May 30, 2018).

Ecology: It grows on sea facing cliffs constituted of Triassic dolomites at 10–100 m a.s.l. The association *Pimpinello lithophilae-Centaureetum lovricii* is characterized by the steno-endemic *Centaurea lovricii* and differentiated by *Brassica incana* and *Pimpinella tragium* subsp. *lithophila*. The latter is found in almost all countries of the northern Mediterranean Basin, while the distribution area of *Brassica incana* includes the western Balkans and Italy (Euro+Med 2006–2023). However, those two taxa are rare along the Dalmatian coast (Bogdanović and Ruščić 2011, Nikolić et al. 2015, Nikolić 2023) and therefore are selected as differential taxa of the new association.

Aurinia leucadea and *Capparis orientalis* were also included among the character-taxa of the *Capparo-Aurinion*. The association includes narrow endemic taxa (e.g., *Centaurea lovricii*, *Campanula teutana*, *Limonium issaeum*) together with some diagnostic taxa of the *Centaureo-Campanuletalia* (*Inula verbascifolia*, *Pimpinella tragium* subsp. *lithophila*, *Sesleria tenuifolia*) and some others of *Crithmo-Staticetea* / *Helichrysetalia italici* (*Crithmum maritimum*, *Helichrysum italicum*, *Allium commutatum*).

Conservation status: According to Bogdanović et al. (2022), the population of *C. lovricii* is estimated as Vulnerable (VU D1) (*sensu* IUCN 2022). However, it grows on very steep and quite inaccessible habitats, which makes the population and plant association unthreatened by any human disturbance. The rarity of this taxon and its associated plant community necessitates the implementation of a comprehensive conservation strategy.

The association name *Pimpinello lithophilae-Centaureetum issaeae* Terzi, Bogdanović, D'Amico et Jasprica 2020 *nom. inval.* (Art. 3 l) was formed from the taxa names *Pimpinella tragium* subsp. *lithophila* and *Centaurea issaea*. Although at that time, the name *Centaurea issaea* Lovrić was in use in the Flora Croatica Database (accessed 1 October 2018, see Terzi et al. 2020), it is a *nomen nudum* for *Centaurea lovricii*. Therefore, the name *Pimpinello lithophilae-Centaureetum issaeae* cannot be validated and is replaced here by *Pimpinello lithophilae-Centaureetum lovricii*. The relevés originally ascribed to the *Pimpinello lithophilae-Centaureetum issaeae* by Terzi et al. (2020: relevés 48–54 of Supplement S3) belong to the original diagnosis of the new association.

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Short communication

Local-scale changes in plant community composition following succession of oak-hornbeam forest after grassland abandonment

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Abstract – In this local-scale synecological study, we investigated the changes in plant community composition throughout secondary succession occurring after cessation of agricultural land use (i.e. grassland abandonment). The successional sequence studied had the following pathway: *Avenula pubescens* (Huds.) Dumort. hay-pastures → *Brachypodium pinnatum* (L.) P.Beauv. successional grassland → *Cornus sanguinea* L. scrubs → late-successional *Populus tremula* L. forest → late-successional oak-hornbeam (*Quercus-Carpinus*) forest. The last forest stage was represented by the association *Epimedio-Carpinetum betuli* (Horvat 1938) Borhidi 1963. Occurrence of plant species throughout secondary succession was mostly stage-specific; only *Fragaria vesca* L., *Ajuga reptans* L., *Cornus sanguinea, Prunus spinosa* L., and *Viola hirta* L. showed survival ability throughout almost all stages.

Keywords: chronosequence, ecosystem development, *Epimedio-Carpinetum betuli*, forest development, temporal community changes

Introduction

Changes in plant community composition following both primary and secondary succession have been studied on a global scale. Some global-scale ecological patterns during successional changes are common and can be detected in many different biomes (see Prach and Walker 2020). However, purely floristic changes during succession can be highly dependent on the region under study, as they depend on the local species pool, land-use history and disturbance type, as well as on environmental filtering effects. Therefore, floristic changes can differ even within a single study area of the same climate and same climax community. This can make it hard to formulate generalizations about the floristic changes during succession, except in the cases when generalizations are made for a specific area of interest. In Croatia, vegetation succession is poorly studied. Only rare data are available on grassland succession (Krstonošić et al. 2016, Kutnjak 2010, Randić 2007), whereas somewhat more comprehensive research on the whole successional sequences has been carried out in the neighboring country of Slovenia (Čarni et al. 2007, Čarni et al. 2021, Dakskobler 2010). In this paper, we depict the local-scale community composition changes following secondary succession of oak-hornbeam forest after cessation of agricultural land-use (i.e. grassland abandonment) in NW Croatia.

