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On the cover:

Melaleuca williamsii subsp. *synoriensis* was found along the banks of the Tiber River in Rome, Italy, representing its first record outside its native range. This finding is reported by Iamonico and Nicolella (pp. 115-118).

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Nomenclature of the Balkan alliance *Romuleion graecae* (*Poetea bulbosae*)

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Abstract – The *Romuleion*, the only alliance of the order *Poetalia bulbosae* (class *Poetea bulbosae*) found on the Balkan Peninsula, represents Mediterranean perennial and ephemeral pastures. It has been found in several Balkan countries, from Greece to the Republic of North Macedonia, Montenegro, Croatia and Bulgaria. However, a revision of its nomenclature according to the fourth edition of the International Code of Phytosociological Nomenclature shows that the name of the alliance was not validly published. In this paper we therefore describe the new alliance *Romuleion graecae*, together with the new association *Plantagini lagopodis-Poetum bulbosae*.

Keywords: Balkan vegetation, ICPN, Mediterranean grassland, nomenclature, phytosociology, *Poetea bulbosae*, syntaxonomy

Introduction

The class *Poetea bulbosae* is accepted in the standard European vegetation classification (EVC), as representing the “Mediterranean and Maghrebinian seasonal perennial and ephemeroid pastures in the thermo- to oro-Mediterranean belts” (Mucina et al. 2016, EVS 2017, FloraVeg.EU 2023, Terzi et al. 2024).

The class includes only one order, *Poetalia bulbosae*, and six alliances, namely *Trifolio-Periballion*, *Plantaginion serrariae*, *Poo bulbosae-Astragalion*, *Ornithogalo corsici-Trifolion subterranei*, *Plantaginion cupanii*, and *Romuleion* (FloraVeg.EU 2023, Fernández-González et al. 2023, Terzi et al. 2024).

The distribution range of most of these alliances is restricted to the western Mediterranean, i.e. the Iberian Peninsula and the Tyrrhenian Islands, and to Italy (i.e. *Plantaginion cupanii*), while only the alliance *Romuleion* has been found on the Balkan Peninsula and its islands (Preislerová et al. 2022). More specifically, the *Romuleion* has been reported in Greece, the Republic of North Macedonia, Croatia, and Montenegro, while its occurrence is considered uncertain in Albania, Bulgaria, and Bosnia and Herzegovina (Oberdorfer 1954, de Bolòs et al. 1996, Čarni et al. 2014, Škvorc et al. 2017, Preislerová et al. 2022, Stanišić-Vujačić et al. 2023, Terzi and Jasprica 2024).

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The *Romuleion* was originally placed in the class "*Thero--Brachypodietea*" (Oberdorfer 1954), and then moved to the *Brachypodio-Chrysopogonetea* (Horvat et al. 1974), the *Saginetea maritimae* (Rodwell et al. 2002), and the *Tuberarietea guttatae* (Čarni et al. 2014). Finally, in the EVC, it has been framed within the *Poetea bulbosae* (Mucina et al. 2016).

Although the *Romuleion* is the only alliance of the class *Poetea bulbosae* occurring in the Balkans, with a potentially rather wide distribution, its original description raises some problems from a nomenclatural point of view (see also Čarni et al. 2014). Therefore, the aim of this paper is to revise the nomenclature of the alliance and thus contribute to the stabilisation of the European vegetation system by the use of correct names.

Material and methods

The revision of the nomenclature of the alliance *Romuleion* is based on the fourth edition of the International Code of Phytosociological Nomenclature (ICPN, Theurillat et al. 2021), whose articles (Art.) are quoted in brackets in the text. The names of the syntaxa, which are given exactly as in the mentioned articles, are given in quotation marks (" ").

In order to assess possible synonymy between some of the names of the associations involved in the validation of the alliance name, Jaccard's similarity index (J, Kent 2012) was calculated between the phytosociological synoptic tables of some of the associations.

The taxonomic nomenclature follows Euro+Med (2006-2023), while the syntaxonomic nomenclature follows FloraVeg.EU (2023) and its updates as proposed by Fernández-González et al. (2023) and Terzi et al. (2024).

To facilitate comparison with the EVC, the names of the syntaxa in the syntaxonomic scheme at the end of the discussion have been supplemented with the codes already assigned in the EVC (three digits for classes, five for orders and six for alliances).

Results and discussion

The "*Romulion*" alliance [recte: *Romuleion*] was originally described by Oberdorfer (1954) with a diagnosis comprising two associations. Another, the "*Biareto-Poetum timoleontis*", is merely mentioned as provisional (Art. 3b) and is moreover invalid as it lacks a sufficient original diagnosis (Art. 2b). The two substantiated associations, "*Tortileto--Poetum timoleontis*" and "*Lagopeto-Poetum timoleontis*", are represented by a synoptic table derived from 14 and 17 relevés, respectively, from the surroundings of the city of Thessaloniki, Thessaly, Isthmus of Corinth, Attica, Southern Macedonia and Thrace (all in Greece). However, this synoptic table contains neither *Poa timoleontis* Boiss. (Fl. Orient. 5: 607. 1884), nor *Poa bulbosa* subsp. *timoleontis* (Boiss.) Hayek (Repert. Spec. Nov. Regni Veg. Beih. 30(3): 260. 1933), but only *Poa bulbosa*. Consequently, these associations were not validly published because one of the name-giving taxa is missing in their diagnosis (Art. 3f), although mentioned in the text (Oberdorfer 1954: 89, "…von *Poa bulbosa* beherrschten Gesellschaften"). Since all associations assigned to the diagnosis of the alliance in Oberdorfer (1954) are thus invalid, the name *Romuleion* Oberdorfer 1954 is also invalid for lack of a sufficient diagnosis (Art. 2b).

In Horvat et al. (1974: 120-121) the two associations described by Oberdorfer (1954) within the *Romuleion* alliance, namely "*Tortileto-Poetum timoleontis*" and "*Lagopeto-Poetum timoleontis*", were united under a single association name, "*Poetum timoleontis*", with two subassociations ("Subass. mit *Stipa tortilis*" and "Subass. mit *Plantago lagopus*"). However, the diagnosis of the *Poetum timoleontis* is still based on the same synoptic relevés published by Oberdorfer (1954), where the name-giving taxon *Poa timoleontis* is missing. Consequently, the names *Poetum timoleontis* Oberdorfer ex Horvat, Glavač et Ellenberg 1974 (Art. 3f) and *Romuleion* Oberdorfer ex Horvat, Glavač et Ellenberg 1974 (Art. 2b) are still invalid names.

Later, Bolòs et al. (1996) renamed the alliance "*Romulion graecae* Oberd. 1954 em. nom. (*Romulion* Oberd.)" and assigned a new association, "*Airo elegantissimae-Trifolietum dalmaticae*", from the Ionian island of Cephalonia (Greece). This association was validly published with a sufficient original diagnosis consisting of seven relevés (Table 12, on page 106). This was not so for the alliance because the name-giving taxon *Romulea linaresii* subsp. *graeca* Bég. (Bot. Jahrb. Syst. 38: 325, 1907) was missing in the only valid element of the diagnosis, namely in the relevés of the new association (the two associations of Oberdorfer (1954), which were also referred to, were invalidly published). The name *Romuleion graecae* Oberdorfer ex de Bolòs, Masalles, Ninot, et Vigo 1996 is therefore invalid under Art. 3f.

Čarni et al. (2014) provided a different interpretation on the validity of the names published by Oberdorfer (1954), considering the alliance *Romuleion* and the two associations "*Tortileto-Poetum timoleontis*" and "*Lagopeto-Poetum timoleontis*" as validly published. They argue that the taxon name "*Poa bulbosa*" given in the table on page 90 ("Liste I") in Oberdorfer (1954) is a printing error for "*Poa bulbosa* coll." as written in the two other tables ("Liste II" and "Liste III") of the work on pages 92 and 94, respectively. In their view, "*Poa bulbosa* coll." would include all subspecies of *Poa bulbosa* (or species of the *Poa bulbosa* aggregate), including *Poa timoleontis* (*Poa bulbosa* subsp. *timoleontis*), and would therefore comply with Art. 3f. The justification of this interpretation would be the statement made by Oberdorfer on page 88 that *Brachypodium ramosum* [recte: *B. retusum*] or *Brachypodium phoenicoides*, which are widespread in the western Mediterranean, are completely receding eastwards where they are replaced by various subspecies ("div. ssp.") of *Poa bulbosa*. To some extent, such an interpretation might have been supported by the content of Art. 3f in the third edition of the ICPN (Weber et al. 2000), which only states that the name-giving taxon should be "indicated in the original diagnosis". This interpretation is no longer valid with the present rules, as they state (Art. 3f Note 1) that the name-giving taxon must be present in the relevés belonging to the "original diagnoses of the associations that have been quoted in the original diagnosis of the alliance". Therefore, the taxon *Poa timoleontis*, which is not explicitly included in the collective species *Poa bulbosa*, is not mentioned in the synoptic table of the two associations. Consequently, the associations *Stipo tortilis-Poetum timoleontis* and *Lagopo--Poetum timoleontis* are not validly published in Oberdorfer (1954). In considering these two association names as validly published, Čarni et al. (2014) also made some corrections. In both names, the first name-giving taxon corresponds to a specific epithet used without the generic name, namely "*Tortileto*" for "*Stipa tortilis*" and "*Lagopeto*" for *Plantago lagopus*. In accordance with Art. 14b, Čarni et al. (2014) corrected the former name to *Stipo tortilis-Poetum timoleontis*. For the latter name, where the specific epithet "*lagopus*" is also a validly published generic name, they retained the association name and corrected the orthographic error ("*Lagopo*" instead of "*Lagopeto*"; Arts. 10a and 41b). Indeed, *Lagopus arvensis* Fourr. (Ann. Soc. Linn. Lyon sér. 2, 17: 140, 1869) is a valid name (International Plant Names Index, https://www.ipni.org/n/32110-1, accessed 3 Oct 2023) for *Plantago lagopus* L., and according to the third edition of the ICPN (Weber et al. 2000) this name should be retained as the name-giving taxon. However, as *L. arvensis* Fourr. is an illegitimate name, this would no longer be the case with the current rules (Art. 44). Čarni et al. (2014, p. 124) also corrected the second name giving-taxon of the name *Lagopo-Poetum timoleontis*, namely *Poa timoleontis*, to *Poa bulbosa*, in accordance with Art. 43, because they considered the presence of the taxon *Poa timoleontis* in the research area to be doubtful. On the other hand, they did not make this correction for the name *Stipo tortilis-Poetum timoleontis*, considering that the taxon *Poa timoleontis* could occur where Oberdorfer had originally sampled the association. Čarni et al. (2014) provided a neotype for the *Lagopo-Poetum timoleontis* Oberdorfer 1954 corr. Čarni et al. 2014, but the latter name was nevertheless not incidentally validated, as it was not reported as new (Art. 3i).

As the name *Lagopo-Poetum* remains invalidly published, we validate it, and correct the name here based on the results of Čarni et al. (2014), namely *Plantagini lagopodis--Poetum bulbosae* Čarni, Matevski, Šilc et Ćušterevska ex Terzi, Jasprica, Čarni, Matevski, Bergmeier et Theurillat ass. nov. hoc loco. The original diagnosis of the new association includes relevés 1-12 of Table 1, on page 112, in Čarni et al. (2014), and its holotypus is relevé 5 in this table, which is the same relevé selected as the neotype for the invalid *Lagopo--Poetum bulbosae*.

A second association, *Romuleo graecae-Poetum bulbosae*, was also validly published by Čarni et al. (2014) and classified in the *Romuleion*. However, the name of this alliance was not validated either, as it was not reported as new (Art. 3i). Recently, Stanišić-Vujačić et al. (2023) maintained the same nomenclatural interpretation for the *Romuleion* as Čarni et al. (2014), and they described two new associations to be included in this alliance, the *Romuleo bulbocodii--Poetum bulbosae* and the *Ornithogalo exscapi-Poetum bulbosae*.

Attempts to find a neotype for the "*Tortileto-Poetum timoleontis*" have failed, as no adequate relevé could be found. Two relevés containing *Poa bulbosa* together with *Stipa capensis* (= *S. tortilis*) were published by Čarni et al. (2014) among the relevés of the *Romuleo graecae-Poetum bulbosae* (relevés 14 and 20, Table 1). However, the floristic composition of these two relevés appears to be more similar to the other relevés of the *Romuleo graecae-Poetum bulbosae* than to those in the synoptic table of the "*Tortileto-Poetum timoleontis*" in Oberdorfer (1954). A comparison of the synoptic table of the original diagnosis of the *Plantagini lagopodis-Poetum bulbosae* in Čarni et al. (2014) (relevés 1-12, Table 1) showed that it is also closer to the *Romuleo graecae-Poetum bulbosae* (J = 0.51) than to the relevés in Oberdorfer's synoptic table of the "*Lagopo-Poetum timoleontis*" (J = 0.27) or the "*Tortileto-Poetum timoleontis*" (J = 0.27). Conversely, a comparison between the two synoptic relevés of Oberdorfer (1954) showed that their floristic composition is very similar (J = 0.78). Therefore, the syntaxonomic interpretation by Horvat et al. (1974), which considers Oberdorfer's two syntaxa to be two subassociations of the same association seems justified, and the *Lagopo-Poetum timoleontis* Oberdorfer 1954 could correspond to a different association than the *Plantagini lagopodis--Poetum bulbosae*. However, in order to verify this hypothesis, relevés from the regions from which Oberdorfer described his two associations are essential.

In terms of alliance, the *Romuleion* currently contains five associations (see the syntaxonomic scheme below). A sixth association (*Festuco valesiacae-Poetum bulbosae*) has recently been described (Terzi and Jasprica 2024), but questions remain about its inclusion in this alliance. Another undescribed association may occur in Crete, according to unpublished relevés of E. Bergmeier. Those new syntaxa are not considered in the present discussion. The name of the alliance is derived from the genus *Romulea* Maratti (Pl. Romul. Saturn. 13, 1772), which is present with two taxa in the original diagnoses of the five described associations, (1) *Romulea bulbocodium* (L.) Sebast. & Mauri (Fl. Roman. Prodr. 17, 1818) and (2) *Romulea linaresii* subsp. *graeca*. *Romulea bulbocodium* is a widespread Mediterranean species (Euro+Med 2006-2023) whereas *Romulea linaresii* subsp. *graeca* is a Balkan-Anatolian taxon occuring in Turkey, Greece, including the Aegean islands, and some Balkan countries (e.g. Hadžiablahović and Bulić 2004, Dimopoulos et al. 2013, Raycheva et al. 2021).

Since the alliance is centred in the southern Balkans, we follow the proposal of Bolòs et al. (1996) to consider *Romulea linaresii* subsp. *graeca* as the name-giving taxon, and we validate the name *Romuleion graecae* Oberdorfer ex Terzi, Jasprica, Čarni, Matevski, Bergmeier et Theurillat all. nov. hoc loco. The nomenclatural type (holotypus) of the new alliance is the *Romuleo graecae-Poetum bulbosae* Čarni, Matevski, Šilc et Ćušterevska 2014 (Čarni et al. 2014, p. 125) from the southern part of the Balkans (Greece). According to Oberdorfer (1954) and Čarni et al. (2014), the characteristic species of the alliance are: *Allium guttatum*, *Alyssum minutum*, *Alyssum repens*, *Campanula ramosissima*, *Gagea reticulata*, *Hedypnois rhagadioloides*, *Hypochaeris cretensis*, *Lagoecia cuminoides*, *Linaria simplex*, *Lotus angustissimus*, *Ornithogalum collinum*, *Ornithogalum armeniacum*, *Picris pauciflora*, *Romulea bulbocodium*, *Romulea linaresii* subsp. *graeca*, *Romulea columnae*, *Sedum aetnense*, *Silene graeca*, *Ziziphora capitata*.

Therefore, we propose the following syntaxonomic scheme (the author citation of the class follows Terzi et al. 2024):

BUL – *Poetea bulbosae* Rivas Goday et Rivas-Martínez ex Navarro Andrés et Valle Gutiérrez 1984

BUL-01 *Poetalia bulbosae* Rivas Goday et Rivas-Martínez in Rivas Goday et Ladero 1970

BUL-01F *Romuleion graecae* Oberdorfer ex Terzi, Jasprica, Čarni, Matevski, Bergmeier et Theurillat all. nov. hoc loco [holotypus: *Romuleo graecae-Poetum bulbosae* Čarni, Matevski, Šilc et Ćušterevska 2014; synonyms: *Romuleion* Oberdorfer 1954 (Art. 2b), *Romuleion* Oberdorfer ex Horvat, Glavač et Ellenberg 1974 (Art. 2b), *Romuleion graecae* Oberdorfer ex de Bolòs, Masalles, Ninot et Vigo 1996 (Art. 3f)]

Plantagini lagopodis-Poetum bulbosae Čarni, Matevski, Šilc et Ćušterevska ex Terzi, Jasprica, Čarni, Matevski, Bergmeier et Theurillat ass. nov. hoc loco [syn. *Lagopo-Poetum bulbosae* Oberdorfer 1954 corr. Čarni, Matevski, Šilc et Ćušterevska 2014 (corr. superfl.)]

Romuleo graecae-Poetum bulbosae Čarni, Matevski, Šilc et Ćušterevska 2014

Airo elegantissimae-Trifolietum dalmatici Bolòs, Masalles, Ninot et Vigo 1996

Romuleo bulbocodii-Poetum bulbosae Stanišić-Vujačić, Stešević, Hadžiablahović et Šilc 2023

Ornithogalo exscapi-Poetum bulbosae Stanišić-Vujačić, Stešević, Hadžiablahović et Šilc 2023

Poetum timoleontis Oberdorfer ex Horvat, Glavač et Ellenberg 1974 (Art. 3f) [incl. *Lagopo-Poetum timoleontis* Oberdorfer 1954 (Art. 3f), *Tortileto--Poetum timoleontis* Oberdorfer 1954 (recte: *Stipo capensis-Poetum timoleontis*) (Art. 3f)]

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Author contribution statement

M.T. and J.-P.T. conceived and wrote the manuscript; all authors critically revised and approved the manuscript.

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The taxonomy and distribution of algae in the thermal springs of Türkiye

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Abstract – The algal flora and physio-chemical parameters of seven thermal springs in Denizli were studied for the first time. Samples for algal analyses were taken monthly between May 2013 and June 2014, while the physio-chemical parameters were measured seasonally. The mean pH value of the thermal springs was 6.3, and temperatures varied between 34–60 °C. The significant differences ($P < 0.001$) among the thermal springs were in their temperature and pH, as well as concentration of Cl^-, Fe^{2+}, K^+, Mg^{2+}, Na^+, Li^+, Ca^{2+}, HCO_3^-, and SO_4^{2-} ions. A total of 43 Cyanobacteria and three Bacillariophyta taxa were determined. The most common taxon was *Spirulina subsalsa* Oersted ex Gomont, sampled from five sampling sites. According to the principal component analysis (PCA), the most important determining factor for the algae was temperature, followed by concentration of K^+ and Cl^- ions.

Keywords: cyanobacteria, diatoms, ecology, principal component analysis, thermal algae, travertine

Introduction

Algae are commonly found in seas and fresh or brackish waters. However, they can survive in deserts, moist soils and stones, thermal springs, snow at the poles, and in all conditions with very little moisture. Thermal springs, in particular, are extreme ecosystems for algae. Certain groups of algae are common in thermal springs, such as Cyanobacteria and Bacillariophyta. Examining the species living in thermal springs is important in determining the factors conducive to life in these environments. Thus, the potential economic value of these species is revealed, and their biotechnological use improves.

Because it is located on fault lines, Türkiye has numerous thermal springs, concentrated mainly in the west of the country. The city of Denizli has numerous and varied thermal springs, which are famous worldwide for travertines and historical and cultural features. Travertines are formed by the precipitation of calcium in layers like thick lime deposits (Güner 1970, Pentecost et al. 1997). The most important characteristic of thermal springs in Denizli is that, although they are very close, they show different physio-chemical parameters (Kozak 2020). These thermal springs are in Pamukkale, Karahayıt, Yenicekent, Inalti, Şanlıalp, Umut, and Gölemezli. While Karahayıt, Yenicekent, and Umut have deep circulation and a high temperature, Pamukkale and Gölemezli have a low temperature and shallow water characteristics (Yaman and Özgür 2005). Pamukkale, or "Hierapolis," is on the UNESCO list of world heritage sites and is perhaps one of the oldest spa centers in Türkiye. The Hellenistic spa town of Hierapolis was a focus of interest for visitors at the end of the second century BC (UNESCO 2022).

The first study in Türkiye about the biology of thermal springs was from Pamukkale (Regel and Skuja 1937), and there are other studies on the algal flora of the Pamukkale thermal spring (Güner 1966, Pentecost et al. 1997, Altunöz et al. 2016, Öztürk Ulcay et al. 2017). Also, Aysel et al. (1992) examined cyanobacteria in the Ilıksu thermal springs (Zonguldak, Türkiye) and reported 33 Cyanobacteria taxa. Öztürk Ulcay et al. (2007) explored the thermal springs in Dikili (İzmir, Türkiye) and identified 19 Cyanobacteria taxa. Öztürk Ulcay and Kurt (2017) identified a total of 27 taxa (21 Cyanobacteria, five Bacillariophyceae and one Conjugatophyceae) in Alangüllü (Aydın, Türkiye). Öztürk (2020) reported 13 Cyanobacteria taxa in the thermal springs in Kütahya (Türkiye). Thermal algae studies have also intensified in the west of Türkiye where many thermal springs are located. Although there are many thermal springs in Denizli, thermal algae studies in this region have only been carried out in the Pamukkale and Karahayıt thermal springs.

The aim of the present study was to provide for the first time a study of the algal flora in the thermal springs of Denizli and their relationship to the ecological conditions of seven thermal springs in Denizli.

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Material and methods

Sampling

Denizli is one of the important thermal regions of Türkiye for health tourism and the touristic thermal springs there were chosen as the study area (Fig. 1). Gölemezli (D1; 37°59' N 29°02' E), Yenicekent (D2; 38°02' N 28°57' E), İnaltı (D3; 37°54' N 28°45' E), Şanlıalp (D4; 37°57' N 29°04' E), Umut (D5; 37°55' N 28°49' E), Pamukkale (D6; 37°55' N 29°07' E) (Fig. 2), and Karahayıt (D7; 37°58' N 29°06' E) (Fig. 3). Samples were taken from several points (thermal water outlet points, natural and artificial pools, travertines, and thermal water channels) of these thermal springs. Sample sites were scattered over approximately 207 km^2, ranging from an altitude of 148 m to 420 m a.s.l.

Algae sampling was done monthly between May 2013 and June 2014 using forceps, a spatula, and a plankton net with a 30 µm mesh size. Two algae samples were taken separately from each sample site in 50 mL falcon tubes. Then, a 4% formaldehyde solution was added to one of the samples for fixing. The second algae sample was used for identification. The algae samples were labeled and brought to the laboratory in the dark. Algae samples were examined under an Olympus BX 50 (phase-contrast) microscope and photographed using a Sony DSC-TX7 camera in the laboratory. The literature was used to identify the algae taxa: Komárek and Anagnostidis (1999, 2005), and Komárek (2013) for Cyanobacteria taxa and Cantonati et al. (2017) for Bacillariophyta. The nomenclature was checked on the AlgaeBase database (Guiry and Guiry 2023).

Water samples were taken simultaneously in sterile bottles during the algae sampling. The pH and temperature were measured *in-situ*, with a Hanna HI 9812-5 Portable Meter. Sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), lithium (Li^+), magnesium (Mg^{2+}), ferrous (Fe^{2+}), chloride (Cl^-), bicarbonate (HCO_3^-), and sulphate (SO_4^{2-}) analyses were made *ex-situ*. The concentrations of Na^+, K^+, Ca^{2+}, Li^+, Mg^{2+}, and Fe^{2+} ions were measured by atomic absorption spectrometer, the concentrations of Cl^- and HCO_3^- ions were measured by titrimetric analysis, the concentration of SO_4^{2-} ion was measured by theoretical methods, and the ion concentration results are given as mg L^{-1}. *In-situ* measurements were made every month (between May 2013 and June 2014), while *ex-situ* measurements were made seasonally (in July and October 2013 and in January and April 2014). The results of *in-situ* and *ex-situ* measurements are given as their means.

Fig. 1. The map of the study area showing the investigated thermal springs, and their locations in Denizli (Türkiye). D1 – Gölemezli, D2 – Yenicekent, D3 – İnaltı, D4 – Şanlıalp, D5 – Umut, D6 – Pamukkale, D7 – Karahayıt.

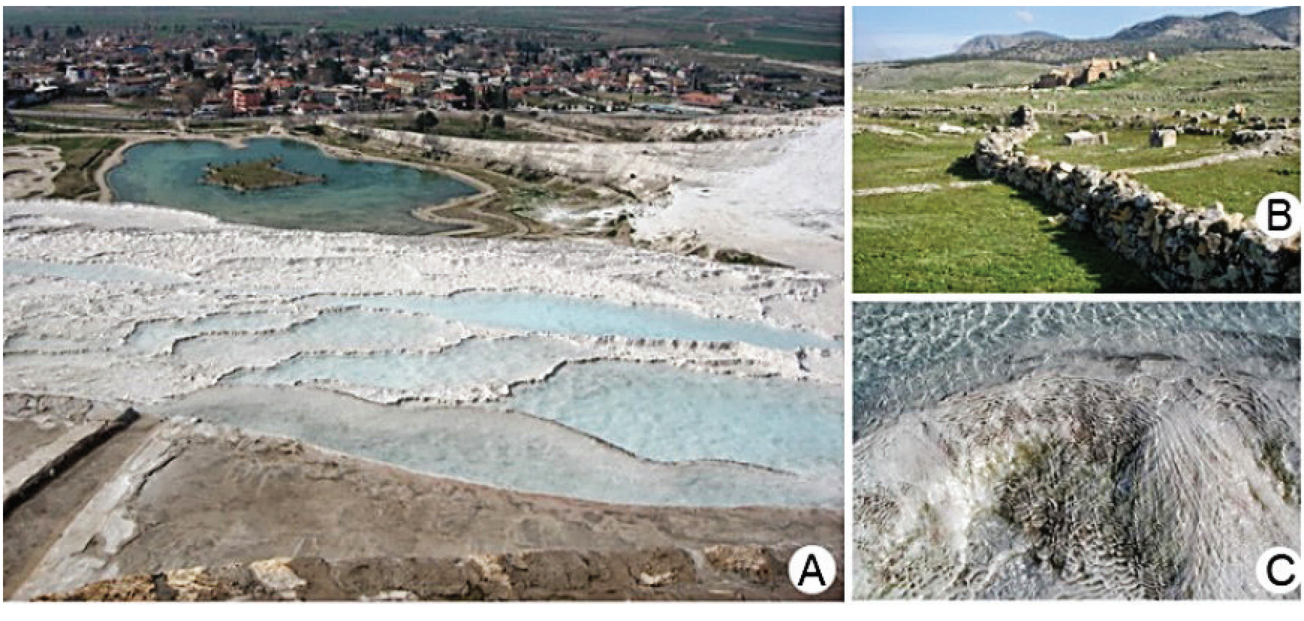

Fig. 2. The Pamukkale thermal spring (D6). A – general view of travertines, B – the Hellenistic spa town of Hierapolis, C – details of the travertines (photo: Sevilay Öztürk).

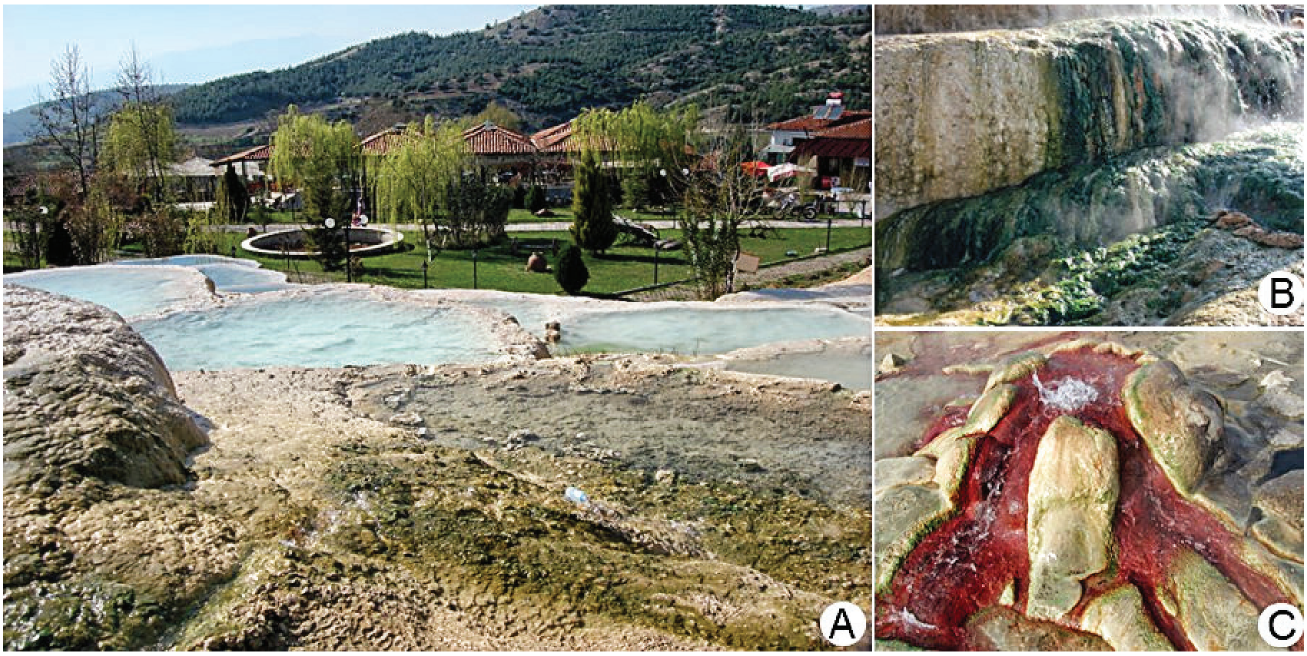

Fig. 3. The Karahayıt thermal spring (D7). A – general view of travertines, B – details of the travertines, C – thermal water outlet points (photo: Sevilay Öztürk).

Statistical analysis

Canoco 5.0 software for Windows (Microcomputer Power, Ithaca, NY, USA) was used to determine the correlation between the physio-chemical characteristics of the thermal springs and algal flora (Ter Braak and Šmilauer 2012). Firstly, detrended correspondence analysis (DCA) was done to determine the gradient length and whether the studied gradient was suitable for linear or unimodal models. The results of the DCA were ideal for principal component analysis (PCA). The algal flora composition (total of 46 taxa from 7 sample sites on 12 sampling dates) with binary data was used in this analysis. Tests of significance of the first and all canonical axes were performed to statistically assess the relation between algal flora composition and physio-chemical characteristics (Monte Carlo test: 499 permutations under the reduced model).

Additionally, the Monte Carlo permutation test was applied to determine the statistical significance of physio-chemical characteristics in explaining the composition of algal flora. This involved a stepwise "forward selection" of explanatory variables, as available in Canoco. The process was begun by selecting the most effective explanatory variable (the one that best explained the overall variance in the data). Subsequent variables were chosen based on their decreasing importance in explaining the total variance in the dataset, in conjunction with the previously selected variables. The statistical significance of each variable was also assessed. The variation in algal flora composition explained by the physio-chemical characteristics included in the analysis was expressed as a percentage, representing the ratio of the sum of all canonical eigenvalues to the total variance (total inertia).

The Kruskal-Wallis H-test was conducted using SPSS 28.0 software to determine the statistical significance of the difference in values of physio-chemical parameters of thermal springs by sample sites. The Kruskal-Wallis test used the 12-month means of pH and temperature values and the seasonal means of Na^+, K^+, Ca^{2+}, Li^+, Mg^{2+}, Fe^{2+}, Cl^-, HCO_3^-, and SO_4^{2-} values of water samples from seven thermal springs. None of these data were transformed. The Kruskal-Wallis test is a non-parametric analysis of variance test used to assess the significance of differences among means of three or more groups in cases where the data does not follow a normal distribution.

Results

Physio-chemical characteristics of the thermal springs

Differences were observed among the physio-chemical parameters of seven thermal springs in Denizli (Tab. 1).

The highest temperature value was measured at D3 as 60 °C, the lowest temperature value was measured at D6 as 34 °C, and the mean temperature value of all sampling sites was 47 °C. In terms of calcium values, D7 had the highest value of 377.10 mg L^{-1}, while D3 had the lowest value of 8.7 mg L^{-1}. With potassium values, D4 had the highest value of 134.0 mg L^{-1}, while D6 had the lowest value of 7.5 mg L^{-1}. A difference in ferrous values was also seen among sample sites, with D7 having the highest value of 2.45 mg L^{-1} and D3 having the lowest value of 0.034 mg L^{-1}. In terms of bicarbonate values, D3 (689.53 mg L^{-1}) is quite low compared to the other sample sites. It was observed that the lithium values of D6 and D7 were considerably lower than those of the other sample sites. The measured sodium values at D4 (1392.2 mg L^{-1}) were twenty times higher than at D6

Tab. 1. The physio-chemical parameters of the thermal springs (D1-D7) in Denizli (Türkiye) and the results of the Kruskal-Wallis test (with SPSS 28.0) were used to reveal the statistical significance of the similarities and differences of the physio-chemical parameters of the sample sites (P < 0.001). D1 – Gölemezli, D2 – Yenicekent, D3 – İnaltı, D4 – Şanlıalp, D5 – Umut, D6 – Pamukkale, D7 – Karahayıt. T – temperature, Ca^{2+} – calcium, Mg^{2+} – magnesium, Fe^{2+} – ferrous, SO^{2-} – sulphate, K^+ – potassium, Na^+ – sodium, Cl^- – chloride, and Li^+ – lithium ions. The Kruskal-Wallis column contains the test values among the sample sites.

Parameters	Sample sites									
	D1 Mean ± SD	D2 Mean ± SD	D3 Mean ± SD	D4 Mean ± SD	D5 Mean ± SD	D6 Mean ± SD	D7 Mean ± SD	Kruskal–Wallis test H	df	P-value
Na^+ (mg L^{-1})	646.4 ± 12.05	725.5 ± 10.69	1019.6 ± 23.37	1392.2 ± 19.48	1235.3 ± 66.93	68.6 ± 1.29	176.5 ± 2.45	26.483	6	0.001
K^+ (mg L^{-1})	80.35 ± 1.32	103.3 ± 1.77	100.5 ± 2.24	134 ± 3.16	117.3 ± 4.04	7.5 ± 0.16	33.5 ± 1.06	26.283	6	0.001
Ca^{2+} (mg L^{-1})	226 ± 10.61	197.8 ± 7.73	8.7 ± 0.25	12 ± 0.84	9.9 ± 0.08	350.275 ± 5.68	377.1 ± 4.4	26.490	6	0.001
Li^+ (mg L^{-1})	1.5 ± 0.04	1.02 ± 0.00	2.35 ± 0.08	5.44 ± 0.08	4.88 ± 0.08	0.16 ± 0	0.35 ± 0	26.643	6	0.001
Mg^{2+} (mg L^{-1})	178.4 ± 3.77	77.3 ± 1.02	38.9 ± 0.62	42.3 ± 0.58	38.9 ± 0.6	150 ± 3.16	167 ± 3.65	26.024	6	0.001
Fe^{2+} (mg L^{-1})	0.07 ± 0.00	2.19 ± 0.02	0.034 ± 0.00	0.07 ± 0	0.047 ± 0	0.06 ± 0	2.45 ± 0.02	26.157	6	0.001
Cl^- (mg L^{-1})	86 ± 1.41	68 ± 1.41	150 ± 2.16	150 ± 1.82	175 ± 3.16	15 ± 0	29 ± 0.81	26.117	6	0.001
HCO_3^- (mg L^{-1})	1293.62 ± 60.91	2007.56 ± 22.66	689.53 ± 8.34	2526.23 ± 31.13	1629.23 ± 57.47	1177.6 ± 10.55	1348.54 ± 25.93	26.195	6	0.001
SO_4^{2-} (mg L^{-1})	1740 ± 37.21	705 ± 4.54	1576 ± 10.42	1336 ± 14.98	1184 ± 53.75	630 ± 7.61	889 ± 7.07	26.483	6	0.001
pH	6.2 ± 0.05	6.7 ± 0.05	6.4 ± 0.08	6.2 ± 0.05	6.2 ± 0.05	59 ± 0.08	6.025 ± 0.05	25.089	6	0.001
T (°C)	46 ± 1.41	55 ± 3.16	60 ± 1.82	54 ± 2.16	50 ± 2.44	34 ± 1.15	54 ± 0.81	23.648	6	0.001

(68.6 mg L^{-1}) (Tab. 1). The D6 and D7 thermal springs were clearly distinguished from the others by their high value of Ca^{2+} concentration and low Cl^-, Na^+, and K^+ concentrations. In this study, it was determined that the most important physio-chemical parameter was temperature, and considering the temperature differences of thermal springs, it was seen that the most significant difference was found for D6, which has the lowest temperature. Another notable point was the differences in the concentration of Fe^+ which was higher in D2 and D7 than in other thermal springs (Tab. 1). A significant difference was found when the Cl^-, Fe^{2+}, K^+, Mg^{2+}, Na^+, Li^+, Ca^{2+}, HCO_3^-, SO_4^{2-}, pH and temperature values of the thermal springs were compared with the Kruskal Wallis test (P < 0.001) (Tab. 1).

Algal flora

A total of 46 algae taxa (43 Cyanobacteria, 3 Bacillariophyta) were identified according to their morphological and ecological characteristics (Tab. 2). Synechococcales was the dominant order with 18 taxa among other Cyanobacteria orders. The D5 sample site had the most biodiversity with 22 taxa. The most common taxon was *Spirulina subsalsa* Oersted ex Gomont, collected from the five sample sites, followed by *Jaaginema geminatum* (Schwabe ex Gomont) Anagnostidis & Komárek, and *Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe from four sample sites. In the present study, three Bacillariophyta taxa were identified from the sample sites of D5 and D6 that had water temperatures of 32–36 °C.

Statistical analysis

First, the DCA was performed to find a suitable analysis, and then gradient lengths were assessed (Axis 1: 0.7356; Axis 2: 0.4883). According to the gradient lengths obtained from the DCA, the data were found to be suitable for PCA. Among the physio-chemical parameters analyzed, nine were included in the forward selection (temperature, Cl^-, SO_4^{2-}, Fe^{2+}, K^+, Mg^{2+}, Na^+, Li^+, and Ca^{2+}). In the PCA, the identified 46 taxa, the investigated thermal springs, and the physio-chemical parameters were used (Fig. 4).

The significance effect was supported by a Monte Carlo permutation test (499 permutations, F-ratio < 0.1, P-value = 1). As a result of the PCA, the total variation was 49.71429, and the first two axes explained 58.59% of the variance (Fig. 4). The most determining factor was the temperature (T), followed by K^+ and Cl^-. Although D5 (with 22 taxa) and D6 (with 16 taxa) had the highest number of taxa, they did not closely correlate with all the parameters (Fig. 4). Interestingly, D6 is partially positively correlated to Ca^{2+} and Mg^{2+} and negatively correlated to the rest. Conversely, D5 is negatively correlated to Ca^{2+} and Mg^{2+}, while it is partially positively correlated to the rest. Additionally, it can be seen that all the other sample sites are positively correlated to almost all the physio-chemical parameters (Fig. 4).

Tab. 2. Taxa, taxa codes and distribution of algal flora determined in thermal springs (D1-D7) in Denizli (Türkiye). D1 – Gölemezli, D2 – Yenicekent, D3 – İnaltı, D4 – Şanlıalp, D5 – Umut, D6 – Pamukkale, D7 – Karahayıt.

Taxa Code	Taxa	Sample Sites
	Cyanobacteria	
1	*Synechococcus nidulans* (Pringsheim) Komárek in Bourrelly	D5
2	*Merismopedia* sp.	D5
3	*Jaaginema* sp.	D4
4	*Jaaginema angustissimum* (West & G.S. West) Anagnostidis & Komárek	D5
5	*Jaaginema subtilissimum* (Kützing ex De Toni) Anagnostidis & Komárek	D4
6	*Jaaginema pseudogeminatum* (G.Schmid) Anagnostidis & Komárek	D6
7	*Jaaginema geminatum* (Schwabe ex Gomont) Anagnostidis & Komárek	D1, D2, D3, D5
8	*Pseudanabaena* sp.	D1
9	*Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe	D1, D2, D5, D7
10	*Pseudanabaena minima* (G.S.An) Anagnostidis	D5, D7
11	*Pseudanabaena catenata* Lauterbor	D1, D5
12	*Leibleinia epiphytica* (Hieronymus) Compère	D6
13	*Leptolyngbya* sp.	D5
14	*Leptolyngbya subtilis* (West) Anagnostidis	D4, D5, D6
15	*Leptolyngbya foveolarum* (Gomont) Anagnostidis & Komárek	D5, D6
16	*Leptolyngbya boryana* (Gomont) Anagnostidis & Komárek	D5
17	*Leptolyngbya gelatinosa* (Woronichin) Anagnostidis & Komárek	D5
18	*Planktolyngbya contorta* (Lemmermann) Anagnostidis & Komárek	D7
19	*Spirulina subtilissima* Kützing ex Gomont	D1, D4
20	*Spirulina major* Kützing ex Gomont	D3, D7
21	*Spirulina subsalsa* Oerstedt ex Gomont	D1, D2, D5, D6, D7
22	*Spirulina robusta* H.Welsh	D5
23	*Chroococcus* sp.	D5
24	*Chroococcus membraninus* (Meneghini) Nägeli	D5
25	*Cyanosarcina* sp.	D5
26	*Gloeocapsa gelatinos*a Kützing	D6
27	*Gloeocapsopsis cyanea* (Krieger) Komárek & Anagnostidis	D5, D6
28	*Geitlerinema* sp.	D1
29	*Kamptonema jasorvense* (Vouk) Strunecký, Komárek & J.Smarda	D6
30	*Kamptonema okenii* (C.Agardh ex Gomont) Strunecký, Komárek & J.Smarda	D1, D4, D7
31	*Kamptonema animale* (C.Agardh ex Gomont) Strunecký, Komárek & J.Smarda	D3, D5, D6
32	*Kamptonema cortianum* (Meneghini ex Gomont) Strunecký, Komárek & J.Smarda	D1, D5, D6
33	*Microcoleus autumnalis* (Gomont) Strunecky, Komárek & J.R.Johansen in Strunecky	D6
34	*Porphyrosiphon versicolo*r (Gomont) Anagnostidis & Komárek	D6
35	*Symploca* sp.	D3
36	*Oscillatoria proboscidea* Gomont	D7
37	*Phormidium* sp. 1	D1
38	*Phormidium* sp. 2	D7
39	*Phormidium terebriforme* (C.Agardh ex Gomont) Anagnostidis & Komárek	D1, D4
40	*Phormidium interruptum* Kützing ex Forti	D6
41	*Komvophoron minutum* (Skuja) Anagnostidis & Komárek	D4
42	*Komvophoron skujae* Anagnostidis & Komárek	D3, D5
43	*Calothrix fusca* Bornet & Flahault	D6
	Bacillariophyta	
44	*Cyclotella* sp.	D5
45	*Diploneis interrupta* (Kützing) Cleve	D6
46	*Pinnularia microstauron* (Ehrenberg) Cleve	D6

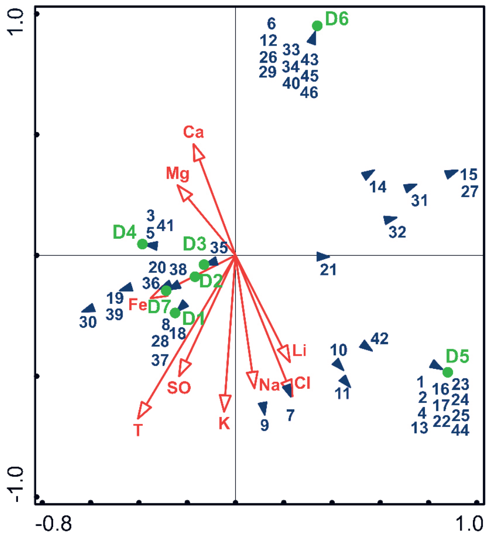

Fig. 4. Principal component analysis (PCA) diagram of the algal flora, the physio-chemical parameters, and the sample sites of thermal springs (D1-D7) in Denizli. The graph shows the algal taxa codes (blue triangles), the sample sites (green circles), and the physio-chemical parameters, temperature, and concentration of different ions (red arrows) with the canonical axes. The algal taxa codes are given in Tab. 2. T – temperature, Ca – calcium, Mg – magnesium, Fe – ferrous, SO – sulphate, K – potassium, Na – sodium, Cl – chloride, and Li – lithium.

Discussion

In this taxonomic study, the algal flora and physico-chemical parameters of seven thermal springs in Denizli were examined. We observed that these thermal springs were different in terms of their physio-chemical properties. As in similar studies in the literature (Ward and Castenholz 2000, Papke et al. 2003, Sompong et al. 2005), the relationship of species diversity in thermal springs and physio-chemical parameters and substrate structure was observed in this study. Accordingly, the correlation between the algal flora of the thermal springs and their physio-chemical parameters was examined statistically by PCA (Fig. 4).