Materials and methods

Study area

The study was conducted in NW Croatia, in the surroundings of the village Brlog Ozaljski (45° 37' 32.37" N, 15° 24' 11.09" E). The sessile oak and common hornbeam forest (association *Epimedio-Carpinetum betuli*) is the dominant vegetation cover of the area. The soil type is calcocambisol on limestone. In the study area, during the last few decades, the abandonment of agricultural land use led to a progression of vegetation succession, resulting in a significant loss of meadow and pasture communities. Today, the remaining meadows are used as hay-pastures, i.e., for obtaining hay and for low-intensity rotational grazing by sheep.

Vegetation survey

The sequence of vegetation succession was studied using the space for time substitution, and five stages of succession

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were included in the study (Fig. 1). The sequence was determined during preliminary field investigations. In the last century, the lands of the study area that are today hay-pastures were used in various ways, mostly as extensive arable fields. The age of succession since abandonment was roughly estimated with preliminary field investigations, imagery from Google Earth Pro 7.3.6. time series and Croatian Geoportal (https://geoportal.dgu.hr/), and by interviews with a local farmer. The approximately estimated age of the studied successional stages following hay-pasture abandonment was as follows: successional grasslands (2–5 years), shrub stages (5–15 years), *Populus tremula* L. stage (15–30 years), and oak-hornbeam stage (> 30 years). The vegetation survey was performed throughout 2019 and 2020 in May, June, July, and August, using five 100 m^2 plots (10 × 10 m) within each successional stage, totaling 25 plots. Species cover in grasslands was recorded using the extended Braun-Blanquet scale, and in scrub and forest stage using the classical Braun-Blanquet scale. Nomenclature of plant taxa followed POWO (https://powo.science.kew.org/).

Data analysis

Differences in community composition between the successional stages were plotted with non-metric multidimensional scaling (NMDS) using a species × site matrix based on Bray-Curtis dissimilarities. This was done in PAST 4.03 software. Prior to the NMDS, cover values of species occurring in more than one vegetation layer were pooled, and all of the cover values were transformed to type I ordinal percentage scale (Van der Maarel 2007).

Results and discussion

The NMDS ordination plot (Fig. 2) provided a very good representation of the community composition differences between the successional stages. The succession direction was in correspondence with the first NMDS axis. Total number of species found in the present study was 160, whereas total species number observed in hay-pasture, successional grassland, *C. sanguinea* scrub, *P. tremula* forest, and oak-hornbeam forest was 76, 72, 59, 46, and 59, respectively. The plant community composition of successional stages is listed in On-line Suppl. Tab. 1.

Spread of *Brachypodium pinnatum* (L.) P. Beauv. after hay-pasture abandonment

After cessation of hay-pasture management, *Brachypodium pinnatum* completely colonized the habitat, reaching a cover of > 75%. These successional grasslands seem to be a part of the order *Brachypodietalia pinnati* Korneck 1974. The only frequent species in *B. pinnatum* grassland were *Dactylis glomerata* L., *Briza media* L., *Filipendula vulgaris* Moench., and seedlings of *Cornus sanguinea* L. and *Prunus spinosa* L. Even though the number of species after the spread of *B. pinnatum* remained somewhat high, it decreased the abundance of many hay-pasture species, some of them completely ceasing after it had spread. Due to its incredible colonization efficiency attributed to clonal propagation, but also efficient generative dispersal (Bąba et al. 2012), *B. pinnatum* spread is considered a threat to plant biodiversity of native calcareous grasslands in Europe (Bobbink and Willems 1987). It can decrease species diversity through light reduction by its dense canopy and litter accumulation (Bobbink and Willems 1987), and its dense root system is likely to be efficient in competing for nutrients. On the other hand, its spread is a natural part of the succession after cessation of agricultural land use in many areas of Croatia. However, it provides low-quality forage due to high silica levels in its tissues and high amount of

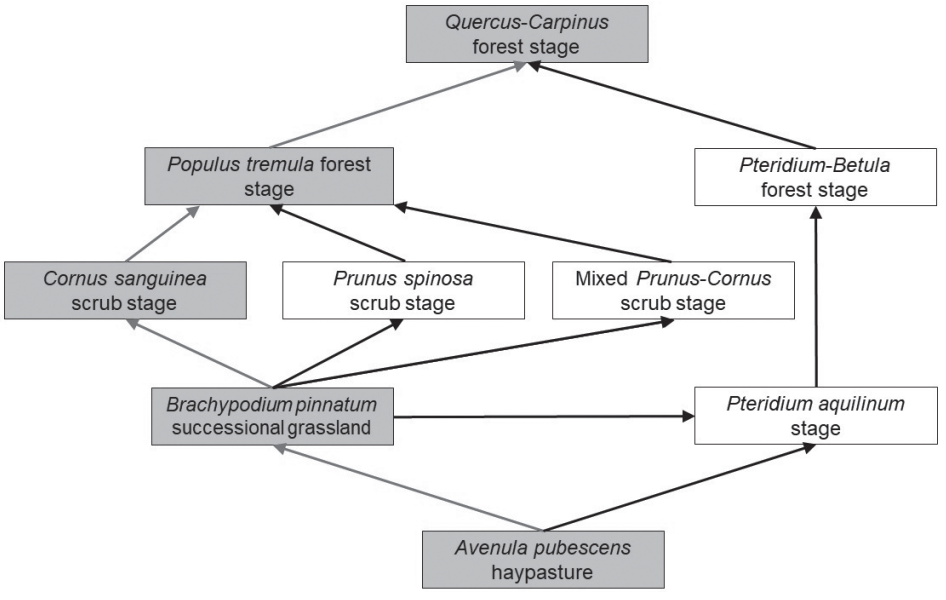

Fig. 1. Different successional pathways of oak-hornbeam forest development after cessation of agricultural land-use (i.e. grassland abandonment) observed in the study area. The successional sequence marked in grey was analysed in the present study.