The critical factor determining the distribution of algal flora in the thermal springs was temperature, with diatoms preferring relatively low temperatures. *Pinnularia microstauron* (Ehrenberg) Cleve, which was sampled from sites with low temperatures in this study (D5 and D6), has been reported from similarly low-temperature thermal springs (Fazlutdinova et al. 2020, Kaštovský and Komárek 2001). Kaštovský and Komárek (2001) defined the region below 36 °C in thermal waters as the mesothermophilic diatom region and stated that in this region, diatoms and cyanobacterial taxa form clusters together. Similar to Kaštovský and Komárek (2001), especially at low temperatures in D6, *P. microstauron* and *Diploneis interrupta* (Kützing) Cleve were observed to form mats with cyanobacteria taxa. The results of the PCA (Fig. 4) showed the negative correlation of these two diatom taxa with temperature, confirming diatom preference for lower temperatures in thermal springs.

Cyanobacteria taxa are common in thermal springs because they can survive at higher temperatures than other algae. *Jaaginema angustissimum* (West & G.S.West) Anagnostidis & Komárek is one of the common taxa of thermal and sulfate (SO_4^{2-}) springs (Güner 1970, Komárek and Anagnostidis 2005, Arman et al. 2014, Öztürk Ulcay and Kurt 2017), and it was determined from D5, which is also characterized by a high SO_4^{2-} value in this study.

Interestingly, although *Gloeocapsa gelatinosa* Kützing was found to have a low correlation with Cl^- in this study (Fig. 4), Roy et al. (2015) reported that the taxon was associated with higher Cl^- by canonical correspondence analysis. In this study, *G. gelatinosa* and *Calothrix fusca* Bornet & Flahault were collected together from the same sample site which is consistent with the report of Lukavsky et al. (2011) that they formed a mat together.

The most common taxon in this study was *Spirulina subsalsa*, which did not have a significant correlation with the physio-chemical parameter results of the PCA. Altunöz et al. (2016) identified this taxon in the Pamukkale thermal spring and reported that this taxon did not show a distribution correlated with physio-chemical parameters, which is consistent with the present study. However, Roy et al. (2015) reported that the combined effect of pH, K^+, and HCO_3^- was effective in the distribution of *S. subsalsa*. As in the study of Kanellopoulos et al. (2016), *S. subsalsa* and *Phormidium terebriforme* (C.Agardh ex Gomont) Anagnostidis & Komárek were sampled together from the travertine substrates in this study (D1). The travertines are frequently colonized with Cyanobacteria mats, commonly composed of more than one taxon (Pentecost 2003, Kanellopoulos et al. 2016). Pentecost (2003) reported that travertines are often covered with a mixture of *Oscillatoria*, *Spirulina*, and *Phormidium* taxa. This result was also observed in this study; *Jaaginema geminatum*, *Spirulina major* Kützing ex Gomont, *S. subsalsa*, and *P. terebriforme* were collected from travertine substrates in D1, D2, D5, and D7. In addition, the D2 and D7 thermal springs had a high value of Fe^{2+} concentrations. Pierson and Parenteau (2000) reported well-developed cyanobacterial mats in high Fe^{2+} concentrations. As can be seen from the PCA (Fig. 4), *Planktolyngbya contorta* (Lemmermann) Anagnostidis & Komárek and *Oscillatoria proboscidea* Gomont were identified from D7 with the highest Fe^{2+} concentration. Also, common taxa of D2 and D7, with high Fe^{2+} concentrations, were *Pseudanabaena mucicola* and *S. subsalsa*, and the distribution of these species in this study overlaps with the related data from the literature. However, *S. subsalsa* does not appear to be associated with Fe^+ concentrations (Fig. 4). The possible reason may be the ecological tolerance of *S. subsalsa*.

Conclusions

This study enlarged the worldwide knowledge of algal flora inhabiting thermal springs, which helps to improve the ecological data on the physio-chemical parameter preferences of Cyanobacteria and Bacillariophyta taxa and contributes to the understanding of the effects of physio-chemical parameters on the diversity of these ecosystems. Our findings also confirm that algal flora diversity is directly affected by temperature, followed by potassium and chloride ions in the water of thermal springs.

Further ecological, physiological, and biotechnology studies are required for an understanding of the potential usability of these thermophilic species of algae. For all these reasons, determining the species diversity in thermal springs can also underpin new economic and industrial uses.

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Author contribution statement

S.Ö. collected algae samples, measured thermal springs, and did the statistical analysis. S.Ö. and O.K. identified the algae samples and prepared the manuscript.

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Does palynotaxonomy contribute to the systematics of the genus? The section *Multicaulia* of the genus *Hedysarum* (Fabaceae) example in Türkiye

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Abstract – The purpose of the current work was to assess the systematic and taxonomic significance of the pollen morphological characteristics of the *Multicaulia* section of *Hedysarum* species found throughout Türkiye using scanning electron microscopy (SEM) and light microscopy (LM) techniques. For this reason, seven different species currently classified in the *Multicaulia* section were collected from various localities. The pollen grains were nonacetolyzed, prepared and directly measured according to the Wodehouse method. Pollen grains were found to be subprolate in *H. nitidum* but prolate in others and the exine sculpturing pattern was determined to be microreticulate-perforate in six taxa while only *H. varium* subsp. *syriacum* had reticulate-perforate sculpture. The minimum exine thickness was measured at 0.96 µm in *H. varium* subsp. *varium*, while among the investigated species, *H. nitidum* had an exine measurement of 1.38 µm. In this study, multivariate analysis was used to investigate nine qualitative and quantitative features. Data cluster analysis revealed that the pollen characteristics were important in helping to distinguish the different species from one another. The results demonstrated that the genus *Hedysarum* of the section *Multicaulia* was divided into two major groups based on variations in pollen dimensions. The usefulness of certain pollen morphological traits was assessed, and the congruency of the palynomorphological investigations was examined. The findings demonstrated the significance of pollen micromorphology in precisely identifying and categorizing the *Multicaulia* section.

Keywords: *Hedysarum*, Leguminosae, palynology, taxonomy, sweet vetch, systematic

Introduction

It is a broadly acknowledged theory that the fields around the large Tethys Sea (which covered part of the present Mediterranean zone during the Tertiary) became a center of speciation for many plant families after the Alpine uplift and Oligocene climatic changes during the Pleistocene and Holocene. Only several legume tribes such as *Hedysareae*, *Genisteae*, *Viciae*, *Loteae*, and *Trifoliae*, were able to endure the severe climatic conditions, surviving and multiplying under extreme pressure (summer drought, winter frost). The Fabaceae family of flowering plants, which includes roughly 19,500 species divided into 751 genera, is regarded as the third largest flowering plant family. This enormous family has demonstrated a high degree of species diversity and evolutionary success in a variety of global ecosystems, including stony grasslands, deserts, seashores, alpine and arctic meadows (Choi and Ohashi 2003, Legume Phylogeny Working Group 2023). The Mediterranean and Irano-Turanic phytogeographical regions have some specific genera such as *Onobrychis*, *Hedysarum*, *Medicago*, *Astragalus*, *Anthyllis*, *Trigonella*, *Coronilla*, *Hippocrepis*, *Ononis*, *Genista*, etc. (Lesins and Lesins 1979, Polhill 1981). Two areas are distinctly bipolar for this speciation: the eastern Mediterranean and Irano-Turanic zone (around the Aegean, Black, and Caspian seas), and the western Mediterranean or lbero-Maghrebi (around the Tyrrhenian and Alboran seas). Within the genus *Hedysarum*, circum-Mediterranean, mostly eastern Mediterranean, species are outstanding (among the 21 taxa recognized for Europe by Chrtková-Zertova, 1968, 13 are eastern Mediterranean). Among the 45 taxa recognized for the circum-Mediterranean areas by Greuter et al. (1989), 33 are present in the eastern Mediterranean.

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In Türkiye alone, 22 taxa (one of which is suspect) of *Hedysarum* were recognized by Hedge (1979), and with the addition of the new taxa, the number of taxa in the genus will reach 24 (Başköse et al. 2018, Aytaç et al. 2020, Hamzaoğlu and Koç 2020, Kandemir et al. 2023). Hedge (1979) separated *Hedysarum* into five sections (*Hedysarum*, *Obscura*, *Multicaulia*, *Subacaulia*, and *Crinifera*) in the Flora of Türkiye. Of these, 11 are endemic to Türkiye, and the endemism ratio of the genus is 52.3%. The classification of sections of *Hedysarum* is also quite problematic. Until recently, it has included four sections: *Hedysarum*, *Membranacea* B. Fedtsch., *Stracheya* (Benth.) B.H. Choi & H. Ohashi, and *Multicaulia* (Boiss.) B. Fedtsch. However, section *Multicaulia* consists of three subsections: *Multicaulia* B.H. Choi & H. Ohashi, *Crinifera* (Boiss.) B.H. Choi & H. Ohashi, and *Subacaulia* (Boiss.) B.H. Choi & H. Ohashi.

Characters such as the size, shape, and number of leaflets, indumentum, flower color, size, shape, and proportions of the standard, wings and keel, and shape and indumentum of the lomentum are extremely variable among the species of the *Hedysarum* genus (Hedge 1979). *Hedysarum varium* is the most common and polymorphic species of the genus in Türkiye. Hedge (1979) also noted that *H. varium* may be extended by hybridization or introgression with *H. pestalozzae*, *H. syriacum*, and *H. nitidum*. The differences between these four species are unsatisfactory, and further work is needed before their interrelationships and taxonomy can be placed on sounder foundations.

The palynomorphology of the many Fabaceae taxa has taxonomic significance (Erdtman 1969, Ohashi 1971, Perveen and Qaiser 1998, Pavlova and Manova 2000, Pınar et al. 2000, Avcı et al. 2013, Çeter et al. 2013, Bagheri et al. 2019). Three apertures, reticulate or suprareticulate ornamentation of exine, oblate-spheroidal or prolate pollen grains were given in those studies. Additionally, the micromorphological (pollen, fruit, and seed) characteristics can contribute to the discrimination of *Hedysarum* species, especially the pollen grains and muri size. However, there has been no taxonomical study conducted on the pollen structures of the subsection *Multicaulia* in Türkiye (Ohashi 1971, Polhill 1981, Faegri et al. 1989, Moore et al. 1991, Choi and Ohashi 1996, Civelek et al. 1999, Pavlova and Manova 2000, Sa et al. 2010, Ghanavati and Amirabadizadeh 2012, Dural and Citak 2015).

In order to enhance the pollen morphological research into the genus *Hedysarum*, the current study was conducted to describe and evaluate in detail, for the first time, the palynomorphological properties of seven taxa distributed in Türkiye using light microscopy (LM) and scanning electron microscopy (SEM). The objectives of this work were to: 1) identify and investigate the features of the pollen grains of species belonging to the *Multicaulia* subsection; and 2) use numerical analyses to clarify the systematic significance of the palynological traits.

Materials and methods

Plant material

The specimens of the section *Multicaulia* were collected from various localities, as listed in Table 1, and stored in the herbarium of the Department of Biology of Selçuk University (KNYA). The taxonomical description of the species followed that of Hedge (1979).

Palynological analysis

Both LM and SEM were used to examine pollen grains of the genus *Hedysarum*. For palynological analysis, pollen samples of specimens were obtained from herbarium materials. To observe and calculate the characteristics of pollen grains, the Wodehouse technique was used (Wodehouse 1935). In this unique and easy technique, pollen grains were separated from the anthers and were stained with glycerin-jelly and covered with coverslips. A Leica DM 1000 light microscope with a Canon 450D camera (Ota City, Tokyo, Japan) and software from the Kameram 21 program (Argenit, Istanbul, Türkiye) was used to measure and observe fifty pollen grains per specimen. The equatorial diameter (E), polar axis (P), colpus length (Clg), colpus width (Clt), apocolpium (t), thickness of the exine and intine, and lumina width (LW) were examined and the P/E ratios were calculated. The mean, standard deviation (SD), minimum–maximum range, and mean values of these pollen characteristics for the taxa under study were given.

The unacetolyzed pollen grains were transferred directly onto aluminum stubs, covered with gold using a Cressington Auto 108 sputter coater (Cressington Scientific Instruments, Watford, Hertfordshire, UK) and photographed

Tab. 1. Localities of specimens of *Hedysarum* from the section *Multicaulia* collected from various localities in Türkiye and stored in KNYA herbarium.

Taxa	Locality	Herbarium no
H. laxum (DC.) Spreng	C5 Adana: Tufanbeyli, 1300 m.	B. Çıtak-366
H. leucocladum Boiss.	B5 Nevşehir: Zelve, 1100 m.	B. Çıtak-150
H. huetii Boiss.	A8 Erzurum: Tortum, 1300 m.	B. Çıtak-377
H. nitidum Willd.	A8 Erzincan: Refahiye, 1500 m.	B. Çıtak-369
H. pestalozzae Boiss.	C4 Karaman: Ayrancı, 1300 m.	B. Çıtak-160
H. varium Willd. subsp. *varium*	C4 Konya: Konya-Beyşehir road, 1300 m.	B. Çıtak-161
	B6 Sivas: Sivas-Erzincan road, 1500 m.	B. Çıtak-367
H. varium subsp. *syriacum* (Boiss.) C.C. Towns.	B5 Aksaray: Aksaray-Nevşehir road, 1150 m.	B. Çıtak-151

using a Zeiss Evo Ls10 scanning electron microscope (SEM) (Carl Zeiss NTS GmbH, Oberkochen, Germany) for the SEM analyses (Dural and Citak 2015).

The pollen terminologies of Punt et al. (2007), and Halbritter et al. (2018) were followed.

Statistical analysis

The determined qualitative and quantitative characters were scored for numerical analysis. Nine pollen characters were used to evaluate *Hedysarum* taxonomic relationships. These characters are equatorial and polar axes, exine, intine, colpus length, colpus width, apocolpium, sculpture and lumen width. Next, the determined qualitative and quantitative characters were turned into a data matrix. For the pollen characters of the seven taxa, the coefficients of correlation were detected and compiled using the clustering analysis method (unweighted pair group method with arithmetic mean (UPGMA), dissimilarity, standardized variables). Using nine characters and seven taxa, a primary matrix was established for the multivariate analysis. The Gower general coefficient similarity (Gower 1971), which can be utilized directly to a variety of character types (binary, qualitative, and quantitative characters), provided a basis for the clustering analysis. The UPGMA method was selected due to its widespread usage, congruence with the classification derived by traditional techniques, and visible accuracy in generating a reflection similarity matrix, as demonstrated by the co-phenetic correlation coefficient of Sokal and Rohlf (1962) and symmetrical hierarchical structure (Sokal and Rohlf 1962, McNeill 1979). The covariance matrix was created using non-standardized, untransformed, and centered data (Citak 2019). All calculations were performed via MVSP 3.22 software (Kovach Computing Services, Anglesey, Wales, Kovach 2013).

Results

Pollen morphology

The pollen grains of the taxa of the subsection *Multicaulia* were determined as monad, radially symmetrical and isopolar. The shapes of the pollen grains were elliptic and not compressed at the poles in equatorial view or orbicular in polar view (Fig. 1, Fig. 2).

Quantitative characteristics of pollen grains from the plant samples are summarized in Table 2.

The general pollen shape in this study was determined as prolate; subprolate pollen shape was observed only in *H. nitidum*. The highest average pollen grain diameter on polar axis was observed in *H. laxum* (22.85 μm), whereas the lowest diameter was observed in *H. huetii* (20.53 μm). Similarly, the maximum pollen grain diameter on the equatorial axis was observed in *H. nitidum* (16.23 μm), while the minimum diameter was examined in *H. laxum* (12.68 μm). The size of the pollen grains was calculated based on the P/E ratio. The highest P/E value was observed in *H. laxum* (1.80), while the lowest value was observed in *H. nitidum* (1.33).

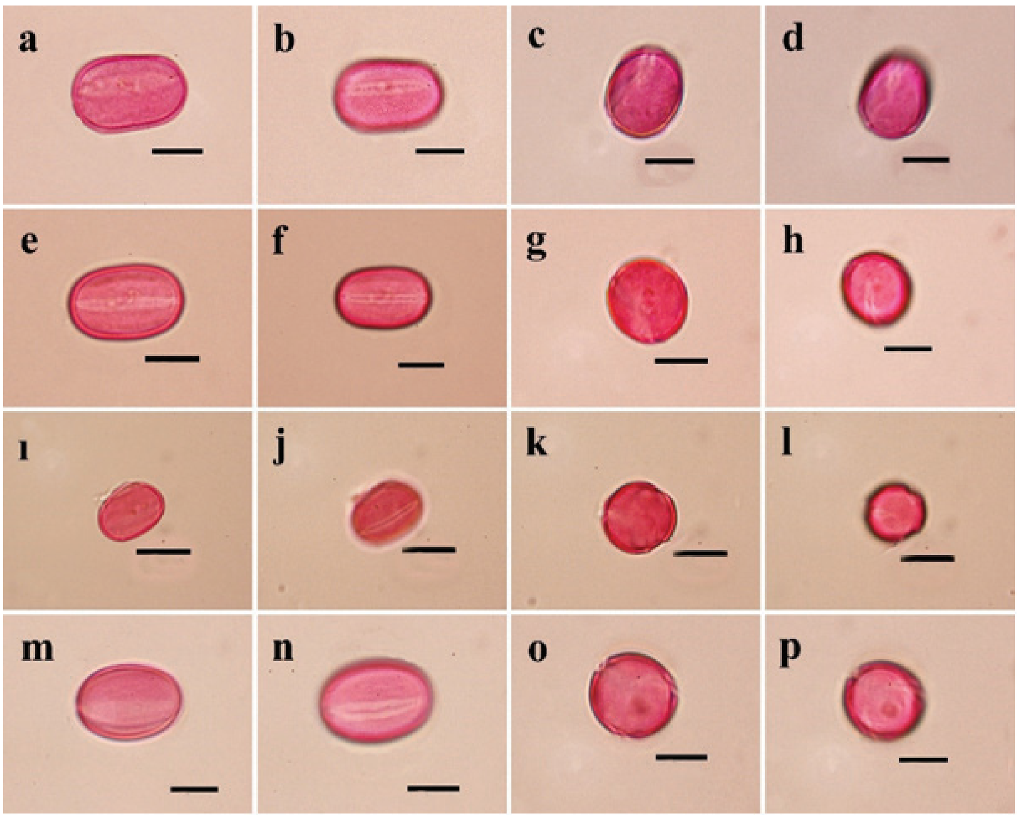

Fig. 1. Pollen microphotographs of species of the *Hedysarum* genus of *Multicaulia*: **a-d.** *H. laxum*, **e-h.** *H. leucocladum*, **ı-l.** *H. huetii*, **m-p**. *H. nitidum*. Scale bar: 10 μm.

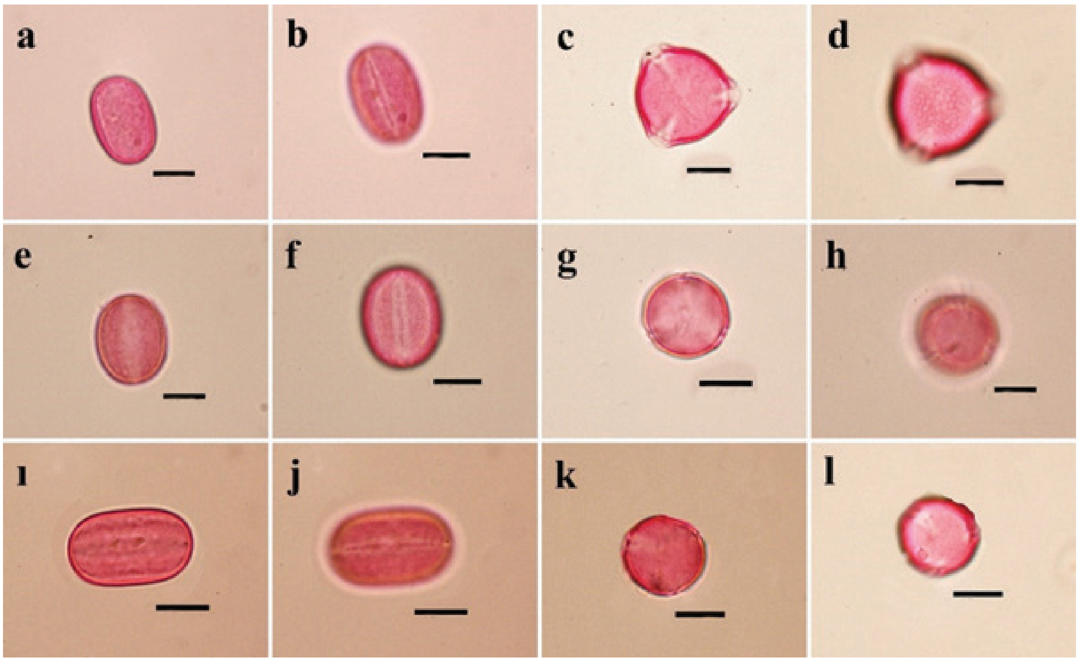

Fig. 2. Pollen microphotographs of species of *Hedysarum* genus of *Multicaulia* **a-d.** *H. pestalozzae,* **e-h.** *H. varium* subsp. *varium* **ı-l.** *H. varium* subsp. *syriacum*. Scale bar: 10 µm.

All of the studied members of the subsection *Multicaulia* had tricolpate apertures. The investigated species had different aperture sizes, with the maximum average colpi length observed in *H. pestalozzae* (19.84 µm), while the minimum was found in *H. huetii* (16.83 µm). In terms of the width, the maximum colpus width was found in *H. nitidum* (3.93 µm), while the minimum width was observed in *H. leucocladum* (2.97 µm). An operculum with psilate ornamentation on the colpus was determined in all pollen grains.

The exine ornamentation was the most distinguishing character of the species. In current study, all of the species had microreticulate-perforate exine sculpturing, except for *H. varium* subsp. *varium*, which had reticulate-perforate exine sculpturing (Fig. 3). The exine thickness ranged from a maximum of 1.38 µm in *H. nitidum* to a minimum of 0.96 µm in *H. varium* subsp. *varium*. The lumina width also varied among the species, and ranged from a maximum of 1.10 µm in *H. varium* subsp. *varium* to a minimum of 0.50 µm in *H. pestalozzae*. The sculpture of pollen grains was microreticulate-perforate or reticulate-perforate in equatorial view; however, only perforate ornamentation was observed in polar view. The walls of the muri were sinuous in the examined species.

Tab. 2. The palynomorphological characters examined in *Multicaulia* subsection (values in µm, except for P/E). PS: pollen shape, P: prolate, SP: subprolate, E: exine, I: intine, Clg: colpus length, Clt: colpus width, t: apocolpium, Sc: sculpturing, MP: microreticulate-perforate, RP: reticulate-perforate, LW: lumina width. Fifty pollen grains per specimen were analyzed. * indicates endemic taxa and **indicates characters used in statistical analysis.

Taxa	Equatorial axes (E)**		Polar axes (P)**		P/E	PS	E**	I**	Clg**	Clt**	T**	Sc**	LW**
	Min – Max	Mean ± SD	Min – Max	Mean ± SD									
**H. laxum*	10.28 – 15.34	12.68 ± 1.81	17.67 – 25.26	22.85 ± 2.37	1.80	P	1.3	0.58	19.23	3.14	3.10	MP	0.57
H. leucocladum	13.26 – 15.76	14.31 ± 0.78	19 – 22.38	21.03 ± 1.09	1.46	P	1.27	0.62	19.55	2.97	2.97	MP	0.53
H. huetii	13.55 – 15.28	14.7 ± 0.7	19.2 – 21.78	20.53 ± 1.09	1.39	P	1.06	0.54	16.83	2.99	4.39	MP	0.59
**H. nitidum*	15.64 – 16.91	16.23 ± 0.47	19.65 – 23.77	21.67 ± 1.13	1.33	SP	1.38	0.63	19.71	3.93	3.25	MP	0.74
**H. pestalozzae*	9.8 – 16.24	13.6 ± 2.04	16.37 – 22.65	20.96 ± 1.54	1.54	P	1.00	0.74	19.84	3.45	3.19	MP	0.50
H. varium subsp. *varium*	10.41 – 18.13	15.76 ± 2.44	19.64 – 23.79	21.57 ± 1.13	1.36	P	0.96	0.66	19.69	3.6	2.74	RP	1.10
H. varium subsp. *syriacum*	14 – 15.44	14.76 ± 0.44	21.03 – 23.67	22.34 ± 0.88	1.51	P	1.16	0.55	19.17	3.82	3.61	MP	0.62

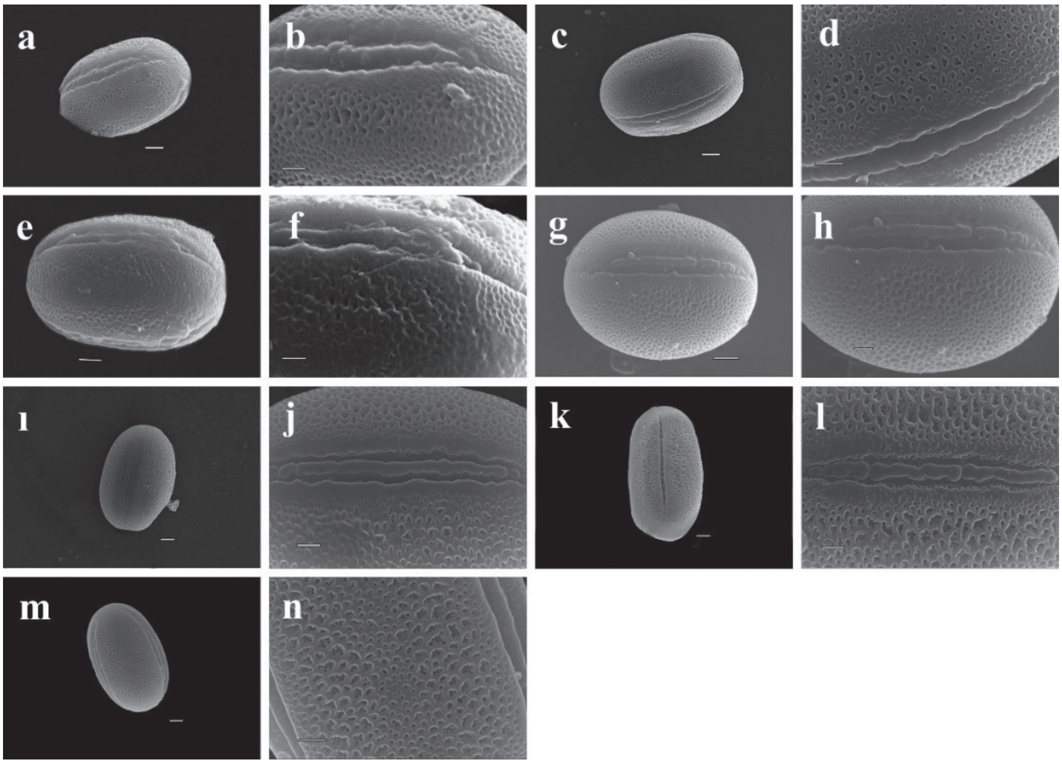

Fig. 3. Electron microphotographs of species of *Hedysarum* genus of *Multicaulia.* **a-b.** *H. laxum,* **c-d.** *H. leucocladum,* **e-f.** *H. huetii,* **g-h**. *H. nitidum,* **ı-j.** *H. pestalozzae,* **k-l.** *H. varium* subsp. *varium,* **m-n.** *H. varium* subsp. *syriacum*. Scale bar (**a, c, e, g, ı, k, m**): 2 µm; scale bar (**b, d, f, h, j, l, n**): 1 µm.

Numerical analysis of the pollen characters traits

A dendrogram produced with the use of the UPGMA in the cluster analysis, taking into account the ten palynological variables (Tab. 2) found in the section *Multicaulia,* is shown in Fig. 4. Similarities among the investigated taxa were reflected in the dendrogram, which revealed two main groups: group A (with 45% similarity) contained only *H. huetii*, which was a distinct species, and group B (with 60% similarity) comprised the remaining 6 taxa. Group B comprised two primary clusters, further denoted as clusters C and D. Two species (*H. pestalozzae* and *H. varium* subsp. *varium*, with a 73% similarity) made up cluster C. Four taxa with two subgroups, clusters D1 and D2, were included in cluster D. Cluster D1 contained *H. laxum* and *H. leucocladum*. Cluster D2 also included 2 species: *H. nitidum* and *H. varium* subsp. *syriacum*.

Discussion

The present study examined the usefulness of using the pollen morphological characteristics of species of the section *Multicaulia* of the genus *Hedysarum* in Türkiye. Using

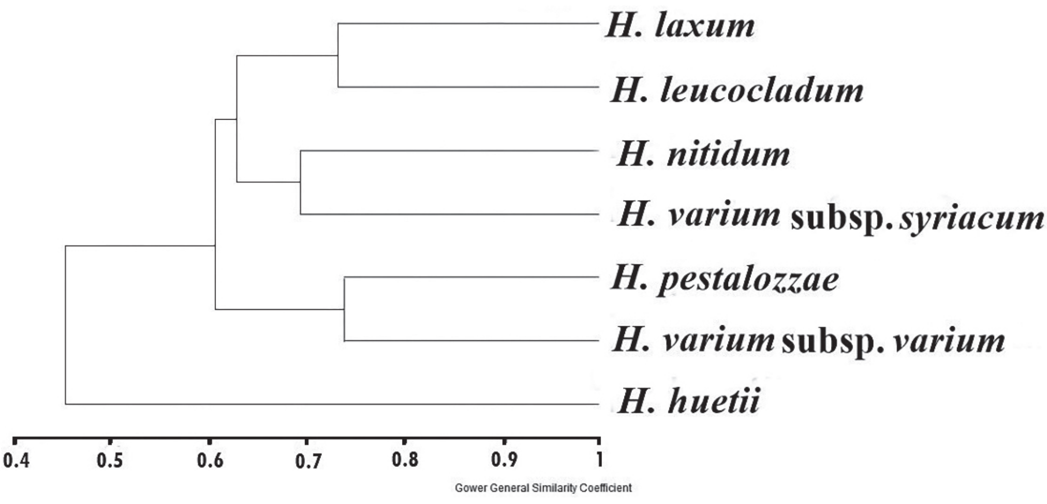

Fig 4. The derived dendrogram based on the palynological characters showing the similarity distance of *Hedysarum* species.

these pollen morphological characters, delimitation of taxa was determined.

The findings of current investigation show that the pollen dimensions (E and P/E ratio), the thickness of the exine and intine, the shape of the polar and equatorial views, and the aperture type of the Türkiyeli representatives of *Hedysarum* were comparatively homogeneous (Tab. 2, Fig. 1). Ohashi (1971), Ferguson and Skvarla (1981), Faegri and Iversen (1989), Moore et al. (1991), Pavlova and Manova (2000), and Ghanavati and Amirabadizadeh (2012) confirmed that the pollen morphological characteristics of the different sections of *Hedysarum* are essentially the same. Polar and equatorial axes were useful pollen characters to group species with the aid of numerical analysis as mentioned before in *Astragalus* (Bagheri et al. 2019). Another study declared that *Onobrychis* genus had oblate-spheroidal pollen shape (Avcı et al. 2013).

All of the species investigated in this research had tricolpate-type pollen grains with microreticulate-perforate exine ornamentation. Only *H. varium* subsp. *varium* was different from others, with reticulate-perforate exine. Reticulate and finely reticulate exine ornamentation were reported previously in the genus *Hedysarum* by Civelek et al. (1999), Pavlova and Manova (2000), and Dural and Citak (2015). The sculpture of exines were determined with three types counted reticulate, suprareticulate and microreticulate in close relatives of *Hedysarum* genus, *Astragalus* and *Onobrychis* (Avcı et al. 2013, Bagheri et al. 2019). Perveen and Qaiser (1998) reported that the ornamentation, and in particular, the shape and size of the muri and lumina, was an important character for Fabaceae (Pavlova and Manova 2000, Ghanavati and Amirabadizadeh 2012, Dural and Citak 2015). The colpus membrane has been identified as being covered with large and small sculptural elements by different researchers in the genus *Hedysarum* (Pavlova and Manova 2000, Dural and Citak, 2015). Moreover, in this study the same sculptural elements were observed in the subsection *Multicaulia* as described for the operculum with psilate sculpture.

The UPGMA dendrogram derived based on the palynological characters discriminated the species of the subsection *Multicaulia*. The positions of the species and the relationships of the subsection *Multicaulia* of the genus *Hedysarum* in the cluster were found to be in agreement with the previous classification made on a large scale (Hedge 1979, Hamzaoğlu and Koç 2020). *H. huetii*, which is a very distinct species, can be easily separated from the others by its fleshy and ovate-orbicular leaflets; hence, it was alone in a different subclade. It was very surprising that *H. pestalozzae* and *H. varium* subsp. *varium* were in the same clade, because of their broad morphological differences, particularly regarding the color of the corolla. In addition, the positions of *H. varium* subsp. *syriacum* and *H. nitidum* in the same clade were surprising due to their morphological differences. This situation raised the question of whether or not new taxonomic regulations for these taxa are required. The last two species, *H. laxum* and *H. leucocladum*, which are local and endemic, were in the same subclade. The distribution of the species of the subsection *Multicaulia* has raised the question of whether or not the relationships of the taxa should be reviewed one more time based on their pollen characteristics. Furthermore, different lumina dimensions, lumina types, and densities among taxa may be associated to their multiple germination requirements or dispersal strategies, which may have developed as a result of an evolutionary adaptation independent of various habitat types or microclimatic regions, particularly for endemics. More data are needed to solve the systematic problems of this section, especially regarding the phylogenetical relationships of the taxa.

Conclusion

To sum up, an in-depth investigation of the pollen characters was highly helpful in classifying the studied taxa. In future work, we propose that the systematic problems still unresolved in *Hedysarum* taxa should perhaps be solved by combining morphological with anatomical, micromorphological, karyological, and additional molecular data.

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Morpho-palynological assessment of the genus *Terminalia* L. (Combretaceae) in Egypt

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Abstract – Eleven *Terminalia* species growing in streets and gardens in Cairo (Egypt) were investigated for their pollen morphology. The study's objectives were to explore the variation among *Terminalia* species' pollen pheno-characters and to categorize the various species within this genus according to these pollen characteristics. The results showed that there are significant variations in the pollen morphological characteristics in the genus under study, and as a result, these traits can be regarded as eurypalynous. Each of the two main categories of *Terminalia* species mono-morphic and di-morphic includes various pollen morphologies, ranging from spheroidal to prolate with hexa-aperture that may be similar or of two types. For each of the eleven species, an identification key and a clustering phenogram for the fifteen major pollen features have been created. The aperture type, aperture similarity, and exine ornamentation are some of the crucial traits for the differentiation of the species examined while the pollen shape class has a poor diagnostic character. According to the unique characteristics of the pollen morphology of the *Terminalia* species, three groups and two subgroups were identified.

Keywords: microscopy, palynology, pollen key, species determination, taxonomy

Introduction

The family Combretaceae is an advanced flowering plant with a worldwide tropical distribution (Hutchinson 1969). The family has a different number of genera, ranging from 16 (Rendle 1956) to 20 (Mabberley 2017). Cronquist (1981) recognized 400 species, while Willis (1966) recognized 600 species within this family. These variations in the number of genera and species are due to a lot of synonyms and the either clumping or splitting of genera (Scott 1979). Classification of Combretaceae has been faced with many opinions since Exell (1954) as the family was divided into two subfamilies, Strephonematoideae with the monotypic genus *Strephonema* and Combretoideae with the rest of the genera. More recent treatments by Exell and Stace (1966) and Stace (2007), divided the family into two tribes, Laguncularieae and Combreteae. The latter tribe was subdivided into three subtribes: Combretinae, Pteleopsidinae and Terminaliinae. *Terminalia* is the second largest genus after the genus *Combretum* under the subtribe Combritenae, tribe Combreteae, family Combretaceae, suborder Myrtineae, order Myrtales, (Stace 2002, Heywood et al. 2007). Species under this genus are shrubs, trees, and creepers distributed in tropical and subtropical regions, especially in Africa and savannas (Arundhati et al. 2020). Many of its species have high economic value as they are used widely in commercial applications and Chinese traditional medicine (Zhang et al. 2019). The genus name came from the word terminal in Latin, as their leaves are crowded at the tops of the shoots. Recognition of the species under the genus *Terminalia* was according to fruit and leaf morphological characters (Chakrabarty et al. 2019, Rayane et al. 2022). The species under this genus differ greatly in their internal and external features in addition to their chromosome numbers (Stace 1965, Ohri 1996). Wickens (1973) and Fyhrquist (2007) found that bark, leaves, and fruit morphology can be used as diagnostic features in the identification of *Terminalia* species. Since species under the genus *Terminalia* are traditionally used in the treatment of some illnesses, precise identification and recognition of the closely related species need clarification.

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Flowers in the genus *Terminalia* are grouped in terminal simple racemes with duplicates of five anthers alternating with five petals. Despite the numerous stamens, only a few pollen morphological studies have dealt with taxonomical issues of the genus, e.g. Erdtman (1966), Patel et al. (1984), El-Ghazali et al. (1998), Krachai and Pornpongrungrueng (2015). Other palynological articles were mostly focused on the family level. The taxonomic disagreement concerning the species in the genus *Terminalia,* compounded by the challenge of accurately identifying certain species based only on macro-morphological characteristics, is the reason for this investigation. The aims of this work are to investigate the variation in the pollen pheno-characters of *Terminalia* species, and to classify the different species under this genus with respect to their pollen characters. Therefore, the subject of this investigation was the pollen grain classification of eleven *Terminalia* cultivated species, planted in Cairo parks, streets, and botanical gardens as ornamental trees.

Material and methods

Mature unopened flowers of eleven species were collected from Egyptian botanical gardens and streets during the flowering season (Tab. 1). The flowers were opened carefully using two needles under a stereo-microscope to release the anthers. The obtained anthers were smeared onto cleaned glass slides with a few drops of glycerol, covered, and sealed with paraffin wax. In order to prevent the harmomegathic effects – which occur when chemicals are exposed to aperturate pollen grains during the acetolysis process and the grains fold in response to changes in humidity (Taia 2022), non-acetolysed pollen grains were examined and then measured and photographed using a Motic (B-150D) light microscope (LM) fitted with a USB digital-video camera and computer software with 10× and 40× objective lenses.

All the measurements were based on 20 to 30 pollen grains. A calibrated oculometer was used to obtain the minimum and maximum measures and calculate the mean and standard deviation of each parameter and the apocolpium index (polar area index), which was calculated as the ratio of the mean distance between the apices of two colpi in both the main and sub apertures to its equatorial diameter. Values are expressed in micrometers.

For scanning electron microscope (SEM) investigation, non-acetolysed pollen grains were sputtered onto double sticks fixed in cleaned, aluminum labeled stubs, then coated with 20 nm gold in a Polaron JFC-1100 coating unit, finally examined and photographed using JEOL-JSM.I T200 Series Scanning Electron Microscope made available in the electron microscope unit, Faculty of Science, Alexandria University, Egypt. The terminology used for the descriptions is that of Punt et al. (2007).

Fifteen pollen morphological characters have been subjected to cluster analyses using PAST 3 programs V.3. designed by Hammer (1999-2013).

To generate a key, the data obtained from the description of pollen characters were subjected to automated key generation using version 4.12 of the DELTA suite of programs (Dallwitz et al. 2000).

Results

General description of *Terminalia* pollen grains

For the accurate identification of members of the *Terminalia* species, the morphological characteristics of pollen – such as size, shape, aperture conditions, and exine ornamentation – are crucial. The morphological properties of pollen, both quantitative and qualitative, derived by LM and SEM analysis are compiled in Tab. 2 and Fig. 1 and Fig. 2. The examined species' pollen grains are eurypalynous, meaning they vary greatly in character, shed in monads, apolar or isopolar and radially symmetric. These species are classified as either monomorphic or dimorphic. *T. arjuna, T. bentzoe, T. catappa, T. mantaly*, and *T. muelleri* are examples of monomorphic plants; on the other hand, the

Tab. 1. The localities of collected specimens, geographic coordinates, and the date of collection of the investigated *Terminalia* species, kept at Cairo University Herbarium (CAI).

No	Species	Locality	Date	Collector
1	*T. arjuna* (Roxb.) Wight & Arn.	Cairo: Zamalek, El-Giza street 30° 02' N 31° 13' E 12	July 2023	Rim Hamdy
2	*T. bellirica* (Gaertn.) Roxb.	Giza: Zoological garden 30° 01' 27.4" N 31° 12' 52.7" E 7	Nov. 2016	Heba Mostafa
3	*T. bentzoe* (L.) L. f.	Giza: Zoological garden 30° 01' 34.0" N 31° 12' 46.0" E 7	July 2016	Heba Mostafa
4	*T. brownii* Fresen.	Giza: Mazhar botanical garden 30° 03' N 31° 08' E 10	Dec. 2016	Heba Mostafa
5	*T. catappa* L.	Giza: Orman botanical garden 30° 01' N 31° 12' E 7	May 2019	Rim Hamdy
6	*T. chebula* Retz.	Giza: Zoological garden 30° 01' 34.0" N 31° 12' 46.0" E 7	Sept. 1963	Mohamed El-Mahdi
7	*T. laxiflora* Engl. & Diels	Cairo: El Zohriya garden 30° 02' N 31° 13' E 16	July 2023	Rim Hamdy
8	*T. mantaly* H. Perrier	Giza: Mazhar botanical garden 30° 03' N 31° 08' E 10	Feb. 2019	Rim Hamdy
9	*T. muelleri* Benth.	Giza: Zoological garden 30° 01' 35.2" N 31° 12' 52.4" E 7	Nov. 2016	Heba Mostafa
10	*T. myriocarpa* Van Heurck & Mull.	Giza Zoological garden 30° 01' 34.8" N 31° 12' 46.0" E 7	Dec. 2019	Heba Mostafa
11	*T. sericea* Burch. ex DC.	Giza: Zoological garden 30° 01' 34.0" N 31° 12' 46.0" E 7	June 1969	Mohamed El-Mahdi

Tab. 2. Pollen morphological data of 11 *Terminalia* species from Cairo, Egypt. AI – apocolpium index, Amb – the outline of pollen grain seen in polar view, Cir – circular, Di – dimorphic, E – equatorial axis diameter (µm), Eth – exine thickness (µm), EO – exine ornamentation, Gr – group, Hlob – hexalobate, M – mean, Mono – monomorphic, Min–Max – minimum and maximum values, No – number, P – polar axis length (µm), P/E – polar axis/equatorial axis, Pr – prolate, RG – rugate granulate, RP – rugate perforate, Sca – scabrate, SD – standard deviation, Sh – shape, SP – sub prolate, Sph – spheroidal, St – striate, StR – striate rugate, Tr – triangular.