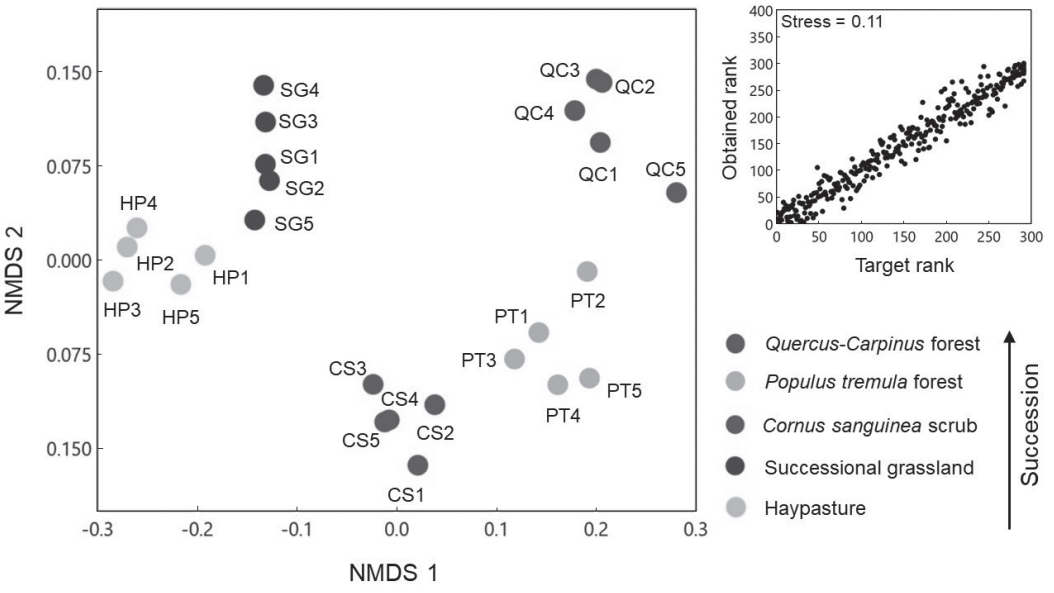

Fig. 2. Non-metric multidimensional scaling ordination (NMDS) of successional stages based on community composition differences obtained by Bray-Curtis dissimilarities. The plot in the upper right corner represents the Shepard plot. The direction of succession progression is well represented by the first two NMDS axes.

structural C (Canals et al. 2017), thus, its spread in our study area has a negative impact on agronomic quality of local hay-pastures.

Cornus sanguinea scrubs (CS)

C. sanguinea gained a complete dominance in this stage, with a cover of 90–100%. Almost all species in the herb layer persisted with a cover of <1%. As its fruits are dispersed by frugivorous birds in high densities, it can easily dominate after cessation of land management (see Kollmann and Grubb 2001). The most frequent species under *C. sanguinea* bushes were *Erigeron annuus* (L.) Desf, *Centaurea jacea* L., *Ajuga reptans* L., *Clinopodium vulgare* L., *Fragaria vesca* L., *Veronica chamaedrys* L., *Prunus spinosa*, and *Rubus fruticosus* L. This stage would likely correspond to the ass. *Corno--Ligustretum* Horvat ex Trinajstić & Z. Pavletić 1991. *Rubus fruticosus* had a somewhat high overall abundance as it was able to intertwine its stems around the branches of *C. sanguinea*, thus acquiring more sunlight. *Carpinus betulus* L. seedlings already appeared in the herb layer of this stage, but *B. pinnatum* and *Populus tremula* were not present in our plots. We noticed that the shrub stages were full of wildlife routes, mainly that of rabbits, roe deer, and wild boars, which might be responsible for the suppression of the herb layer. For *P. tremula*, another possibility is that the saplings were not yet present in the shrub stage, and their growth may occur sometime later by cloning via suckers of the nearby trees (Worrell 1995).

Late-successional *Populus tremula* forest stage (PT)

P. tremula was dominant in the tree layer, accompanied by *Betula pendula* and *Carpinus betulus*. *Cornus sanguinea* remained present in the shrub layer, but its dominance was low compared to that in the scrub stage. *Corylus avellana* L. was also abundant in some plots. In the herb layer, *C. betulus* seedlings were frequent, and those of *Cornus sanguinea* and *Prunus spinosa* still survived in these shaded conditions. *Epimedium alpinum* L. *and Hedera helix* L., the species specific for climax forest, were already present in this stage. It could be considered that this stage resembles some form of ass. *Populo tremulae-Betuletum pendulae* (Glišić 1950) Trinajstić 2004, even though the uniqueness of this association is questionable (Vukelić 2012), as it probably highly depends on the successional context.

Late-successional oak-hornbeam forest stage (QC)

Carpinus betulus was the dominant tree in this stage, accompanied by *Quercus robur* L. In the shrub layer, only *Corylus avellana* appeared in four plots, whereas in the herb layer, *Epimedium alpinum*, *Rubus hirtus* Roxb., and the seedlings of *C. betulus* occurred in all plots. *R. hirtus* occasionally occurred in the previous successional stages, but became frequent in the herb layer of this stage, whereas *E. alpinum,* a species of Illyrian-Balkan chorotype diagnostic for the Illyrian oak-hornbeam forests, dominated the herb layer. This stage was only an approximation to the climax of Illyrian oak-hornbeam forests (ass. *Epimedio-Carpinetum betuli*) that were once present in the study area.

General remarks on the studied succession

Species survival throughout succession differed. *Ajuga reptans* and *Fragaria vesca* survived in all stages. These two species are known to have a high ecophysiological plasticity to changes in environmental conditions, especially in relation to light availability. *A. reptans* can grow well in high light exposure, but in shaded conditions such as under the forest canopy, it can maintain itself likely due to efficient use of nitrogen from the humus in order to increase chlorophyll

synthesis, and consequentially, light capture (Golovko and Dymova 1999). Under canopies, *F. vesca* is known to allocate more energy to aboveground biomass production at the expense of root biomass (Hancock and Bringhurst 1978), which can lead to increase of leaf area in order to expand light capture efficiency. *Viola hirta* was not present in hay-pastures, but it did appear in successional grasslands and persisted throughout the succession. *Veronica chamaedrys* and *Clinopodium vulgare* survived all the way to the *Cornus sanguinea* scrub stage. In the herb layer, *C. sanguinea* and *Prunus spinosa*, being highly dispersed by frugivorous birds, persisted from successional grassland to the late-successional *Populus tremula* stage.

Conclusion

In the studied area, after hay-pasture management is abandoned, succession begins with the spread of *Brachypodium pinnatum*, after which many hay-pasture species decrease in abundance or completely disappear. Succession further proceeds to a *Cornus sanguinea*-dominated scrub stage, followed by a forest stage with *Populus tremula* as a dominant tree. Succession ends with an oak-hornbeam forest (association *Epimedio-Carpinetum betuli*). Only five generalist species survived in almost all succession stages.

Acknowledgments

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Commentary

Data on species plasticity and stable characters have an overall importance in identification keys: comments on Brullo et al. (2022) article

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The genus *Elatine* L. consists of ephemeral species of wetland habitats that live in the temperate regions of both hemispheres. Because of their relatively fast life cycle and small habit, they lead inconspicuous lives, which is probably why they have been relatively little studied in European botany. Although the botanists of the 19th and early 20th century discovered all the currently recognised taxa, there were only a few studies that specifically addressed questions on their biology. This lack of detailed knowledge triggered the more recent interest in this genus (especially the European members), and several papers provided a huge amount of data and evidence about the species' ecology, phenotypic plasticity, biogeography, karyology and molecular taxonomy.

In their recently published paper, Brullo et al. (2022) aimed to lectotypify *Elatine macropoda* Guss. and *E. gussonei* (Sommier) Brullo et al., two enigmatic members of the genus. They gave an overview of the taxonomic issue of these species, identified the type specimens, and gave a comprehensive description of both species, also aiming to clarify open questions in the nomenclature of these taxa. However, some of the points raised by Brullo et al. (2022) are in contrast to previously published scientific evidence, leading them to reach different taxonomic conclusions. In our view, this contrast is the result of (i) misinterpretation of some key findings published in our works, and (ii) adhering to preconceptions on the distribution and specific characteristics of these species. Given the importance of scientific discussion, we here attempt to shed light on contested points to help a better understanding of the taxonomy of this genus in Europe.

It was interesting to note that Brullo et al. (2022) reported hybridisation to be uncommon in *Elatine*, and suggested that it may be a rare phenomenon due to the prevalent autogamous nature of *Elatine* species. In their support of this statement, Brullo et al. (2022) cited Razifard et al. (2017), who reported the allopolyploid hybrid origin of *E. americana* (Pursh) Arn. and *E. hexandra* DC. in their work titled 'Reticulate evolution in *Elatine* L. (Elatinaceae), a predominantly autogamous genus of aquatic plants.' We acknowledge the reference made by Brullo et al. (2022) to Razifard et al. (2017) as an example of hybridisation in *Elatine* species. Furthermore, our own results demonstrated the presence of hybrid lineages in *Elatine* section *Elatinella* subsection *Macropodae*, which includes the focal species of the work of Brullo et al. (2022). Given our findings, along with the previous reports by Sramkó et al. (2016) and Takács et al. (2017), which were also cited by Brullo et al. (2022), it is possible that hybridisation in this genus may be more common than accepted. While the authors may have been aware of the presence of hybridisation in their focal group, we appreciate their analysis and interpretation of the available data, but we must disagree with them: hybridisation is not so rare in this genus.