No	Species	State		P	E	P/E	Sh	Amb	Eth	EO	Main apertures				Sub apertures			
											No	Type	Length	AI	No	Type	Length	AI
1	*T. arjuna*	Mono	Min – Max M ± SD	24.8 – 33.6 30.2 ± 3.4	23.8 – 30.2 28.7 ± 1.5	1.12	SP	Hlob, Cir	2.6	StR	3	Colpate	23.8 – 32.6 29.8 ± 2.8	0.25	3	Colpate	21.2 – 30.2 28.4 ± 1.8	0.34
2	*T. bellirica*	Di	Min – Max M ± SD	24.8 – 32.2 29.2 ± 3.0	21.8 – 28.2 26.5 ± 1.7	1.15	SP	Tr	2.0	StR	3	Colpate	22.8 – 30.2 29.2 ± 3.0	0.22	3	Colporate	21.5 – 29.8 26.2 ± 1.6	0.30
			Min – Max M ± SD	25.2 – 30.4 28.2 ± 1.8	24.8 – 30.6 29.0 ± 1.6	1.0	Sph	Tr	2.2	StR	3	Colpate	22.0 – 25.4 24.2 ± 1.6	0.21	3	Colporate	20.2 – 24.2 22.8 ± 1.4	0.28
3	*T. bentzoe*	Mono	Min – Max M ± SD	23.8 – 28.2 25.6 ± 2.6	22.4 – 26.0 25.2 ± 1.8	1.1	Sph	Hlob	2.2	Sca	3	Colpate	21.2 – 26.4 22.6 ± 3.8	0.19	3	Colporate	20.4 –2 2.6 22.2 ± 0.4	0.22
4	*T. brownii*	Di	Min – Max M ± SD	24.2 – 31.6 28.4 ± 3.2	23.2 – 29.8 27.6 ± 2.2	1.12	Sp	Tr	2.2	St	3	Colporate	23.2 – 29.6 23.8 ± 5.8	0.16	3	Colporate	22.8 – 28.2 23.2 ± 5.0	0.19
			Min – Max M ± SD	25.0 – 32.2 29.8 ± 2.4	21.8 – 29.8 27.6 ± 2.2	1.26	Pr	Tr	2.2	St	3	Colporate	24.2 – 31.2 29.4 ± 1.8	0.14	3	Colporate	22.8 – 30.2 26.6 ± 3.6	0.20
5	*T. catappa*	Mono	Min – Max M ± SD	25.8 – 34.6 33.6 ± 1.0	23.2 – 29.8 27.4 ± 2.4	1.25	Pr	Tr	2.5	RP	3	Colporate	23.4 – 32.8 30.2 ± 2.6	0.21	3	Colporate	21.2 – 30.6 28.6 ± 2.0	0.25
6	T. chebula	Di	Min – Max M ± SD	22.4 – 27.6 25.2 ± 2.4	20.0 – 24.2 23.2 ± 1.0	1.12	SP	Cir	2.6	Sca	3	Colpate	20.4 – 24.8 22.8 ± 2.0	0.24	3	Colporate	16.8 – 18.2 17.0 ± 1.2	0.36
			Min – Max M ± SD	22.2 – 27.2 26.2 ± 1.0	22.6 – 27.8 26.0 ± 1.8	1.0	Sph	Cir	2.4	Sca	3	Colpate	19.2 – 24.8 22.0 ± 2.8	0.28	3	Colporate	15.4 – 18.2 17.2 ± 1.0	0.41
7	*T. laxiflora*	Di	Min – Max M ± SD	21.8 – 26.8 24.2 ± 2.6	20.0 – 24.8 23.8 ± 1.0	1.1	Sp	Tr	2.2	RG	3	Colpate	19.2 – 24.8 23.6 ± 1.2	0.25	3	Colporoidate	19.0 – 24.2 23.6 ± 0.6	0.26
			Min – Max M ± SD	24.8 – 33.2 29.2 ± 4.0	19.8 – 26.4 24.2 ± 2.2	1.22	Pr	Hlob	2.2	RG	3	Colpate	21.8 – 30.2 28.2 ± 2.0	0.26	3	Colporoidate	20.8 – 29.6 26.8 ± 2.8	0.28
8	*T. mantaly*	Mono	Min – Max M ± SD	24.4 – 32.2 30.2 ± 1.0	23.8 – 30.4 29.2 ± 1.2	1.12	Sph	Hlob	2.2	StR	3	Colpate	20.2 – 28.2 27.8 ± 0.4	0.28	3	Colporate	19.2 – 27.2 25.8 ± 1.4	0.32
9	*T. muelleri*	Mono	Min – Max M ± SD	24.6 – 31.8 29.2 ± 2.6	23.4 – 30.8 28.2 ± 2.6	1.12	Sp	Hlob	2.6	StR	3	Colpate	22.2 – 29.6 27.2 ± 2.4	0.28	3	Colporoidate	20.2 – 26.4 24.8 ± 1.6	0.32
10	*T. myriocarpa*	Di	Min – Max M ± SD	24.8 – 28.2 25.8 ± 2.4	23.2 – 26.4 24.8 ± 1.6	1.1	SP	Cir	2.4	RP	3	Colpate	22.2 – 26.4 23.6 ± 2.8	0.34	3	Colporate	20.0 – 24.2 22.6 ± 1.6	0.38
			Min – Max M ± SD	27.8 – 34.5 32.4 ± 2.1	24.2 – 30.8 28.6 ± 2.0	1.25	Pr	Cir	2.4	RP	3	Colpate	24.2 – 30.8 28.2 ± 2.6	0.32	3	Colporate	20.2 – 27.6 25.8 ± 1.8	0.36
11	T. sericea	Di	Min – Max M ± SD	18.6 – 24.4 22.0 ± 2.2	17.2 – 23.4 21.2 ± 2.2	1.12	SP	Hlob	2.2	RP	3	Colpate	18.8 – 20.4 19.2 ± 1.2	0.32	3	Colporoidate	15.2 – 19.8 18.6 ± 1.2	0.42
			Min – Max M ± SD	20.2 – 24.4 23.2 ± 1.2	20.0 – 24.6 23.2 ± 1.4	1.0	Sph	Cir	2.2	RP	3	Colpate	19.0 – 21.2 20.4 ± 1.2	0.34	3	Colporoidate	18.2 – 21.0 19.8 ± 1.6	0.41

dimorphic species were represented by *T. bellirica, T. brownii, T. chebula, T. laxiflora, T. myriocarpa* and *T. sericea*; indicating that they have two distinct pollen shapes, sizes, apertures, and even exine ornamentation within the pollen grains that are gathered from the same flower.

Shape and size

In their polar view, the pollen grains were triangular, hexa-lobed or circular (Fig. 1 and Fig. 2). In the equatorial view, they were spheroidal (*T. bellirica*, *T. bentzoe*, *T. chebula, T. mantaly* and *T. sericea*), subprolate (*T. arjuna, T. bellirica, T. brownii, T. chebula, T. laxiflora, T. muelleri, T. myriocarpa* and *T. sericea*) and prolate (*T. brownii, T. catappa, T. laxiflora* and *T. myriocarpa*). *T. catappa* had the largest pollen diameter on the polar axis (P) at 34.6 µm, while *T. sericea* had the smallest (18.6 µm). Similarly, *T. muelleri* and *T. myriocarpa* had the biggest pollen diameter on the equatorial axis (E) (30.8 µm), whereas *T. sericea* had the smallest diameter (17.2 µm). Based on the ratio between the lengths of the polar axis (an imaginary straight line connecting the two poles) to the equatorial diameter (P/E ratio), pollen sizes and shape variations were examined; *T. brownii* had the highest P/E value (1.26), while *T. bellirica, T. chebula,* and *T. sericea* had the lowest values (0.1).

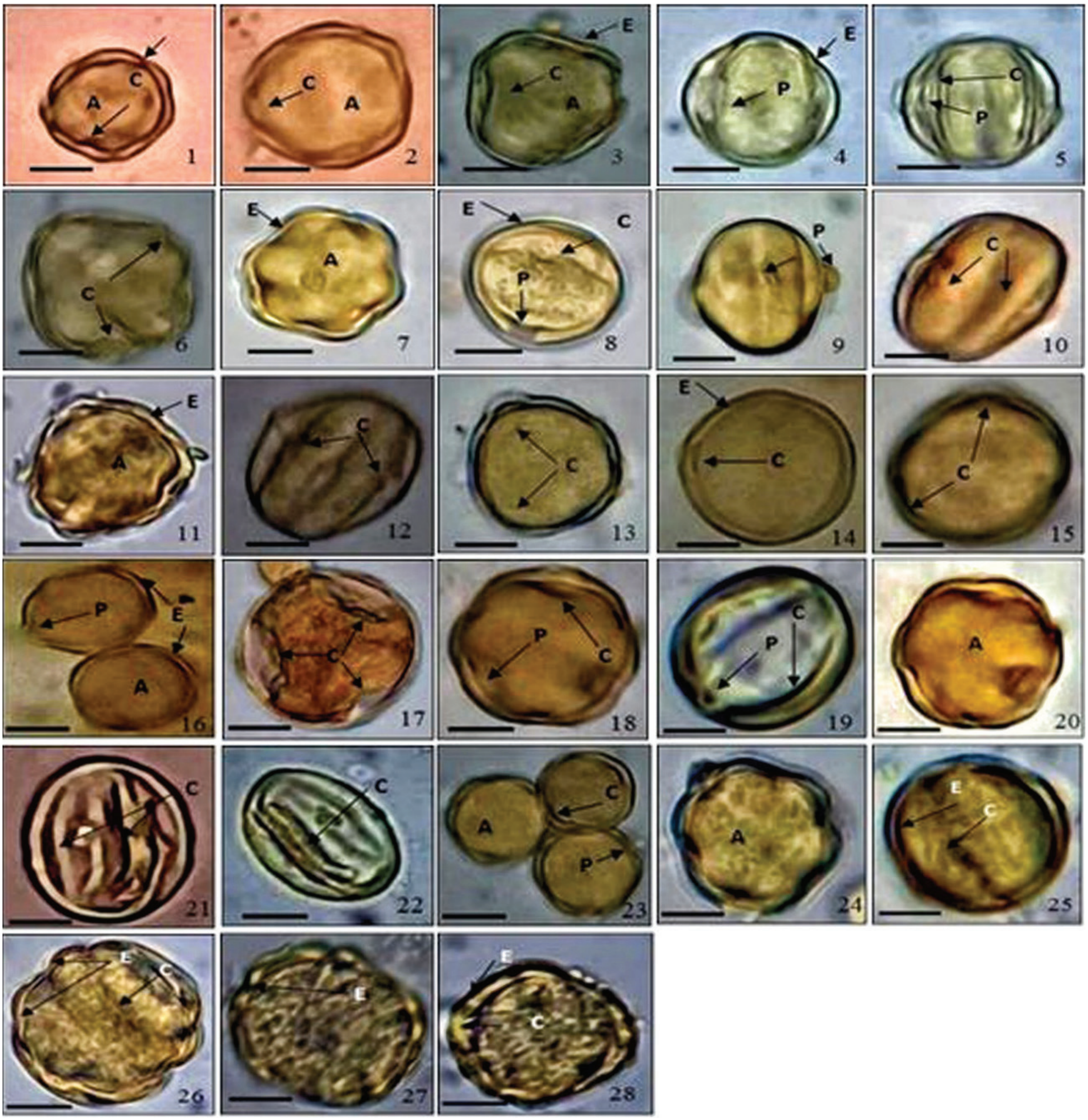

Fig. 1. Light microscopy micrographs of the studied *Terminalia* species. (1-2) *T. arjuna*, (3-6) *T. bellirica*, (7-8) *T. bentzoe*, (9-10) *T. brownii*, (11- 12) *T. catappa*, (13- 16) *T. chebula*, (17-19) *T. laxiflora*, (20) *T. mantaly*, (21-22) *T. muelleri*, (23-25) *T. myriocarpa*, (26-28) *T. sericea*. (A – amb, C – colpus, E – exine, P – pore, scale bar = 10 µm).

Aperture conditions

Apertures are the first characteristic to take into account when identifying pollen. In *T. arjuna*, *T. brownii*, and *T. catappa*, the apertures are iso-colpate; in the other investigated species, the apertures are hetero-colpate; they have two or more types of colpi, one of which varies in length and/or in whether endoapertures are present. The six apertures seen on the pollen grains of the *Terminalia* species

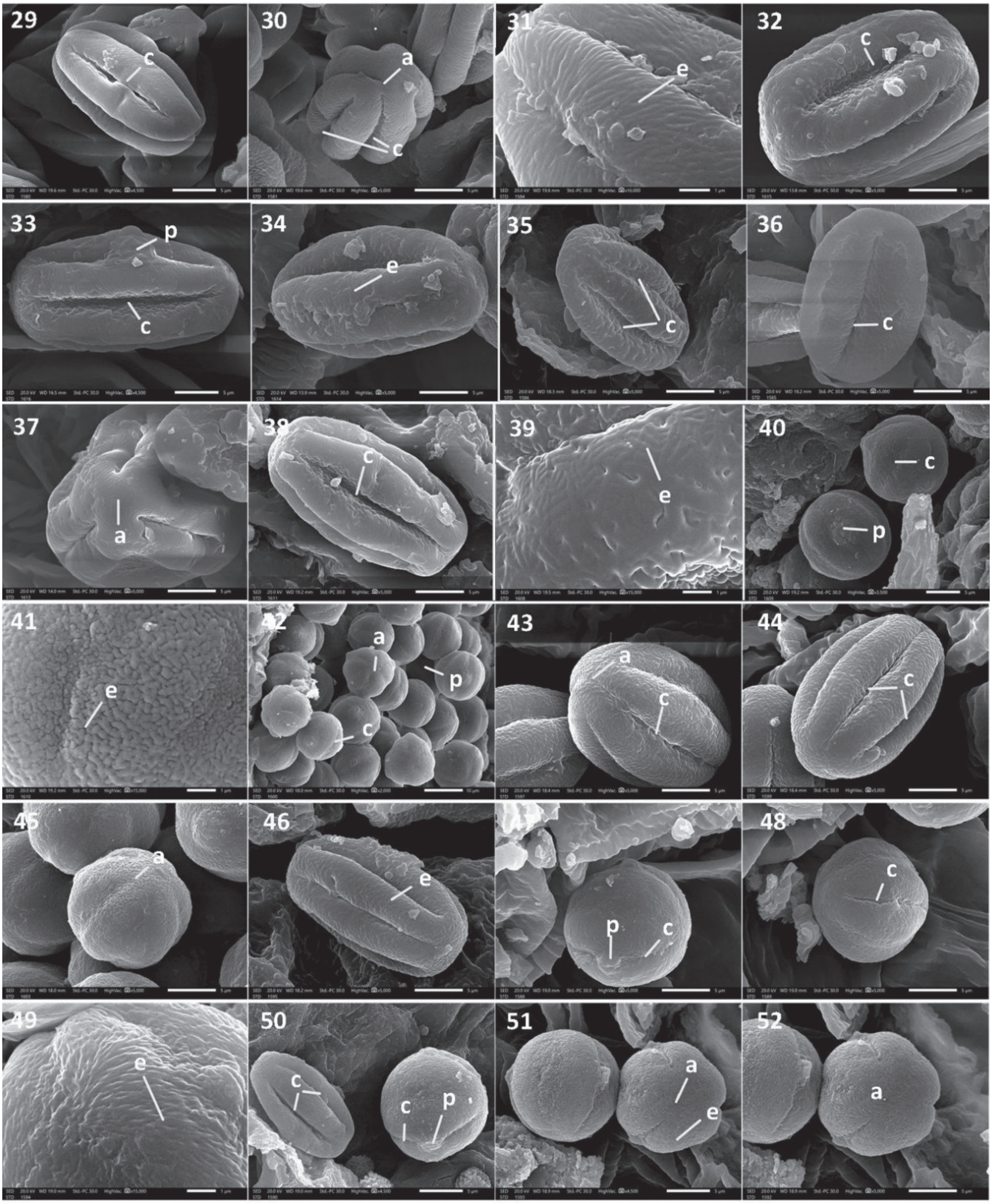

Fig. 2. Scanning electron microscope microphotographs showing the different pollen characters within the studied *Terminalia* species. (29-31) *T. arjuna*, (32-34) *T. bellirica,* (35) *T. bentzoe*, (36-37) *T. brownii*, (38-39) *T. catappa*, (40-41) *T. chebula*, (42-46) *T. laxiflora*, (47-49) *T. mantaly,* (50-52) *T. myriocarpa*. (a – amb, c – colpus, e – exine, p – pore). Scale bar = 1 µm (31, 39, 41, 49), 5 µm (29, 30, 32-38, 40, 43-48, 50-52), 10 µm (42).

under study are composed of three primary, long apertures that alternate with three smaller, possibly distinct, but somewhat shorter apertures.

Pollens of the species *T. arjuna* are hexa-colpate in which the main apertures and the sub-apertures are of the same type. The pollen grains of the species *T. bellirica*, *T. bentzoe, T. chebula, T. mantaly* and *T. myriocarpa* are hexa-colpate in which the main apertures are colpate and the sub-apertures are colporate. Also, hexa-colpate pollen grains are observed in *T. laxiflora, T. muelleri* and *T. sericea* in which the main apertures are colpate and the sub-apertures are colporoidate. In *T. brownii* and *T. catappa* the pollen grains are hexa-colporate in which the main apertures and the sub-apertures are of the same type.

The colpi are not connected at the poles and the apocolpium indices varied between the studied species from 0.14 in *T. brownii* to 0.42 in *T. sericea*. The aperture membranes are either psilate or granulate. In the case of composite apertures, the pores are either small and in the same colpi direction (lolongate) or oval shaped covered by granulated exinous layer (colporoidate). According to the state of the pollen grains, momo-or di- morphic and aperture types the studied species are grouped mainly in two main groups and four subgroups (Tab. 2).

Exine ornamentation

Exine sculpturing is the most distinguishing character of the species; in this study, the species have some variation in exine ornamentation; the exine is generally tectate, scabrate in *T. bentzoe,* and *T. chebula*; rugate in *T. laxiflora*; rugate perforate in *T. catappa, T. myriocarpa* and *T. sericea* with thin ectexine and thick endexine and intine layers as observed by light microscope investigation. Striate ornamentation is observed only in *T. brownii* while striate-rugate ornamentation is recorded in *T. arjuna, T. bellirica, T. mantaly* and *T. muelleri* (Tab. 2). Exine thickness ranges from the minimum (2.0 μm) in *T. bellirica* to the maximum (2.6 μm) in *T. arjuna, T. chebula* and *T. muelleri* (Fig. 1).

Clustering analysis

The phenogram resulting from the cluster analysis of fifteen palynological characters (On-line Suppl. Tab. 1) after coding according to their states (On-line Suppl. Tab. 2) divided the studied taxa into three groups at similarity distance 0.936. Group 1 has one species only, *T. sericea* (11). Group 2 holds eight species, subdivided into two subgroups at similarity distance 0.952, 2A with three species; *T. brownii* (4), *T. laxiflora* (7), *T. myriocarpa* (10); and subgroup 2B with five species; *T. catappa* (5), *T. mantaly* (8), *T. muelleri* (9), *T. arjuna* (1), *T. bellerica* (2). Group 3 includes two species, *T. bentzoe* (3) and *T. chebula* (6) (Fig. 3).

Computer-generated key

1. Pollen shape subprolate or prolate. 2
   Pollen shape subprolate or spheroidal. 3
   Pollen shape prolate . . . . ***T. catappa***
   Pollen shape spheroidal 4
   Pollen shape subprolate 5
2. Amb shape triangular or hexalobate; Exine ornamentation rugate-granulate. . . . . ***T. laxiflora***
   Amb shape triangular; Exine ornamentation striate . . . . ***T. brownii***
   Amb shape circular; Exine ornamentation rugate-perforate. . . . . ***T. myriocarpa***
3. Amb shape circular or hexalobate; Exine ornamentation rugate-perforate . . . . ***T. sericea***
   Amb shape triangular; Exine ornamentation striate-rugate . . . . ***T. bellirica***
   Amb shape circular; Exine ornamentation scabrate . . . . ***T. chebula***
4. Exine ornamentation scabrate . . . . ***T. bentzoe***
   Exine ornamentation striate rugate . . . . . . ***T. mantaly***
5. Amb shape circular or hexalobate; Sub aperture type colpate. . . . . ***T. arjuna***
   Amb shape hexalobate; Sub aperture type colporoidate . . . . ***T. muelleri***

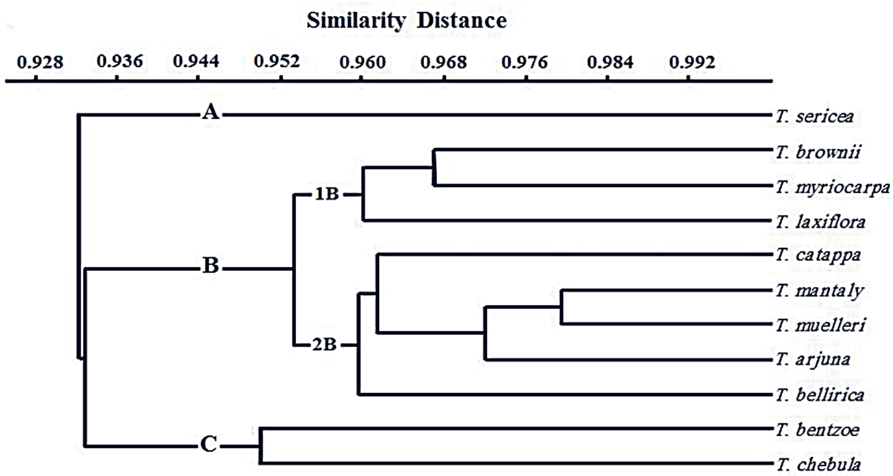

Fig. 3. Phenogram based on 15 pollen characters showing the possible relationships among the studied *Terminalia* species using percent similarity distances.

Discussion

Morpho-palynological studies have proved to be of great importance for plant identification and taxonomic classification. In this study, different palynological features such as variations in pollen characters in the same flower (morphism), polar and equatorial outline and their ratio (P/E), type of aperture, presence or absence of pores, beside exine thickness and ornamentation were investigated in eleven *Terminalia* (Combretaceae) species belonging to 7 sections from Egypt using LM and SEM. Based on palynological features, a taxonomic key was created for simple identification and species delimitations.

Mignot et al. (1994) pointed to the importance of aperture type in angiosperm recognition at any taxonomic level, even down to the intra individual level. Based on the results, *Terminalia* species may be divided into two main groups: monomorphic and dimorphic. Each group has a variety of pollen shapes, ranging from spheroidal to prolate, with many shapes seen in the pollen sampled from the same flower. Pollen hydration status and environmental conditions may contribute to heterogeneity in pollen shape and size within the same taxon (Grant-Jacob et al. 2022, Taia et al. 2023). El Ghazali (2022) noted differences in the sizes and forms of pollen among the same taxa within the family Combretaceae. Despite this, he defined their pollens as stenopalynous, meaning that their characteristics are either stable or slightly changed. According to this investigation, the *Terminalia* under study exhibit significant variety in their pollen morphological characteristics, and as a result, they can be classified as eurypalynous.

The highest PA length is seen in *T. myriocarpa* (27.8-34.5 µm), whereas *T. sericea* has the smallest PA length (18.6-23.2 µm). El Ghazali et al. (1998) have documented these differences in pollen size and shape. Given that multiple shape types have been observed in a single flower, shape class is determined to have less diagnostic value within the taxa in the material under examination. Taia et al. (2023) have noted this investigation; as a result, it does not play a significant role in the taxonomic discrimination of the examined taxa, except in the case of *T. sericea*, which is grouped separately in the clustering analysis (group A), this separation being due to it having the smallest PA and ED lengths and the smallest main aperture and sub-aperture lengths. This result supports the classification of this species as it belongs to section Psidioides Exell.

Four monomorphic species (*T. catappa*, *T. mantaly*, *T. muelleri*, and *T. arjuna*) were gathered by the mono-morphic character within the same taxa and placed in subgroup 2B in close proximity to the dimorphic taxa (*T. bellerica*). This grouping strengthens the taxonomic status of *T. catappa*, *T. mantaly*, *T. muelleri* as they belong to the same section Eucatappa Engl. & Diels. Despite *T. arjuna* being a separate section Pentaptera (Roxb.) Engl. & Diels, it is grouped with the former three species according to the mono-morphic pollen character.

According to El Ghazali and Krzywinski (1989), the apertures can be classified as iso- or hetero-colpate, meaning that they have two or more types of colpi, with one type differing in length and/or having endoapertures or not. This previously acknowledged observation was discovered in *Rosa sericea* (Ullah et al. 2022). It was also discovered in *Fagonia* species (Taia et al. 2021). El-Ghazali et al. (1998) noted the heterocolpate pollen grains within the taxa under study. This study noted variations in the length and width of the colpi as well as the existence and dimensions of endo-apertures; this result is in line with previous studies mentioned.

Since *T. brownii*, *T. laxiflora*, and *T. myriocarpa* have triangular or circular amb, the triangular amb gathered them in subgroup 1B. Amb shape was determined to be a valuable character in the building of the identification key. This result is partially in agreement with the taxonomy of *T. brownii*, *T. laxiflora* as they belong to the same section, Platycarpae Engl. & Diels, but does not agree with the classification of *T. myriocarpa* as a separate section Myriocarpae. A triangular amb is also present in *T. bellirica*, although it is distinct from the preceding three species in terms of other pollen characteristics and is located in subgroup 2B, albeit remaining closely linked to them. Certain species could be distinguished using the hexagonal, triangular, or circular amb, as the identification key illustrates. The two species; *T. bellirica* and *T. bentzoe* are belonging to section Billiricae Engl. & Diels, however, the study separated them in different groups; this separation is due to the difference between them in pollen state and amb shape.

Exine thickness ranged from 2.0 µm to 2.6 µm; however, there were significant differences in exine ornamentation. The rugate-perforate pattern was seen in *T. myriocarpa* and *T. sericea*, while the striate or striate-rugate pattern was seen in the majority of the investigated species. Only *T. laxiflora* has rugate-granulate ornamentation, but *T. bentzoe* and *T. chebula* have scabrate exine ornamentation; for this reason, the former two species were grouped together in group C although they belong to different sections; *T. bentzoe* sec. Belliricae Engl. & Diels, *T. chebula* sec. Myrobalanus (Gaertn.) Eichl. As a result, exine thickness varies very little among the taxa, whereas exine ornamentation, displayed by the key, is more helpful in identifying the taxa. This observation is consistent with every palynological study conducted since that of Erdtman (1966).

Conclusion

Based on the study's findings, three groups and two subgroups can be identified based on the unique characteristics of the pollen morphology of the *Terminalia* species under investigation. The pollen morphism, aperture homogeneity, amb form, and exine ornamentation are the most crucial characteristics in classifying the investigated taxa.

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First record of the woody *Melaleuca williamsii* s.l. (Myrtaceae) out of its native range

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Abstract – *Melaleuca williamsii* subsp. *synoriensis* (Myrtaceae) has been found in Rome (central Italy) along the banks of the river Tevere, representing the first discovery outside its native range (E-Australia). Description, distribution in Italy, phenology, and original photographs are provided.

Keywords: Europe, exotic species, *Melaleuca*, morphology, original material

Introduction

During the last decade, there has been increasing evidence of the negative impacts of alien species on the environment, the economy, and human well-being in all parts of the world (see e.g. Vilà et al. 2011, Rumlerová et al. 2016, Sohrabi et al. 2023). Provision of data about the distribution and status of the naturalization of alien plants is useful in preventing or reducing these impacts (see e.g. Gallardo et al. 2019, Pyšek et al. 2020,).

In this context, a recent investigation of the genus *Melaleuca* L. by ter Huurne et al. (2023) showed how several species (widely planted around the world mainly for ornamental and pharmaceutical uses) spread rapidly and have significant ecological impacts on autochthonous flora.

Melaleuca (Myrtaceae Juss.) is a genus that has been accepted without controversy for over 200 years, but was recently shown to be polyphyletic (Ladiges et al. 1999, Brown et al. 2001, Edwards et al. 2010). Edwards et al. (2010) demonstrated that eight out of the nine genera of the tribe Melaleuceae recognized by Wilson et al. (2005) fall within *Melaleuca* which was accepted as a single species-rich genus including 330–350 taxa (106 nomenclatural changes were proposed by Craven et al. 2014). According to POWO (2024) *Melaleuca* is native to Australia, while some taxa are aliens in Africa, Asia, the Americas and as far as Europe is concerned, only in the former Yugoslavia.

As part of an ongoing study on the alien flora of Italy and the Mediterranean area (see e.g. Iamonico 2010, 2022, Iamonico et al. 2014, Iamonico and Sánchez Del Pino 2016, Sukhorukov et al. 2016, Iamonico and El Mokni 2017, El Mokni and Iamonico 2018), we found in Rome (central Italy) the woody *Melaleuca williamsii* Craven subsp. *synoriensis* Craven, which represents the first records of the taxon out of its native range. Because of the difficulty we had in the identification of the species of taxon, and with the aim of helping European botanists in understanding this complicated genus, we examined in depth the species found and similar ones by studying also their protologues and associated types and original material.

Material and methods

The work is based on field surveys, analysis of relevant literature (protologues are included), and examination of specimens preserved in the following herbaria: CANB, GOET, K, MEL, NWS, P, and RO (acronyms according to Thiers 2024+).

The articles cited throughout the text are referred to the Shenzhen Code (hereafter reported as “ICN”; Turland et al. 2018).

Results

Melaleuca williamsii Craven subsp. ***synoriensis*** Craven, Novon 19: 452–453. 2009 ≡ *Callistemon pungens* subsp. *synoriensis* (Craven) Udovicic & R.D.Spencer, Muelleria 30: 24 (2012)

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Fig. 1. *Melaleuca williamsii*. A – habitat along the Tevere River (Rome, Italy), B – leaf, C – fruiting branch, D – inflorescence, E – flower (Photo: G. Nicolella).

Holotype: Australia, New South Wales, Ca. 200 NE Tom Cabin, NE part of New England Natl. Park, ca. 12 km SE of Ebor, 04 December 1993, P. J. Lepschi & J. Mowatt 1411 (CANB467657!, image of the holotype available at https://plants.jstor.org/stable/viewer/10.5555/al.ap.specimen.canb467657); isotypes: BRI (*non vidi fide* Craven 2009: 452), MEL2456739! (https://plants.jstor.org/stable/viewer/10.5555/al.ap.specimen.mel2456739), NE (*non vidi fide* Craven 2009: 452), NSW (*non vidi fide* Craven 2009: 452).

Native distribution area: Eastern Australia, from Queensland to New South Wales (Craven 2006, POWO 2024).

Occurrence in Italy: the plants found in Italy (February 2023) grow among the cracks in the quay along the right bank of the river Tevere in Rome (Latium region, central Italy) (Fig. 1).

Flowering and fruiting times: late winter to late spring (February–June). The discovery is not only the first one for Italy and Europe, but also the first out of the native range of the species (see Craven 2006, Uotila 2011, POWO 2024 and literature therein). We consider *Melaleuca williamsii* subsp. *synoriensis* as casual alien in Italy.

Notes: currently, three subspecies are accepted for *Melaleuca williamsii*, i.e. subsp. *williamsii*, subsp. *fletcheri* Craven, and subsp. *synoriensis* Craven (POWO 2024, WFO 2024). These subspecies can be distinguished from each other based on the bark texture, hairiness of leaves and hypanthium, color of filaments, and shape of cotyledons (Craven 2009). The Roman plants are identifiable as *M. williamsii* subsp. *synoriensis*, having leaves and hypanthium pubescent and staminal filament red [no data about the bark was reported by Craven (2009) for subsp. *synoriensis*, whereas the author distinguished subsp. *williamsii* and subsp. *fletcheri* in having barks with, respectively, papery and fibrous texture], whereas the other two subspecies display leaves lanuginose (subsp. *williamsii*) and filaments white, pink, or mauve (subsp. *fletcheri*).

Discussion

Although several taxonomic issues on *Melaleuca* were solved, some others, concerning the species rank, still need to be addressed. Indeed, we had difficulties in identifying the *Melaleuca* plants found in Rome even when we referred to the important diagnostic key to Australian *Melaleuca* taxa prepared by Craven et al. (2016). Issues arise for the leaf features, i.e. shape (some statements are reported in both the key phrases of step no. 5 of Australian *Melaleuca*'s key) and width. Roman plants have leaves 0.3–0.6 cm wide, a range that overlaps the choice of the step no. 5 in diagnostic key of Australian *Melaleuca* ("Leaves less than 4 mm wide" *vs*. "Leaves 4 mm or more wide"). By choosing both the phrases of step no. 5, we reached the following candidates: *M. linearis*, *M. subulata*, and *M. williamsii*. However, to attain a certain identification, we decided to examine carefully these three species, especially by studying the protologues, types, and original material.

The results follow:

1) *Melaleuca linearis* [described by Wendland and Schrader 1796: 19, Tab. XI" (image available at http://www.plantillustrations.org/illustration.php?id_illustration=246556); lectotype designated by Dowe et al. (2019) on a specimen preserved at GOET]: leaf blade has a ratio length/width of at least 10 (up to 15).

2) *Melaleuca subulata* [≡ *Callistemon subulatus* (described by Cheel 1925: 259); holotype: NSW139989 (image available at https://plants.jstor.org/stable/viewer/10.5555/al.ap.specimen.nsw139989?loggedin=true); a further Cheel's specimen (not isotype, found by us) at K (barcode K000793339, image available at https://plants.jstor.org/stable/viewer/10.5555/al.ap.specimen.k000793339): ratio length/width of the leaf blade ranging from 10 to 16.

3) *Melaleuca williamsii* [proposed by Craven (2006: 474) as *nomen novum pro Callistemon pungens* Lumley & R.D.Spencer *non Melaleuca pungens* Schauer (see Arts. 6.9 and 53.1 of ICN); holotype at MEL; isotypes at K (found by us), NE, NWS, and CANB; a good illustration by Lumley and Spencer (1990: 254, Fig. 1)]: ratio length/width of the leaf blade ranges from 3 to 7.

Based on the ratio length/width of the leaf blade, the Roman plants (ratio 5.5–10.0) overlap *Melaleuca williamsii* (3–7), whereas *M. linearis* and *M. subulata* have higher ratios (10–15 and 10–20, respectively).

In addition to the spontaneous Australian species, we also considered the *Melaleuca* species that are currently used as ornamentals in Italy (personal observations made in plant nurseries), i.e. *M. citrina* (native to E-Australia and alien in India, Kenya and Tanzania, former Yugoslavia, U.S.A., and Mexico; POWO 2024) and *M. viminalis* (native to E-Australia and alien in India, Kenya and Tanzania, U.S.A., and Mexico; POWO 2024):

1) *Melaleuca viminalis*: it has stamens fused (*vs*. free and inserted just in a ring on the hypanthium rim in the Roman plants) and hypanthium glabrous (*vs*. pubescent) (Craven et al. 2016).

2) *Melaleuca citrina*: this species cannot be ascribed to Roman plants (based on Craven et al. 2016) because of the length of the longest stamens [up to 25 mm long (range 17–25 mm) in *M. citrina vs*. up to 20 mm long (range 13–20 mm) in our plants] and the width of the inflorescences (45–70 mm in *M. citrina vs*. 38–47 mm wide). Concerning the ratio length/width of the blades, we studied the protologue of *M. citrina* and examined the original material. Curtis (1794: [260] validly published the name *Metrosideros citrina* (basionym of *Melaleuca citrina*) also providing a coloured illustration (Plate no. 260, original material). Curtis' Botanical Magazine is an illustrated publication (first issue published on 1 February 1787), the longest-running botanical magazine (currently referred to as the journal Curtis's Botanical Magazine) and including drawings of ornamental and exotic plants cultivated at Kew Gardens. According to the HUH-Index of Botanists (2013), Curtis' herbarium is unknown. No specimen useful for lectotypification was traced. Therefore, Curtis' Plate no. 260 is the only extant original material for *Metrosideros citrina* and the ratio length/width of the blades ranges from 2 to 6 (*vs*. 5.5–10.0 in our plants). Since the length of the stamens and the width of the inflorescence cannot be measured on Curtis' Plate no. 260 (a scale bar is lacking), we examined specimens collected by L. A. Craven and/or B. J. Lepschi (the experts in the genus *Melaleuca* and authors of the Australian key) and verifying that longer stamens are 21–23 mm long (*vs*. up to 20 mm long), whereas inflorescence are 53–55 mm wide (*vs*. 38–47 mm).

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Changes in grassland vegetation on the island of Plavnik (Croatia) over 100 years

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Abstract – The changes in the grassland vegetation that have occurred over the last almost 100 years on the northeastern Adriatic island of Plavnik (Croatia) were studied. For this purpose, 29 phytosociological relevés of standard plot size were collected using the Braun-Blanquet phytosociological approach and compared with those of 100 years ago. With the aim of defining the differences among the communities and identifying the main environmental gradients, cluster analysis (Ward's method on a Chord distance matrix) and non-metric multidimensional scaling ordination were applied. Taxa score-weighted means of the Ellenberg-type indicator values of main environmental variables were used to assess ecological differences among communities, and disturbance-related indicator values were calculated to assess the disturbance regime in shaping plant communities. According to the syntaxonomic framework for the grassland associations, three associations and two subassociations were found. Among them the *Festuco valesiacae-Poetum bulbosae* (*Poetalia bulbosae, Poetea bulbosae*), the *Helichrysetum italici salvietosum officinalis* (*Scorzoneretalia villosae, Festuco--Brometea*), and the *Hedypnoido rhagadioloidis-Asphodeletum ramosi* were proposed as new. The *Festuco valesiacae--Poetum bulbosae* documents for the first time, based on phytosociological data, the occurrence of the *Poetea bulbosae* in Croatia. Three associations were considered extinct, possibly as a result of changes in grazing pressure and type.

Keywords: eastern Adriatic, grazing pressure, *Helichrysum italicum*, Mediterranean Islands, *Poetea bulbosae*, *Scorzoneretalia villosae*, syntaxonomy, vegetation changes

Introduction

The Mediterranean basin is considered a biodiversity hotspot on account of its exceptional concentration of endemic species and the loss of habitats suitable for them (Myers et al. 2000). A significant component of this biodiversity is preserved in the Mediterranean islands due to the presence of steno-endemic taxa and distinctive plant assemblages (Médail 2017, Vargas 2020). The islands of the eastern Adriatic, with its multitude of archipelagos, are particularly important from this point of view, and they are characterised by a very high plant richness, comprising almost 1800 vascular taxa (Nikolić et al. 2008).

The phytosociological investigation of the north-eastern Adriatic using the approach of Braun-Blanquet (Westhoff and van der Maarel 1980) began in the 1930s, with the pioneering studies of Croatian botanist Stjepan Horvatić on some islands, such as Pag and Rab (Trinajstić 2001 and references therein). On those islands, one of the most widespread aspects of vegetation was represented by open Mediterranean-sub-Mediterranean grasslands the particular floristic composition of which justified their attribution to an endemic alliance (*Chrysopogono grylli-Saturejion subspicatae* Horvat et Horvatić 1934 *nom. inval.*). Subsequently, that alliance was classified in an order (*Scorzonero villosae-Chrysopogonetalia grylli* Horvatić et Horvat in Horvatić 1963) and class also endemic to the Western Balkans (*Brachypodio-Chrysopogonetea* Horvatić 1963) (see Terzi 2011).

According to the current European syntaxonomic framework, the so-called EuroVegChecklist (EVC, Mucina et al. 2016), those grasslands mostly belong to the western Balkan alliances *Chrysopogono grylli-Koelerion splendentis* and *Scorzonerion villosae*, and to the trans-Adriatic order

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Scorzoneretalia villosae, included in turn in the class *Festuco-Brometea* (but see also Terzi et al. 2023).

The first survey – the subject of Horvatić's doctoral thesis (Trinajstić 2001) – concerned the vegetation of the island of Plavnik, in the Kvarner area (Croatia), where Horvatić (1927) described five grassland syntaxa, namely *Festucetum valesiacae*, *Asphodeletum microcarpi*, *Helichrysetum italici*, "*Helichrysetum salviosum*" and *Andropogonetum grylli*. For each of them, a diagnosis was provided consisting of a variable number (from 5 to 10) of quadrats of 1 square metre each, with a complete list of taxa accompanied by quantitative scores. Although each vegetation type was sampled on only a small area, Horvatić (1927) gave a fairly precise idea of the main dominant and codominant taxa present at that time.

Quite surprisingly, neither Horvatić's later papers (e.g. Horvatić 1963, 1973) nor the subsequent syntaxonomic revisions of Croatian grasslands of the order *Scorzoneretalia villosae* (e.g. Horvat et al. 1974, Trinajstić 2008, Terzi 2015) mentioned these five syntaxa. Moreover, the herbaceous vegetation of the island of Plavnik has never been studied subsequently.

This island presents a rather interesting situation from a vegetation point of view because it has remained uninhabited for a long period of time, being used as a hunting reserve and only occasionally visited by hunters and tourists (along the coastal areas). The conspicuous presence of animals, sheep and fallow deer, left in the wild, has presumably significantly increased the grazing pressure on the grassland ecosystems.

The present work therefore aims to (i) provide new relevés for the herbaceous vegetation of the island of Plavnik, using a more reliable plot size than that used by Horvatić (1927), (ii) assess the changes in vegetation that have occurred over almost 100 years, (iii) provide a syntaxonomic framework for the grassland associations occurring on the island, and (iv) identify the main ecological drivers of grassland diversity.

Materials and methods

Study area

The island of Plavnik (surface area 8.63 km^2, coastline length 18.47 km) belongs to the north-eastern Adriatic group of islands in the Kvarner Bay, Croatia (Fig. 1). It is located between the islands of Cres and Krk and it stretches in a northwest-southeast direction for a length of 6.3 km. The coastline of the island is not indented: the northern coast is very steep and poorly accessible, while the southern is flat, gentle, accessible and with several coves.

The island has high terrain ruggedness. The northern and central part is a karstic plateau with an elevation of approximately 90–180 m a.s.l., bordered by steep rocky slopes exceeding 30°. The highest peak reaches an elevation of 193.6 m a.s.l. To the south-east, the karstic plateau gradually passes into lowlands with altitudes lower than 30 m a.s.l. (Fig. 2).

The bedrock consists mainly of carbonate rocks (Fuček et al. 2015). The Upper Cretaceous carbonate deposits can be recognized in different types of vertically alternating lithotypes. Multi-layered and massive recrystallized limestones with radiolithid-chondrodotic lithosomes are developed on both western and eastern coasts, while in the central part there are medium-thick layered shallow-sea mudstones and granular limestones with benthic foraminifera, radiolithid rudists and chondrodonts. The brown soils on limestone are predominant on the island (Vukadinović 2021).

The area is influenced by both Mediterranean and continental climates and characterized by intrusions of cold air during the winter and convective precipitation in the summer. These specific conditions result in the lack of a distinct dry period typical of the Mediterranean climate (Šegota and Filipčić 2003). The average annual air temperature is 15.3 °C and precipitation averages 1253.7 mm yr^{-1} (data from the town of Krk station for 1998-2022, Croatian Meteorological and Hydrological Service). The highest monthly average

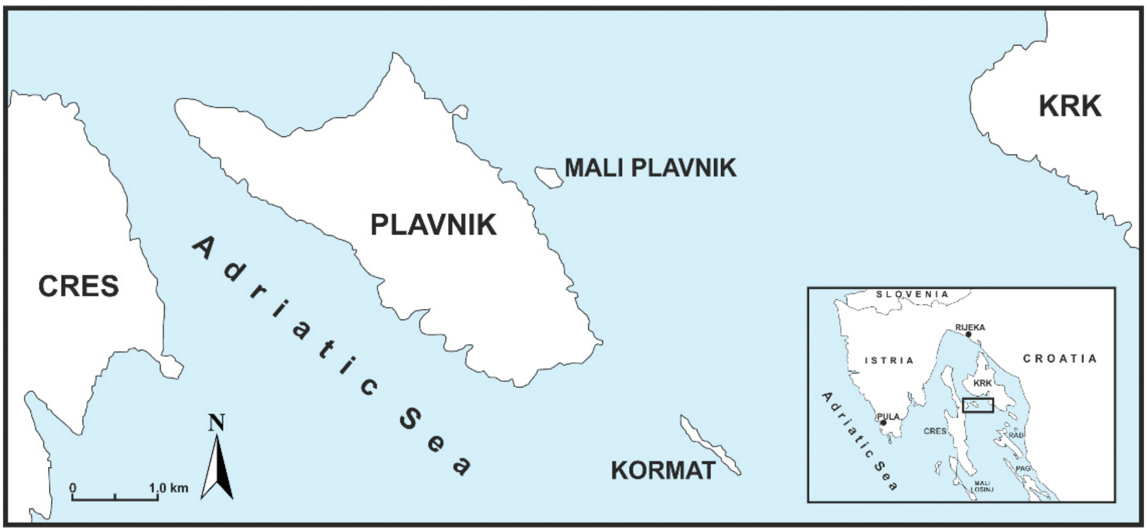

Fig. 1. The island of Plavnik is located between the islands of Cres and Krk in the Kvarner Bay (Croatia). The square on the map in the lower right corner shows the study area in the NE Adriatic context.

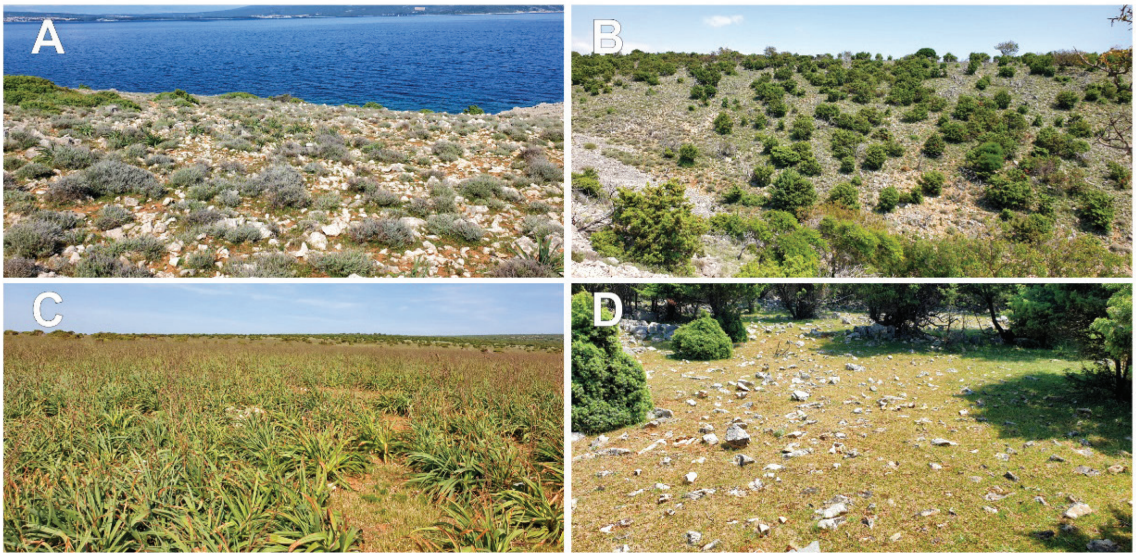

Fig. 2. Physiognomy of the plant associations on the island of Plavnik in May 2023: A – *Helichrysetum italici* Horvatić 1927 on the island's eastern coast, B – *Helichrysetum italici salvietosum officinalis subass. nov.* covers the open areas on the island's western slopes, C – *Hedypnoido rhagadioloidis-Asphodeletum ramosi ass. nov.*, in the southernmost part of the island, D – *Festuco valesiacae-Poetum bulbosae ass. nov.* occurs on the karstic plateau in the north-central part of the island.

temperature is 30.5 °C in July, and the lowest 2.5 °C in January. The absolute maximum of 38.8 °C was recorded on 21 July 2015, and the absolute minimum (-9.0 °C) on 24 January 2006. The greatest rainfall comes in September, October and November with averages of 155.6, 162.0 and 178.0 mm, respectively. In the period from June to August the total rainfall is 184.2 mm. Northern winds (NW, ENE, NE) prevail throughout the year. This area has 2521 hours of sunshine per year (data for the island of Rab station for 1998-2022). On average the relative air humidity is 70%.

Phytoclimatic indices were calculated according to Rivas-Martínez et al. (2011): annual positive temperature (Tp) is 1,834; continentality index (Ic) is 18.9; thermicity index (It) is 238; ombrothermic index (Io) is 0.68. According to these indices, the neighbouring Krk station lies within the temperate macrobioclimate, meso-temperate thermotype and semiarid ombrotype.