In light of this, it is not appropriate to consider it a "surprising consequence" that two species of *Elatine* live in sympatry on the island of Sicily, because hybridisation – which does not seem as rare as suggested by Brullo et al. (2022) – requires the close encounter of different species at least at some point during their evolutionary history. It would have been more important in this respect to make a reference to the admixed lineage made up of *E. gussonei* from Lampedusa and Malta (Sramkó et al. 2016, Takács et al. 2017). The introgressed nature of these samples may explain some morphological differences of these populations from the

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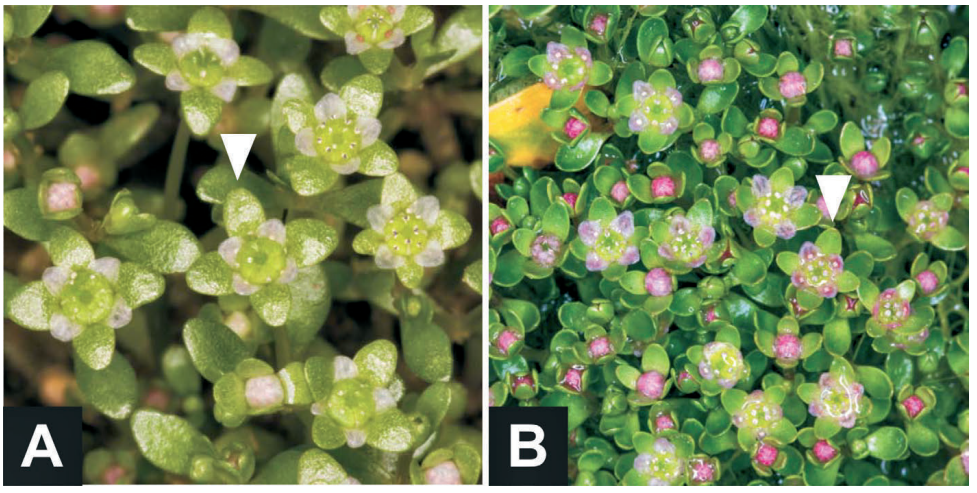

Fig. 1. Flowering specimens of *Elatine gussonei*: A – cultivated (*in vitro*) from Lampedusa, B – indigenous (*in situ*) from Malta. Petal/sepal ratio is occasionally <1. According to Brullo et al. (2022) erroneous assumption this is a distinguishing character that is specific to *E. macropoda* (see Brullo et al. 2022, Fig. 2). (Photo: B.A. Lukács).

rest of the distribution area of *E. gussonei*, which may be behind the view of seeing these populations as "different" by various scholars. In this respect, it is noteworthy to refer to the unfortunate use of a Lampedusa plant, without characterising its nuclear genome, as the lectotype of *E. gussonei* now selected by Brullo et al. (2022), which can also be an introgressed plant.

Not only is there the problem of hybridisation, but our detailed study (Molnár et al. 2015) of the well-known phenotypic plasticity of vegetative characters in this amphibious genus (Mason 1956, Mifsud 2006) is another key piece in the literature that is overlooked by Brullo et al. (2022). Although they refer to the existence of phenotypic plasticity, in this context they failed to cite the results of Molnár et al. (2015) on the stability of seed characteristics and instability of vegetative and floral characters. This explains why they refer to the length of the petal and the sepal as one of the key characters separating the species *E. macropoda* and *E. gussonei*. Although Gussone (1827), Sommier (1907) and Pignatti (2017) emphasised the relevance of floral characters in the taxonomy of *Elatine*, we must take it into account that Mifsud (2006) has already documented the instability of these characters, which is simply rejected by Brullo et al. (2022) on the basis of a subjective evaluation ("*In our opinion, the floral traits cannot be linked exclusively to environmental conditions or flowers age*") and claim the opposite, citing their observations without measured and tested dataset ("*based on our observations, E. gussonei (Lampedusa and Malta) is morphologically distinct from the typical E. macropoda*"). Sommier (1907) has already emphasized that *E. gussonei* differs from *E. maropoda* by its more curved seeds. In line with Sommier's and Mifsud's work, our measured dataset and statistical analyses demonstrate that seed morphology, especially its shape and surface ornamentation remains stable under different environmental conditions (Molnár et al. 2015), hence these are the most obvious morphological characters to differentiate species of *Elatine*, at least on the studied area. Moreover, this study also showed that the amount of light alone has a significant effect on the morphology of the vegetative and floral parts of the plants. Compared to plants growing under natural light conditions, the internodes, pedicels, caulin- and sepal leaves of *in vitro* grown individuals exposed to less intense artificial light are longer (Fig. 1A and Fig. 1B).