The observed global and regional warming has been unequivocal since the middle of the 20th century (IPCC 2014) and is present along the eastern Adriatic coast (Branković et al. 2013). Climate change shows a tendency to manifest in drier conditions for the Adriatic (Patarčić et al. 2014, WBG 2021), which is a common feature for the broader European part of the Mediterranean (EEA 2019). However, for example, the precipitation change is regionally highly variable and further analyses are needed to define the assessment of climate change impacts at the regional and local scale (Gajić-Čapka et al. 2018).

From the phytogeographic point of view, the eastern and southwestern part of the island is covered by stands of the *Fraxino orni-Ostryion* alliance i.e. *Querco pubescenti-Carpinetum orientalis* Horvatić 1939 association. The meso-Mediterranean evergreen vegetation of the *Fraxino orni-Quercion ilicis* alliance is developed only in the north-western part of the island. However, woodlands have been significantly changed due to the long-term anthropogenic influences and nowdays only degraded vegetation types, i.e. maquis, garrigue and grasslands, may be found. Large areas of trees were felled between the two world wars to obtain open pastures, intended for sheep rearing. The island is classified among Croatia's Important Plant Areas (IPAs) and is part of the Natura 2000 ecological network (Topić and Vuković 2010, Official Gazette 2019).

The island has had no permanent residents or settlements during the last centuries. Between the two world wars, only a few herding groups lived on the island to provide living conditions for livestock, mainly sheep, while goats made up only 10-20% of the total number of head (Dumančić 1988). After the Second World War, such a simple nomadic way of life gradually faded away. The sheep population has drastically decreased, while goats, in general, have practically disappeared on the eastern coast of the Adriatic. There are no available data on the number of sheep in Plavnik over last century. In addition, the non-native European fallow deer (*Dama dama*) was introduced in 1995. According to the data of the local hunting association Farma Plavnik d.o.o. (personal communication), a number of the fallow deer, with regular annual shooting of ca. 50 nap, are stable and amounts to about 160 nap. The maximum nutritional requirements for females are during lactation, and this period coincides with the maximum development of vegetation.

Vegetation sampling and data analysis

The map of the distribution areas of the five types of plant communities on the island of Plavnik provided by Horvatić (1927: p. 44) was compared with the most recent satellite images available on Google Earth Engine, to identify areas still covered by herbaceous vegetation. Those areas, and in particular the locations mentioned in that paper, were surveyed. The vegetation types were identified in the field based on their dominant and codominant taxa. Only for the "*Andropogonetum grylli*", whose dominant taxon should have been *Chrysopogon gryllus*, we were unable to find any phytocoenosis clearly dominated by this plant, which is nowadays rather rare on the island.

The remaining four vegetation types were sampled through 29 relevés, made in May 2023 according to the phytosociological approach (see Westhoff and van der Maarel 1980). The relevés were sampled in quadrats of 25 m^2 (5 m × 5 m). All vascular plants in the plots were recorded and their cover-abundance values were visually estimated using the Braun-Blanquet cover-abundance scale with the subdivisions 2m, 2a and 2b as proposed by Barkman, Doing and Segal (in: van der Maarel 1979). This extended Braun-Blanquet scale (i.e. r, +, 1, 2m, 2a, 2b, 3, 4, 5) was replaced by the 9-point ordinal scale (i.e. 1-9, see Westhoff and van der Maarel 1980), before the statistical analyses. The main physical features of the plots (e.g., geographic coordinates, slope, exposure, vegetation cover, rockiness, and stoniness) were also recorded. Stoniness is defined here as the percentage of plot coverage by stones (incoherent material), regardless of their size. Rockiness, on the other hand, indicates the percentage of coverage of the plot determined by the outcropping rocky substrate.

The data matrix (29 relevés × 115 taxa) was subjected to hierarchical clustering using Ward's method on a Chord distance matrix (i.e., Euclidean distance on pre-transformed data through normalization by sites). Four interpretable clusters were subjectively defined by visual examination of the dendrogram.

To visualize the floristic and ecological relationships among the four clusters of relevés, nonmetric multidimensional scale (NMS) ordination was performed by using the Chord distance. Both cluster analysis and ordination were performed through the PcOrd 6.22 software package (McCune and Mefford 2011), the NMS with the "slow and thorough" option of the autopilot mode.

To assess ecological differences among the four plant communities, taxa score-weighted means of the Ellenberg-type Indicator Values (IVs) of temperature (T), soil reaction (R), moisture, and nutrient content were calculated for each relevé, based on the EIVs provided for Italy – which is the closest country geographically and ecologically to the study area – in Tichý et al. (2023).

In addition, given the presence of high numbers of sheep and fallow deer on the island of Plavnik and the possible importance of the disturbance regime in shaping the different plant communities, three additional disturbance-related IVs, taken from Midolo et al. (2023), were considered. In more detail, the following IVs, were calculated on taxa score-weighted means for each relevé: Disturbance severity (Ds), Disturbance frequency (Df), and Grazing pressure (Gr). Joint plots were used to represent linear correlations between IVs and ordination scores, with an r^2 cut-off of 0.3.

Indicator species analysis (ISA) (Dufrêne and Legendre 1997) was used to identify the indicator taxa of the four main clusters of relevés obtained by cluster analysis, and their combinations in larger groups (see De Caceres et al. 2010). The indicator values were calculated for all taxa present in at least three relevés. The statistical significance ($P < 0.01$) of the indicator values was tested by a Monte Carlo test with 10000 permutations. The ISA was run in R software (R Core Team 2023) through the package indicspecies 1.7.12 (De Cáceres and Legendre 2009).

In order to verify whether the relevés from the island of Plavnik in which *Asphodelus ramosus* was dominant (i.e. cluster 'He' in Figs. 3 and 4) could represent an association independent of the others described so far in the eastern Adriatic, we compared these relevés with the associations *Bromo erecti-Chrysopogonetum grylli* Horvatić 1934 and *Narcisso tazettae-Asphodeletum microcarpi* Šegulja 1969. In more detail, a second data matrix (55 relevés × 283 taxa) was obtained by adding the seven relevés reported in Tab. 3 to the other 48 relevés carried out by i) Horvatić (1934, rels. 11-23 of table XXVIII) on the island of Pag, Croatia; ii) Stanišić-Vujačić et al. (2022, rels. 1-17 of table 1) at Ćemovsko polje, Montenegro, iii) Šegulja (1970, rels. 1-8 of table II) in Labin, Istria, Croatia, and iv) Hećimović (1984, *Narcisso tazettae-Asphodeletum microcarpi sisymbrietosum officinalis* Hecimović 1984, rels. 1-10 of table 5) on the islets of Bobara and Mrkan, in southern Croatia.

Using the same methods as described above, an NMS ordination was performed on that matrix to visualise the floristic relationships between the relevés, and then an indicator species analysis was run to identify the taxa that distinguish the *Asphodelus ramosus* community on Plavnik Island from those elsewhere.

The taxonomic nomenclature follows Euro+Med (2006-2023). *Bromopsis erecta* and *B. condensata* were treated under the same tag, the latter being more abundant and frequent on the island of Plavnik.

Syntaxonomic nomenclature of alliances, orders and classes follows the EuroVegChecklist (https://floraveg.eu/vegetation/, retrieved on 10 October 2023, as modified by Terzi et al. 2024a), whose correct names are given without author citations. Syntaxon names not included in the EVC, but are instead provided with author citations. Names reported exactly as mentioned in the original papers are reported in quotation marks.

Results

The dendrogram (Fig. 3) and further NMS (Fig. 4) showed four main clusters of relevés corresponding to the plant communities dominated by (Fe) *Festuca valesiaca* and *Poa bulbosa*, (As) *Asphodelus ramosus*, (He) *Helichrysum italicum*, and (Sa) *Salvia officinalis* and *Helichrysum italicum*.

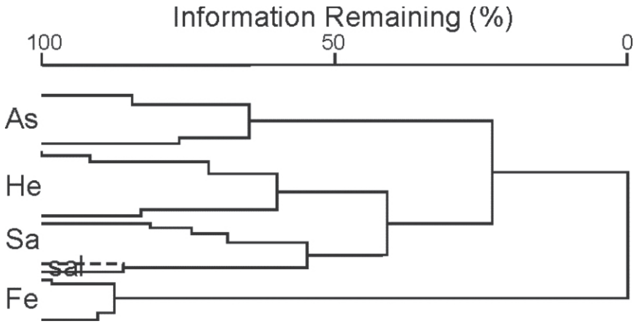

Fig. 3. Hierarchical clustering (Ward's method, Chord distance) shows four main groups of relevés corresponding to the plant communities: As – *Hedypnoido rhagadioloidis-Asphodeletum ramosi*, He – *Helichrysetum italici*, Sa – *Helichrysetum italici salvietosum officinalis*, "sal" indicates a group of relevés clearly dominated by *Salvia officinalis*, Fe – *Festuco valesiacae-Poetum bulbosae*.

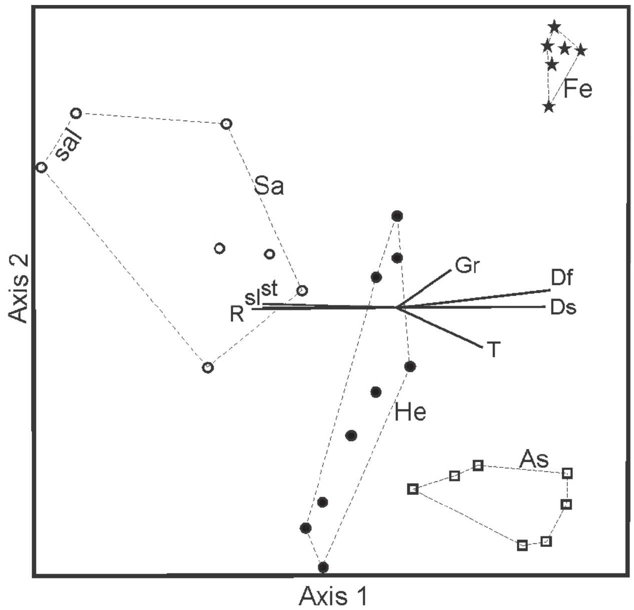

Fig. 4. Non-metric multidimensional scaling of releves in the dataset. Abbreviations: As – *Hedypnoido rhagadioloidis-Asphodeletum ramosi*, He – *Helichrysetum italici*, Sa – *Helichrysetum italici salvietosum officinalis*, "sal" indicates a group of relevés clearly dominated by *Salvia officinalis*, Fe – *Festuco valesiacae-Poetum bulbosae*. Ds – disturbance severity, Df – disturbance frequency, Gr – grazing pressure, T – temperature, R – soil reaction, st – stoniness, sl – slope.

The cluster Fe groups together relevés from the summit plateau of the island, at more than 150 m a.s.l., where Horvatić (1927) had previously described the association *Festucetum valesiacae*. The mean number of taxa is 33 whereas in all the relevés of this cluster 53 taxa were found. In addition to *F. valesiaca* and *P. bulbosa* many species were recorded with high frequency (see Tab. 1).

Tab. 1. *Festuco valesiacae-Poetum bulbosae ass. nov.* on the island of Plavnik. Life-forms (LF): Ch – chamaephytes, G – geophytes, H – hemicryptophytes, P – phanerophytes, T – therophytes. Asterisk (*) indicates holotypus (rel. 1).

Rel. number	LF	1*	2	3	4	5	6
Exposition (°)		0	0	0	0	0	0
Slope (%)		0	0	0	0	0	0
Rockiness (%)		30	40	30	60	40	20
Stoniness (%)		10	30	10	10	20	30
Vegetation cover (%)		70	60	65	50	60	70
Festuco valesiacae-Poetum bulbosae							
Poa bulbosa	H	6	6	6	5	6	4
Festuca valesiaca	H	5	6	6	5	6	6
Poetalia bulbosae, Poetea bulbosae							
Trifolium suffocatum	T	6	4	4	4	3	3
Plantago lanceolata	H	3	2	2	2	2	3
Erodium cicutarium	T	2	2	2	2	2	2
Trifolium subterraneum	T	3	2	.	2	3	2
Trifolium scabrum	T	2	2	2	2	.	2
Stipo-Trachynietea distachyae							
Trifolium campestre	T	4	4	5	5	2	5
Filago germanica	T	4	4	3	3	4	2
Galium murale	T	3	3	3	2	3	2
Sherardia arvensis	T	3	2	3	2	2	3
Gastridium ventricosum	T	2	2	3	2	2	3
Sideritis romana	T	2	2	2	3	3	2
Catapodium rigidum	T	2	2	2	2	2	2
Ornithogalum collinum	G	2	2	.	2	.	.
Ononis reclinata	T	2	.	.	2	.	.

Tab. 1. continued

Rel. number	LF	1*	2	3	4	5	6
Tuberarietea guttatae							
Crepis neglecta subsp. *neglecta*	T	2	3	2	2	2	2
Vulpia ciliata	T	2	2	3	3	2	2
Medicago minima	T	3	2	2	2	2	2
Galium divaricatum	T	.	2	3	2	2	2
Aira elegantissima	T	2	2	2	2	.	2
Asterolinon linum-stellatum	T	.	2	.	2	2	2
Plantago bellardii	T	.	.	3	3	2	.
Minuartia hybrida	T	4	2	.	2	.	.
Lotus hispidus	T	.	2	.	.	2	.
Festuco-Brometea							
Koeleria splendens	H	3	4	2	2	3	3
Thymus longicaulis	Ch	2	2	3	.	2	3
Teucrium capitatum subsp. *capitatum*	Ch	2	2	2	.	2	2
Carduus nutans subsp. *micropterus*	H	.	.	.	2	.	2
Eryngium amethystinum	H	.	.	.	2	.	2
Salvia pratensis	H	2	2	.	.	.	.
Other taxa							
Petrorhagia saxifraga	H	3	2	2	2	2	2
Anagallis arvensis	T	2	.	2	2	2	2
Scorzoneroides autumnalis	H	2	2	2	.	.	2
Cerastium pumilum subsp. *glutinosum*	T	2	2	2	2	.	.
Torilis nodosa	T	2	2	.	.	2	.
Geranium molle	T	2	.	2	.	2	.
Marrubium incanum	H	.	.	2	2	2	.
Medicago monspeliaca	T	.	.	2	.	2	.
Sedum acre	Ch	2	.	2	.	.	.
Euphorbia helioscopia	T	2	.	.	.	2	.

Date, coordinates and sporadic species (with 2) of the relevés: Rel. 1 (holotypus): 28.V.2023, lat. 44° 58' 41", long. 14° 31' 27", *Arenaria serpyllifolia*, *Linum tenuifolium*; Rel. 2: 28.V.2023, lat. 44° 58' 42.5", long. 14° 31' 23", *Asparagus acutifolius*, *Brachypodium sylvaticum*, *Euphorbia exigua*, *Veronica arvensis*; Rel. 3: 28.V.2023, lat. 44° 58' 41", long. 14° 31' 21", *Euphorbia cyparissias*, *Helichrysum italicum*; Rel. 4: 28.V.2023, lat. 44° 58' 39", long. 14° 31' 23", *Valantia muralis*, *Achnatherum bromoides*, Rel. 5: 28.V.2023, lat. 44° 58' 38", long. 14° 31' 27.5", *Ficaria verna*; Rel. 6: 28.V.2023, lat. 44° 58' 35", long. 14° 31' 32", *Carex halleriana*.

The indicator species associated to this cluster are *Trifolium suffocatum*, *Erodium cicutarium*, *Crepis neglecta* subsp. *neglecta, Petrorhagia saxifraga*, and *Marrubium incanum*.

The second partitioning level separates the cluster As. This cluster represents the vegetation clearly dominated by *Asphodelus ramosus*, the latter being always recorded with a cover greater than 75%, except in one relevé. This vegetation type is well represented in the southern part of the island. The indicator taxa of this cluster are *Asphodelus ramosus*, *Hedypnois rhagadioloides*, *Crepis zacintha*, and *Centaurium maritimum*. The mean number of taxa per relevé is 32.

The third cluster He represents the most widespread type on the island: open vegetation dominated by *Helichrysum italicum* and *Koeleria splendens*, and with other taxa typical of the *Festuco-Brometea* class occurring with high frequency (e.g. *Bromopsis erecta*, *Euphorbia cyparissias*, Tab. 2).

The indicator species of this cluster are *Reichardia picroides*, *Bupleurum veronense*, *Plantago holosteum*, and *Hippocrepis comosa*. This vegetation type usually develops on rocky and stony ground whereas on steep slopes on the eastern part of the island, in some places, it gives way to a *Salvia officinalis-Helichrysum italicum* community, represented by the cluster Sa. The latter corresponds to the "*Helichrysetum salviosum*" described by Horvatić (1927) and includes seven relevés, with a mean number of taxa of 21. This cluster is actually heterogeneous with some relevés which represent a variant of the *Helichrysetum italici* due to the presence and co-dominance of both *Helichrysum italicum* and *Salvia officinalis*, whereas two relevés (indicated as "sal" in Figs. 3 and 4) are clearly dominated by the latter. *Salvia officinalis* is the only indicator species of this cluster.

The NMS (Fig. 4) resulted in a two-axis solution, with a final stress 8.96, indicating a fairly good ordination.

Tab. 2. *Helichrysetum italici typicum* (rels. 1-10) and *Helichrysetum italici salvietosum officinalis* (rels. 11-14); transition to *Stipo--Salvietum officinalis* (rels. 15-16) on the island of Plavnik. For abbreviations of Life-forms [LF], see caption of Tab. 1. Asterisk (*) indicates neotypus (rel. 3), and holotypus (rel. 13), respectively.

Rel. number	**LF**	**1**	**2**	**3***	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13***	**14**	**15**	**16**
Exposition (°)		20	50	0	130	230	50	140	0	160	70	160	150	270	50	270	60
Slope (%)		5	10	10	2	3	10	2	0	2	2	15	20	10	20	30	25
Rockiness (%)		30	40	40	10	70	30	20	10	40	40	40	40	30	30	30	30
Stoniness (%)		40	50	30	40	20	70	20	40	40	40	70	40	40	50	70	70
Vegetation cover (%)		60	60	70	60	60	70	60	70	70	75	50	55	60	60	70	60
Helichrysetum italici																	
Helichrysum italicum	Ch	7	7	8	6	6	7	7	8	7	7	6	6	6	5	2	2
Salvia officinalis	Ch	.	.	.	.	.	.	.	.	.	6	5	4	7	7	8	8
Chrysopogono grylli-Koelerion splendentis, Scorzoneretalia villosae																	
Bromopsis condensata (+ *Br. erecta*)	H	4	4	3	2	4	3	2	4	3	5	2	2	5	3	4	3
Koeleria splendens	H	5	5	5	4	5	4	3	4	5	4	.	.	2	2	.	.
Festuca valesiaca	H	.	.	2	2	.	.	2	2	4	3	.	.	3	2	.	.
Bupleurum veronense	T	2	2	2	2	2	2	.	.	.	.	.	.	.	.	.	.
Carduus nutans subsp. *micropterus*	H	.	.	.	.	2	.	.	.	.	.	.	.	2	2	2	.
Centaurea tommasinii	H	2	2	2	.	.	.	.	.	.	.	.	2	.	.	.	.
Teucrium montanum	Ch	2	.	.	.	.	.	.	.	.	2	2	.	2	.	.	.
Aethionema saxatile	Ch	.	.	.	.	.	.	.	.	.	.	2	2	2	.	.	.
Medicago prostrata	H	.	.	2	2	.	.	.	.	2	.	.	.	.	.	.	.
Chrysopogon gryllus	H	.	.	.	.	.	.	.	2	2	.	.	.	.	.	.	.
Eryngium amethystinum	H	.	.	.	.	.	.	.	.	.	2	.	.	.	2	.	.
Genista sylvestris subsp. *dalmatica*	Ch	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Stipa eriocaulis	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
Festuco-Brometea																	
Euphorbia cyparissias	H	4	3	2	2	3	5	2	.	2	2	.	.	.	.	.	.
Linum tenuifolium	Ch	2	2	.	2	2	2	.	.	2	2	2	.	2	.	.	.
Melica ciliata	H	.	.	2	.	.	.	2	2	2	2	2	2	.	.	2	2
Salvia pratensis	H	2	.	3	2	3	2	2	2	.	2	.	.	.	.	.	.
Sanguisorba minor	H	2	2	2	.	2	3	2	2	.	2	.	.	.	.	.	.
Sedum acre	Ch	2	.	2	2	2	2	.	.	.	.	.	.	.	.	.	.
Thymus longicaulis	Ch	2	.	2	2	2	.	.	2	.	.	.	.	.	.	.	.
Convolvulus cantabrica	H	.	.	.	2	.	2	2	.	.	.	.	2	.	.	.	.
Hippocrepis comosa	H	2	2	.	2	.	2	.	.	.	.	.	.	.	.	.	.
Plantago holosteum	H	4	4	4	.	.	4	.	.	.	.	.	.	.	.	.	.
Achnatherum bromoides	H	.	.	.	.	.	.	2	2	.	.	.	.	.	.	.	.
Carex halleriana	H	.	.	.	.	.	.	.	.	.	.	.	2	4	.	.	.
Centaurium erythraea	H	.	.	2	2	.	.	.	.	.	.	.	.	.	.	.	.
Dorycnium pentaphyllum subsp. *herbaceum*	H	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Galium corrudifolium	H	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Knautia purpurea	H	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.
Pilosella piloselloides	H	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.
Teucrium chamaedrys	Ch	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.
Stipo-Trachynietea distachyae																	
Catapodium rigidum	T	3	2	2	2	2	3	2	3	3	2	2	2	2	3	.	.
Trifolium campestre	T	2	2	3	3	3	2	4	4	5	3	2	3	2	2	.	.
Trachynia distachya	T	3	2	3	3	3	2	2	2	3	2	4	3	2	.	.	.

Tab. 2. continued

Rel. number	**LF**	**1**	**2**	**3***	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13***	**14**	**15**	**16**
Euphorbia exigua	T	2	2	2	2	2	.	2	2	2	2	3	3	2	.	.	.
Gastridium ventricosum	T	2	2	2	2	2	2	3	2	2	2	.	2	2	.	.	.
Sideritis romana	T	2	.	2	5	2	2	2	2	2	2	2	2	.	2	.	.
Medicago minima	T	2	2	2	3	2	.	3	3	3	2	2	3	.	.	.	.
Valantia muralis	T	3	3	2	3	2	4	2	.	2	.	2	2	.	2	.	.
Trifolium scabrum	T	2	2	2	3	.	.	3	3	3	2	.	2	2	.	.	.
Filago germanica	T	.	.	2	2	2	.	2	2	.	.	.	.	.	.	2	.
Sherardia arvensis	T	.	.	2	2	.	.	2	3	2	.	.	.	.	2	.	.
Asterolinon linum-stellatum	T	.	2	2	2	.	.	.	.	2	.	.	2	.	.	.	.
Ononis reclinata	T	.	.	2	2	2	.	.	.	2	2	.	.	.	.	.	.
Plantago bellardii	T	.	.	2	3	3	.	.	2	.	.	.	.	.	.	.	.
Crepis neglecta subsp. *neglecta*	T	.	.	2	.	.	.	.	.	.	.	.	.	.	2	2	.
Galium murale	T	.	.	.	.	.	.	2	2	.	2	.	.	.	.	.	.
Ornithogalum collinum	G	2	2	.	.	.	.	.	2	.	.	.	.	.	.	.	.
Valerianella eriocarpa	T	.	.	2	2	.	.	2	.	.	.	.	.	.	.	.	.
Other taxa																	
Dactylis glomerata subsp. *hispanica*	H	2	2	2	2	.	3	2	3	2	3	.	3	2	2	3	.
Linum trigynum	T	3	2	2	2	2	2	2	2	2	3	2	.	3	2	.	.
Anagallis arvensis	T	3	2	2	2	2	2	2	2	2	2	2	2	.	.	.	.
Carlina corymbosa	H	2	.	2	2	2	2	.	2	2	2	.	.	2	.	.	.
Reichardia picroides	H	2	2	2	2	2	2	2	.	.	2	.	.	2	.	.	.
Juniperus oxycedrus	P	2	2	2	2	.	.	.	.	2	2	.	.	.	4	2	.
Plantago lanceolata	H	3	2	2	2	2	2	2	.	2	.	.	.	.	.	.	.
Sonchus bulbosus subsp. *bulbosus*	G	.	.	.	.	.	.	3	4	2	2	.	2	2	2	2	.
Teucrium capitatum subsp. *capitatum*	Ch	2	.	2	.	2	.	2	2	2	2	.	2	.	.	.	.
Geranium purpureum	T	.	.	2	.	.	.	2	2	2	2	.	.	2	2	.	.
Orobanche minor	T	.	.	.	2	2	.	2	2	2	.	.	.	2	.	.	.
Torilis nodosa	T	.	.	.	.	.	.	2	2	2	2	.	2	.	2	.	.
Petrorhagia saxifraga	H	.	.	.	2	.	.	2	2	2	.	.	2	.	.	.	.
Poa bulbosa	H	.	.	2	2	.	.	3	3	2	.	.	.	.	.	.	.
Scorpiurus muricatus	T	.	2	2	.	3	3	.	.	.	.	.	2	.	.	.	.
Aira elegantissima	T	.	.	2	.	.	.	2	2	.	.	.	.	.	.	.	.
Allium sp.	G	2	.	.	.	.	.	.	.	.	.	2	.	.	2	.	.
Asparagus acutifolius	P	.	.	.	.	.	.	2	.	2	.	.	.	2	.	.	.
Cerastium pumilum subsp. *glutinosum*	T	.	.	.	.	.	.	.	2	.	.	.	2	.	2	.	.
Galium divaricatum	T	.	.	.	2	.	.	.	2	2	.	.	.	.	.	.	.
Lotus corniculatus	H	2	2	2	.	.	.	.	.	.	.	.	.	.	.	.	.

Dates, coordinates and sporadic species (with 2): Rel. 1: 25.V.2023, lat. 44° 58' 28", long. 14° 32' 25", *Euphorbia peplus*, *Thesium divaricatum*; Rel. 2: 25.V.2023, lat. 44° 58' 13", long. 14° 32' 36", *Thesium divaricatum*, *Anthyllis vulneraria* subsp. *rubriflora*; Rel. 3 (neotypus): 25.V.2023, lat. 44° 58' 00", long. 14° 32' 42", *Asphodelus ramosus*, *Hedypnois rhagadioloides*, *Leontodon crispus*, *Pilosella officinarum*, *Convolvulus althaeoides* subsp. *tenuissimus*; Rel. 4: 25.V.2023, lat. 44° 57' 54", long. 14° 32' 47", *Asphodelus ramosus*, *Crepis zacintha*, *Hedypnois rhagadioloides*, *Leontodon tuberosus*, *Crepis foetida*; Rel. 5: 25.V.2023, lat. 44° 57' 50", long. 14° 32' 56", *Pilosella officinarum*; Rel. 6: 25.V.2023, lat. 44° 57' 55", long. 14° 32' 54", *Lotus hispidus*, *Rubus ulmifolius*; Rel. 7: 27.V.2023, lat. 44° 58' 04", long. 14° 31' 30", *Clematis vitalba*, *Vulpia ciliata*, *Oxalis corniculata*, *Quercus ilex*, *Stachys officinalis*; Rel. 8: 27.V.2023, lat. 44° 58' 09.5", long. 14° 31' 15", *Clematis vitalba*, *Cynosurus echinatus*, *Euphorbia peplus*, *Geranium molle*, *Minuartia hybrida*, *Vulpia ciliata*, *Trifolium suffocatum*, *Ficaria verna*; Rel. 9: 27.V.2023, lat. 44° 58' 13.5", long. 14° 31' 02", *Paliurus spina-christi*, *Scorzoneroides autumnalis*; Rel. 10: 28.V.2023, lat. 44° 58' 49", long. 14° 31' 42", *Crepis zacintha*, *Minuartia hybrida*, *Paliurus spina-christi*, *Sonchus asper*, *Carthamus lanatus*; Rel. 11: 26.V.2023, lat. 44° 57' 27.5", long. 14° 31' 59", *Urospermum picroides*; Rel. 12: 26.V.2023, lat. 44° 57' 28", long. 14° 31' 55", *Cynosurus echinatus*; Rel. 13 (holotypus): 27.V.2023, lat. 44° 58' 11", long. 14° 31' 13", Rel. 14: 28.V.2023, lat. 44° 58' 42", long. 14° 31' 38", *Geranium molle*; Rel. 15: 27.V.2023, lat. 44° 58' 06.5", long. 14° 31' 07", *Sesleria autumnalis*, *Sonchus asper*, *Myosotis arvensis*, *Phillyrea latifolia*; Rel. 16: 28.V.2023, lat. 44° 58' 39", long. 14° 31' 39", *Clematis flammula*.

The two axes accounted for 79.5 of the total variation (axis 1 = 65.3%, axis 2 = 14.2%). The four clusters occupy different parts of the ordination space without overlapping each other. Axis 1 separates the clusters He and Sa among them and from Fe and As. This axis is positively correlated with Df, Ds, Gr and temperature whereas it is negatively correlated with slope, stoniness and reaction. The correlation strengths of the EIVs of moisture and nutrient content with the ordination axes were lower than the selected r^2 cut-off and we will not comment on those variables further. Therefore, the Sa cluster groups relevés taken on steeper slopes than the other clusters and with high percentages of stones. The Fe and As clusters have the highest values of disturbance, in terms of both severity and frequency. The lowest reaction values for these two clusters are probably related to disturbance. Axis 2 clearly separates Fe from As, the former with greater values of grazing, the second with greater values of temperature, being in the southern part of the island at the lowest altitude and mostly on the south-facing side.

The NMS ordination of the relevés of the As cluster together with those of the associations *Bromo erecti-Chrysopogonetum grylli* and *Narcisso tazettae-Asphodeletum microcarpi* resulted in a three-axis solution with a final stress of 7.5 (Fig. 5).

Most of the variance is represented by axis 1 (55.1 %) whereas axes 2 and 3 account for 22.6 % and 2.5 %, respectively. The relevés of the two associtions *Bromo erecti-Chrysopogonetum grylli* (BC1 and BC2 in Fig. 5) and *Narcisso tazettae-Asphodeletum microcarpi* (NA1 and NA2) are separated on axis 1, whereas the relevés of the cluster As are in the middle. The latter are clearly separated from the others on axis 3 (Fig. 5). Interestingly, the relevés of the two phytosociological tables of the *Bromo erecti-Chrysopogonetum grylli* (i.e. BC1 and BC2) and those of the two tables of the *Narcisso tazettae-Asphodeletum microcarpi* (i.e. NA1 and NA2) form four well separated groups of relevés.

The cluster As was found to be well distinguished in the species-space from the other associations with which it has no overlap. The indicator species analysis indicated the following 11 taxa as diagnostic for the As cluster (indicator values in brackets): *Hedypnois rhagadioloides* (74), *Linum trigynum* (72.3), *Galium murale* (70.6), *Euphorbia cyparissias* (68.9), *Geranium purpureum* (67.2), *Sideritis romana* (62.4), *Vulpia ciliata* (60.8), *Centaurium maritimum* (56.3), *Plantago bellardii* (56.3), *Scorpiurus muricatus* (56.3), and *Lotus hispidus* (42.3).

Discussion

A direct quantitative comparison of Horvatić's data with the new data is not completely possible because the plot sizes used in the two surveys are too different (see also Dengler et al. 2009). For example, the five plots used by Horvatić (1927) to represent the *Festucetum valesiacae* sample a total area of 5 m^2 and include 17 taxa. The new dataset comprises six relevés of the same area – each with a plot size of 25 m^2 and containing an average of 33 taxa – for a total number of taxa of 53. Furthermore, the quantitative values of the taxa in Horvatić's article are estimated on a scale comprising at least 10 (undefined) values (-, -1, 1, 1-2, 2, 2-3, 3, 3-4, 4, 5), which are not directly comparable with the 9-points scale used in this paper. However, on the basis of qualitative considerations, it can be deduced whether the plant communities remained fairly similar or not during the 100 years.

As written above, although Horvatić (1927) described five grassland syntaxa on the island of Plavnik, we were on-

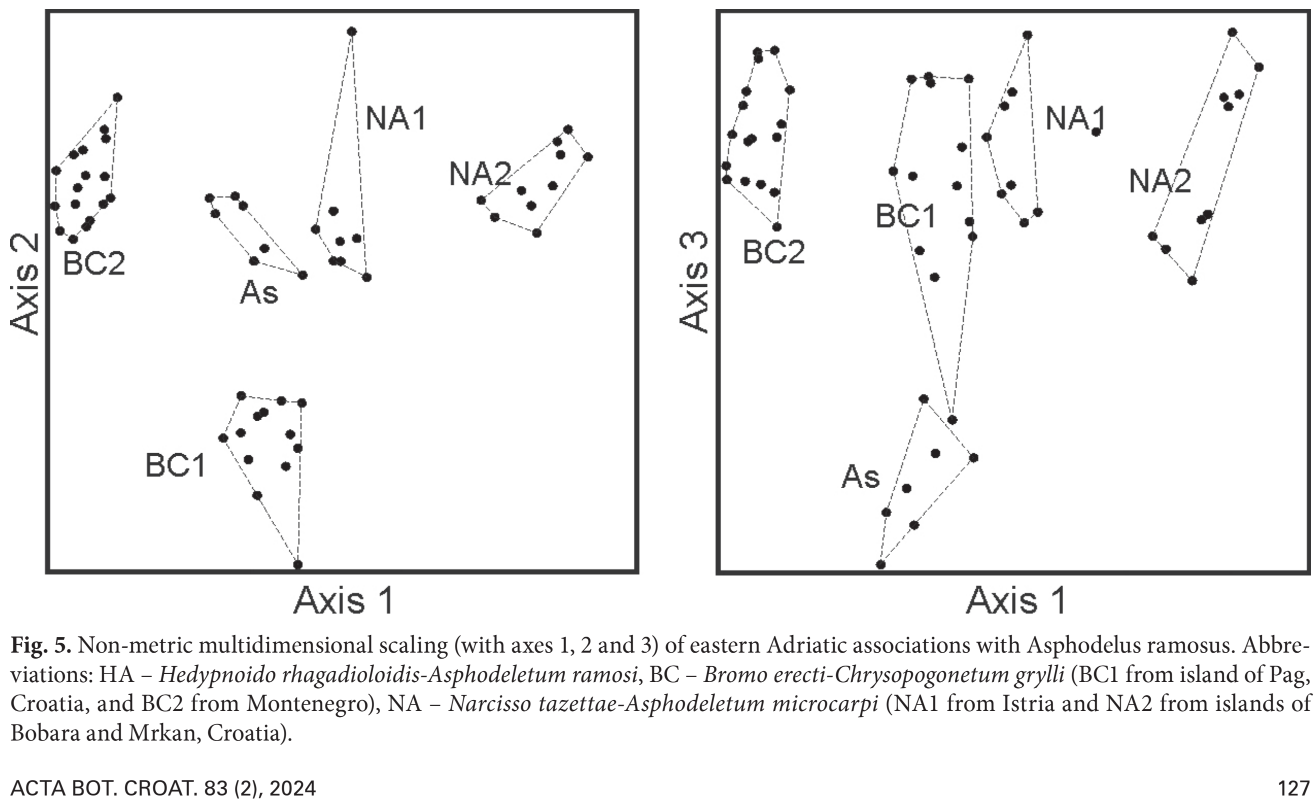

Fig. 5. Non-metric multidimensional scaling (with axes 1, 2 and 3) of eastern Adriatic associations with Asphodelus ramosus. Abbreviations: HA – *Hedypnoido rhagadioloidis-Asphodeletum ramosi*, BC – *Bromo erecti-Chrysopogonetum grylli* (BC1 from island of Pag, Croatia, and BC2 from Montenegro), NA – *Narcisso tazettae-Asphodeletum microcarpi* (NA1 from Istria and NA2 from islands of Bobara and Mrkan, Croatia).

ly able to find four grassland community types. In fact, the area originally covered by *Andropogonetum grylli* is completely occupied by a plant community dominated by *Asphodelus ramosus*, whereas *Chrysopogon gryllus* – i.e. the dominant taxon of the *Andropogonetum grylli* – is quite rare on the island.

Festuco valesiacae-Poetum bulbosae

The *Festucetum valesiacae* described by Horvatić (1927) for the continuous herbaceous expanses on the summit of the island is no longer present. This association was dominated by *Festuca valesiaca*, followed as cover by *Bromopsis erecta* and *Plantago holosteum*, and included several other species of the class *Festuco-Brometea* class (e.g. *Thymus longicaulis*, *Chrysopogon gryllus*, *Koeleria splendens*), to which it clearly belonged. In that area, the physiognomic situation appears quite different nowadays, with herbaceous phytocoenoses interspersed with shrubs and trees of *Juniperus oxycedrus*, generally on flat surfaces, where sheep and fallow deer find shelter (Fig. 2D). The herbaceous vegetation is dominated by *Poa bulbosa* and *F. valesiaca*, followed by some *Trifolium* species (*T. campestre*, *T. suffocatum*, *T. subterraneum*, *T. scabrum*), and *Koeleria splendens*. The results show that this plant community is clearly distinct from the others in terms of floristic composition and ecology. Indeed, it is the most disturbed in terms of intensity and frequency of disturbance (Ds and Df in Fig. 3) together with *Asphodeletum ramosi* but, unlike the latter, it has a higher grazing pressure (Gr). Therefore, the plant community dominated by *Poa bulbosa* and *Festuca valesiaca* (Fe in Fig. 3) is here considered to be a new association, the *Festuco valesiacae-Poetum bulbosae ass. nov. hoc loco* (holotypus rel. 1 in Tab. 1), possibly derived from *Festucetum valesiacae* – of which *Festuca valesiaca*, *Koeleria splendens*, and other taxa are still present – as a consequence of the prolonged stay of sheep and deer and intensive grazing.

The presence with high cover and/or frequency of *Poa bulbosa*, *Trifolium suffocatum*, *T. subterraneum*, *Erodium cicutarium*, *Plantago lanceolata*, and other species, indicates that the *Festuco valesiacae-Poetum bulbosae* belongs to the *Poetea bulbosae*, i.e. a class representing heavily grazed pastures, which are mainly present in the western Mediterranean basin (e.g. Rivas Goday and Rivas-Martínez 1963). This class comprises a single order, *Poetalia bulbosae*, and several alliances, none of which occur in Croatia (Preislerová et al. 2022), with the exception of the *Romuleion* (Škvorc et al. 2017). This alliance was originally invalidly described by Oberdorfer (1954, see Terzi et al. 2024b) for the south and southeast of the Balkan Peninsula, and was identified by 20 character species, none of which are present in our relevés (at least in the phenological stage of vegetation at the time of sampling). The alliance was originally classified in the class "*Thero-Brachypodietea*" (Oberdorfer 1954), and then moved to the "*Brachypodio-Chrysopogonetea*" (Horvat et al. 1974), "*Saginetea maritimae*" (Rodwell et al. 2002), and *Tuberarietea guttatae* (Čarni et al. 2014). The EVC has instead classified the *Romuleion* in the *Poetea bulbosae* (EVC) to represent "Macedonian seasonal perennial pastures on acidic substrates". The *Poetea bulbosae*, indeed, are most frequently found on acid substrates (Oberdorfer 1954, Čarni et al. 2014).

The numerous taxa of the *Stipo-Trachynietea distachyae* found in the *Festuco valesiacae-Poetum bulbosae* are linked to the basic substratum of the island of Plavnik, while those of the *Tuberarietea guttatae* highlight the dynamic relationships with the oligotrophic grasslands of this class, where the *Romuleion* was framed by some authors (e.g. Čarni et al. 2014). Two associations of the *Poetalia bulbosae* were recently described near Podgorica, Montenegro, on fluvio-glacial deposits and calcareous rocky substrate and framed in the *Romuleion* (Stanišić-Vujačić et al. 2023). The floristic composition of those associations, however, includes only three character species of this alliance (*Romulea linaresii* subsp. *graeca*, and the very rare *Hypochaeris cretensis* and *Hedypnois rhagadioloides*). The associations from Montenegro were found in an area where rocky grasslands of the alliance *Chrysopogono grylli-Koelerion splendentis* are widespread and indeed at least one of those associations (*Romuleo bulbocodii-Poetum bulbosae* Stanišić-Vujačić et al. 2023) is characterized by the presence of numerous *Festuco-Brometea* species (Stanišić-Vujačić et al. 2022, 2023). The *Festuco valesiacae-Poetum bulbosae*, derived from the *Festucetum valesiacae*, also has dynamic relationships with that alliance and in fact shares several species (e.g., *Koeleria splendens*, *Thymus longicaulis*) with the Montenegrin associations. However, the natural potential vegetation of the Plavnik area is different from that of the surrounding of Podgorica, in Montenegro.

The floristic relationships of the *Festuco valesiacae-Poetum bulbosae* with the *Romuleion* are, however, even weaker, given that none of the character species of this alliance were detected in our relevés. For this reason, the *Festuco valesiacae-Poetum bulbosae* is only provisionally classified in the *Romuleion*, pending the expansion of knowledge about the variability of the *Poetalia bulbosae* in the Balkans by new phytosociological studies. It is indeed plausible to admit that a second alliance of the *Poetalia bulbosae*, in addition to the *Romuleion*, is present in the Western Balkans and that it is dynamically linked to a degradation of the grasslands of the *Scorzoneretalia villosae* (not present in Greece, where the *Romuleion* was originally described), but a large-scale comparison is needed to confirm this hypothesis, which is beyond the scope of this paper. In any case, the *Festuco valesiacae-Poetum bulbosae* documents for the first time, based on phytosociological data, the presence of *Poetalia bulbosae* and *Poetea bulbosae* in Croatia.

Helichrysetum italici

The most frequent vegetation type on the island is represented by the *Helichrysum italicum* dominated plant community ("He" in Figs. 3 and 4), which grows on rocky and stony substrates throughout the island. *Helichrysum italicum* is widespread in southern Europe with several subspecies and the nominal one, *Helichrysum italicum* subsp. *italicum*, which is the only one found on the island of Plavnik, has a range in Europe that includes southern France, Italy and the Balkans (Euro+Med 2023). This subspecies grows in different types of vegetation, such as thermophilic scree communities of the *Scrophulario-Helichrysetalia* (Brullo and Spampinato 1991), and coastal sub-aerohaline dwarf scrub vegetation of the *Helichrysetalia italici* (Foucault 2020). In the Balkans, it has been considered a character taxon of the "*Brachypodio-Chrysopogonetea*" (Horvatić 1963, Horvat et al 1974). *Helichrysum italicum* is a suffruticose chamaephyte and, where it is dominant, the vegetation physiognomy takes on the appearance of a chamaephytic garrigue (Horvatić 1927). In the Dinaric Arc mountain range, this type of vegetation is often rich in many species typical of grasslands, so that it is not always possible to distinguish chamaephytic garrigues from 'pure' grasslands from a syntaxonomic point of view. In fact, the alliance *Chrysopogono grylli-Koelerion splendentis*, of the order *Scorzoneretalia villosae*, includes vegetation types dominated by both hemicryptophytes and dwarf chamaephytes, such as *Helichrysum italicum* and *Salvia officinalis*.

The same situation can be observed on the island of Plavnik where the *Helichrysetum italici* includes *Helichrysum italicum*, as the dominant, together with many grassland taxa, such as *Bromopsis condensata*, *Trifolium campestre*, *Dactylis glomerata* subsp. *hispanica*, *Salvia pratensis*, *Euphorbia cyparissias*, *Koeleria splendens*, *Hippocrepis comosa*. This plant community grows on incoherent substrata with a floristic composition that resembles that sampled by Horvatić (1927) a hundred years ago, except for the absence of *Chrysopogon gryllus*, which has evidently become rather rare on the island (Fig. 2A). Although the total number of species found in the new relevés is far higher than that recorded by Horvatić (1927), this difference is almost certainly due to the different sizes of the plots used in the two sampling surveys. The species that shape the physiognomy of the *Helichrysetum italici* are *Helichrysum italicum*, *Bromopsis condensata*, *Koeleria splendens*, and *Trifolium campestre*. The association clearly belongs to the *Chrysopogono grylli-Koelerion splendentis* due to the presence of characteristic species of the class *Festuco-Brometea* (e.g. *Bromopsis condensata*, *Euphorbia cyparissias*, *Linum tenuifolium*, *Festuca valesiaca*, *Hippocrepis comosa*), of the order *Scorzoneretalia villosae* and of the alliance (e.g. *Thymus longicaulis*, *Centaurea tommasinii*, *Plantago holosteum*, *Bupleurum veronense*) (see Terzi 2015). Although *Helichrysum italicum* is quite common in other associations of this alliance, the specific floristic composition of the *Helichrysetum italici* is different from that of all the other associations already described. In any case, even if this association was considered as a syntaxonomic synonym of another association belonging to the alliance, the name *Helichrysetum italici* would retain priority over names described later.

The *Helichrysetum italici* occurs in its typical aspect (subass. *typicum*, represented by rels. 1-9, in Tab. 2) on most of the island, and only on some spots does it associate with *Salvia officinalis* to give rise to the subassociation described in the next section. The typical aspect corresponds to that described by Horvatić (1927) with 10 plots of 1 square meter each. The size of these individual plots thus appears to be much smaller than that normally used to sample the plant communities of the *Scorzoneretalia villosae*, which varies in 90% of cases between 10 and 150 square meters (Terzi 2015). Because of the small area of the plots sampled by Horvatić (1927), and perhaps also because of the omission of some species, each of those plots counts only a very limited number of taxa. Moreover, the quantitative values associated with these taxa are on a scale hardly comparable with those routinely used in modern phytosociological studies.

Although none of the 10 relevés originally published by Horvatić (1927), if taken alone, can be considered a representative relevé of the *Helichrysetum italici* for the reasons expressed above, the association is nevertheless validly described because at least the synoptic column of the association, i.e. the last column of table IV ("Skrižaljka IV", on p. 33, in Horvatić 1927), constitutes a sufficient original diagnosis according to Article 7 of the International Code of Phytosociological Nomenclature (ICPN, Theurillat et al. 2021).

Therefore, we do not designate a lectotype, which would be incomplete and unrepresentative of the typical aspect of the association. Thus, not having a suitable relevé of the original diagnosis to be selected as lectotype of the association (see also Def. VIII of the ICPN), we find it more correct and useful to designate the relevé 3 in Tab. 2 as neotypus hoc loco of the *Helichrysetum italici* and of its subassociation *typicum*.

Helichrysetum italici salvietosum officinalis

Along some steep, mainly north-facing slopes on incoherent substrates or on limestone outcrops, the typical aspect of the *Helichrysetum italici* gives way to another type of vegetation where *Salvia officinalis* becomes dominant or co-dominant with *Helichrysum italicum* (Fig. 2B). This aspect was erroneously described by Horvatić (1927) as "*Helichrysetum salviosum*" (Art. 3e of the ICPN, Theurillat et al. 2021), to indicate a subtype of the *Helichrysetum italici*. Its floristic composition indeed retains many species of the *Helichrysetum italici* with the addition of a few others, including the dominant *Salvia officinalis*, thus forming the subassociation *Helichrysetum italici salvietosum officinalis subass. nov. hoc loco* (holotypus rel. 13 of Tab. 2). This subassociation has fewer species than the typical one.

The grassland vegetation of the Western Balkans dominated by *Salvia officinalis* belongs to different vegetation types, mainly framed in the alliances *Chrysopogono grylli-Koelerion splendentis* and *Saturejion subspicatae* (Terzi et al. 2023). In our case, the *Helichrysetum italici salvietosum officinalis* definitely belongs to the first alliance and is perhaps transitional to the *Stipo-Salvietum officinalis*, whose nomenclatural type was recorded on the island of Unije, also located in Kvarner, about 50 km (as the crow flies) from Plavnik. In particular, two relevés could belong to this association (subcluster 'sal' in Fig. 3), which show the lowest number of taxa, the clear dominance of *Salvia officinalis*, and the presence of *Helichrysum italicum* with very low coverage values.

Hedypnoido rhagadioloidis-Asphodeletum ramosi

The vegetation with *Asphodelus ramosus* develops mainly in the southern part of the island, with large and surprising expanses absolutely dominated by this species, with a cover usually greater than 90% (Fig. 2C). Two relevés recorded in the vicinity of the *Helichrysetum italici* distribution area represent a transition towards this association and indeed there *Helichrysum italicum* is the second dominant species with a cover of 25-50%. A very different situation is described by Horvatić (1927) who reports local patches of *Asphodeletum microcarpi* interspersed in a matrix of other associations (*Andropogonetum grylli* and *Helichrysetum italici*) and sharing several species with them. He notes that this sharing of species between the *Asphodeletum microcarpi* and the other associations is even more evident during the winter, when *Asphodelus ramosus*, the main dominant species, survives belowground. In the plots sampled by Horvatić (1927), *Asphodelus ramosus* was recorded with a score of "2" (or "3" in only one case) together with other species with similar or greater cover, so that some of its plots look like local variants of the *Andropogonetum grylli*.

On the other hand, as written above, during our surveys on the island we found few plants of *Chrysopogon gryllus* for which it was not possible to identify the *Andropogonetum grylli* nor transitions between this association and the *Asphodeletum microcarpi*. In the most typical situation, *Asphodelus ramosus* is by far the dominant species, and with the exception of the cases representing a transition with the *Helichrysetum italici* (rels. 5 and 6 in Tab. 3), in all other relevés the second dominant species never scored higher than 2a (i.e. up to 12% cover).

This plant community comprises numerous annual taxa of the classes *Stipo-Trachynietea distachyae* and *Tuberarietea guttatae*. The presence of so many annual plants is not a novelty for this kind of vegetation and has been observed in many other areas of the Mediterranean (see Terzi 2023 and references therein). The plant community dominated by *Asphodelus ramosus* was found to be the most disturbed grassland type on the island, together with the *Festuco valesiacae-Poetum bulbosae*. The thriving of *Asphodelus ramosus* is usually associated with intense grazing pressure and frequent fires. These factors might also be the ecologi-

Tab. 3. *Hedypnoido rhagadioloidis-Asphodeletum ramosi ass. nov.* on the island of Plavnik. For abbreviations of Life-forms [LF], see caption of Tab. 1. Asterisk (*) indicates holotypus (rel. 2).

Rel. number	**LF**	**1**	**2***	**3**	**4**	**5**	**6**	**7**
Exposition (°)		90	0	0	40	0	0	0
Slope (%)		5	0	0	2	0	0	0
Rockiness (%)		3	10	0	0	20	20	10
Stoniness (%)		3	20	5	0	40	30	10
Vegetation cover (%)		90	100	100	95	98	85	90
Hedypnoido rhagadioloidis-Asphodeletum ramosi								
Asphodelus ramosus	G	9	9	9	9	9	7	9
Helichrysum italicum	Ch	2	3	2	3	7	7	5
Crepis zacintha	T	.	4	3	3	2	.	2
Hedypnois rhagadioloides	T	2	2	2	2	2	2	.
Centaurium maritimum	T	.	.	2	3	.	2	2
Stipo-Trachynietea distachyae								
Gastridium ventricosum	T	2	3	4	3	4	2	3
Trifolium campestre	T	2	2	3	3	3	2	3
Trifolium scabrum	T	3	3	2	2	2	.	3
Sherardia arvensis	T	2	2	2	2	2	2	2
Catapodium rigidum	T	3	2	2	.	2	3	2
Sideritis romana	T	2	.	2	.	2	5	2
Filago germanica	T	2	2	2	3	.	.	2
Galium murale	T	2	2	3	2	.	.	2
Trachynia distachya	T	.	2	.	.	3	3	3
Medicago minima	T	2	.	.	.	2	3	2
Euphorbia exigua	T	.	.	2	2	2	.	2
Trifolium subterraneum	T	2	.	2	2	.	.	.
Valantia muralis	T	.	2	.	.	.	3	.

Tab. 3. continued

Rel. number	LF	1	2*	3	4	5	6	7
Tuberarietea guttatae								
Linum trigynum	T	2	3	3	2	2	2	2
Galium divaricatum	T	2	2	3	3	2	.	2
Plantago bellardii	T	2	2	2	2	2	.	2
Vulpia ciliata	T	5	2	4	3	.	.	2
Scorpiurus muricatus	T	2	2	2	.	2	2	.
Aira elegantissima	T	.	3	3	3	.	.	.
Lotus hispidus	T	4	2	3	.	.	.	.
Festuco-Brometea								
Euphorbia cyparissias	H	5	4	4	2	2	.	3
Sanguisorba minor	H	2	2	2	2	2	2	2
Festuca valesiaca	H	3	2	2	.	2	2	2
Linum tenuifolium	Ch	2	2	.	2	2	2	2
Bromopsis condensata (+ *Br. erecta*)	H	.	.	.	2	2	4	2
Convolvulus cantabrica	H	.	2	2	.	2	2	.
Koeleria splendens	H	.	2	.	.	3	2	.
Salvia pratensis	H	.	2	.	3	.	2	.
Thymus longicaulis	Ch	.	.	.	.	2	2	2
Galium corrudifolium	H	.	.	.	.	2	4	.
Bupleurum veronense	T	.	.	.	.	.	2	.
Medicago prostrata	H	.	.	.	.	.	.	2
Chrysopogon gryllus	H	.	.	.	.	2	.	.
Melica ciliata	H	.	.	.	.	.	.	2
Other taxa								
Dactylis glomerata subsp. *hispanica*	H	4	.	2	2	2	3	4
Anagallis arvensis	T	2	3	2	2	2	2	2
Poa bulbosa	H	3	.	2	2	2	2	2
Geranium purpureum	T	.	2	2	2	2	2	2
Teucrium capitatum subsp. *capitatum*	Ch	2	2	.	.	2	2	2
Plantago lanceolata	H	.	.	.	2	.	2	.
Orobanche minor	T	.	.	.	.	.	2	2

Date, coordinates and sporadic species (with 2, except where indicated) of the relevés: Rel. 1: 25.V.2023, lat. 44° 58' 02", long. 14° 32' 41", *Daucus carota*; Rel. 2 (holotypus): 26.V.2023, lat. 44° 57' 26", long. 14° 33' 01", *Carlina corymbosa*; Rel. 3: 26.V.2023, lat. 44° 57' 28.5", long. 14° 32' 59"; Rel. 4: 26.V.23, lat. 44° 57' 36", long. 14° 32' 58", *Scorzoneroides autumnalis* (3); Rel. 5: 26.V.2023, lat. 44° 57' 31", long. 14° 32' 51"; Rel. 6: 25.V.2023, lat. 44° 57' 51", long. 14° 32' 52", *Euphorbia peplus*, *Leontodon tuberosus*, *Ononis reclinata*, *Anisantha madritensis*, *Asterolinon linum-stellatum*, *Sedum acre*; Rel. 7: 26.V.2023; lat. 44° 57' 33", long. 14° 32' 42", *Urospermum dalechampii*.

cal drivers on the island of Plavnik, where we hypothesise that at least one of them (grazing or fires) has presumably occurred on the island with greater intensity and/or frequency than was the case 100 years ago. Increased grazing pressure is plausible due to the high number of sheep easily observed on that side of the island, while it is more difficult to assess the role of fires as data on their intensity and frequency are not available.

Thus, the present plant community dominated by *Asphodelus ramosus* on Plavnik Island differs both floristically and ecologically from the *Asphodeletum microcarpi* described by Horvatić (1927). It is also clearly different from the *Bromo erecti-Chrysopogonetum grylli* (i.e. *Asphodelo-Chrysopogonetum grylli* Horvatić 1963, cf. Terzi 2015) and *Narcisso tazettae-Asphodeletum microcarpi*. In this case, the Plavnik plant community differs in physiognomy, given the clear dominance of *Asphodelus ramosus*, and floristic composition (Fig. 5). The indicator species that distinguish the plant community of Plavnik Island from those of other locations in the eastern Adriatic are mainly annual species, some of which indicate a slightly acidic to almost neutral substrate (e.g. *Centaurium maritimum*, *Linum trigynum*), as is to be expected in heavily grazed karst areas.

In view of the above, the plant community dominated by *Asphodelus ramosus* on the island of Plavnik is considered here a new association: *Hedypnoido rhagadioloidis-Asphodeletum ramosi ass. nov. hoc loco* (holotypus rel. 2 of Tab. 3). However, its classification at a higher syntaxonomic rank is rather difficult (see Terzi 2023 and references therein). Biondi et al. (2016) proposed a new class, order and alliance to classify the vegetation dominated by *Asphodelus ramosus* (and other geophytes) but they have not been accepted because a comprehensive syntaxonomic review on the subject is still lacking (Biurrun and Willner 2020, Terzi 2023). In Montenegro, *Asphodelus ramosus* is included within communities of the *Artemisietea vulgaris*, although it is most abundant in the *Bromo erecti-Chrysopogonetum grylli* (Stanišić-Vujačić et al. 2022). As a result of this as yet undefined syntaxonomic framework, we refrain from classifying the new association into one of the accepted classes.

With the exception of *Festucetum valesiacae*, *Andropogonetum grylli* and *Asphodeletum microcarpi*, which can be considered extinct, the other plant communities on Plavnik Island can be classified as in the following syntaxonomic scheme:

Festuco-Brometea Br.-Bl. et Tx. ex Soó 1947
- *Scorzoneretalia villosae* Kovačević 1959
  - *Chrysopogono grylli-Koelerion splendentis* Horvatić 1973
    - *Helichrysetum italici* Horvatić 1927 *typicum*
    - *Helichrysetum italici salvietosum officinalis subass. nov. hoc loco*

Poetea bulbosae Rivas Goday et Rivas-Mart. in Rivas-Mart. 1978
- *Poetalia bulbosae* Rivas Goday et Rivas-Mart. in Rivas Goday et Ladero 1970
  - *Romuleion* Oberd. 1954 *nom. inval.* (?)
    - *Festuco valesiacae-Poetum bulbosae ass. nov. hoc loco*

Class: ?
- *Hedypnoido rhagadioloidis-Asphodeletum ramosi ass. nov. hoc loco*

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Author contribution statement

MT and NJ conceived the study, sampled the vegetation, made the statistical analysis, and share first authorship.

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Effects of hormopriming and pretreatment with gibberellic acid on fenugreek (*Trigonella foenum-graecum* L.) seed germination

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Abstract – Various approaches are used to improve crop production. Seed priming is one of the simplest and least expensive methods currently used to ensure rapid and uniform yields. Our study highlights the role of priming and imbibition in improving seed germination. The objective of this study was to investigate the effect of seed imbibition and hormopriming with 0.1 mM gibberellic acid (GA_3) on germination performance and biochemical changes in fenugreek (*Trigonella foenum-graecum* L.) radicles. The results showed that hydropriming and imbibition with GA_3 significantly improved germination performance and radicle growth. Concurrently, treatments induced stimulation of the antioxidant activities of superoxide dismutase, ascorbic peroxidase, catalase and guaiacol peroxidase, and decreased lipid peroxidation, stimulated an increase in total non-enzymatic antioxidant capacity and reduced glutathione content. Accumulation of hydrogen peroxide and cytochemical analysis of reactive oxygen species (ROS) *in situ* confirmed the role of imbibition in stimulating ROS. Interestingly the effects of imbibition with gibberellic acid were more effective then hormopriming, probably due to the partial degradation of GA_3 during dehydration process.

Keywords: hormopriming, germination, gibberellic acid, radicle, reactive oxygen species, *Trigonella foenum-graecum*

Introduction

Plant production and productivity are strongly determined by seed germination, which is a critical step in the life cycle of higher plants (Cheng and Bradford 1999). Seed germination may be asynchronous and consequently plant performance may be far from satisfactory. There are currently several approaches to overcoming this problem, including priming. Seed priming is a pregermination technique that ensures rapid, uniform and synchronized germination and improves seedling vigour and growth under normal and adverse environmental conditions (Varier et al. 2010). The priming process involves the imbibition of seeds to allow pregermination metabolic activation, followed by dehydration prior to the reversible phase (avoiding radicle breakthrough) to avoid radicle emergence (Finch-Savage and Leubner- Metzger 2006). This technique is economical and environment-friendly. There are different priming methods for improving seed germination performance, such as hydropriming (imbibing seeds in water), osmopriming (imbibing in an osmotic solution), halopriming (imbibing in a saline solution), chemopriming (imbibing in chemical solutions), and hormopriming (imbibing with phytohormones). Hormopriming, the technique used in this study, is based on treatment with plant hormones such as auxins, abscisic acid, cytokinins, and gibberellins. This type of treatment results in a higher germination performance and the resulting plants can be more tolerant to abiotic stresses including water stress (Singh and Maheswari 2017). Several authors have shown that the positive effects of priming are associated with various physiological, biochemical, cellular, molecular and genetic changes such as mobilization of reserves, degradation of albumen, stimulation of osmolyte synthesis and activation of the cell cycle and some abiotic stress tolerance genes. The activation of antioxidant enzyme systems has been extensively studied in primed seeds (Varier et al. 2010, Boucelha et al. 2019a). The signalling roles of reactive oxygen species (ROS) in seed ger-

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mination and dormancy have been well documented and it is assumed that ROS accumulation is beneficial for seed germination and seedling growth (see the review by Bailly et al. 2008 and Bailly, 2023). In a more precise way, Bailly et al. (2008) proposed the concept of the "oxidative window for germination", which restricts the occurrence of the onset of germination to a critical range of ROS level. Thus, ROS homeostasis regulates the beginning of germination, which is why activity of ROS-scavenging systems plays an important role. Furthermore, several authors also studied the involvement of ROS in the priming phenomenon (Boucelha et al. 2019a, Ren et al. 2023).

The role of ROS in seed dormancy control is due to their interaction with plant hormones that have central functions in seed dormancy and germination such as gibberellic acid (GA) (see review by Bailly et al. 2008). At low levels applied to the seed, GA stimulates germination by breaking dormancy through the activation of hydrolytic enzymes such as amylases, which promote the breakdown of reserves that provide energy for germination, resulting in rapid cell division and radicle elongation (Gubler et al. 1995). ROS have been reported to stimulate GA biosynthesis through a transcriptional regulation (Li et al. 2018) and GA treatment has been shown to induce ROS production (Cembrowska-Lech et al. 2015) by modifying the redox status of aleurone proteins, a process which might be related to ROS accumulation (Maya-Ampudia and Bernal-Lugo 2006).

Few studies have focused on the redox status of root radicles following treatment with GA, particularly applied as hormopriming (Ellouzi et al. 2023). Thus, the objective of this work was to study the effect of GA applied in two different ways, by seed imbibition in GA followed by rinsing (pretreatment with GA) and by hormopriming (GA treatment followed by redehydration) on the redox status of *Trigonella foenum-graecum* L. radicles by investigating enzymatic and non-enzymatic antioxidant systems. Fenugreek (*Trigonella foenum-graecum*) is an annual herb that belongs to the family Leguminosae cultivated worldwide, especially in southwest Asia, Middle East and Mediterranean. Due to its strong flavour and aroma fenugreek in one of those plants whose leaves and seeds are widely consumed as a spice in food preparations, and as an ingredient in traditional medicine. Its seeds are used for their carminative, tonic and aphrodisiac effects (Chopra et al. 1986) and it is assumed to have antidiabetic effect, hypocholesterolemic influence, antioxidant potency, digestive stimulant action, and hepatoprotective effect (Srinivasan 2006).

Materials and methods

Plant material

Our study was carried out on fenugreek seeds (*Trigonella foenum-graecum* L.), variety Halba from southern Algeria. Homogeneous seeds were selected from the same lot and rinsed with 5% hypochlorite solution for disinfection.

Treatments, seed germination and measurement of seedlings radicle growth

For pretreatment seeds were treated by imbibition in distilled water or 0.1 mM GA_3 for 8 hours and then rinsed before germination. Hormopriming and hydropriming were performed by seed soaking in 0.1 mM GA_3 or in distilled water, respectively, for 8 h and then rinsed and dehydrated under ventilation for 48 h before germination. Control seeds were not treated in any way before germination. Treated and control seeds were germinated in Petri dishes (9 cm in diameter) on four layers of absorbent paper soaked in distilled water. Three Petri dishes, with 50 seeds each, were used per treatment. For the first 24 h the germination experiment was performed in an oven at 25 °C, after which the Petri dishes were moved to a 14 h photoperiod of natural daylight and room temperature (24 ± 1 °C) for the next two days. After three days radicles were sampled for ROS detection, biochemical analyses and measurement of activity of antioxidant enzymes.

The length of radicle obtained from treated and untreated seeds was measured by graph paper each day for three days and expressed in cm. The fresh weight of treated and untreated radicles was measured after the 3rd day and expressed in grams.

Cytochemical detection of ROS

Hydrogen peroxide (H_2O_2) *in situ* was detected by the method of Thordal-Christensen et al. (1997), using a cytochemical method with 3,3'-diaminobenzidine (DAB). A brown colour is visible at the site of reaction of DAB with hydrogen peroxide, due to polymerisation of the DAB molecule.

Superoxide anions ($O2^{\cdot -}$) *in situ* were detected by a cytochemical method using nitro blue tetrazolium (NBT) (Rao and Davis 1999). The superoxide radicals present in tissue reduce the NBT to a stable formazan of blue-indigo colour (Beyer and Fridovich 1987).

Biochemical analyses

Hydrogen peroxide (H_2O_2) was measured according to the method described by Alexieva et al. (2001) after reaction with 1 M potassium iodide (KI) in 100 mM potassium phosphate buffer (pH 7.0). The reaction was developed for 1 h in darkness (yellowish colour) and absorbance measured at 390 nm. The amount of hydrogen peroxide per fresh weight (FW) was calculated using a standard curve prepared with known concentrations of H_2O_2 (0 to 10 nM) and expressed as nmol g^{-1} FW.

Lipid peroxidation was measured by quantification of the coloured malondialdehyde complex (MDA-TBA) detected at a wavelength of 532 nm, as described by Popham and Novacky (1991). The MDA content was expressed as µmol g^{-1} FW. This was calculated using the molar extinction coefficient of MDA ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$).

Reduced glutathione (GSH) was measured according to Moron et al. (1979) by using 5,5'- dithiobis-2-nitrobenzoic acid (DTNB) (Ellman's reaction) to give a yellow-coloured product that absorbs at 412 nm. The measurement was calculated from the molar extinction coefficient (ε = 13.3 mM^{-1} cm^{-1}) and expressed as μmol g^{-1} FW.

The total non-enzymatic antioxidant capacity (TAC) was estimated by the phosphomolybdenum method (Prieto et al. 1999). TAC is expressed in mg equivalents of ascorbic acid per g of dry weight. A standard curve was constructed using 0-300 μg mL^{-1} ascorbic acid.

Activities of antioxidant enzymes

For extraction of catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX), 100 mg of radicles were cold ground in extraction buffer (0.1 M Tris-HCl, pH 8.1). The guaiacol peroxidase (GPOX) was extracted from 100 mg of radicles ground in 0.1 M potassium phosphate buffer (KH_2PO_4 / K_2HPO_4, pH 6.5) under cold conditions. The enzyme activity was measured in protein extracts by spectrophotometry. Total soluble proteins were determined by the Bradford method (Bradford 1976).

CAT activity was determined following the decomposition of H_2O_2 at 240 nm using the method described by Anderson et al. (1995). The activity was expressed as μmol of H_2O_2 degraded per minute per mg of protein. This activity was calculated using the molar extinction coefficient of H_2O_2 (ε = 36 mM^{-1} cm^{-1}).

GPOX activity was determined according to the method of MacAdam et al. (1992), slightly modified by Boucelha et al. (2019b). The activity is measured by a colorimetric technique based on the increase in absorbance at 470 nm due to the polymerisation of guaiacol to tetraguaiacol (oxidation), which gives an orange colour in the presence of hydrogen peroxide. The activity was expressed in μmol of oxidised guaiacol per minute per mg of protein, using the molar extinction coefficient of tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}).

APX activity was measured according to the method of Nakano and Asada (1981) by following the oxidation of ascorbate by hydrogen peroxide, which absorbs at a wavelength of 290 nm. The enzymatic activity is expressed as μmol of ascorbate oxidised per minute per mg of protein. This activity was calculated using the extinction coefficient of ascorbate (ε = 2.8 mM^{-1} cm^{-1}).

SOD activity was measured by method reported by Marklund and Marklund (1974) and slightly modified by Boucelha et al. (2019a). It is based on the competition between the oxidation reaction of pyrogallol by superoxide ions and the dismutation by SOD. The increase in absorbance at 420 nm was due to the auto-oxidation of pyrogallol. An enzymatic unit was defined as the quantity of enzyme capable of inhibiting 50% of the autooxidation of pyrogallol under the conditions of the assay. The activity of SOD was expressed in units per minute and per mg of protein.

Decomposition kinetic of GA_3

To study the stability of GA_3 in water used for imbibition, we studied the spontaneous decomposition kinetic of 0.1 mM GA_3 by spectrophotometry at 254 mM, measuring the appearance of gibberellenic acid, a degradation product, according to the method of Pérez et al. (1996).

Statistical analysis

The ANOVA test was used to compare the results. The statistical significance between the results was assessed according to the Tukey post-hoc test and values were considered statistically significant at $P < 0.05$. The analysis of data was performed through STATISTICA 6.0 software (Stat Soft, Inc.). All values were expressed as mean and standard deviation.

Results

Radicles growth

Fenugreek seed pregermination treatments significantly improved radicle length, following the same, almost linear, kinetics for all treatment types (Fig. 1A). However, this improvement was closely related to the type of treatment applied. In fact, we found that GA imbibition (without redehydration) showed the best growth, with an increase of about 66% on day 3 compared to the control batch. This was

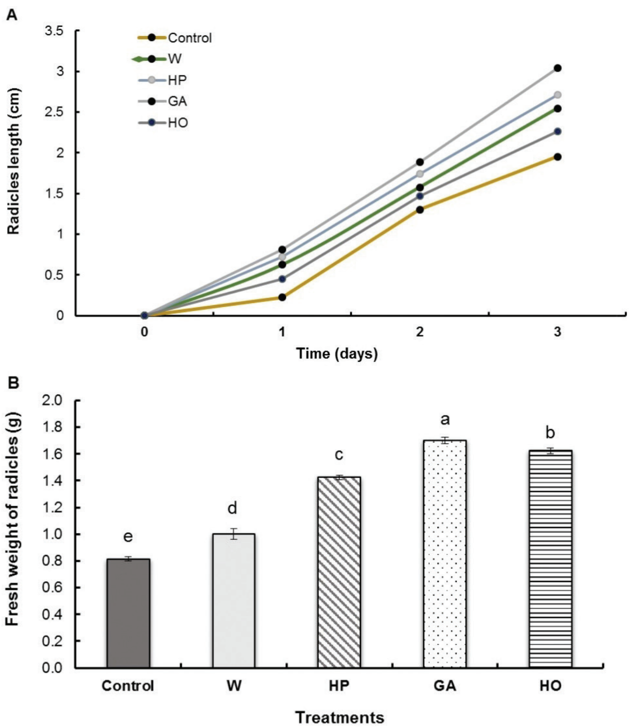

Fig. 1. Radicle length (A) and fresh weight (B) of fenugreek seedlings after different treatments of seeds. W – imbibition with water, HP – hydropriming, GA – imbibition with 0.1 mM gibberellic acid, HO – hormopriming with 0.1 mM gibberellic acid. Results are expressed as means (N = 3) and error bars represent standard errors. Different alphabetical letters indicate a significant difference (P < 0 .05) between means.

Fig. 2. Detection of reactive oxygen species in fenugreek radicles after different treatments of seeds: hydrogen peroxide (H_2O_2) after 24 h of germination (A) and superoxide anion ($O_2^{\cdot-}$) after 48 h of germination (B). W – imbibition with water, HP – hydropriming, GA – imbibition with 0.1 mM gibberellic acid, HO – hormopriming with 0.1 mM gibberellic acid.

followed by the hydropriming (HP) with an increase of 49%, then the imbibition with water (W) and hormopriming (HO) with an increase of 36% and 20%, respectively, compared to the control. All treatments induced an increase in the fresh weight of fenugreek radicles (Fig. 1B). The greatest increases are recorded for imbibition with GA (107%) and hormopriming (95%). For the hydropriming, the increase was 75% while for imbibitions, it was only 23%.

ROS production *in situ*

DAB assay revealed that the degree of H_2O_2 accumulation in tissue varied according to the type of treatment applied. Control seed radicles were characterized by the lowest production of H_2O_2 (Fig. 2A). A high accumulation of H_2O_2 was observed in the radicles of GA and hydropriming treated seeds, especially in the region of the root cap and elongation. In contrast, a low H_2O_2 accumulation was observed in the radicles resulting from hormone priming and water imbibition treatments.

Fenugreek radicles treated with NBT showed an accumulation of superoxide anions in the tissue (Fig. 2B). In the control radicles, the accumulation of superoxide anions was lower than in the radicles from treated seeds. Gibberellic acid imbibition showed the highest production of superoxide anions localized throughout the radicle. Hydropriming treatment also induced an accumulation of superoxide anions. However, in water imbibition and hormopriming treatments the presence of superoxide anions was only slightly visible.

Hydrogen peroxide and malondialdehyde content

Results indicate that the pregermination treatments of fenugreek seeds induced a significant increase in H_2O_2 concentration in the radicles except for water imbibition (Fig. 3A). Indeed, hydropriming and GA imbibition resulted in almost the same increase in H_2O_2 content (101% and 98%,

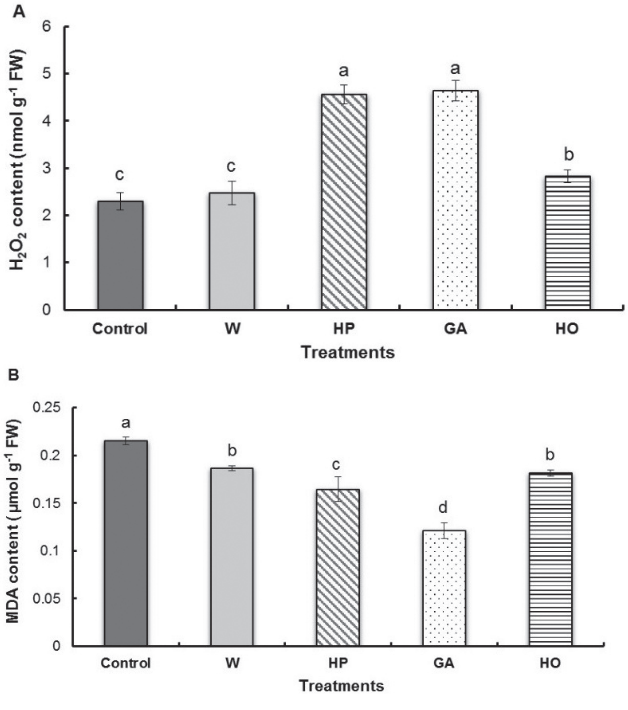

Fig. 3. Contents of hydrogen peroxide (H_2O_2) (A) and malondialdehyde (MDA) (B) in the radicles of fenugreek seedlings after different treatments of seeds. W – imbibition with water, HP – hydropriming, GA – imbibition with 0.1 mM gibberellic acid. Results are expressed as means (N = 3) and error bars represent standard errors. Different alphabetical letters indicate a significant difference (P < 0 .05) between means.

respectively), as the control. An increase of 23% was observed in the case of the hormopriming treatment compared to the control.

Pregermination treatments of fenugreek seeds showed a significantly higher MDA content of control radicles than the radicles of treated seeds (Fig. 3B). Seed imbition with gibberellic acid, favoured a decrease in MDA content for 44% compared to the control. This was followed by the hydropriming treatment (decrease of 24%), then the hormopriming treatment (decrease of 16 %) and finally the water imbibition treatment (decrease of 14%) compared to the control.

Activities of antioxidative enzymes

The pregermination treatment of fenugreek seeds induced a significant increase in antioxidant enzyme activities in some treatments (Fig. 4).

We observed significantly increased activity of catalase in seedlings from the gibberellic acid and hydropriming treated seeds, with increases of 88% and 33%, respectively, compared to the control. However, a less significant increase was observed in the water-imbibition (15%) and hormopriming treatment (12%) (Fig. 4A).

GPOX activity was significantly higher in fenugreek radicles pretreated with gibberellic acid, with an increase of 46% compared to the control. Hydropriming had a less prominent effect on the activation of this enzyme, with an increase of 16% over the control. In contrast, hormopriming and water-imbibition of the seeds had no effect on GPOX activity in the radicle (Fig. 4B).

The results of the APX activity showed that the pretreatments caused a very significant activation of this enzyme. Indeed, GA imbibition and hydropriming caused the significantly highest increase compared to the control (252% and 215% respectively), while in hormopriming and water-imbibition, the increase was of 188% and 148% respectively (Fig. 4C).

The antioxidant enzyme activity of SOD in the radicle revealed that GA imbibition and hormopriming induced respectively significant increases of 29% and 17% over the control. A less significant increase was observed in the activity of this enzyme for hormopriming while water-imbibition treatments did not show any significant change from the control (Fig. 4D).

Total antioxidant capacity and reduced glutathione content

All seed treatments significantly stimulated antioxidant capacity in the radicles of fenugreek seedlings; however, this increase was dependent on the type of treatment applied (Fig. 5A). Indeed, we observed that radicles soaked with gibberellic acid were characterized by the highest value in comparison to control (45%) followed by hydropriming (32%), hormopriming (22 %) and water-imbibition (10%) compared to the control.

Gibberellic acid and hydropriming treatments of fenugreek seeds caused a high increase in reduced glutathione content in the radicles (Fig. 5B) with percentages of 72% and 55% respectively compared to the control. However, we observed that hormopriming induced a weaker increase with 15% while water-imbibition treatment did not present any significant difference with the control.

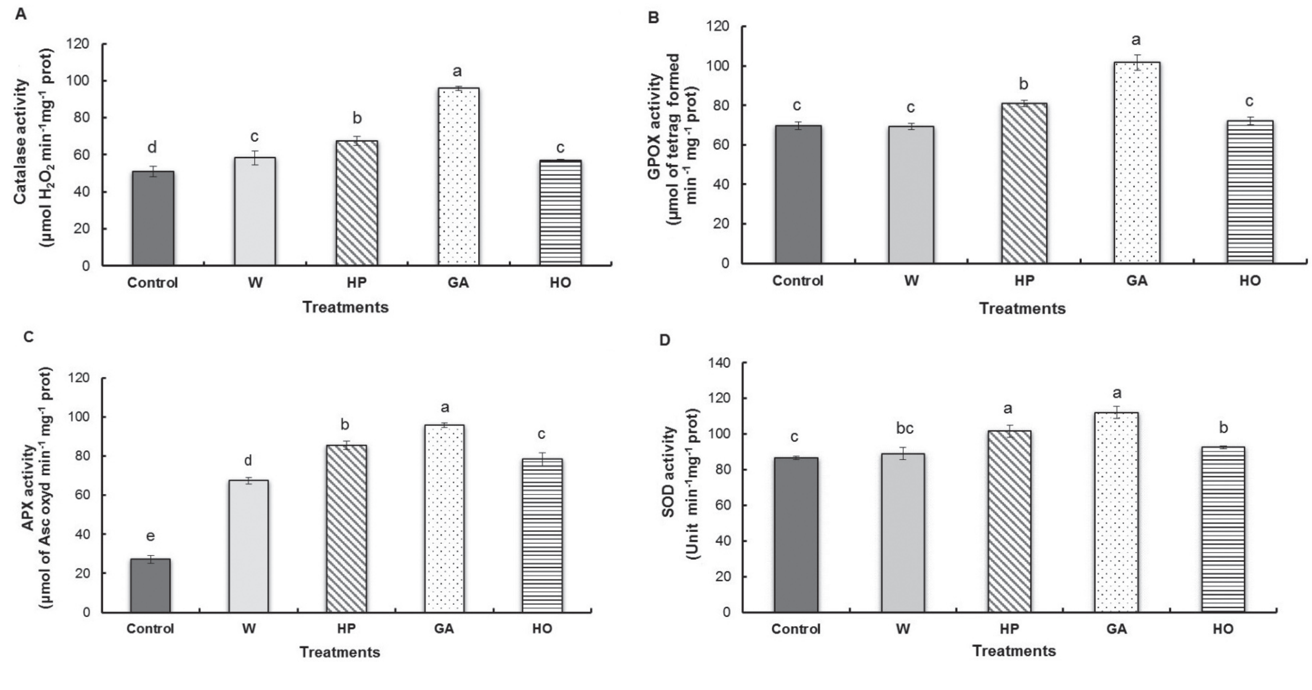

Fig. 4. Antioxidant activities of catalase (A), guaiacol peroxidase – GPOX (B), ascorbate peroxidase – APX (C) and superoxide dismutase – SOD (D) in fenugreek radicles after different treatments of seeds. W – imbibition with water, HP – hydropriming, GA – imbibition with 0.1 mM gibberellic acid, HO – hormopriming with 0.1 mM gibberellic acid. Results are expressed as means (N = 3) and error bars represent standard errors. Different alphabetical letters indicate a significant difference (P < 0 .05) between means.

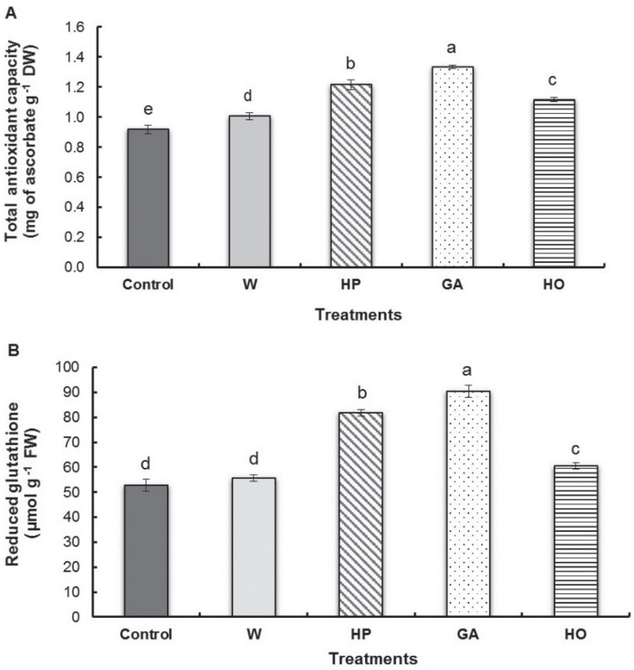

Fig. 5. Total antioxidant capacity (A) and reduced glutathione content (B) of fenugreek radicles after different treatments of seeds. W – imbibition with water, HP – hydropriming, GA – imbibition with 0.1 mM gibberellic acid, HO – hormopriming with 0.1 mM gibberellic acid. Results are expressed as means (N = 3) and error bars represent standard errors. Different alphabetical letters indicate a significant difference (P < 0 .05) between means.

Gibberellic degradation

In order to follow the stability of gibberellic acid in an aqueous medium, we followed its spontaneous degradation at 25 °C. From the curve obtained (Fig. 6), we were able to deduce that the gibberellic acid solution decomposes spontaneously into gibberellinic acid (inactive form).

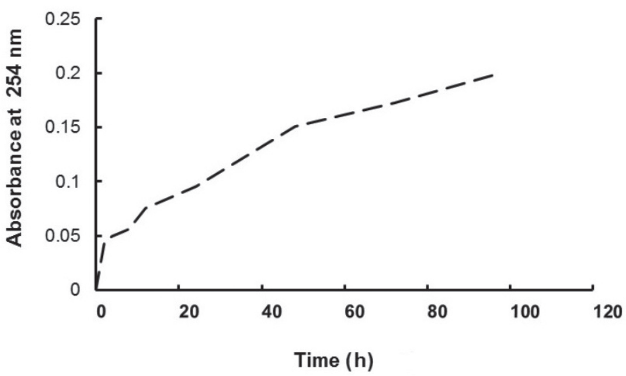

Fig. 6. Kinetics of gibberellic acid degradation at 25 °C represented by gibberellinic acid appearance.

Discussion

In this study, we found that pregermination treatments of fenugreek seeds, particularly imbibition with gibberellic acid, induced biochemical changes in the radicles derived from these fenugreek seeds and consequently better seed germination and seedling growth.

A significant improvement in germination performance was observed in *Trigonella foenum-graecum* L. seeds that had undergone pregermination treatments, particularly in the gibberellic acid and hydropriming treatments, followed by the hormopriming and water imbibition.

Hydropriming is known to improve seed germination performance and has been reported for rice (Hussain et al. 2015), sunflower (Hussain et al. 2006), wheat (Ahmadi et al. 2007), maize (Janmohammadi et al. 2008), bean (Ghassemi-Golezani et al. 2010), lentil (Saglam et al. 2010) and black-eyed bean (Boucelha et al. 2019a). These authors have shown that priming was an effective method for improving germination performance, resulting in uniform and homogeneous cultures. Several authors have suggested that this improvement is related to changes at the seed level, such as accelerated water uptake (Gelormini 1995), increased respiration intensity Corbineau et al. (2000), nucleic acid synthesis, strong degradation of reserves (Varier et al. 2010), activation of antioxidant enzyme activities (Amooaghaie and Vaviland 2011) and strong protein synthesis under genetic control (Varier et al. 2010). All these phenomena could be the consequence of a "memorization by the embryo" of events that occurred during the redehydration imposed during priming. It has been shown that a plant can store information when exposed to stress events and can use this memory to aid responses when these events reoccur (see review by Kinoshita and Seki, 2014). It has been demonstrated that epigenetic mechanisms are essential for stress memory and adaptation in plants (Chen and Arora 2013). Gibberellic acid is known to play a role in stimulating seed germination and is involved in many physiological and biochemical processes in plants (Mirheidari et al. 2022). Moreover, gibberellic acid is widely used in the laboratory and greenhouse to trigger the germination of some seeds that would otherwise remain dormant (Riley 1987). Thus, several works have reported the use of gibberellic acid as an exogenous treatment to enhance germination (Seandhalaksmi et al. 2022). Based on the work of Ogawa et al. (2003), exogenous GA enters the seed and is added to endogenous GA. Indeed, these authors showed that GA- deficient *Arabidopsis thaliana* seeds could not germinate without the addition of exogenous GA. This improvement has been linked to the synthesis and activation of amylases (Vieira et al. 2002) and lipases (Jridi et al. 2004), allowing accelerated germination with faster division cells (Li et al. 2018). Thus, seeds treated with gibberellic acid showed significant cell elongation compared to the other treatments. This cell expansion would also be due to the GA activation of aquaporins, membrane channels involved in water transport, which increases cell turgidity (Ogawa et al. 2003).

The treatments applied to fenugreek seeds stimulated the production of ROS in embryonic tissues, more precisely in the meristematic zone. This ROS formation, as evidenced by cytochemical tests and increased H_2O_2 content, was more pronounced in the gibberellic acid and hydropriming treatments. These effects are in line with the concept of the "oxidative window" proposed by Bailly et al. (2008) and confirmed in the case of hydropriming by Boucelha et al. (2019a). According to Bailly et al. (2008), for seed germination it is necessary that ROS content be within a range that allows signalling, while lower or higher amounts of ROS would lead to inability to germinate. Published data have shown that ROS are key players in several physiological processes in seeds such as seed dormancy control due to their interaction with plant hormones like gibberellic acid (Finkelstein et al. 2008) and the perception and transduction of environmental conditions during imbibition (Bailly 2019). In dry seeds, i.e at low moisture content, ROS accumulation would probably result mainly from non-enzymatic reactions. In this physiological state, glucose and amino groups derived from amino acid and nucleic acids are condensed to form Amadori and Maillard products, which are major sources for ROS production (Sun and Leopold 1995) and lipid peroxidation (McDonald 1999). However, during imbibition, the reactivation of metabolism causes an enhanced accumulation of ROS, generally resulting from electron leakage within the mitochondrial electron transport chain (Kranner et al. 2010).

Bailly et al. (2008) proposed a mechanism for the control of dormancy and germination through a dialogue between ROS and hormone. Indeed, according to these authors, there is an interaction between ROS and the gibberellic acid signalling pathway during germination. Under imbibition conditions, NADPH oxidase and β-oxidation increase ROS levels, which in turn repress the DELLA protein responsible for the negative regulation of GA synthesis. This induces the synthesis of gibberellic acid, which then triggers the activation of GA-inducible transcription factors GAMYB which in turn induces the transcription of α-amylase in the aleurone layers of many seeds (Gubler et al. 1995, Kaneko et al. 2002).

Membrane lipid peroxidation, which causes damage to cell membranes, is a good indicator of the presence of reactive forms of oxygen and thus allows assessment of cellular oxidative stress intensity. Oracz et al. (2007) supported the hypothesis of an inverse correlation between MDA content and seed dormancy since they observed that the MDA content increased during dormancy alleviation. Our results showed a more pronounced decrease in MDA content in the gibberellic acid and hydropriming treatments compared to the control as well as hormopriming and water imbibition where the decrease was less prominent. A reduction in lipid peroxide content in hydroprimed seeds has already been reported by El-Araby and Hegazi (2004) for tomato and Sharma et al. (2014) for okra seeds. This decrease was also observed in gibberellic acid-treated seeds, as shown in the work of Li et al. (2013) and Ahmad (2010). The reduction in MDA accumulation in primed seeds could be explained by improved membrane repair during the priming process and induction of antioxidant enzymes (Nawaz et al. 2013). For all treatments in this study, the decrease of MDA content correlated with the increase of the different antioxidative enzymes activities as well as increased level of glutathione, but did not correlate with the level of ROS. Therefore, the MDA content could be the result of the enhanced antioxidative defence which prevents oxidative damage despite higher level of ROS. Thus, cells maintain ROS homeostasis during germination (Li et al. 2013).

Fenugreek seed treatment stimulated the activation of antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase at the radicle level, with the most significant effect in the gibberellic acid pretreatment. These results are in agreement with those of different authors who showed that treatment of seeds with gibberellic acid leads to an increase in enzymatic antioxidant activity (Li et al. 2013). The increase in antioxidant enzymes activities was also observed in fenugreek radicles after hydropriming treatment although less prominently. Several studies showing that hydroprimed seeds of several crop species are characterised by very high antioxidant activities (Varier et al. 2010, Boucelha et al. 2019a, Melzi Ou Mezzi et al. 2021). Thus, several works have linked germination improvement to increased antioxidant enzymatic activities, which allow the elimination of free radicals, and, then, the restoration of the homeostasis of the redox status (Varier et al. 2010, Boucelha et al. 2019a, Boucelha et al. 2021).

Total antioxidant capacity corresponds to the presence of natural antioxidants capable of preventing oxidative damage (Priando et al. 1999). These non-enzymatic antioxidants include glutathione, ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids and phenolic compounds including flavonoids (Asada 2006). TAC levels reflect the reduced state of these molecules. Our results showed that radicles from seeds soaked in gibberellic acid had the highest total non-enzymatic antioxidant activity, which is in agreement with lower MDA content. Few studies have measured non-enzymatic antioxidant activities after pregermination treatments. Boucelha et al. (2019a) showed no significant changes in hydroprimed *Vigna unguiculta* seeds, while the results of Melzi Ou Mezzi et al. (2021) suggested that hydropriming stimulates total antioxidant activities in fenugreek radicles.