Brullo et al. (2022) were selective in their choice when accepting the taxonomic importance of seed "ornamentation" (i.e., the shape of epidermal pits on the surface of the seed), but deny the utility of seed curvature, although our results (Molnár et al. 2015) clearly demonstrated the taxonomic value of this character (Fig. 2). It may be noted here that our very recently published paper (Łysko et al. 2022) emphasises this role even more: we tested several analytical methods on the discriminatory power of seed morphometry in the genus, where seed shape and ornamentation were found to be highly discriminatory. Regardless of this new result, however, Brullo et al. (2022) are wrong when they refer to Sramkó et al. (2016) as a source of information that seed morphology is a „*quite variable trait even within the single populations.*" In fact, our study summarised the seed morphology of different populations at the species level (given the main goal of reconstructing the evolutionary history of the genus).

In order to demonstrate the usefulness of the preferred seed morphological characters, Brullo et al. (2022) published scanning electron microscope (SEM) images of seeds of *E. macropoda* (Brullo et al. 2022: Fig. 4) and *E. gussonei* (Brullo et al. 2022: Fig. 6), plus a comparative close-up image on epidermal pit shape of both species (Brullo et al. 2022: Fig. 5) where we can see pits of "rectangular or slightly hexagonal" shape as typical of *E. macropoda* (Brullo et al. 2022: Fig. 5A), and pits of "more or less isodiametric and usually hexagonal" shape as typical of *E. gussonei* (Brullo et al. 2022: Fig. 5B). Although the authors do not provide us with any measurement data for a statistically sound comparison, the visual inspection of their Fig. 4 would leave most observer with the impression that Fig. 4C and Fig. 4D