Imbibition in gibberellic solution (without redehydration) and hydromopriming induced separately increases in almost all parameters (radicles growth, H_2O_2 content, catalase, GPOX, APX and SOD activities, TAC, GSH content). However, hormopriming in which seeds were dehydrated after treatment with gibberellic acid resulted in values more like those obtained for seeds imbibed in water. This could mean that some of the exogenous gibberellic acid that entered the seed during imbibition may be degraded to gibberellenic acid during drying. Pérez et al. (1996) reported that gibberellic acid loses its biological activity in aqueous alkaline solutions over time by degradation to gibberellenic acid via an isomeric form *iso*-GA (degradation intermediate), both of which are inactive and do not induce amylase activity in barley endosperm. On the basis of these observations and our results, we suggest that drying the seeds for 48 h caused a partial degradation of GA to inactive forms, which was unable to exert a beneficial effect on the seeds.

Conclusion

Pretreatment of seeds with gibberellic acid and hydropriming resulted in improved germination performance and balanced redox status of fenugreek (*Trigonella foenum-graecum* L.) seeds. These physiological and biochemical changes at the radicle level would be the result of the activation of certain cell signalling pathways leading to a change in gene expressionthat needs to be further elucidated.

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Effects of acetic acid treatment on growth and pigment contents in barley

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Abstract – Acetic acid (AA) is an organic acid and has been widely used as food preservative and a dietary spice in vinegar form. In addition to its therapeutic uses in its vinegar form, AA attenuates inhibitory effects of stress in plants. However, in some plant species toxic effects of AA have been found. Therefore, in this study, 0, 2.5 and 5 mM concentrations of AA were applied to 2-day-old barley (*Hordeum vulgare* L. cv. Bornova-92) seedlings for 2 days in order to investigate the possible toxic effects of AA. After treatments, seedlings were grown in AA-free conditions for 2 days to recover. AA inhibited root and shoot growth; decreased water content, fresh weight, chlorophyll, pheophytin, and carotenoid contents. However, anthocyanin and flavonoid contents, as well as the levels of UV-absorbing compounds and UV-B marker increased in the leaves of AA-treated plants. AA increased hydrogen peroxide (H_2O_2) content in shoots and induced cell death in roots. Soluble carbohydrate content decreased in roots of AA-treated plants while insoluble carbohydrate content increased. Our results demonstrate that AA in young barley seedlings can exhibit its toxic effects through oxidative stress, which induced antioxidative response in the form of molecules with antioxidative activities. These effects persisted for 2 days after the removal of AA.

Keywords: acetic acid, anthocyanins, cell death, hydrogen peroxide, UV-absorbing compounds

Introduction

Soil organic acids are a water-soluble fraction of organic molecules in the rhizosphere. Organic acids (OAs) in soil may be produced by plants and microorganisms or are the result of organic matter decomposition (Adeleke et al. 2017). The concentration of monocarboxylic OAs in soil can reach up to 1 mM. Acetic acid (AA), a monocarboxylic OA, is the smallest OA and is less effective in mobilizing minerals than high molecular weight OAs. Half-life of AA can be a few days (Adeleke et al. 2017). AA, also known as ethanoic acid or vinegar, absorbs moisture; is corrosive to metals, and is widely used in industry and household cleaning. Once AA enters the cell, it dissociates into an acetate anion and a proton. Acetate is converted by acetyl-CoA synthetase (ACSS) to produce acetyl-coenzyme A (acetyl-CoA), an essential molecule produced in carbohydrate metabolism and histone acetylation (Pietrocola et al. 2015). Lynch (1977) reported that 0.1-1 mM AA treatment stimulates root growth in barley seedlings. Recent papers have suggested that AA can protect plants against biotic (Chen et al. 2019) and abiotic stress (Utsumi et al. 2019) through jasmonic acid (JA) and abscisic acid (ABA) synthesis. AA treatment induces *de novo* JA synthesis and enhanced survival during drought stress is linked to histone acetylation (Kim et al. 2017). AA-treated plants show increased leaf water content and pigment levels, as well as upregulation of stress-response and stress--tolerance genes (Chen et al. 2019, Utsumi et al. 2019). AA supplementation ameliorates the toxic effects of seawater in mung bean by increasing uptake of Ca^{2+} and Mg^{2+} and decreasing uptake of Na^{+}, enhancing antioxidant activity, water use efficiency and the contents of several metabolites *e.g.* soluble sugars, phenolics and flavonoids (Rahman et al. 2019). AA also enhances drought tolerance in maize and *Arabidopsis* through proline metabolism (Mahmud et al. 2023) and in soybean through improved antioxidant defence and photosynthesis and accumulation of soluble sugars and free amino acids (Rahman et al. 2021). However, besides these positive effects there are some reports about negative effects of AA on plants. In cassava plants, application of

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20–50 mM AA induced wilting (Utsumi et al. 2019) while in maize seedlings root and shoot growth was inhibited by AA at doses as low as 10 mM (Allen and Allen 2020). In reed (*Phragmites australis*) root growth was reduced at 0.3 mM, and entirely inhibited at 1.7 mM acetic acid (Armstrong et al. 1996). In *Chlamydomonas reinhardtii* AA can even cause cell death (Zuo et al. 2012).

In this study, the effects of AA treatment on early seedling growth were investigated in barley. For this purpose, root and shoot length, fresh (FW) and dry (DW) weight and water content (WC) of seedlings, as well as pigment and H_2O_2 content in leaves, and soluble and insoluble carbohydrate contents in roots were measured. Possible cytotoxic effects of AA treatment were investigated by measuring cell viability in roots by Evans Blue staining, which can penetrate into dead cells and therefore discriminates between viable and non-viable cells. Results of our study show that AA treatment inhibited growth and caused decreases in the levels of photosynthetic pigments *e.g.* chlorophyll, carotenoid, and pheophytin, while increasing the contents of anthocyanin, flavonoids, UV-absorbing compounds and UV-B marker. AA treatment caused the accumulation of H_2O_2 in leaves and decreased cell viability in roots. Insoluble carbohydrate content was enhanced by AA treatment, while soluble carbohydrate content decreased.

Materials and methods

Plant material, growth conditions and treatment

Barley (*H. vulgare* cv. Bornova-92) mature seeds were obtained from the Aegean Agricultural Research Institute (AARI, Izmir, Türkiye).

Ten seeds were placed between two filter papers in a 9-cm-diameter Petri dish containing 6 mL water for overnight imbibition at 6 °C and then germinated in the dark (16/8 h, 25/18 °C, 70 ± 5% humidity, darkness) for 3 days. Uniformly-germinated seedlings were subsequently placed on a filter paper containing 6 mL of the test solution containing AA for 2 days under a light intensity of 1400 μmol m^{-2} s^{-1} (16/8 h, 25/18 °C, 70 ± 5% humidity). At the end of the treatment, seedlings were rinsed, transferred to dishes containing 6 mL of water, and then further incubated for 48 h for recovery at the same growth conditions. Test solutions contained 0, 2.5 mM (0.0143% v/v) or 5 mM (0.0286% v/v) AA (glacial, Merck). Plant samples were harvested immediately after AA treatment (day 0) or 2 days after AA was removed (day 2). Day 2 group represents recovery plants.

Measurement of seedling growth and water content

Seedlings were briefly soaked on filter paper and weighed to determine FW. To determine DW, seedlings were dried at 65 °C until the weight became constant. FW and DW were expressed as milligrams (mg). Water content was calculated according to the formula WC = ((FW–DW) / FW) × 100 and expressed as a percentage. Root and shoot lengths were expressed as centimetres (cm).

Determination of pigment content

To estimate the content of chlorophyll, carotenoid and pheophytin, leaves were homogenized in 80% acetone, incubated at –20 °C and, centrifuged at 6000 × g for 5 minutes at 4 °C. Supernatants were used to determine chlorophyll *a* and *b*, carotenoid and pheophytin levels. The absorbances of the extracts at 470, 655, 663 and 666 nm were measured in a glass cuvette (104.002-OS, Hellma) using Nanodrop (2000C, Thermo Fisher) due to the large volume of the samples and expressed as mg g^{-1} FW (Lichtenthaler 1987, Costa et al. 2006).

To estimate the content of UV-B-absorbing compounds, leaves were homogenized in a methanol : HCl solution (99:1) and centrifuged at 10000 × g for 15 min at 4 °C. To determine the levels of UV-B-absorbing compounds and flavonoids, the absorbances (A) of the supernatants were measured at 300 and 350 nm in a quartz cuvette (104.002-QS, Hellma) using Nanodrop. To determine the levels of anthocyanins, the absorbances of the supernatants were measured at 535 and 657 nm. Anthocyanin contents were calculated according to the formula $((A_{535} – (0.25 × A_{657}))$ using a molar extinction coefficient of 38000 L mol^{-1} cm^{-1}. Flavonoid contents were calculated using a molar extinction coefficient of 20000 L mol^{-1} cm^{-1}. Anthocyanin and flavonoid contents were expressed as μmol g^{-1} FW and μmol mg^{-1} FW, respectively. Content of other UV-B absorbing compounds measured at A_{300} was expressed as A_{300} g^{-1} FW (Cicek et al. 2012).

Determination of hydrogen peroxide and UV-B marker content

Leaves were homogenized in 0.1% trichloroacetic acid and centrifuged at 10000 × g for 15 min and the supernatants were collected. The absorbances of the supernatants at 440 nm were measured to determine UV-B marker content which was expressed as A g^{-1} FW (Cicek et al. 2012).

For the determination of H_2O_2, supernatants were mixed with 0.1 M Tris-HCl and 1 M potassium iodide and incubated at room temperature (RT) for 90 min. The absorbances were read at 390 nm and the H_2O_2 amounts of the unknown samples were estimated according to the standard curve (0–330 nmol) of H_2O_2 (Merck) and expressed as nmol g^{-1} FW (Cicek et al. 2012).

Evans Blue staining

Cell viability in roots was measured according to Baker and Mock (1994). Root samples were immersed in 0.25% Evans Blue stain for 20 min at RT. Then, roots were rinsed with water for 30 min, and 10 root tips (1 cm long) were incubated in 1% SDS : 50% methanol at 50 °C for 1 h. The absorbances of the methanol : SDS solution containing stain released from cells were measured at 595 nm.

Determination of carbohydrate content

Carbohydrate extractions were performed according to Sonjaroon et al. (2018). Briefly, roots were homogenized in 80% ethanol, incubated at 75 °C for 15 min and centrifuged at 6000 × g for 5 min. After the collection of the supernatant, the extraction was repeated twice. The supernatants were combined and used to measure soluble sugar content. The pellet phase containing ethanol-insoluble material was used for starch analysis.

Starch extraction was performed according to McCready et al. (1950) and Sonjaroon et al. (2018). Briefly, the pellet was dried at RT to evaporate EtOH completely and then dissolved in 52% perchloric acid at 6 °C for 30 min. After centrifugation at 2000 × g for 5 minutes and recovery of the supernatant, extraction was repeated twice. The combined supernatants were used to estimate starch content. Soluble sugar and starch (insoluble carbohydrate) contents were measured according to an optimized phenol-sulphuric acid method (Masuko et al. 2005). Fifty µL of the sample was mixed with 150 µL of H_2SO_4 and 30 µL of 5% phenol solution (a generous gift from Chembio, Türkiye) incubated at 90 °C for 5 min and then cooled to RT. The absorbances were read at 490 nm. The glucose amount of the unknown samples was estimated according to the standard curve (0–100 µg) of glucose. For the estimation of starch, the concentration value was multiplied by 0.9. Soluble sugar and starch contents were expressed as µg mg^{-1} FW.

Statistical analysis

All experiments were conducted as independent triplicates. Each datapoint is the arithmetic mean of biological triplicates (N = 3) and the technical triplicates were also included in each experiment. Data were analysed by 2-Way ANOVA, and Tukey's multiple comparison test using Graphpad Prism (version 8.0.1.244). The ANOVA included 2 independent variables as time (day 0 and day 2) and AA concentration (0, 2.5 and 5 mM).

Results

Seedling growth and water content

AA treatment significantly (P < 0.01) decreased root (Fig. 1A) and shoot (Fig. 1B) lengths. At day 0, immediately after AA treatment with 2.5 and 5 mM concentration for 2 days 33.79% and 45.42% decreases in root lengths, respectively, were observed. At day 2, in seedlings recovering for 2 days under non-AA conditions, decreases were 37.07% and 45.15%, at 2.5 and 5 mM concentrations, respectively. For shoot lengths, AA treatment caused 13.73% and 12.63% decreases at day 0 and 18.47% and 22.70% decreases at day 2 for 2.5 and 5 mM concentrations, respectively.

AA-treated plants could not restore shoot and root growth after AA was removed. The effects of AA on root and shoot length were time-independent (P > 0.05).

AA treatment significantly decreased (P < 0.01) FW (Fig. 2A) and WC (Fig. 2B) without affecting (P > 0.05) DW (Online Suppl. Fig. 1A). AA-treated plants could not restore FW after AA was removed. At day 0, AA treatment caused 10.02% and 19.58% decreases in FW at 2.5 and 5 mM concentrations, respectively. At day 2, these decreases were

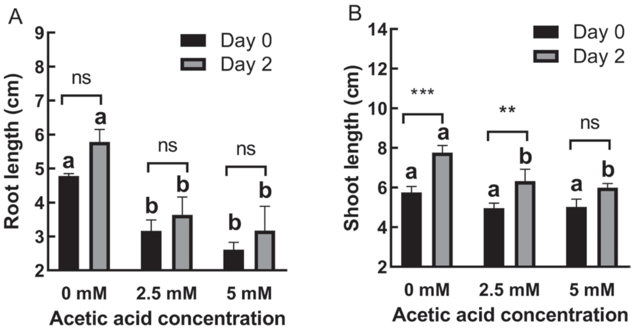

Fig. 1. Changes in root (A) and shoot (B) lengths in 2-day old barley seedlings under acetic acid (AA) treatment. Results are expressed as means ± standard errors (N = 3). Columns indicated by different letters are statistically different at the same timepoint (P < 0.05). Data representing the same concentration at different timepoints are indicated as not significant (ns, P > 0.05), ** for P < 0.01 and *** for P < 0.001.

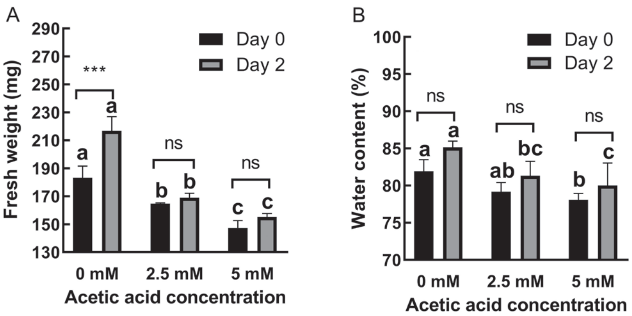

Fig. 2. Changes in fresh weight (A) and water content (B) in 2-day old barley seedlings under acetic acid (AA) treatment. Results are expressed as means ± standard errors (N = 3). Columns indicated by different letters are statistically different at the same timepoint (P < 0.05). Data representing the same concentration at different timepoints are indicated as not significant (ns, P > 0.05) and *** for P < 0.001.

22.08% and 28.43%, respectively. AA treatment also caused 3.32% and 4.68% decreases in WC at day 0 and 4.48% and 6.04% decreases at day 2 for 2.5 and 5 mM concentrations, respectively.

The effects of AA on FW were time-dependent (P < 0.01), while its effects on WC were not time dependent (P > 0.05).

Chlorophyll, carotenoid and pheophytin content

AA treatment decreased chlorophyll *a* (Fig. 3A), total chlorophyll (Fig. 3B), carotenoid (Fig. 3C), and pheophytin (Fig. 3D) levels but significantly (P < 0.01) at day 2, in the recovery group of plants.

At day 0, AA treatment caused 13.77% and 12.29% decreases in chlorophyll *a* content at 2.5 and 5 mM concentrations, respectively. At day 2, these decreases were 43.82% and 36.89%, respectively. For total chlorophyll content, AA treatment caused 11.74% and 10.68% decreases at day 0 and 41.22% and 33.84% decreases at day 2 for 2.5 and 5 mM concentrations, respectively. At day 0, AA treatment caused 23.33% and 8.92% decreases in carotenoid content at 2.5 and

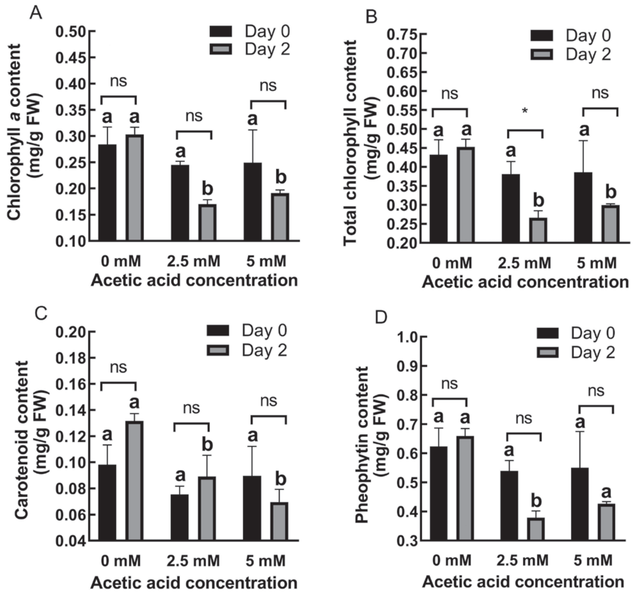

Fig. 3. Changes in chlorophyll *a* (A), total chlorophyll (B), carotenoid (C) and pheophytin (D) content in shoots of 2-day old barley seedlings under acetic acid (AA) treatment. Results are expressed as means ± standard errors (N = 3). Columns indicated by different letters are statistically different at the same timepoint ($P < 0.05$). Data representing the same concentration at different timepoints are indicated as not significant (ns, $P > 0.05$) and * for $P < 0.05$.

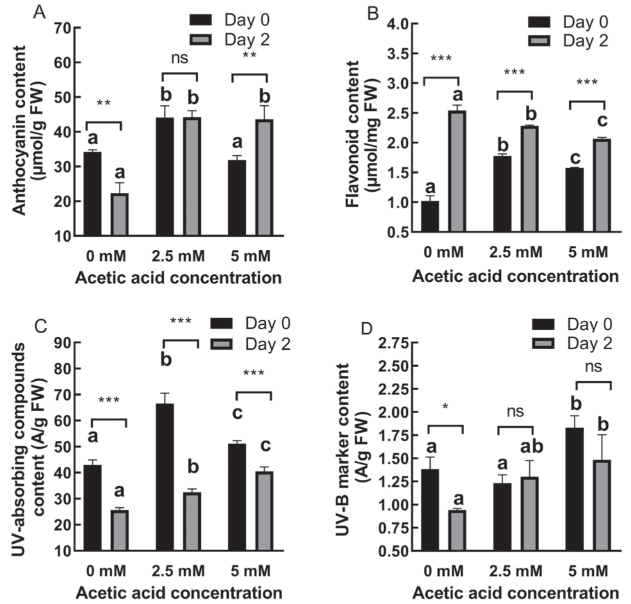

Fig. 4. Changes in anthocyanin (A), flavonoid (B), UV-absorbing compounds (C) and UV-B marker (D) content in shoots of 2-day old barley seedlings under acetic acid (AA) treatment. Results are expressed as means ± standard errors (N = 3). Columns indicated by different letters are statistically different at the same timepoint ($P < 0.05$). Data representing the same concentration at different timepoints are indicated as not significant (ns, $P > 0.05$), * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

5 mM concentrations, respectively. At day 2, these decreases were 32.29% and 47.18%, respectively. For pheophytin content, AA treatment caused 13.54% and 11.70% decreases at day 0 and 42.51% and 35.21% decreases at day 2 for 2.5 and 5 mM concentrations, respectively. Chlorophyll *b* content (On-line Suppl. Fig. 1B) was not significantly affected ($P > 0.05$).

The effects of AA on photosynthetic pigments were found to be time-dependent ($P < 0.05$).

Content of anthocyanins, flavonoids, UV-absorbing compounds and UV-B marker

AA treatment mostly increased ($P < 0.01$) content of anthocyanins (Fig. 4A), flavonoids (Fig. 4B), UV-absorbing compounds (Fig. 4C) and UV-B marker (Fig. 4D) in shoots. At day 0, AA treatment caused a 28.98% increase and a 6.89% decrease in anthocyanin content at 2.5 and 5 mM concentrations, respectively. At day 2, both concentrations caused increases (97.94% and 95.37%, respectively). For flavonoid content, AA treatment caused 73.89% and 54.61% increases at day 0 while at day 2, 10.02% and 18.65% decreases for 2.5 and 5 mM concentrations, respectively, were noticed.

For UV-absorbing compounds, AA treatment caused 54.80% and 19% increases at day 0 and 26.93% and 58.04% increases at day 2 for 2.5 and 5 mM concentrations, respectively.

At day 0, AA treatment caused an 11.06% decrease and a 32.28% increase in UV-B marker content at 2.5 and 5 mM concentrations, respectively. At day 2, both concentrations caused increases (38.14% and 57.66%, respectively).

AA-treated plants accumulated all pigments except flavonoids even after AA was removed. The effects of AA on UV-protective pigments were time-dependent ($P < 0.05$).

H_2O_2 content and cell viability

AA treatment dramatically increased ($P < 0.01$) H_2O_2 content in shoots (Fig. 5A) while in roots, increase in A_{600} (Fig. 5B), indicated reduced cell viability, and these effects persisted even after AA was removed. With respect to H_2O_2 content, AA treatment caused 44.22% and 29.71% increases

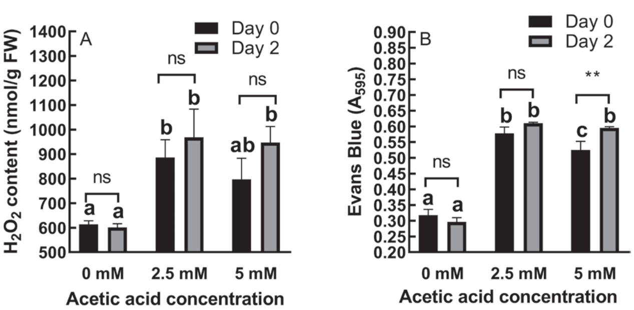

Fig. 5. Changes in H_2O_2 content in barley shoots (A) and cell viability measured by Evans Blue stain (B) in roots of 2-day old barley seedlings under acetic acid (AA) treatment. Results are expressed as means ± standard errors (N = 3). Columns indicated by different letters are statistically different ($P < 0.05$). Data representing the same concentration at different timepoints are indicated as not significant (ns, $P > 0.05$) and ** for $P < 0.01$.

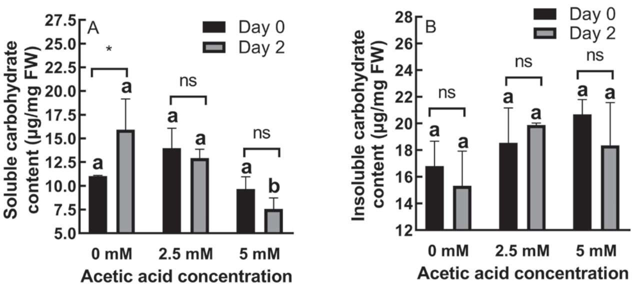

Fig. 6. Changes in soluble (A) and insoluble carbohydrate (B) contents in roots of 2-day old barley seedlings under acetic acid (AA) treatment. Results are expressed as means ± standard errors (N = 3). Columns indicated by different letters are statistically different (P < 0.05). Data representing the same concentration at different timepoints are indicated as not significant (ns, P > 0.05) and * for P < 0.05.

at day 0 and 61.08% and 57.61% increases at day 2 for 2.5 and 5 mM concentrations, respectively.

AA treatment caused 81.76% and 65.09% increases in A_{600} values at day 0 and 105.96% and 100.89% increases at day 2 for 2.5 and 5 mM concentrations, respectively.

The effects of AA on cell viability were time-dependent (P < 0.01), while its effects on H_2O_2 content were not time-dependent (P > 0.05).

Carbohydrate content

AA treatment had significant (P < 0.05) effects on carbohydrate content in roots. At day 0, AA treatment caused a 26.66% increase and a 12.43% decrease in soluble carbohydrate content at 2.5 and 5 mM concentrations, respectively. At day 2, both concentrations caused decreases (18.83% and 52.54%, respectively) (Fig. 6A).

With respect to insoluble carbohydrate content, AA treatment induced 10.42% and 23.15% increases at day 0 and 29.78% and 19.76% increases at day 2 for 2.5 and 5 mM concentrations, respectively (Fig. 6B).

The effects of AA on soluble carbohydrate content were time-dependent (P < 0.05) while its effects on insoluble carbohydrate content were not time dependent (P > 0.05).

Discussion

Stress conditions inhibit plant growth in terms of root and shoot length, DW and leaf area (Rahman et al. 2019). Under stressful conditions plants synthesize various phytoprotectant metabolites that help them defend against stress (Parvin et al. 2022). These metabolites can also be applied exogenously *e.g.* salicylic acid (SA) and phenolic compounds and alleviate stress-induced decreases in yield and productivity by affecting several processes including plant growth, photosynthesis, membrane permeability, antioxidant systems and synthesis of various molecules (Parvin et al. 2022). OAs were also reported to accumulate in plants under stress as these molecules, besides being intermediates in carbon metabolism, are important metabolically active solutes for osmotic adjustment, the balance of cation excess or in coping with nutrient deficiencies and metal tolerance (López-Bucio et al. 2000). AA, an OA, was shown to confer tolerance in various plant species, including tomato, cassava, Arabidopsis, rapeseed, maize, rice and wheat against several stressors (Kim et al. 2017, Chen et al. 2019, Rahman et al. 2019, Utsumi et al. 2019, Rahman et al. 2021, Mahmud et al. 2023). AA-sprayed mung bean plants exhibited enhanced shoot and primary root length and shoot DW under salt conditions (Rahman et al. 2019). The same group later showed that foliar application of AA resulted in improvement of root biomass and leaf area in soybean exposed to drought (Rahman et al. 2021). Utsumi et al. (2019) reported the maintenance of leaf relative WC in drought-treated cassava plants. AA resulted in higher WC and elevated water use efficiency due to decreased stomatal conductance in drought stressed *Cunninghamia lanceolate* plants (Li et al. 2022). A recent paper by Mahmud et al. (2023) similarly stated that AA restored shoot and root growth in maize under drought stress. Interestingly, in our study AA treatment of barley seedlings inhibited plant growth, especially root growth and decreased FW and WC but did not affect DW. These detrimental effects mostly could not be repaired after the 2 day-recovery period. Thus, it can be inferred that, when applied directly to the young seedlings through roots, AA might be regarded as a stressor and the roots, being the first exposed to the effects of AA, are more affected than the shoots. Our results are different than those of most papers, which reveal the positive effects of AA (Kim et al. 2017, Li et al. 2022, Mahmud et al. 2023). However, these studies were performed on different species, with higher concentrations of AA and for longer periods and demonstrated its effects in plants growing in a culture or soil. In the present study, a shorter treatment duration (2 days) with lower concentrations (2.5 and 5 mM) of AA was tested on very young barley seedlings growing on moist filter papers. Lynch (1977) reported that the growth promoting effects of AA depend on pH and plant species. In an interesting study, Allen and Allen (2020) suggested that adjusting pH of the medium can compensate the harmful effects of AA in maize roots. They demonstrated that under unbuffered conditions, such as during germination on filter paper, acetic acid exists in the membrane permeable undissociated form which caused maize seedling root inhibition. Similarly, in our study the pH of the water with AA was not adjusted, which could explain the toxic effects of AA on barley seedlings, even at low concentrations.

Severe stress conditions cause degradation of chlorophyll, while low stress stimulates chlorophyll content, to allow plants to cope with stress (Agathokleous et al. 2020). Under stressful conditions, degradation of free chlorophyll is necessary to prevent cell damage (Takamiya et al. 2000) and chlorophyll content can reflect the damage caused by stress (Agathokleous et al. 2020). Exogenously applied AA reduced the impact of drought stress in cassava plants by increasing chlorophyll and carotenoid amounts (Utsumi et al. 2019). AA also improved chlorophyll fluorescence in *C. lanceolate* under drought stress by maintaining higher chlorophyll contents due to delay of degradation of pigments or induction of their synthesis (Li et al. 2022). Hawrylak-Nowak et al. (2015) stated that AA had slight effects on carotenoid content in the first leaf of sunflower plant. In the present study, AA decreased chlorophyll and carotenoid contents as well as pheophytin content. A decrease in chlorophyll and carotenoid could be correlated with decreased growth and WC, but we expected accumulation of pheophytin in leaves of AA-treated seedlings because of the acidic environment in which the seedlings had grown. Pheophytin, a breakdown product of chlorophyll is observed mainly during leaf senescence (Lin and Charng 2021) or under oxidative stress (Szafrańska et al. 2017). Acidity replaces magnesium ions in the chlorophylls with hydrogen atoms and chlorophylls are converted to pheophytins (Kusmita et al. 2015). This unexpected result can be explained by the severity of stress, which caused reduced pheophytin content, as it did in duckweed plants exposed to UV-B radiation for 7 days (Farooq et al. 2005).

The growth-restricting effects of AA observed in our study suggests that AA may affect cell viability particularly in roots, which were more susceptible to AA than shoots. We also investigated H_2O_2 content in shoots, in which AA-induced reductions were less prominent than in roots. H_2O_2 is a type of ROS characterized by low reactivity. It serves as a signalling molecule due to its long life span and small size (Khan et al. 2018). ROS are produced as byproducts of various metabolic pathways and scavenged by antioxidant defence systems. Under certain (stress or non-stress) conditions, ROS production is elevated in plants (Apel and Hirt 2004). Higher concentrations of H_2O_2 deplete the ascorbic acid and glutathione pool, cause damage to proteins, nucleic acids and lipids and compromise cell integrity and eventually result in cell death (Khan et al. 2018). Stress conditions increase H_2O_2 content and decrease cell viability in plants, while compounds that confer stress tolerance usually act inversely (Cikili et al. 2019). Hawrylak-Nowak et al. (2015) reported that AA and particularly malic acid promoted cell viability in roots of Cd-treated plants due to increased high dehydrogenase activity. They also observed that AA decreased the content of H_2O_2 in leaves and roots of sunflower plants subjected to Cd stress. However, in our study, we observed the accumulation of H_2O_2 in shoots and decreased cell viability in roots after AA treatment, even after recovery step. Consistently with our findings, Armstrong et al. (1996) reported necrosis of the roots of reed subjected to 1.67 mM AA. These results suggest that AA can induce the oxidative stress in shoots and reduce the root length due to cell death and imply that AA, even at low concentrations, acts as a stressor in barley. In the study by Hawrylak-Nowak et al. (2015) where opposite results were found, 7-day-old sunflower seedlings were treated with 0.25 or 0.5 mM AA in 1.5-times strength Hoagland's II nutrient solution for 14 days. Different plant species and different experimental conditions, especially concerning the pH of the medium may explain the contradictory results.

Plants accumulate phenolic compounds (PCs) when exposed to stress. PCs can be found in several intracellular locations; and stimulate stress tolerance in plants by performing diverse functions such as scavenging of ROS, enhancement of cell division and improvement of photosynthesis (Parvin et al. 2022). The most abundant group of PCs is that of flavonoids, containing anthocyanins as a subgroup (Parvin et al. 2022). Flavonoids, anthocyanins, and other UV-absorbing compounds accumulate under stress conditions, although UV-B exposure was shown to be the most efficient in increasing the levels of UV-absorbing compounds and UV-B marker (Cicek et al. 2012). Depending on the plant species and its genotype, abiotic stress such as drought affects contents of flavonoids, which act as signalling molecules, antioxidant molecules and UV protectant and regulate hormones (Shin et al. 2021, Parvin et al. 2022, Kumar et al. 2023). Molecules that can enhance stress tolerance of plants can stimulate synthesis of secondary metabolites *e.g.* flavonoids (Kumar et al. 2023). In the present study, AA treatment increased not only anthocyanin and flavonoid contents but also UV-absorbing compounds (A_{300}) and the UV-B marker (A_{440}). Except for the flavonoids, the increasing trend persisted after recovery step. AA treatment immediately and directly impacted UV-absorbing pigments probably as a result of increased H_2O_2 which induced synthesis of various phenolic compounds to act as ROS scavengers.

Because AA is a precursor of acetyl-CoA, an essential molecule of carbohydrate metabolism, we decided to investigate how AA treatment affects sugar contents in roots. There are contradictory reports on the effects of acetate on starch synthesis. Starch degradation is usually activated under abiotic stress to redirect carbon for stress responses, but starch can also accumulate when growth is inhibited more than photosynthesis (Ribeiro et al. 2022). Fan et al. (2012) reported acetate-induced starch accumulation in *C. reinhardtii*. However, Rengel et al. (2018) observed that overexpression of chloroplastic ACSS under nutrient replete conditions enhanced starch content in *C. reinhardtii*, while acetate treatment did not. Under nitrogen starvation, excess acetyl-CoA was stored as triacylglycerol. Interestingly, Arabidopsis plants with reduced activity of ATP-citrate lyase, which is responsible for conversion of mitochondria--derived citrate to acetyl-coA, have higher amounts of anthocyanin and starch (Fatland et al. 2005). Huang et al. (2017) found that microalgae *Chlorella sorokiniana* GXNN01 favours AA as a carbon source over glucose under normal and high light conditions. Use of AA as carbon source resulted in higher biomass with high lipid and low starch contents. In this study, soluble carbohydrate contents decreased in AA-treated roots, while insoluble carbohydrate contents increased, which is consistent with the findings of Fan et al. (2012). Moreover, changes in the content of soluble carbohydrates were more prominent than those of insoluble carbohydrates. Soluble sugars in seeds serve as fast-use reserves for energy production but they can also be efficient in protecting membrane integrity during stress conditions (Ferreira et al. 2009).

In conclusion, even at low concentrations, AA treatment can induce dramatic and persistent changes in non-stressed plants that endure even after AA is removed. Previous studies focused on the amelioration of stress, particularly drought, by AA treatment that was usually applied to plants growing in a culture medium or soil. In this study, AA was applied to young (2-day-old) seedlings growing on a filter paper and exhibited its toxic effects *i.e.* restriction of growth, decrease in WC, photosynthetic pigments and viability in root cells and the accumulation of UV-absorbing pigments and H_2O_2 in shoots and insoluble carbohydrates in roots.

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Trehalose-induced metabolic responses in basil (*Ocimum basilicum*) seedlings under salt treatment

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Abstract – Trehalose (Tre) is an osmoprotectant known to be an important player in regulating response to salinity. In this research, the effect of Tre foliar application on the growth rate, the content of photosynthetic pigments, accumulation of metabolites, and activity of antioxidant enzymes of basil seedlings under salt stress has been investigated. Basil seedlings were factorially treated twice a week with levels of 0, 25, 50, 100, and 150 mM NaCl and weekly with concentrations of 0 and 5 mM Tre for 4 weeks. Growth characteristics, the content of photosynthetic pigments including chlorophyll and carotenoid, and starch content decreased in response to salt stress, while the activity of antioxidant enzymes and the accumulation of metabolites including soluble sugars, proteins, amino acids, and proline increased. Tre treatment caused severe inhibition of plant growth, further reduction of photosynthetic pigments, and amount of soluble proteins during salinity stress. Also, starch, total protein, amino acids, and proline were hyperaccumulated in response to Tre. These results indicate that Tre not only does not reduce the detrimental effects of salinity in basil seedlings but also inhibits plant growth possibly by diverting carbon to other metabolic pathways.

Keywords: proline, salt stress, soluble sugars, starch, trehalose

Introduction

Salinity is one of the most important factors limiting the yield of crops, especially in arid, semi-arid, and coastal areas (Mostofa et al. 2015). This stress negatively affects plant physiology by imposing several major limitations leading to reduced crop growth and productivity (Ali et al. 2021, Zhang et al. 2021, Khan et al. 2023). Salinity causes osmotic and ionic stress, and production of reactive oxygen species (ROS) in plant cells (Ahammed et al. 2018, Khan et al. 2023, Colin et al. 2023). High production of ROS induces oxidative stress, which can destroy membrane components and biomolecules and, at higher levels, cause cell, tissue, and plant death (Kaur et al. 2022, Khan et al. 2023, Peng et al. 2023). Salinity tolerance is a complex process that involves various molecular, physiological, and biochemical mechanisms (Zhang and Shi 2013, Islam et al. 2023).

The use of osmoprotectants can augment stress tolerance mechanisms in plants (Chen and Jiang 2010, Islam et al. 2023). These compounds, which are known as compatible solutes, are highly soluble compounds with low molecular weight that directly or indirectly protect plants against stresses through various mechanisms such as regulating cellular osmosis, preventing membrane damage, stabilizing proteins and enzymes, and eliminating ROS (Singh et al. 2015, He et al. 2018). Application of exogenous osmoprotectants can be considered an alternative approach to improve plant productivity under saline conditions (Nakayama et al. 2005). The high concentration of compatible solutes can balance the salts entering the cell and at the same time deal with the high concentration of salts inside the cell (Türkan and Demiral 2009).

Sugars are one of the most important compatible solutes that, in addition to the role of signal molecules, can act as a source of metabolic energy and play a role in regulating metabolism in plants (Islam and Mohammad 2021). Trehalose (Tre) is a non-reducing disaccharide consisting of two glucose molecules with a chemically non-reactive nature that makes it compatible with cellular metabolism even at high concentrations, and plays an important role as an osmoprotectant in stress tolerance of some plants (Ali and Ashraf 2011, Lunn et al. 2014). It is naturally distributed in many organisms, from bacteria and fungi to plants and invertebrates (Nounjan et al. 2012, Kosar et al. 2019), where it is used for carbon storage and transport as well as being an osmoprotectant (Lunn et al. 2014). Among plants, Tre synthesis has been reported in some resurrection plants and other plants such as *Arabidopsis* (Muller et al. 1999, Lunn et al. 2014). It has been shown that the expression of plant genes involved in Tre metabolism undergoes remarkable changes

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in response to various abiotic stresses (Nakashima et al. 2009). Tre and trehalose-6-phosphate, a phosphorylated intermediate in Tre biosynthesis, act as signal molecules in carbohydrate metabolism (Wingler et al. 2000, Lunn et al. 2014). Tre induces sucrose synthase activity in soybean (Muller et al. 1998) and trehalose-6-phosphate acts as a specific signal of sucrose status in *Arabidopsis*, and may be seen as part of a homeostatic mechanism to control the level of sucrose, as well as part of regulatory networks in sucrose export in source organs and in growing sink organs (Lunn et al 2014). Tre has also a key role in the control of metabolism during plant growth and development, and reduces the effects of stress through mechanisms such as membrane protection, stabilization of proteins and enzymes, detoxification and removal of ROS, molecular signaling, and increasing the accumulation of osmolytes (Luo et al. 2010, Abdallah et al. 2016, Islam and Mohammad 2021). Most studies on the role of osmoprotectants in enhancing stress tolerance have focused on proline and glycine betaine, but there is relatively little knowledge about the effects of Tre on higher plants under stress conditions. Some studies have shown that the exogenous application of Tre on plants has induced tolerance to salinity stress (Nounjan et al. 2012, Abdallah et al. 2016, Rohman et al. 2019). In contrast, it has been found that Tre can have an inhibitory effect on the growth of some plants (Schluepmann et al. 2004, O'Hara et al. 2013) and algae under salinity (Panjekobi and Einali 2021). However, in most plants, endogenous Tre cannot adequately ameliorate the harmful effects of stress (Mostofa et al. 2015).

Basil (*Ocimum basilicum* L.) is a medicinal and aromatic plant that is used in the pharmaceutical and food industries due to its abundant essential oils, high content of phytomedicines and natural antioxidant molecules, and is usually produced for economic purposes (Kwee and Niemeyer 2011, Caliskan et al. 2017). It is commonly used in traditional medicine and herbal therapy (Bahcesular et al. 2020, Farouk and Omar 2020). To date, no study has been conducted on the role of Tre in the physiological and biochemical responses of basil, an important agricultural and medicinal plant, to salinity stress. Accordingly, basil was taken as a model system to determine the effect of exogenous Tre on growth, accumulation of osmolytes, and the activity of some enzymes of the antioxidant system under salt stress.

Materials and methods

Plant material and experimental design

Basil seeds (*Ocimum basilicum* L.) obtained from the Botanic Garden at the University of Sistan and Baluchestan were sown in a greenhouse with a temperature of 29 ± 1 °C in trays containing moist cocopeat. After germination, seedlings with two or three leaves that had grown uniformly were selected and each seedling was transferred to a 14 x12 cm plastic pot containing 1 kg of cocopeat and placed at the same temperature. Seedlings were irrigated with 1/2 Hoagland's standard nutrient solution at three-day intervals until reaching the 6-8 leaf stage. Three days before salt treatment, the seedlings were divided into two parts. By foliar spraying on the seedlings, one part was treated with a concentration of 5 mM Tre and the other part received only distilled water. Salinity treatment was applied by adding sodium chloride (NaCl) in concentrations of 0, 25, 50, 100, and 150 mM to the nutrient solutions of each plant group. Tre-untreated plants, which received only 1/2 Hoagland's solution without NaCl, were considered as the control for salinity experiments. Seedlings were treated twice a week with salinity and every week with Tre for four weeks. After this period, seedlings were harvested and studied to investigate the morphological and biochemical responses to salt stress and Tre treatment. Each morphological or biochemical experiment was performed individually with three biological replicates.

Morphological traits

The length of root and shoot along with the length, width, and number of leaves of basil seedlings were measured. To determine the amount of biomass, first the fresh weight (FW) of each shoot and root was measured, and after exposure to 70 °C for 72 h, the dry weight (DW) was determined. Shoot water content (SWC) of basil seedlings was obtained using the following formula:

$$SWC = ((\text{shoot FW} - \text{shoot DW})/\text{shoot FW}) \times 100$$

Determination of photosynthetic pigments content

Photosynthetic pigments including chlorophyll (Chl) and total carotenoids (Car) were extracted from 1 g of fresh leaf tissue with 80% acetone. The resulting mixture was filtered through filter paper (Whatman No. 1) and the residue was saved to measure soluble sugars and total protein. The absorbance of the filtrate was recorded at 663, 645, and 652 nm, and the amounts of Chl_s *a*, *b*, and total were measured using the following equations (Arnon 1949):

$$\text{Chl } a \text{ (mg mL}^{-1}) = 0.0127 \times A_{663} - 0.00269 \times A_{645}$$

$$\text{Chl } b \text{ (mg mL}^{-1}) = 0.0229 \times A_{645} - 0.00468 \times A_{663}$$

$$\text{Total Chl (mg mL}^{-1}) = A_{652}/34.5$$

Total Car content was measured at 470 nm using the following equation (Lichtenthaler and Buschmann, 2001):

$$\text{Total Car } (\mu\text{g mL}^{-1}) = (1000 \times A_{470} - 1.82 \times \text{Chl } a - 85.02 \times \text{Chl } b)/198$$

The content of pigments was expressed as mg per g FW.

Determination of soluble sugars and starch content

Soluble sugars, including reducing and non-reducing sugars, were extracted from 40 mg of acetone powder (resulting from the extraction of photosynthetic pigments) using 80% ethanol (Einali and Valizadeh 2017). Ethanol extracts were used to determine the content of soluble sugars. Reducing sugars (RS) were determined by the method of Miller (1959) and non-reducing sugars (NRS) were measured by Handel's (1968) method. The residues obtained from the extraction of soluble sugars were used to extract and measure the amount of starch (McCready et al. 1950). The content of soluble sugars and starch was expressed as mg per g FW.

Determination of total proteins, total amino acid, and proline content

Total proteins refer to proteins extracted with a sample buffer containing 60 mM Tris–HCl buffer (pH 6.8), 10% (v/v) glycerol, and 2% (w/v) sodium dodecyl sulfate (Stone and Gifford (1997). Extraction of total proteins from 20 mg of acetone powder was done with 0.5 mL of sample buffer at 90 °C for 1 h, followed by centrifugation at 10000 g for 15 minutes (Alisofi et al. 2020). The amount of total proteins was measured at a wavelength of 750 nm by the method of Markwell et al. (1981) and expressed as mg per g FW.

Free amino acids and proline were extracted from 0.2 g of fresh leaf tissue using 80% ethanol at 70 °C for 10 min, followed by centrifugation at 2000 g for 10 min (Einali and Valizadeh 2017). The extraction process was repeated four more times. The concentrated ethanol extract was decolored by chloroform (1:5, v/v). The ninhydrin (1% (w/v) ninhydrin and 0.06% (w/v) KCN in acetone) method using a glycine calibration curve at 570 nm was used to determine total free amino acid (Yemm and Cocking 1955). Proline content was measured at 520 nm by another ninhydrin (1% (w/v) ninhydrin in 60% (v/v) acetic acid) method using proline as a standard (Bates et al. 1973). The content of free amino acids and proline was expressed as µmol per g FW.

Soluble protein extraction and enzyme assays

Crude enzyme extract was prepared from 0.2 g of fresh leaf tissue with 3 mL of enzyme extraction buffer containing 100 mM cold potassium phosphate buffer (pH 7.0), 10% glycerol, 1 mM EDTA, 10 mM KCl, 1 mM $MgSO_4$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone (PVPP) as described elsewhere (Alisofi et al. 2020). Extraction was done in a cold mortar with pestle. The extraction buffer for ascorbate peroxidase (APX) determination contained 5 mM ascorbic acid as well. The homogenate was filtered through four layers of cheesecloth and incubated at 4 °C for 1 h. The amount of 10 mg of charcoal was added to the filter to remove the extracted pigments, and then it was centrifuged at 12000 g for 10 min at 4 °C. The supernatant, containing soluble protein fraction, was used for enzyme assays. Soluble proteins refer to proteins extracted in the absence of sodium dodecyl sulfate. Soluble protein content was measured by Bradford's (1976) method using the albumin standard curve and expressed as mg per g FW.

APX reaction mixture (1 mL) consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2, 0.5 mM ascorbic acid, and 50 µL enzyme extract. The oxidation of ascorbate to dehydroascorbate was monitored at 290 nm and the activity of the enzyme was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} and expressed as µmol of oxidized ascorbate per min per g FW (Chen and Asada 1992).

The activity of pyrogallol peroxidase (PPX) was measured in a reaction mixture (1 mL) containing 50 mM potassium phosphate buffer (pH 7.0), 40 mM pyrogallol, 1 mM H_2O_2, and 50 µL enzyme extract. The conversion rate of pyrogallol to purpurogallin was monitored at 430 nm and the enzyme activities were calculated using the extinction coefficient 2.47 mM^{-1} cm^{-1} and expressed as µmol of purpurogallin produced per min per g FW (Nakano and Asada 1981). Polyphenol oxidase (PPO) activity was measured in the same way as PPX at 430 nm using the extinction coefficient 2.47 mM^{-1} cm^{-1}, except that the reaction mixture was without H_2O_2 (Nakano and Asada 1981).

Statistical analysis

All results obtained from plant growth and biochemical studies were expressed as mean and standard deviation (SD) of three independent measurements. The statistically significant difference between treatments was determined in the form of factorial design using two-way ANOVA and Duncan's test at the level of 5% ($P < 0.05$).

Results

Effect of salinity and Tre on plant growth

Salt treatment at low concentration (25 mM) caused a clear 15% increase in shoot length compared to the control, but higher salt concentrations gradually decreased these values (Tab. 1).

Tab. 1. Longitudinal growth and leaf characteristics of trehalose-treated (+Tre) or untreated (−Tre) basil seedlings in response to different salinity concentrations. Values are the mean ± standard deviation of three separate measurements. Different letters in each column indicate significant differences between the various treatments at P < 0.05 according to the Duncan test. LL – leaf length; LW – leaf weight.

Salt treatment (mM)		Plant length (cm)		LL (cm)	LW (cm)	LL/LW	LL × LW (cm^2)	Number of leaves
		Shoot	Root					
0	−Tre	26.00 ± 1.00[b]	19.67 ± 2.08[a]	3.77 ± 0.46[a]	1.93 ± 0.12[a]	1.95 ± 0.20[a]	7.30 ± 1.18[a]	14.00 ± 1.00[ab]
	+Tre	23.33 ± 0.58[c]	22.67 ± 2.08[a]	2.67 ± 0.29[bc]	1.63 ± 0.06[b]	1.64 ± 0.21[a]	4.35 ± 0.41[c]	14.00 ± 0.00[a]
25	−Tre	30.00 ± 1.00[a]	20.00 ± 2.00[a]	4.33 ± 0.76[a]	2.10 ± 0.17[a]	2.07 ± 0.39[a]	9.12 ± 1.84[a]	16.00 ± 0.00[a]
	+Tre	24.00 ± 2.65[c]	21.00 ± 1.00[a]	2.93 ± 0.31[bc]	1.70 ± 0.10[b]	1.73 ± 0.18[a]	4.99 ± 0.69[bc]	14.67 ± 1.15[ab]
50	−Tre	26.33 ± 2.08[b]	21.00 ± 1.00[a]	4.03 ± 0.45[a]	2.23 ± 0.21[a]	1.80 ± 0.07[a]	9.07 ± 1.80[a]	15.33 ± 1.15[a]
	+Tre	21.33 ± 1.53[d]	21.33 ± 1.53[a]	3.13 ± 0.15[b]	1.67 ± 0.15[b]	1.89 ± 0.15[a]	5.23 ± 0.63[bc]	14.67 ± 1.15[ab]
100	−Tre	24.33 ± 0.58[c]	23.00 ± 2.65[a]	3.87 ± 0.32[a]	2.13 ± 0.21[a]	1.81 ± 0.03[a]	8.29 ± 1.46[a]	15.33 ± 1.15[a]
	+Tre	20.33 ± 2.08[d]	19.33 ± 1.15[a]	3.20 ± 0.10[b]	1.77 ± 0.06[b]	1.81 ± 0.08[a]	5.65 ± 0.26[b]	14.67 ± 1.15[ab]
150	−Tre	21.33 ± 1.53[d]	21.00 ± 0.00[a]	3.50 ± 0.30[ab]	1.93 ± 0.12[a]	1.81 ± 0.19[a]	6.77 ± 0.72[a]	13.33 ± 1.15[b]
	+Tre	14.67 ± 1.53e	19.67 ± 1.53[a]	2.70 ± 0.17[c]	1.37 ± 0.15[c]	1.98 ± 0.11[a]	3.71 ± 0.63[d]	13.33 ± 1.15[b]

Tab. 2. Shoot and root biomass accumulation and water content of trehalose-treated (+Tre) or untreated (–Tre) basil seedlings in response to different salinity concentrations. Values are the mean ± standard deviation of three separate measurements. Different letters in each column indicate significant differences between the various treatments at P < 0.05 according to the Duncan test. FW – fresh weight; DW – dry weight; SWC – shoot water content.

Salt treatment (mM)		FW (g)		DW (g)		FW/DW		SWC (%)
		Shoot	Root	Shoot	Root	Shoot	Root	
0	-Tre	2.19 ± 0.08^{a}	0.43 ± 0.04^{a}	0.60 ± 0.01^{a}	0.05 ± 0.01^{c}	3.64 ± 0.08^{c}	9.50 ± 2.20^{a}	72.49 ± 0.58^{d}
	+Tre	1.06 ± 0.16^{c}	0.18 ± 0.02^{d}	0.23 ± 0.07^{de}	0.08 ± 0.01^{b}	4.76 ± 1.04^{b}	2.26 ± 0.43^{e}	78.35 ± 4.49^{cd}
25	-Tre	1.99 ± 0.31^{ab}	0.31 ± 0.03^{b}	0.52 ± 0.01^{b}	0.10 ± 0.01^{b}	3.83 ± 0.62^{bc}	3.21 ± 0.21^{d}	73.41 ± 4.20^{d}
	+Tre	1.12 ± 0.25^{c}	0.26 ± 0.04^{bc}	0.25 ± 0.05^{de}	0.06 ± 0.01^{c}	4.61 ± 0.91^{b}	4.66 ± 0.82^{b}	77.67 ± 4.90^{cd}
50	-Tre	2.29 ± 0.31^{a}	0.32 ± 0.02^{b}	0.43 ± 0.06^{c}	0.12 ± 0.01^{a}	5.29 ± 0.09^{b}	2.74 ± 0.30^{e}	81.09 ± 0.31^{c}
	+Tre	1.64 ± 0.14^{b}	0.16 ± 0.03^{de}	0.27 ± 0.03^{d}	0.06 ± 0.02^{c}	6.19 ± 0.79^{b}	2.80 ± 0.55^{e}	83.65 ± 2.26^{c}
100	-Tre	1.71 ± 0.21^{b}	0.24 ± 0.04^{c}	0.41 ± 0.08^{c}	0.09 ± 0.01^{b}	4.28 ± 0.91^{c}	2.52 ± 0.28^{e}	76.00 ± 4.75^{cd}
	+Tre	1.38 ± 0.15^{b}	0.29 ± 0.05^{bc}	0.23 ± 0.02^{e}	0.06 ± 0.00^{c}	6.03 ± 0.60^{b}	4.79 ± 0.94^{b}	83.31 ± 1.57^{bc}
150	-Tre	1.10 ± 0.04^{c}	0.13 ± 0.02^{e}	0.30 ± 0.01^{d}	0.07 ± 0.01^{c}	3.73 ± 0.20^{c}	1.84 ± 0.02^{f}	73.13 ± 1.41^{d}
	+Tre	1.01 ± 0.04^{c}	0.08 ± 0.01^{f}	0.11 ± 0.02^{f}	0.02 ± 0.00^{d}	9.56 ± 0.96^{a}	3.86 ± 0.39^{c}	89.47 ± 1.11^{a}

However, salinity did not affect root length and leaf characteristics including length (LL), width (LW), LL/LW ratio, LL × LW production, and number of leaves. Tre treatment alone or under salt stress had a significant negative effect on the length of the shoot and some leaf characteristics compared to untreated controls, but it did not change the root length and the number of leaves (Tab. 1).

Although SWC remained unchanged, the effect of salinity on fresh and dry weight of shoots and fresh weight of roots was negative and caused a decrease proportional to salt concentration, while the dry weight of roots increased significantly in response to different salt treatments compared to the control (Tab. 2).

Tre application in most salt treatments caused a decrease in fresh and dry weight of shoot and root, so that the decrease in dry weight was more intense. In contrast, Tre treatment caused a 23% increase in SWC in seedlings exposed to 150 mM NaCl compared to the untreated control (Tab. 2).

Effect of salinity and Tre on photosynthetic pigments

The amount of Chl and Car in response to salinity of 50 mM and above decreased significantly compared to the control (Fig. 1).

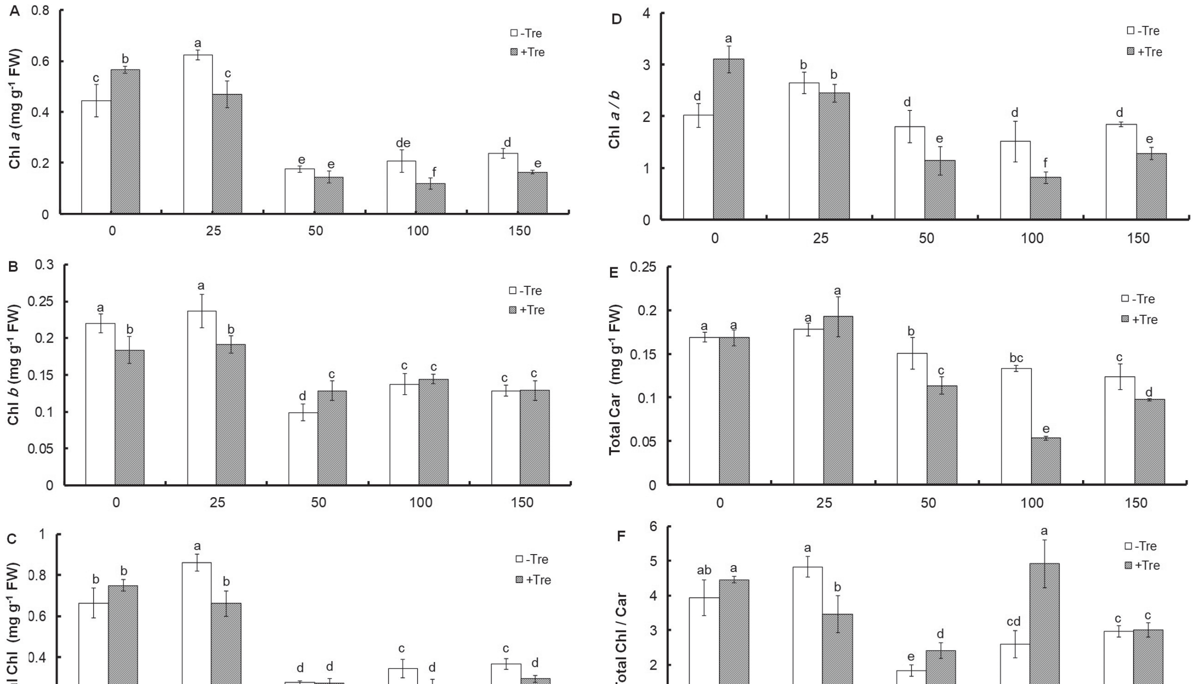

Fig. 1. Effect of different salinity concentrations on content of Chl *a* (A), Chl *b* (B), and total Chl (C), as well as on Chl *a* / *b* ratio (D), total Car (E), and Chl / Car ratio (F) in trehalose-treated (+Tre) or untreated (-Tre) basil seedlings. Results are the mean ± standard deviation of three separate measurements. Different letters indicate significant differences between the various treatments at P < 0.05 according to the Duncan test.

Although the 25 mM concentration of NaCl had no effect on the amount of Chl *b* and total Car compared to the control (Fig. 1B, 1D), it caused a significant increase in the content of Chl *a* and total Chl by 41 and 30%, respectively (Fig. 1A, 1C). A significant or non-significant increase in response to 25 mM salinity was observed in Chl *a/b* and Chl/Car ratios, respectively, which decreased or remained unchanged at higher salinities compared to the control (Fig. 1D, 1E). Tre treatment resulted in a significant decrease in pigments amount and Chl *a/b* ratio at most salt levels compared to untreated controls (Fig. 1A-1E). However, the Chl/Car ratio increased in salt treatments with a NaCl concentration above 25 mM (Fig. 1F).

Effect of salinity and Tre on soluble sugars and starch contents

All salt treatments caused a sharp increase in the content of soluble sugars, including reducing, non-reducing and total sugars, compared to the control (Fig. 2A-2C), so that total soluble sugars reached 145.81 mg g^{-1} FW under 150 mM NaCl (Fig. 2C).

However, a significant decrease in RS/NRS ratio and starch content was found under all salinities compared to control (Fig. 2D, 2E). Tre treatment increased soluble sugars in non-stress or salinity conditions up to 50 mM NaCl concentration compared to untreated plants, but at higher salt concentrations, it caused a slight increase in NRS and a significant decrease in RS (Fig. 2A-2C). Tre alone increased the ratio of RS/NRS by 22% compared to the untreated control, but did not change this ratio at mild salinity (25 and 50 mM NaCl) or even decreased it to the same extent at 100 and 150 mM NaCl (Fig. 2D). These changes were associated with the accumulation of starch in Tre-treated seedlings at salt concentrations of 50 mM and higher (Fig. 2E).

Effect of salinity and Tre on total proteins, total amino acid, and proline contents

The positive effect of salinity on total protein content was observed only at 25 mM salt concentration with a 75% increase and no significant change was found in other salt treatments, while all salinity levels significantly increased soluble proteins, especially up to 10.88 mg g^{-1} FW under 50 mM NaCl (Fig. 3A, 3B).

Salinity also caused the accumulation of amino acids and proline so that this increase was proportional to the salt concentration and reached 151 and 247% of control for amino acids and proline in 150 mM NaCl concentration, respectively (Fig. 3C, 3D). Tre treatment at all salinity concentrations caused a strong increase in total proteins, amino acids, and proline contents, which was associated with a decrease in the concentration of soluble proteins (Fig. 3).

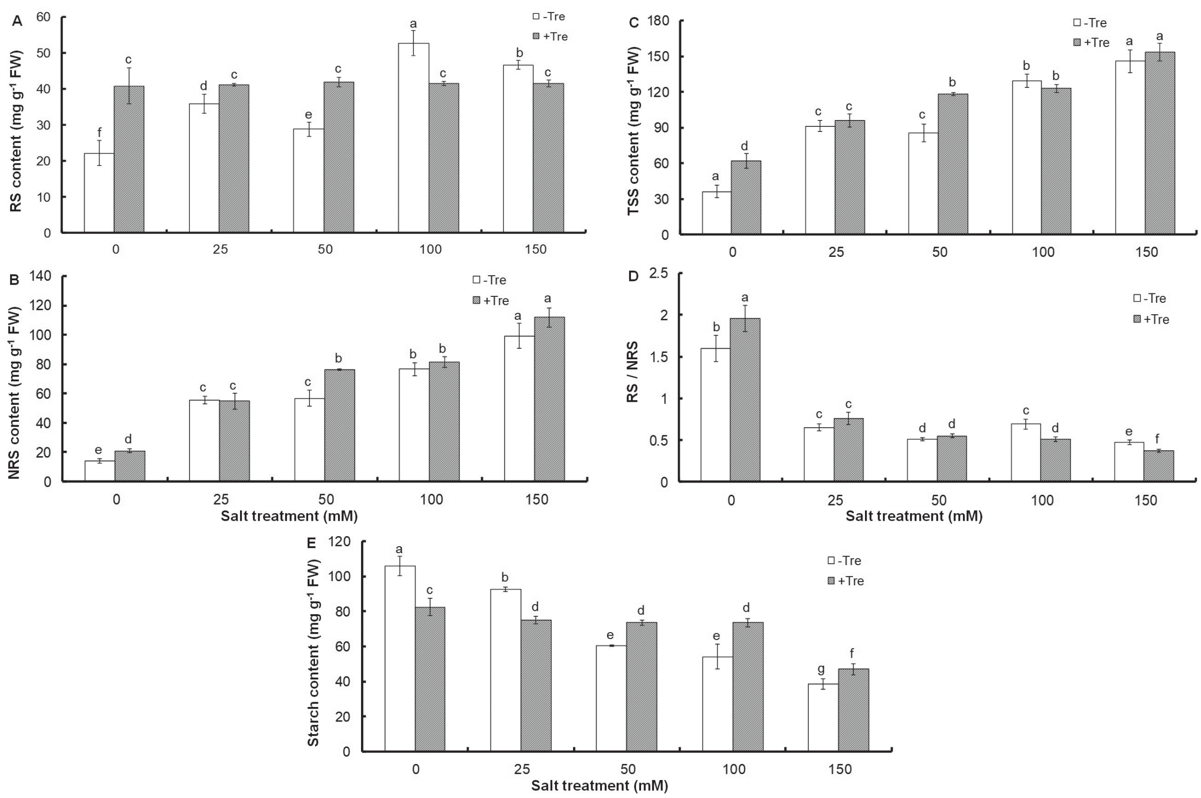

Fig. 2. Effect of different salinity concentrations on content of reducing sugars, RS (A), non-reducing sugars, NRS (B), and total soluble sugars, TSS (C), as well as on reducing / non-reducing sugars, RS/NRS ratio (D), and starch content (E) in trehalose-treated (+Tre) or untreated (-Tre) basil seedlings. Results are the mean ± standard deviation of three separate measurements. Different letters indicate significant differences between the various treatments at P < 0.05 according to the Duncan test.

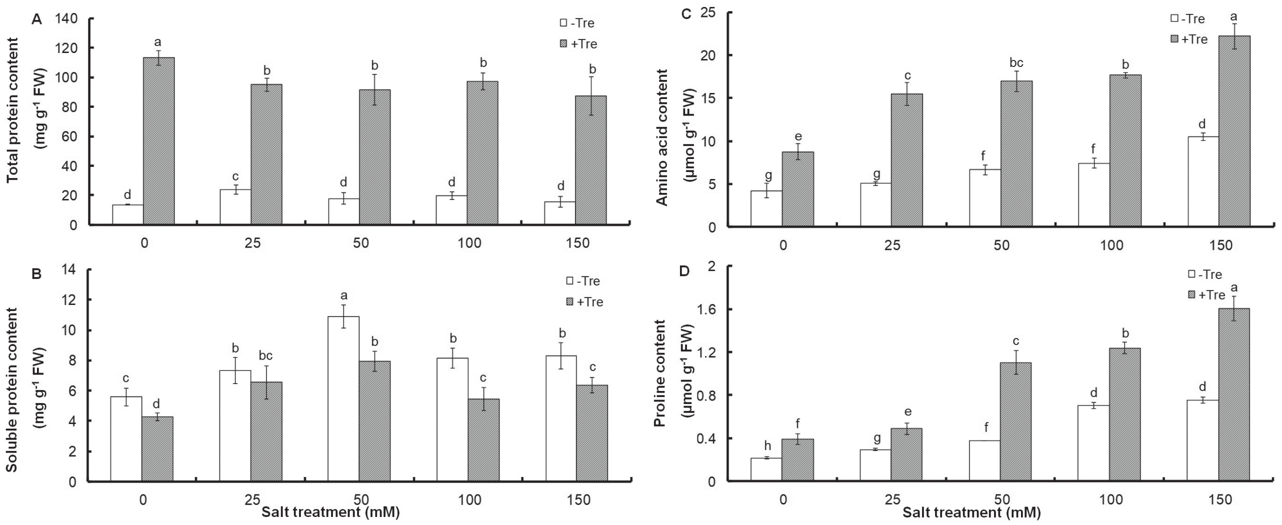

Fig. 3. Effect of different salinity concentrations on contents of total proteins (A), total soluble proteins (B), total amino acids (C), and proline (D) in trehalose-treated (+Tre) or untreated (-Tre) basil seedlings. Results are the mean ± standard deviation of three separate measurements. Different letters indicate significant differences between the various treatments at P < 0.05 according to the Duncan test.

Effect of salinity and Tre on enzyme activities

The activity of antioxidant enzymes including APX, PPX, and PPO increased strongly with salt concentration and reached 144, 179, and 218% at 150 mM NaCl compared to the control (Fig. 4).

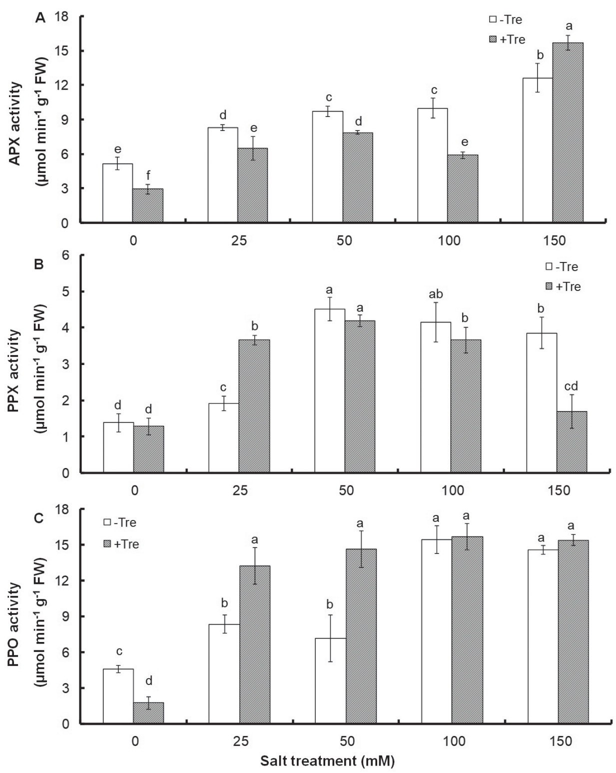

Fig. 4. Effect of different salinity concentrations on the activity of ascorbate peroxidase, APX (A), pyrogallol peroxidase, PPX (B), and polyphenol oxidase, PPO (C) in trehalose-treated (+Tre) or untreated (-Tre) basil seedlings. Results are the mean ± standard deviation of three separate measurements. Different letters indicate significant differences between the various treatments at P < 0.05 according to the Duncan test.

Tre treatment alone or with concentrations of up to 100 mM salt strongly decreased APX activity but increased its activity at 150 mM salt concentration by 24% compared to untreated control (Fig. 4A). PPX enzyme activity did not change in plants treated with Tre alone, but it was strongly increased at 25 mM salinity by 92%, slightly decreased at higher salt concentrations, and significantly decreased at 150 mM salinity by 56% compared to untreated control (Fig. 4B). The activity of PPO enzyme showed a 62% decrease in Tre-treated seedlings compared to untreated ones under non-stress conditions, but it increased significantly by 58 and 104% at concentrations of 25 and 50 mM salt, respectively, and remained unchanged at higher salinities (Fig. 4C).

Discussion

Much research has documented the negative effect of salinity on plant growth and biomass (Ahmad and Jhon 2005, Yoon et al. 2009, Qiu et al. 2014, Ahmad et al. 2018, Scagel et al. 2019, Sheyhakinia et al. 2020). Despite the reduction of some growth indices in basil seedlings under saline conditions, root length, leaf characteristics, and shoot water content did not change and root dry weight increased. Considering the lack of change in the length of the root and also the decrease in the ratio of fresh weight to dry weight of the root under salinity, which is due to a decrease in fresh weight and increase of dry weight, the increase of root biomass can be caused by the accumulation of nutrients in the organ during stress. In fact, salinity causes a change in the pattern of carbon allocation between roots and shoots. This change in the pattern of carbon distribution during salinity is associated with the reduction of shoot biomass. An increase in root biomass and a decrease in shoot biomass under salt stress have been reported (Bernstein and Kafkafi 2002, Imada et al. 2015). Since this process is similar to the responses of roots and shoots to soil water availability (Zhang et al. 2005, McCarthy and Enquist 2007, Imada et al. 2008), one of the reasons is probably the reduction of soil moisture absorption (McCarthy and Enquist 2007). In fact, the inhibition of growth induced by salinity may be related to the reduction of water absorption due to the reduction of soil osmotic potential. Such a phenomenon leads to cell dehydration and loss of turgor pressure, which leads to growth arrest (Zhao et al. 2021). In addition, salinity leads to the accumulation of Na^+ and Cl^- ions, which reduces the absorption and transport of nutrients due to competitive interactions with their transporters (Zhao et al. 2021, Gao et al. 2022). However, in our results, salinity-induced growth reduction was observed without a change in shoot water content, indicating that this reduction is associated with continuous water uptake and maintenance of shoot turgor pressure, as previously documented (De Costa et al. 2007, Alisofi et al. 2020, Sheyhakinia et al. 2020). Accumulation of proline in basil seedlings under salinity can explain the uninterrupted water uptake and thus the maintenance of shoot water content despite growth limitation. Therefore, changes in carbon partitioning and accumulation of osmolytes to continue water absorption by plants can be the main reason for growth inhibition under saline conditions. The results of studies on *Hibiscus sabdariffa* (Sheyhakinia et al. 2020) and *Momordica charantia* (Alisofi et al. 2020) under salt stress are in good agreement with our findings.

Contrary to previous studies on the effect of Tre on plant growth under salinity stress (Mostofa et al. 2015, Abdallah et al. 2020), Tre treatment in most salinities decreased shoot length and fresh and dry weight of shoots and roots in basil seedlings, so that the dry weight reduction was more severe. Early experiments on the effect of Tre on higher plants through inhibition of trehalase (a Tre-degrading enzyme) in species with very low trehalase activity showed that Tre accumulation is toxic or at least Tre acts as a plant growth inhibitor possibly through inhibition of cell wall biosynthesis (Veluthambi et al. 1981, Veluthambi et al. 1982a). This effect is associated with a disturbance in carbohydrate metabolism as indicated by a decrease in sucrose content (Veluthambi et al. 1982b). Therefore, the reduction of growth in basil seedlings due to Tre treatment can be related to the low activity of this enzyme. Meanwhile, in plant species with high trehalase enzyme activity, such as *Raphanus sativus, Quamoclit phoenicea, and Zea mays*, toxicity and inhibition of growth by Tre were not observed (Veluthambi et al. 1981). In transgenic *Arabidopsis* seedlings in which trehalase enzyme activity was overexpressed, there was no effect of growth inhibition in the presence of Tre, unlike the control (Schluepmann et al. 2004). Therefore, the decrease of growth induced by Tre can be due to the plant's inability to metabolize it, which leads to the accumulation of trehalose-6-phosphate and subsequently to the reduction of the glucose-6-phosphate pool (Schluepmann et al. 2004). In addition, it has been found that trehalose-6-phosphate induces plant defense responses, which are associated with growth reduction (Reignault et al. 2001, Brodmann et al. 2002, Renard-Merlier et al. 2007). However, Tre did not affect SWC in basil seedlings up to 100 mM NaCl, but increased it in seedlings exposed to 150 mM NaCl. This event is associated with proline hyperaccumulation, so it can be concluded that changes in carbon partitioning and directing it to proline biosynthesis are the reason for Tre-induced growth inhibition under saline conditions. Considering the role of Tre and its metabolism in the regulation of growth and development (Nunes et al. 2013, O'Hara et al. 2013), it is likely that this metabolite is involved in the growth regulation processes of basil seedlings. In this regard, it has recently been found that methyl jasmonate treatment not only does not reduce the harmful effects of salinity stress in radish (*Raphanus sativus*) but can even inhibit plant growth (Henschel et al. 2023). This shows that signal molecules and osmolytes such as Tre, which usually increase tolerance to environmental stresses, do not work in the same way in all plants and their performance against different stresses can depend on the type of plant species.

It has been found that the concentration of photosynthetic pigments is sensitive to environmental stresses and in most plants, it decreases significantly in response to salinity (Ahmad and Jhon 2005, Gunes et al. 2007, Yoon et al. 2009, Qiu et al. 2014). However, a study on tomato has shown that 0.3 M salt concentration induces Chl production per unit of leaf area (Agong et al. 2004). This shows that very low salinity induces Chl biosynthesis in basil, as observed in the concentration of Chl *a* and total Chl at 25 mM NaCl. In addition, the observed decrease in Chl *a/b* and Chl / Car ratios in response to salinity shows the greater sensitivity of Chl *a* compare to Chl *b* and Chl to Car. Contrary to current studies on the role of Tre in increasing the photosynthetic pigments of different plants under salinity (Theerakulpisut and Phongngarm 2013, Abdallah et al. 2016, 2020), treatment of basil seedlings with Tre in most salinities showed a significant decrease in pigment content compared to untreated plants. The increase in the ratio of Chl *a/b* in Tre-treated seedlings under non-stress conditions or not changing it at the concentration of 25 mM indicates the greater sensitivity of Chl *b* to Tre. It has been found that the biosynthesis of Chl *b* is carried out through the oxidation of a methyl group to a formyl group on the B ring of the Chl *a* molecule (Porra et al. 1994). Therefore, it can be assumed that the enzyme(s) involved in Chl *a* biosynthesis are more active than Chl *b* due to Tre treatment alone or in combination with low salinity. However, the sharp decrease of this ratio in salt concentrations of 50 mM and higher shows the greater sensitivity of Chl *a* to Tre. The change pattern of the Chl to Car ratio also shows the lower sensitivity of Car to Tre in non-stressed conditions or in combination with low salinity and its greater sensitivity in higher salinity concentrations. Considering the negative effect of Tre on the growth of basil seedlings under salt stress, the reduction of photosynthetic pigments can be attributed to the inability of plants to properly metabolize Tre or direct carbon to other metabolic pathways.

Accumulation of sugars or osmotic regulators in plant cells under salinity is a strategy to control plant water content and inhibit water loss (Chaves et al. 2009). The role of the accumulation of soluble sugars in salinity stress tolerance has been widely studied (Mishra et al. 2008, Yin et al. 2010). This indicates the type of basil strategy in salinity tolerance that is done through the accumulation of soluble sugars. Considering the sharp decrease in the ratio of RS/NRS under salt stress compared to the control, it can be concluded that the accumulation of NRS occurs at a higher rate than RS during salt treatment. In addition, the numerical comparison of the amount of these sugars in response to different salt concentrations shows that the accumulation of NRS is almost twice as much as RS. This indicates the important role of non-reducing sugars in basil seedlings for salinity tolerance. On the other hand, the increase of soluble sugars during salinity stress is associated with the decrease in starch content. This suggests that a change in photosynthetic carbon partitioning occurs in basil seedlings during salinity stress, leading to more sucrose synthesis and less starch accumulation. The higher accumulation of NRS than RS confirms this. However, starch breakdown can also increase soluble sugars during salt stress. The conversion of starch to sugars, especially NRS, and a change in their metabolism, which has been introduced as a common defense strategy against water stress, is possible through increasing the activity of starch hydrolyzing enzymes such as amylases and simultaneous decrease of sucrose hydrolyzing activities (Kumari and Asthir 2016). The unremarkable increase of NRS content in Tre-treated seedlings under salinity compared to the untreated controls indicates that this metabolite does not accumulate in basil seedlings. This finding can confirm the hypothesis that Tre accumulates in some Tre-treated plants in another form such as trehalose-6-phosphate (Schluepmann et al. 2004). This proves that the effects of Tre on growth are due to changes in carbohydrate metabolism. Evidence to support this hypothesis comes from the observation that the simultaneous addition of sucrose in the presence of Tre and starch accumulation restores growth in response to Tre feeding (Wingler et al. 2000). Tre feeding leads to an increase in ADP-glucose pyrophosphorylase gene expression as well as an increase in its enzyme activity, which is crucial in starch biosynthesis (Wingler et al. 2000). In addition, trehalose-6-phosphate also plays a role in the activation of this enzyme (Kolbe et al. 2005). Various studies have shown that growth was impaired after an increase of the gene expression of this enzyme in potato plants, but with the addition of sucrose, growth returned to the normal state (Stark et al. 1992). Accordingly, one of the reasons for Tre-induced growth inhibition during salt treatment can be due to the reduction of carbon for export to growth areas because of starch accumulation.

Environmental stresses, including salinity, generally cause protein degradation or reduced synthesis due to the acceleration of the aging process (Mishra et al. 2008, Misra and Saxena 2009). The results of our study on basil seedlings showed the opposite of this finding, which could indicate an increase in the synthesis of stress-specific proteins or enzyme proteins during the salinity period. The observed decrease in soluble protein levels, which was accompanied by a significant increase in total protein content in Tre-treated seedlings, could be attributed to a change in their solubility due to Tre treatment. This suggestion is supported by the fact that the level of soluble protein is positively correlated with protein solubility (Afify et al. 2012, Ma et al. 2019, Ebert et al. 2020). Therefore, the increase in total protein while the soluble protein decreased can be due to the role of Tre in reducing the solubility of proteins. The response of basil seedlings to Tre treatment can confirm that the pathway of carbon partitioning and its metabolism is changed due to Tre treatment, which in turn reduces growth in Tre-treated seedlings under salinity conditions. Similar results regarding the effects of Tre on salinity tolerance and its role in carbon partitioning have also been reported in *Dunaliella bardawil* (Panjekobi and Einali 2021).

Accumulation of amino acids and proline is considered a common response during environmental stress, and is often associated with the improvement of plant tolerance to stress conditions (Claussen 2005, Khadri et al. 2006, Yoon et al. 2009). However, some studies have shown that the level of accumulated proline indicates the severity of stress symptoms when plants are exposed to different types of abiotic stresses (Metwally et al. 2003, Mostofa et al. 2014, 2015). A study on *Cathranthus roseus* under salt stress showed that proline accumulation under stress conditions is negatively correlated with relative water content, biomass, and potassium accumulation (Chang et al. 2014). However, the lack of change in SWC in saline conditions or its increase due to Tre treatment and high salt dose, which is associated with proline accumulation, can indicate the increase of this metabolite in order to maintain the water content of the plant. Therefore, in our study, the excessive accumulation of amino acids and proline that occurred especially due to Tre treatment is related to the maintenance of water content, which is caused by Tre-induced metabolic changes during salt stress. In this way, the increase of these metabolites in seedlings treated with Tre alone also indicates a kind of stressful condition. On the contrary, studies on plants treated with Tre under salt (Nounjan et al. 2012, Mostofa et al. 2014, Sadak, 2019) and drought stress (Ali and Ashraf 2011) show a lower level of proline or amino acid in these plants, which could indicate a lower demand for proline or a compensatory mechanism for Tre, because both can act as an osmoprotectant.

The increased activity of antioxidant enzymes in response to salinity can be considered as an indication of increased ROS production and a common protective mechanism to reduce oxidative damage caused by salinity. These results are consistent with other studies on the role of ROS-scavenging enzymes under salt stress (Ahmad et al. 2018, Alisofi et al. 2020, Sheyhakinia et al. 2020). Different activities of antioxidant enzymes in response to Tre under salt stress have been reported in various plants. Tre treatment decreased the activity of peroxidase, catalase, and superoxide dismutase enzymes in rice under salt stress (Rohman et al. 2019). In another study, the treatment of *Chenopodium quinoa* with Tre under salt stress increased the activity of APX, catalase and superoxide dismutase enzymes (Abdallah et al. 2020). In addition, an increase in the activity of antioxidant enzymes has been observed with Tre treatment under different stress conditions (Zhao et al. 2019, Liu et al. 2020). However, in all these studies, Tre-treated plants showed tolerance to salt stress, which can indicate the existence of different strategies in plants to cope with salinity in the presence of Tre. In contrast, our results showed that the changes in enzyme activity patterns induced by Tre treatment do not indicate increased tolerance to salt stress, because these patterns are not correlated with other indicators showing stress reduction.

Conclusion

The results of this study show that salt stress without change in SWC decreases growth characteristics, photosynthetic pigments, and starch content, but increases the activity of ROS-scavenging enzymes and the accumulation of metabolites including soluble sugars, proteins, free amino acids, and proline. Tre treatment not only does not reduce the adverse effects of salinity but even causes more severe inhibition of plant growth, further reduction of photosynthetic pigments and soluble proteins along with the excessive accumulation of free amino acids and proline. Therefore, Tre treatment is not effective in salinity tolerance of basil seedlings and reduces their growth possibly through diverting carbon to other metabolic pathways rather than growth processes. However, due to the effect of Tre on the change in carbon partitioning, the role of this molecule in various metabolic and physiological pathways is obvious, the detailed understanding of which requires further research on the physiological effects of Tre under stress and non-stress conditions.

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Author contribution statement

R. Karamzehi carried out all the laboratory research. A. Einali designed the experiment, provided all the technical support during the laboratory work, analyzed data and wrote the manuscript. All authors have read and approved the submitted manuscript.

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Mitigation of cadmium toxicity stress by magnetopriming during germination of soybean

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Abstract – Cadmium (Cd) is a highly toxic heavy metal that poses a significant threat to food safety and agricultural production worldwide. Its solubility enables easy entry into plants, leading to reductions in seed germination, growth and crop yield. Thus, it is crucial to discover methods to alleviate the harmful impact of Cd on plant growth. Magnetopriming (MP) is a non-invasive and cost-effective technique that has been proposed to improve plant growth under abiotic stress conditions. The current study investigated the potential of MP to improve the seed germination, early seedling growth and biochemical responses of soybean under Cd toxicity. Soybean seeds were magnetoprimed with static magnetic field (SMF) strength of 200 mT for 1 hour before germination. The unprimed and magnetoprimed seeds were grown under different concentrations of cadmium chloride (0, 25, 50, 75, 100, 200 and 300 μM $CdCl_2$) in Petri plates for five days in the dark at 25 + 1 °C. The results revealed that Cd toxicity significantly reduced the germination percentage and inhibited the seedling growth parameters and increased oxidative stress, as determined by malondialdehyde (MDA) content in soybean seedlings from unprimed seeds. The inhibitory effect was increased with increasing concentration of Cd. However, MP remarkably increased the germination percentage, seedling growth parameters, activities of total amylase and protease, and hydrogen peroxide (H_2O_2) content and decreased MDA and proline content in germinating soybean seedlings at all concentrations of Cd. These findings suggest that MP can alleviate the adverse effects of Cd stress in soybean seedlings and increase the tolerance index towards Cd toxicity by enhancing the activity of amylotic and proteolytic enzymes and reducing oxidative stress.

Keywords: cadmium toxicity, germination, lipid peroxidation, magnetopriming, tolerance index

Introduction

Abiotic stressors, which include temperature extremes, water availability, salinity, heavy metals, and UV radiation, can have detrimental effects on plants and result in significant crop yield losses (Saharan et al. 2022). Heavy metals such as arsenic, cadmium, lead, and mercury are toxic to plants and animals even at low concentrations and can accumulate in the environment from human activities such as mining, industrial discharge, and use of pesticides and fertilizers (Alengebawy et al. 2021, Singhal et al. 2023). Cadmium (Cd) is a nonessential, heavy metal element and a widespread environmental contaminant that is persistent, non-biodegradable, bio-accumulative and highly toxic even at low concentrations (Hussain et al. 2019, Mahawar and Shekhawat 2023). Cd toxicity, can affect many physiological and morphological processes in plants, including plant growth, respiration, water and nutrient uptake, and root growth (Chen et al. 2011, Liu et al. 2012, Sheirdil et al. 2012). Cd causes adverse effects on germination, seedling vigour index, and plant growth (Sheirdil et al. 2012, He et al. 2014). Plants exposed to Cd have damaged photosynthetic apparatus and inhibited chlorophyll biosynthesis, which negatively impacts growth and biomass, leaf chlorophyll fluorescence, and photosynthetic parameters. (Ci et al. 2010, Xue et al. 2014, Yang et al. 2015, Mahawar et al. 2021). Cd contamination in agricultural soils limits crop production, which is a major concern given the increasing demand for food worldwide.

Soybean (*Glycine max* (L.) Merr.) is the most significant legume crop in the world, as 20% of the seed is oil and 40% is high-quality protein (Ferguson and Gresshoff 2009). It plays a significant role in the global oilseed agriculture landscape because of its high productivity, profitability, and essential role in preserving soil fertility. The increase in Cd concentration significantly inhibited seed germination, root

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and shoot growth in soybean plants (Sheirdil et al. 2012). Therefore, it is crucial to explore the approaches to alleviating heavy metal toxicity in soybean. Seed priming has proven to be an effective strategy for increasing seed vigour and germination, as well as seedling growth and field establishment in unfavourable conditions of heavy metal toxicity (Prajapati et al. 2020). It appears that seed priming is a helpful method for reducing cadmium toxicity in plants (Singhal et al. 2023). In the field of seed technology, physical techniques have proven to be much superior to conventional protocols of osmo, chemical, hydro and hormonal priming (Bilalis et al. 2012). One of the most researched physical pre-sowing seed treatments in agriculture is based on the use of magnetic field (MF) for the alleviation of heavy metal toxicity (cadmium, arsenic and mercury) in plants (Chen et al. 2011, Fatima et al. 2020, Prajapati et al. 2023).

Magnetopriming (MP) has a significant effect on a number of processes, including morpho-structural elements (seed germination, plant growth, and yield) and modifications in the expression of genes linked to nitrogen metabolism and seed germination in non-stress and abiotic stress conditions (Kataria et al. 2020, Sarraf et al. 2020, Raipuria et al. 2021). According to some theories, MF could attract iron particles, growth hormones, and carbohydrates (Xue et al. 2014). It may also have an impact on transcription-related factors and gene expression processes (Stutte et al. 2006, Kataria et al. 2020). Additionally, it might immediately trigger the production of calcium ions (Ca^{2+}) in seed embryos (Kataria et al. 2023). The germination of seeds and the physiological growth of tomato plants are significantly impacted by neodymium earth magnets by the stimulation of seed germination and speed of germination by 50% and production of larger leaf areas and more root hairs in the growing plantlets (Abhary and Akhkha 2023). It was earlier reported that MP mitigates the adverse effect of cadmium stress in mung beans by reducing the level of malondialdehyde, nitric oxide, hydrogen peroxide and superoxide radical content and increased the growth and photosynthetic performance in mung bean plants (Chen et al. 2011). However, the mitigating effect of MP during seed germination has not been reported for heavy metal toxicity especially for cadmium in soybean seedlings. Hence the objective of the present study is to examine the impact of MP on seed germination, seedling growth and biochemical parameters in soybean under cadmium toxicity and to reveal if MP could enhance tolerance of soybean towards Cd toxicity during early seedling stage.

Materials and methods

Plant material

Breeder seeds of soybean (*Glycine max* [L.]) var. JS-9560 (commonly grown variety of soybean in the Malwa region, Indore, Madhya Pradesh, India), were obtained from the ICAR-Indian Institute of Soybean Research, Khandwa Road, Indore, M.P., India.

Magnetic field treatment

To pre-treat the seeds with a static magnetic field (SMF), we used an electromagnet (AETec), fabricated by the Academy of Embedded Technology in Delhi, India. The pole components measured 16 cm in length and 9 cm in diameter while the coil consisted of 3000 turns and a resistance of 16 Ohms. Nearly 100 dry seeds of soybean at a time were placed in a cylindrical sample holder made of thin transparent plastic between the two poles of the electromagnet for magnetopriming, while maintaining the temperature at 25 ± 1 °C. The distance between the two poles was 5 cm. The SMF was developed using a direct current (DC) power supply (80 V/10 A) with a continuously changing output. Based on previous studies, soybean seeds were treated with 200 mT of SMF for an hour (Kataria et al. 2020). The SMF strength was obtained by adjusting the current and voltage by power supply and the obtained SMF strength was measured using a digital gauss meter (AETech model DGM-102) with a probe consisting of an indium arsenide crystal encapsulated by a 5×4×1 mm non-magnetic sheet. The local geomagnetic field was less than 10 mT in the north to south direction. With the exception of the SMF treatment (200 mT for 1 hour) for primed seeds, all other conditions were the same for unprimed and magnetoprimed seeds. Every experiment was carried out concurrently with unprimed seeds (which were used as controls) stored at room temperature (25 ± 1 °C) and kept away from the magnetic field (less than 5 mT).

Treatment to induce cadmium toxicity

To conduct the germination experiment, both magnetoprimed and unprimed soybean seeds were first surface--sterilized with 0.01% $HgCl_2$ for 2 minutes, followed by washing three times with distilled water. The fifteen seeds were then allowed to germinate on wet Whatman filter paper in each Petri plate (15 cm diameter) in three replications with 10 mL of different concentrations of $CdCl_2$ (25, 50, 100, 200, and 300 µM) while 10 mL of distilled water was used as a control (0 µM). The seeds were kept for germination in darkness at 25 ± 1 °C for 5 days in the incubator. Germination percentage was calculated based on the number of normal seedlings, as the ratio of the number of germinated seeds to the total number of seeds on the third day of imbibition in different concentrations of $CdCl_2$.

Early growth characteristics of seedlings

To estimate the effect of magnetopriming on soybean seedling growth, ten seedlings were randomly selected from each treatment (magnetoprimed and unprimed seeds germinated under different concentrations of $CdCl_2$) in biological triplicates (N = 3). The length of shoot and root, along with the length of the whole seedling, were measured for each selected seedling. Fresh weight was also measured. For dry weight determination, the seedlings were dried in an oven at 80 °C for 72 h and weighed. The vigour index was calculated using the formulae proposed by Abdul-Baki and Anderson (1973):

Vigour index I = germination % × seedling length (root + shoot) cm

Vigour index II = germination % × seedling dry weight (root + shoot) g

Vigour index I is the multiple of the percentage of germination and seedling length, and Vigour index II is the multiple of percentage of germination and seedling dry weight.