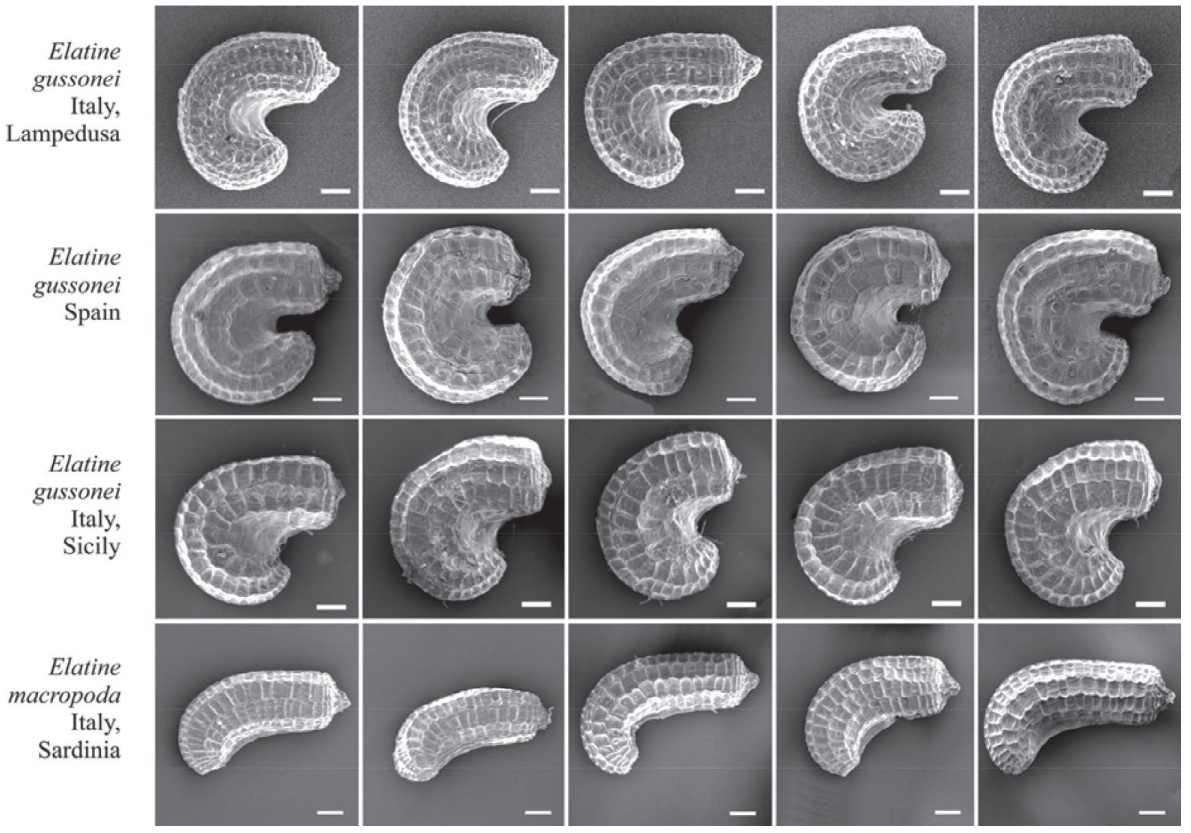

Fig. 2. Comparison of seeds of *Elatine gussonei* and *E. macropoda*. Scale bars = 0.1 mm. (SEM photo: A. Popiela).

(Sicilian plants from Modica and Ispica, respectively) are more similar to Fig. 5B, therefore, should be identified according to their epidermal seed pit shapes as *E. gussonei*.

In fact, both the seed curvature and epidermal structure clearly suggest the correct identification of the plants shown from Modica and Ispica as *E. gussonei*. Although Brullo et al. (2022) argue for the importance of longer petal length as a distinctive character that defines *E. gussonei*, it is rather easy to find *Elatine* plants with short petals on the island of Malta (see Fig. 1B) where – according to Brullo et al. (2022) – only *E. gussonei* lives. Such plants were also presented by Mifsud (2006) and further examples can be seen in his photographic collection (https://maltawildplants.com/ELTN/Elatine_gussonei.php). If Brullo et al. (2022) were to consider the role of seed morphological characters in the genus and take into account the numerous publications that discuss the plastic nature of vegetative and floral characters (Molnár et al. 2014, 2015, Sramkó et al. 2016, Takács et al. 2017, Łysko et al. 2022) as well as the phylogenetic results (Sramkó et al. 2016, Razifard et al. 2017) in greater detail, they might find it easier to accept the presence of *E. gussonei* in Sicily and other Mediterranean areas. We must note here it is common in taxonomy for researchers to rely on different sets of characters and hold differing taxonomic opinions.

We accept, however, that this contradicts the well-established view on the very limited distribution and endemic nature of *E. gussonei* (Brullo et al. 1988, 2022), and would also necessitate the conservation re-evaluation of this species (Takács et al. 2017). Having said that we also think this species will still remain one of the key characteristic species of temporary Mediterranean ponds that – quite correctly! – enjoy the highest level of conservation interest in the European Union. Therefore, the taxonomic re-interpretation of *E. gussonei* and the consequently larger distribution area (it is still a Mediterranean endemic!) is not of concern for this plant from a conservation point of view. Instead, a better understanding of taxonomy is a fundamental prerequisite of well-established species conservation (Mace 2004).

In summary, Brullo et al. (2022) downplay i) the importance of seed shape as an identification character and ii) the environment (primarily light intensity and water availability) and phenology dependent nature of floral characters. While their results, which lack any report of detailed statistical analyses, are based on seed pit morphology, it is important to note that these values can be compared and verified. However, it is necessary to maintain scientific rigor, and the lack of detailed measurements and rigorous statistics in their report may limit the verifiability of the findings. Considering the contradictions between our previously presented coherent works and the recent claims of Brullo et al. (2022), we cannot accept their statements on the morphology and distribution of *Elatine macropoda* and *E. gussonei*. We further claim that their identification key for European (and not Mediterranean, as they indicated) *Elatine* species is misleading, since it focuses on phenotypically plastic characters and thus we recommend using the key presented in Popiela et al. (2017) to identify European species of *Elatine*.

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