Enzyme activities during germination

Total amylase and protease activities were measured in the soybean seedlings derived from unprimed and magnetoprimed seeds after 5 days (120 h) of imbibition under different $CdCl_2$ concentrations. After 5 days of imbibition, the fresh seedlings with root, shoot and cotyledons were taken for analysis of enzymes and biochemical parameters. Each experiment was repeated in three biological replicates with three technical replicates.

The assay of total amylase activity was performed using the method of Sawhney et al. (1970). The seedling homogenate (100 mg) was mixed with 5 mL of 80% chilled acetone and centrifuged at 15000 rpm for 10 min at 4 °C. The resulting pellet was dissolved in 10 mL of 0.2 M phosphate buffer (pH 6.4) and centrifuged again at 15000 rpm for 20 min at 4 °C. The amylase activity in the obtained supernatant was then determined by the addition of 2 mL of phosphate buffer (pH 6.4), 1 mL of starch (1%), and incubation for 30 min at room temperature. Subsequently, 1 mL of 0.1 M HCl and 0.1 mL of 0.1 M potassium iodide were added to the reaction mixture and the absorbance was measured at 660 nm using a spectrophotometer. The total amylase activity was expressed as mg starch hydrolysed g^{-1} fresh weight of seedlings h^{-1}.

The assay of protease activity was carried out according the method of Kunitz (1947) modified by Kataria et al. (2023). The 1.0 g seedlings were crushed in 0.2 M phosphate buffer (pH 7.4) and centrifuged at 13800 rpm at 4 °C for 30 min. To 0.5 mL of supernatant, 0.5 mL of casein (1%) prepared in 0.2 M carbonate buffer (pH 9.2) was added and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by the addition of 1 mL of 10% trichloroacetic acid (TCA) and centrifugation at 13800 rpm for 10 min at 4 °C. After centrifugation, 2.5 mL of carbonate buffer (0.44 M, pH 9.5) was added and the development of a blue colour was observed upon addition of 0.5 mL Folin's reagent followed by incubation for 30 min at room temperature. The protein content was measured at 660 nm against carbonate buffer (0.44 M, pH 9.5) and Folin's reagent taken as a blank and the protease activity was represented as mg protein hydrolysed g^{-1} fresh weight of seedlings.

Determination of biochemical parameters

Thiobarbituric acid reactive substances (TBARS) were used to measure the level of malondialdehyde (MDA), according to the method described by Heath and Packer (1968). Soybean seedlings (100 mg) were homogenized in 1 mL of 0.1% (w/v) TCA, and the resulting homogenate was centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was used for the TBARS assay. Specifically, 0.5 mL of the supernatant was mixed with 1 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA) prepared in 20% TCA and incubated in a boiling water bath for 30 min. The reaction was stopped by placing the tube in an ice bath, and the mixture was then centrifuged at 12000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm, and the value for the non-specific absorbance read at 600 nm was subtracted. TBA (0.5%) in TCA (20%) was taken as blank. The amount of MDA was calculated using an extinction coefficient ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$) and expressed as μmol MDA mg^{-1} fresh weight of seedlings.

The proline content was calculated using the method of Bates et al. (1973). Using a mortar and pestle, 500 mg of soybean seedlings were homogenised in 10 mL of 3% (w/v) aqueous sulphosalicylic acid; the homogenate was then centrifuged for 5 min at 4 °C at 10000 rpm. The proline was estimated using the supernatant. Two mL each of glacial acetic acid, acid ninhydrin reagent, and supernatant were combined. The mixture was boiled at 100 °C for 1 h. The reaction was terminated by cooling in an ice bath and 4 mL of toluene was added. Following thorough mixing, the toluene-containing chromophore was isolated, and the red colour generated absorbance was measured at 520 nm using toluene as the blank. The proline content was calculated through the standard curve prepared in the range of 5–25 μg proline and expressed as μg proline g^{-1} fresh weight of the seedlings.

The estimation of hydrogen peroxide (H_2O_2) was carried out by the method described by Mukherjee and Choudhuri (1983), which involves the formation of a titanium-hydroperoxide complex. First, 0.5 g of soybean seedlings were crushed in chilled acetone (5 mL) and filtered using Whatman No.1 filter paper. The filtrate (5 mL) was mixed with titanium reagent (2 mL; containing 5% (w/v) titanium oxide and potassium sulphate digested in concentrated sulphuric acid) and ammonium hydroxide solution (2.5 mL) to precipitate the titanium-hydroperoxide complex. The resulting precipitate was centrifuged at 13800 rpm for 15 min at 4 °C, and the pellet was resuspended in concentrated sulphuric acid (2 M) and centrifuged again. The absorbance of the supernatant was then measured at 415 nm against sulphuric acid (2 M) using a Shimadzu Spectrophotometer (UV-1800) and the amount of H_2O_2 was calculated and expressed as μmol g^{-1} fresh weight.

Tolerance index (TI)

Tolerance index (in terms of root length) of seedlings from unprimed and magnetoprimed seeds grown under different concentrations of $CdCl_2$ was determined with the following formula given by Iqbal and Rahmati (1992):

Tolerance index (T.I.) = mean root length in metal solution / mean root length in control × 100

Statistical analysis

Three replications and fully randomised designs were used to arrange the samples. The mean ± standard error (S.E.) of the three biological replicates (N = 3) was used to show the data. Ten seedlings were taken for each replication in order to measure the many aspects of seedling growth, including length of seedlings, root and shoot, fresh and dry weight of seedling, and vigour indices. The mean of three seedlings in each replica serves as the data for the biochemical analysis. The data were analysed by Student's t-test and significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as following $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistically significant differences between magnetoprimed and unprimed seedlings grown in non-stress as well as Cd-stress conditions are marked as $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

Results

Effect of MP on seed germination and seedling development under cadmium treatments

Germination percentage decreased with increasing $CdCl_2$ concentration in magnetoprimed and unprimed seeds of soybean. A remarkable decrease of 50% was observed at 100 µM $CdCl_2$ and 72% was observed at 200 and 300 µM $CdCl_2$ in unprimed seeds (Fig. 1a). Magnetopriming of seeds enhanced percentage germination at all the concentrations of $CdCl_2$ used as compared to respective unprimed seeds. Maximum enhancement in percentage germination by MP (41%, 66% and 33%) was obtained at 100, 200 and 300 µM $CdCl_{2,}$ respectively (Fig. 1a).

Root, shoot and seedling lengths were reduced with increasing $CdCl_2$ concentration in magnetoprimed and unprimed seeds of soybean (Fig. 1b-1d). The decrease in root length was more prominent than the decrease in shoot length. Maximum decreases of 75% and 93% were observed in root length of seedlings from unprimed seeds at 100 and 200 µM $CdCl_2$ and root growth was completely inhibited at 300 µM $CdCl_2$ (Fig. 1b). Magnetoprimed soybean seeds demonstrated higher shoot and root length at all the concentrations of $CdCl_2$ used as compared to corresponding controls (Fig. 1b, 1c). Magnetopriming caused increases of 18, 19 and 24% in shoot length of seedlings at 50, 100 and 200 µM $CdCl_{2,}$ respectively (Fig. 1b). Similarly, significant promotion in root length by 55, 113 and 240% at 50, 100 and 200 µM $CdCl_2$ respectively was obtained in seedlings from magnetoprimed seeds as compared to the seedlings of unprimed seeds (Fig. 1c).

There was substantial decrease in length of seedlings observed at a higher concentration (300 µM) of $CdCl_2$ (Fig. 1d). The $CdCl_2$ at 100, 200 and 300 µM concentrations caused significant inhibition of seedling length by 51%, 73% and 80% respectively. On the other hand, a maximum increase of 31% was observed at 100 µM $CdCl_2$ followed by a 59% increase at 200 µM $CdCl_2$ in magnetoprimed seeds (Fig. 1d).

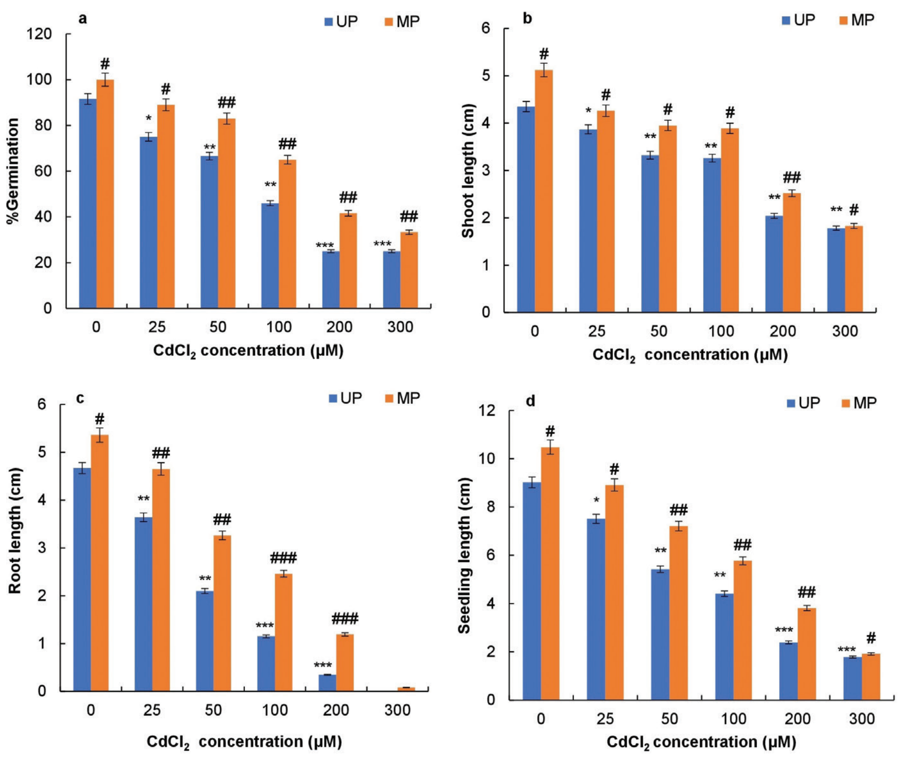

Fig. 1. Effect of magnetopriming on percentage germination (a), shoot length (b), root length (c) and seedling length (d) of soybean seedlings under Cd toxicity. The vertical lines on the bar indicate ± S.E. for mean of triplicates (N = 3). Significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistically significant differences between magnetoprimed (MP) and unprimed (UP) seedlings grown in non-stress as well as Cd-stress conditions are marked as $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

Effect of MP on fresh weight and dry weight of soybean seedling under cadmium treatments

Fresh and dry weight of unprimed and primed seeds gradually decreased with increasing $CdCl_2$ concentration (Fig. 2a, 2b). $CdCl_2$ treatments caused reduction in fresh weight by 26%, 35% and 44% respectively at 100, 200 and 300 µM $CdCl_2$ concentration as compared to the controls (Fig. 2a). Further, there was significant increase in fresh and dry weight of seedling observed in primed seeds as compared to unprimed ones at all the concentrations of Cd used. Maximum of 24, 37, 42 and 21% promotion in fresh weight of seedlings was found in magnetoprimed as compared to unprimed seed respectively at 50, 100, 200 and 300 µM $CdCl_2$ (Fig. 2a). Similar results were observed for dry weight of seedlings (Fig. 2b).

prominently by 75%, 92% and 94% respectively at 100, 200 and 300 µM $CdCl_2$ concentration in unprimed seeds compared to their controls (Fig. 3a). Similarly, vigour index-II decreased severely at 200 and 300 µM $CdCl_2$ concentrations by 83% and 87% respectively in seeds compared to the control (Fig. 3b). However, magnetoprimed seeds showed higher vigour indices at all $CdCl_2$ levels as compared to unprimed seeds. There was prominent increase in vigour index-I at 50, 100 and 200 µM $CdCl_2$ concentration (84%, 165% and 43% respectively) in magnetoprimed seeds as compared to unprimed seeds (Fig. 3a). Similarly, vigour index-II increased significantly at 100, 200 and 300 µM $CdCl_2$ concentration by 106, 147 and 135% respectively in magnetoprimed seeds as compared to unprimed seeds (Fig. 3b).

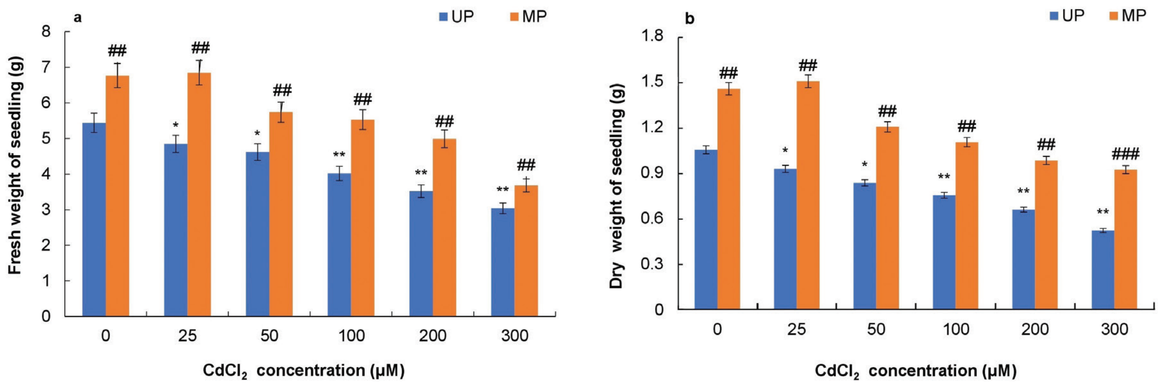

Fig. 2. Effect of magnetopriming on fresh weight (a) and dry weight (b) of soybean seedlings under Cd toxicity. The vertical lines on the bar indicate ± S.E. for mean of triplicates (N = 3). Significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as follows: $^{*}P < 0.05$, $^{**}P < 0.01$. Statistically significant differences between magnetoprimed (MP) and unprimed (UP) seedlings grown in non-stress as well as Cd-stress conditions are marked as $^{\#\#}P < 0.01$.

Effect of MP on vigour indices of soybean seedling under cadmium treatments

Treatment of soybean seeds with different concentrations of $CdCl_2$ reduced the seed vigour indices gradually from 0 to 100 µM $CdCl_2$, thereafter significant decrease was observed at 200 and 300 µM in both primed and unprimed seeds (Fig. 3a, 3b). Vigour index-I was found decreased

Effect of MP on total amylase and protease activities in soybean seedlings under cadmium treatments

When soybean seeds were stressed with $CdCl_2$, total amylase and protease activities decreased slightly in the seedlings from unprimed seeds as compared to control seedlings. However, primed seeds showed increased amylase activity by 49%, 51%, 64% and 70% at 0, 100, 200 and 300 µM

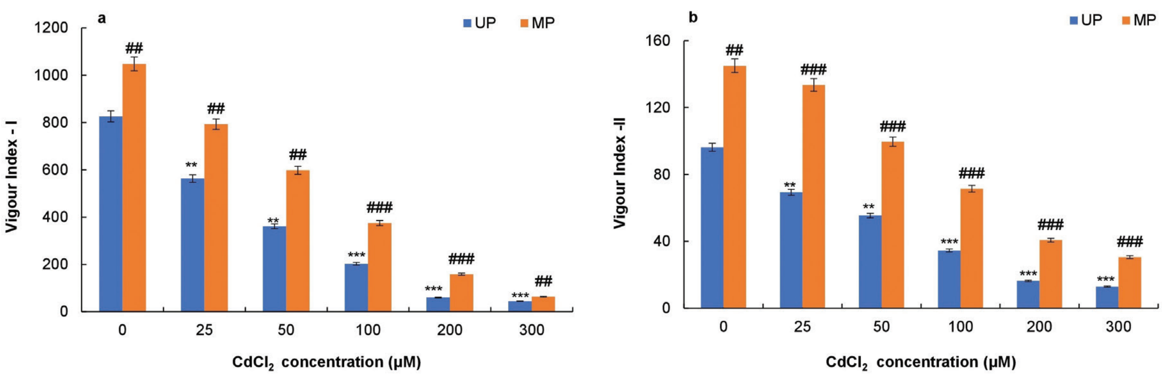

Fig. 3. Effect of magnetopriming on vigour index-I (a) and vigour index-II (b) of soybean seedlings under Cd toxicity. The vertical lines on the bar indicate ± S.E. for mean of triplicates (N = 3). Significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as follows: $^{**}P < 0.01$, $^{***}P < 0.001$. Statistically significant differences between magnetoprimed (MP) and unprimed (UP) seedlings grown in non-stress as well as Cd-stress conditions are marked as $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

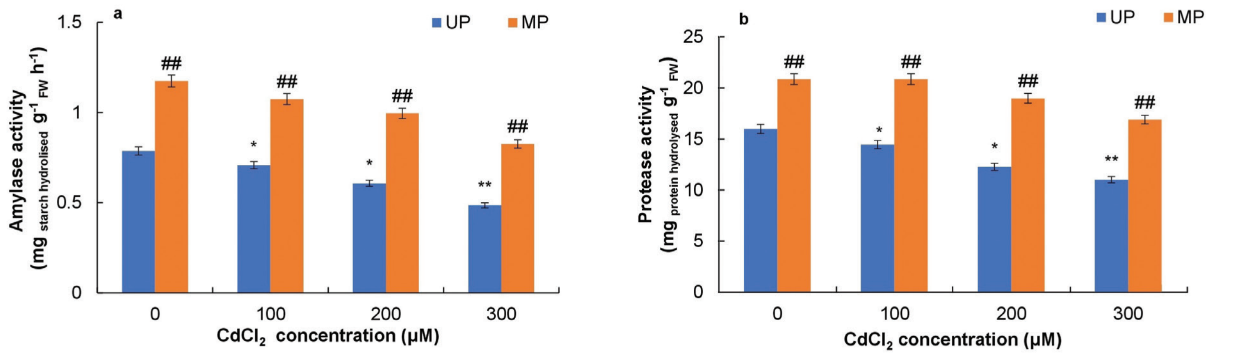

Fig. 4. Effect of magnetopriming on amount of starch hydrolysed (a) and protein hydrolysed (b) in soybean seedlings under Cd toxicity. The vertical lines on the bar indicate ± S.E. for mean of triplicates (N = 3). Significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as follows: *P < 0.05, **P < 0.01. Statistically significant differences between magnetoprimed (MP) and unprimed (UP) seedlings grown in non-stress as well as Cd-stress conditions are marked as ##P < 0.001. FW – fresh weigh.

$CdCl_2$ concentrations, respectively (Fig. 4a). Further, magnetoprimed seeds showed increased protease activity by 30, 44, 54 and 53% at 0, 100, 200 and 300 µM $CdCl_2$ concentrations, respectively (Fig. 4b).

Effect of MP on proline, MDA and H_2O_2 content in soybean seedlings under cadmium treatments

MDA content was found to rise gradually with increasing concentration of $CdCl_2$ in unprimed as well as primed seedlings. However, the level of MDA was higher in seedlings emerged from unprimed seeds at all the concentrations of $CdCl_2$ used (Fig. 5a). MDA content was decreased in the seedlings from SMF-primed seeds as compared to the seedlings from unprimed seeds at all the concentrations of $CdCl_2$ (0 to 300 µM) (Fig. 5a). A remarkable decrease in MDA content (by 37%) was observed at 300 µM $CdCl_2$ concentration in the seedlings from primed seeds as compared to the seedlings of unprimed seeds.

Proline content was considerably increased in seedlings of unprimed seeds at all the concentrations of $CdCl_2$ (Fig. 5b). The enhancement in proline content was 39, 92 and 100% in seedlings of unprimed seeds at 100, 200 and 300 µM $CdCl_2$ concentrations as compared to the control seedlings. Magnetopriming of seeds lowered the proline quan-

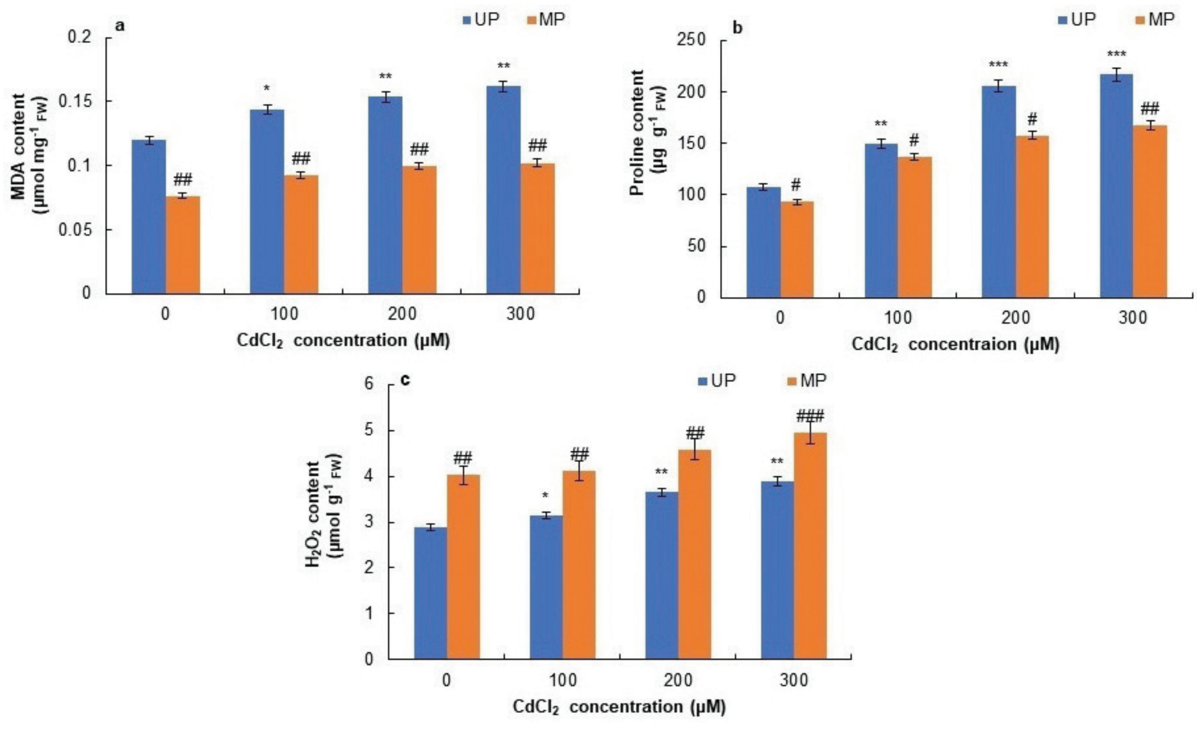

Fig. 5. Effect of magnetopriming on malondialdehyde – MDA (a), proline (b) and H_2O_2 (c) content of soybean seedlings under Cd toxicity. The vertical lines on the bar indicate ± S.E. for mean of triplicates (N = 3). Significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Statistically significant differences between magnetoprimed (MP) and unprimed (UP) seedlings grown in non-stress as well as Cd-stress conditions are marked as #P < 0.05, ##P < 0.01. FW – fresh weight.

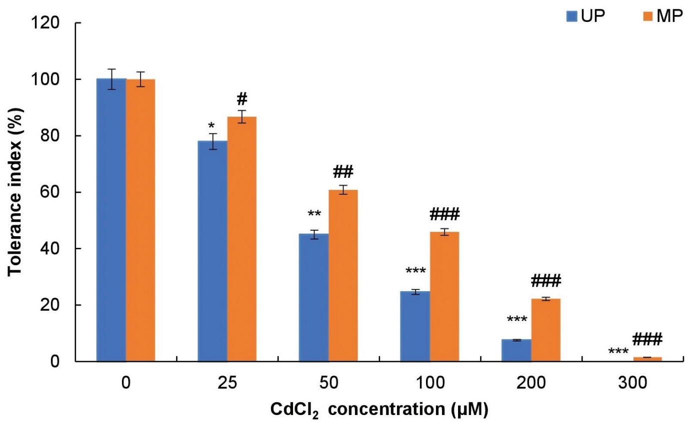

Fig. 6. Effect of magnetopriming on tolerance index of soybean seedlings in terms of root length under Cd toxicity. The vertical lines on the bar indicate ± S.E. for mean of triplicates (N = 3). Significant differences between non-stressed and Cd-stressed soybean seedlings that emerged from unprimed seeds are marked as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Statistically significant differences between magnetoprimed (MP) and unprimed (UP) seedlings grown in non-stress as well as Cd-stress conditions are marked as #P < 0.05, ##P < 0.01, ###P < 0.001.

tity at all concentrations of $CdCl_2$. There was 23% decrease in proline content at 200 µM and 300 µM $CdCl_2$ concentration in primed seeds (Fig. 5b).

The cadmium treatments increased the H_2O_2 content by 26% (200 µM $CdCl_2$) and 35% (300 µM $CdCl_2$) in the seedlings of unprimed seeds as compared to their controls (Fig. 5c). Magnetopriming also caused a further increase in H_2O_2 content in soybean seedlings in comparison to unprimed seeds at all the cadmium chloride concentrations.

Effect of MP on tolerance index (TI) of soybean seedlings under cadmium treatments

TI was 100% for control seedlings and it decreased with an increase in $CdCl_2$ toxicity (Fig. 6). The TI after treatment with 25, 50, 100, 200 and 300 µM $CdCl_2$ was 78, 45, 25, 8 and 0%, respectively, for soybean seedlings obtained from unprimed seeds (Fig. 6). Whereas seeds pre-treated with MP showed higher values of TI at all the levels of $CdCl_2$ i.e. 87, 61, 46, 22 and 2% TI compared to values from unprimed seeds at 25, 50, 100, 200 and 300 µM $CdCl_2$, respectively.

Discussion

Amongst heavy metals, Cd is a toxic environmental contaminant, which has detrimental effect on plants, animals and humans. It is often found in industrial and agricultural waste and can contaminate soil, water, and food (Yin et al. 2021). The germinating seed serves as the first point of interaction between the developing plant and its surrounding environment, making it particularly susceptible to the toxic effects of Cd (Stefanello 2019, Mahawar et al. 2021). Cd significantly inhibited the seed germination of various plant species such as swiss chard, lettuce, wheat, basil, chia, beans and spinach (Ahmad et al. 2012, Bautista et al. 2013, El Rasafi et al. 2016, Gharebaghi et al. 2017, Stefanello 2019).

The results of the present investigation also showed that, in seedlings derived from unprimed seeds, Cd treatments significantly inhibit seed germination and early seedling growth parameters (root, shoot, and seedling length, fresh and dry weight of seedlings, and vigour indices); a dose-response inhibition was noted for every parameter examined. Even in the presence of cadmium, however, MP of soybean seeds with SMF at 200 mT for an hour before germination improved the seeds' early development characteristics. The length of both shoot and root is critical for efficient material exchange and nutrient acquisition (Bewley and Black 1994, Stefanello 2019). The inhibition was more pronounced for root length in the seedlings from unprimed seeds grown under Cd treatments. At a higher concentration of Cd (300 mM $CdCl_2$), the root growth was completely inhibited. Interestingly, MP with SMF enhanced root length to a greater extent than the shoot length. In the present study, positive effects of MP were observed in soybean seedlings under Cd toxicity, such as increased seed germination percentage, root, shoot, and seedling length, as well as enhanced fresh/dry weight and vigour indices of seedlings. The inhibitory effects of Cd on shoot and root growth lead to stunted seedlings as Cd toxicity disrupts the water and nutrient uptake and transport (Liu et al. 2012, Sheirdil et al. 2012, Stefanello 2019). The increase in root length induced by MP can contribute to enhanced water and nutrient uptake and provide necessary resources for seedlings to cope with the toxic effects of Cd. However, the exposure of seeds to MF has earlier been shown to increase their capacity to absorb moisture and their nutrient content in soybean (Kavi 1977, Radhakrishnan and Kumari 2012). These effects on moisture absorption and physiological activity can contribute to improved germination and seedling vigour. There have also been reports on the effects of very low-frequency alternating magnetic fields on cell membrane ionic permeability (Khizhenkov et al. 2001, Thomas et al. 2013). Studies by Kataria et al. (2019, 2020, 2021, 2023) have confirmed that applying MP with SMF-pretreatment to the seeds can have a positive effect on seed germination and seedling vigour under salt stress. Our results are consistent with the findings of several earlier studies, including those on rice, wheat, maize, soybean and barley seeds, which have also revealed that MP can improve seed germination percentage, seedling growth and vigour index (Carbonell et al. 2000, Florez et al. 2007, Martinez et al. 2009, Shine et al. 2012, Kataria et al. 2015, Ercan et al. 2022). The tolerance index represents the ability of seedlings to withstand and survive under $CdCl_2$ toxicity, decreased with increasing concentrations of $CdCl_2$ in both unprimed and magnetoprimed seedlings. However, the seedlings from magnetoprimed seeds exhibited a higher tolerance index than unprimed seed at all the concentrations of Cd. This suggests that MP helped to mitigate the inhibitory effects of cadmium on seedling growth and increased their tolerance to Cd toxicity. Similarly, Kataria et al. (2020, 2022) have reported that the salt tolerance index of soybean seedlings was increased by MP due to the higher nitric oxide production by SMF pre-treatment, which helps to maintain the balance of abscisic acid (ABA) and gibberellic acid (GA) for higher seed germination and seedling growth under salt stress conditions.

Furthermore, high concentrations of Cd have negative effects on hydrolyzing enzymes including acid phosphatases (ACPs) and α-amylases, they prevent reserved carbohydrates from being hydrolyzed and from moving from the endosperm to the growing embryonic axis. As a result, the embryo that is germinating becomes starved (Kuriakose and Prasad, 2008). Amylase is an enzyme that breaks down the stored carbohydrate reserves of a seed during germination. Also, it has been shown that Cd's interactions with proteolytic enzymes limit store protein catabolism, which results in the slowing of seedling growth (Gianazza et al., 2007). The magnetoprimed soybean seeds maintained a higher total amylase and protease activity than unprimed seeds under both non-stress and in cadmium stressed conditions. This suggests that the MP may have influenced the biochemical mechanisms involved in seed germination and growth, including amylase and protease activity. Our finding is consistent with the results of previous studies. For instance, Vashisth and Nagarajan (2010) reported that magnetoprimed wheat and sunflower seeds had significantly higher amylase activity than their controls. Previous studies have reported higher α-amylase and protease activity in magnetoprimed soybean and maize seedlings under various stress conditions such as salt, mercury toxicity and UV-B (Kataria et al. 2017, 2019, 2020, 2023, Raipuria et al. 2021, Prajapati et al. 2023). The higher protease activity in magnetoprimed seeds could contribute to the breakdown of stored proteins into amino acids (Rajendra et al. 2005) that can be utilized for energy and growth during seedling development. The results suggest that MP can enhance the seedling's ability to cope with stress conditions by increasing total amylase and protease activities, which might improve the crop tolerance under Cd toxicity. Theoretical studies have shown that a magnetic field may enhance the density of ions passing through the cellular membrane of a seed, so affecting the osmotic pressure in a way that favours water entering into the cell (Reina et al. 2001). As previously indicated, seeds exposed to either a static or an oscillating magnetic field had higher levels of enzyme activity, particularly α-amylase (Kataria et al. 2017, 2020). The enzymes α-amylase which help the seed use its stored starch energy, are essential for seed germination. Therefore, a larger amount of solvent would be supplied to the seed's germination enzymes more quickly by the magnetic field's osmotic pressure action (Diehl 2022).

Interestingly, MP was found to lower the levels of MDA and oxidative stress marker (proline) and enhanced the H_2O_2 content in soybean seedlings in the presence of Cd toxicity. This suggests that MP may play a protective role against oxidative damage caused by $CdCl_2$ stress in plants. Similar findings were reported by Chen et al. (2011), where MF treatment was found to reduce the toxic effects of Cd on mung bean by increasing the rate of photosynthesis, nitric oxide concentration, and nitric oxide synthase activity, and reducing the lipid peroxidation. Chen et al. (2017) found the alleviation of the adverse effect of Cd and lead (Pb) through MP due to enhanced lipid peroxidation and antioxidant defence system in wheat seedlings. According to Prajapati et al. (2023), MP reduced lipid peroxidation at 200 mT (1 h) while increased the H_2O_2 content in soybean seedlings grown under mercury toxicity. This implies that MP alters membrane stability and H_2O_2 content to regulate antioxidant activity. Fatima et al. (2020) also observed that MP of soybean seeds mitigated the arsenic toxicity in terms of growth, photosynthesis, and water uptake through increase in the thickness of the midrib of leaves.

Several studies have reported changes in proline content in plants under different stress conditions and magnetic treatments. Banerjee and Roychoudhury (2015) stated that proline serves as an osmoprotectant in plants cultivated in a range of stressful conditions. For instance, cadmium toxicity caused a significant increase in proline accumulation in the shoots of *Brassica juncea, Triticum aestivum,* and *Vigna radiata,* indicating that proline plays an important role in the response to cadmium stress (Alia and Saradhi 1991, Sharmila et al. 2017). Mercury treatments raised the proline content in *Melissa officinalis* (Safari et al. 2019) and *Eichhornia crassipes* (Malar et al. 2015), which is consistent with our findings. However, magnetic treatment with SMF has been found to decrease the proline content in soybean seedlings under mercury toxicity (Prajapati et al. 2023). Chickpea plants (Hozayn et al. 2022), S*alvia* (Khosrojerdi et al. 2023) and orange seedlings (Mahmoud et al. 2019) treated with magnetic water also showed decreased amount of proline under saline soils. Okba et al. (2022) reported a decrease in proline content with magnetic water treatment, suggesting a positive role of magnetic water in reducing the detrimental effects of water deficit stress. These authors suggested that proline is especially important during stressful situations because it helps in regulation of the osmotic pressure of the cytosol and the vacuole with that of the external environment, which improves the uptake of water and nutrients. Plants have the capability to produce ROS in response to stress, which can have both positive and negative effects on their growth and survival (Hasanuzzaman et al. 2020). H_2O_2 production was enhanced markedly by Cd toxicity in a number of plant species (Ahmad et al. 2011, Mahawar et al. 2021). However, Shine et al. (2012) have shown that priming with SMF can increase the content of ROS which can serve as key signalling agents, influencing critical events in seed life, including seed germination by inducing ABA catabolism and gibberellic acid (GA) biosynthesis. Magnetoprimed seeds showed a decrease in ABA concentration and a rise in GA content in soybean under salinity suggesting that MP actively contributes to seed germination and the release of dormancy (Kataria et al. 2022). Thus the production of ROS such as H_2O_2 induced by MP of seeds represents one of the mechanisms through which seed invigoration and enhanced seedling growth can be achieved under nonstress as well as under abiotic stress conditions (Kataria et al. 2017, 2021, 2023, Raipuria et al. 2021). According to Barba-Espin et al. (2010), during the germination of pea seeds, H_2O_2 plays a direct role as a signalling molecule within the phytohormone network, causing the induction of proteins linked to plant signalling and development processes. Therefore, H_2O_2 functions as a central node that harmonizes and integrates phytohormone interactions to improve magnetoprimed soybean seed germination and vigour under Cd toxicity. Therefore, MP with SMF could be a promising technique for improving germination of seeds and seedling growth by alleviating cadmium stress through improving the tolerance of soybean seedlings. But further investigation is required to completely comprehend the processes behind the advantageous effects of-MP in the presence of heavy metal toxicity.

Conclusion

In conclusion, the present study revealed that the presence of Cd had a detrimental effect on seed germination and seedling growth, and this effect was found to be concentration dependent. Total amylase and protease activity declined under Cd toxicity, suggesting that it might be the result of disruptions in the reserve mobilisation process from cotyledons to the developing embryonic axis. The tolerance index of seedlings emerged from magnetoprimed seeds indicated that MP mitigates the toxic effects induced by Cd and enhances the tolerance in soybean seedlings by promoting root length, seedling vigour, total amylase and protease activity, H_2O_2 and reducing MDA and proline content as compared to the seedlings from unprimed seeds under Cd toxicity. Overall, the study suggested the possibility that use of a magnetic field might mitigate the harmful effects of Cd toxicity in soybean. Further research in this area could explore the underlying mechanisms and optimize the magnetic field parameters for improved crop productivity and environmental sustainability.

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Short communication

Isoëtes gymnocarpa and *Utricularia* × *neglecta* – new taxa for Montenegro

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Abstract – One lycophyte genus and species (*Isoëtes gymnocarpa*), and one aquatic magnoliophyte species (*Utricularia* × *neglecta*) new for the flora of Montenegro are reported. It is suggested that the new population of *Isoëtes gymnocarpa* and its habitat should be protected.

Keywords: aquatic magnoliophytes, the Balkans, flora, lycophytes, Montenegro, new records

Introduction

The flora of Montenegro (SE Europe) is easily recognized as one of the richest in Europe by the ratio of the number of species per square km (Pulević 2022). This is a reflection of evident habitat heterogeneity and richness resulting in large amounts of different ecological niches available for plants. A long tradition of botanical research has resulted in the more than 3,600 species and subspecies of vascular plants known from the Montenegrin flora (Rohlena 1942, Pulević 2005, 2022, Stešević et al. 2008, Stešević & Caković 2013, 2021). However, some habitat types, e.g., small temporary and permanent water bodies and wetlands in coastal region, have received less attention (cf. Bubanja 2016, Bubanja et al. 2016). A lot of them can be referred to "Mediterranean temporary ponds", a priority habitat according to the European Union 92/43 Habitats Directive, showing a clearly regressive trend. Distributional patterns of plants associated with these habitats in the Balkan Peninsula are still poorly known.

Materials and methods

Some localities in the coastal region and Zeta plain of Montenegro were surveyed during 2022 and 2023 as potential habitats for charophytes (Characeae), focussing on species of plants associated with them. All types of water bodies and wetlands encountered were checked, including Mediterranean temporary ponds, inundated sand pits, quarries, and artificial depressions, excavated ponds, drainage channels, puddles, concrete ponds, temporary and permanent streams and springs and the water bodies associated with them. The strong seasonality in the traits of water bodies and the appearance of aquatic plants as well as habitat heterogeneity are remarkable for the area surveyed. The specimens were collected by hand and studied in a living state. Voucher specimens were deposited in the Herbarium of the Natural History Museum of Montenegro (NHMM) and the Herbarium Mediterraneum Panormitanum (PAL). The nomenclature for lycophytes follows Troia & Greuter (2014), for *Utricularia*, Bobrov et al. (2022).

Results and discussion

Fifteen species of charophytes, belonging to the genera *Chara* L., *Lamprothamnium* J.Groves, *Nitella* C.Agardh, *Tolypella* (A.Braun) A.Braun, and *Sphaerochara* Mädler, were found in the research area. Our efforts aimed at the listing of all associated vascular plant species resulted in the finding of one genus and two species, new for the flora of Montenegro.

Isoëtes gymnocarpa (Gennari) A.Braun (Fig. 1A): North of the Nature Park Ulcinjska Solana (US), unshaded drainage channel within farmland moderately damaged by livestock and probably briefly inundated during winter and early spring, 41.92966 N, 19.27361 E, 3 m a.s.l., 17 IV 2023,

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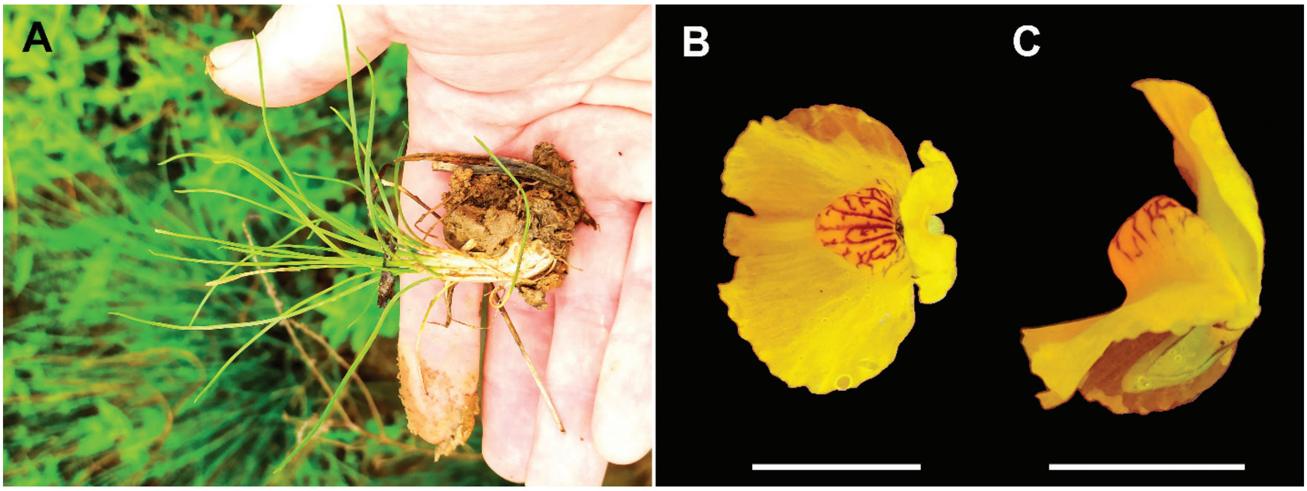

Fig. 1. New genus and species records for Montenegro: A – *Isoëtes gymnocarpa*, general appearance of unearthed plant, B, C – *Utricularia* × *neglecta*, B – isolated corolla showing large flat lower lip, view from above, C – isolated corolla showing short straight spur and nearly right angle between upper and lower lips, lateral view. Scale: B, C – 1 cm. All photos were taken from the voucher specimens by R. E. Romanov.

leg: Roman Romanov (RR) and Snežana Dragićević, det: Angelo Troia (NHMM, PAL).

This habitat is characterized by the specific water regime, which implies an alternation of wet (winter, spring) and dry phases (summer and autumn), just like that in the meadows in the immediate vicinity with mosaic habitats and communities of wet grasslands and pastures which host Mediterranean therophytes and geophytes. These agricultural lands are pastures grazed by livestock (sheep, cows) or partly arable land, with other negative impacts in the immediate environment (like cutting of surrounding trees and shrubs and the associated physical disturbance). The cover abundance of *Isoëtes* was low, not exceeding 20%. The plants were spotted within an area of less than 10 square meters.

The locality is currently outside the Nature Park Ulcinjska Salina, but because of the presence of this rare species of *Isoëtes*, it should be protected by the Park area being extended to include it.

Isoëtes gymnocarpa is close to *I. histrix* Bory (for its terrestrial habitat, and for the presence of phyllopodia and tuberculate megaspores), the main diagnostic character being the length of phyllopodia lateral teeth (not longer and thinner than the central tooth as in *I. histrix*, but as long and as thick as the central tooth, cf. Troia & Greuter 2014, 2015a, 2015b). The group needs further investigation, but we follow here the taxonomic view of Troia & Greuter (2015a) with *I. gymnocarpa* = *I. sicula* Tod. = *I. subinermis* (Gennari) Cesca & Peruzzi. The taxon so circumscribed is spread around the Mediterranean area, from the Iberian Peninsula to Anatolia: the population here reported is the northernmost along the Adriatic coasts, both in the Balkan Peninsula and in Italy. A previous report of *I. histrix* for southern Albania (Barina et al. 2013) should be checked to assess if it belongs to *I. histrix* or to *I. gymnocarpa*.

Isoëtes gymnocarpa should be recognized as indigenous to Montenegro because no cases of non-native species are known for this genus. The inconspicuous general habit of terrestrial *Isoëtes*, having the appearance of sterile monocots, can be a possible explanation why species of this genus were not spotted in Montenegro before.

Utricularia × *neglecta* Lehm. (Fig. 1B, C): Morača River, the lower reaches, the island of Vranjina, the wetland of Bakine Tigle (BT), 19 VI 2023, 42.28217 N, 19.15145 E, 4 m a.s.l., 19 VI 2023, leg: RR, det: RR (NHMM); BT, small floodplain pool, 42.28746 N, 19.15261 E, 5 m a.s.l., 19 VI 2023, leg: RR, det: RR (NHMM). The cover abundance was low, not exceeding 20 % in both cases.

This hybrid is widely distributed in temperate and tropical regions of the Old World (Taylor 1989, Uotila 2013, Bobrov et al. 2022, http://www.plantsoftheworldonline.org/). For a long time, it was reported as *U. australis* R.Br. It was mentioned from Montenegro, from Lake Skadar, based on images of sterile plants, not showing the leaf-teeth bristle character (https://www.inaturalist.org/observations/31338617), a trait only visible with microscopy. Moreover, the use of solely this character can be unreliable (Taylor 1989). Therefore, our records are either the first verified ones for Montenegro, or else can be recognized as a confirmation of an earlier uncheckable record. The infrequent and short period of flowering, usually associated with habitats difficult of access, hampers reliable recording of *Utricularia* species. This hybrid seems to be indigenous to Montenegro; no cases of introduction are known for it in the Balkan Peninsula.

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Author contribution statement

Roman E. Romanov – field studies, preparation and identification of specimens, manuscript writing; Snežana Dragićević – field studies, preparation of specimens, manuscript writing; Angelo Troia – preparation and identification of specimens, manuscript writing.

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Book Review

Chlorophyll *a* fluorescence measurements in Croatia • the first twenty years

Edited by Hrvoje Lepeduš, Marija Viljevac Vuletić, Zvonimir Zdunić. 2023, 189 pp, ISBN 978-953-7843-11-3 (Print), ISBN 978-953-7843-12-0 (eBook)
Publisher: Agricultural Institute Osijek

Chlorophyll fluorescence (ChlF) techniques are sensitive, rapid and non-invasive and provide a wide range of data on plant health. ChlF has been used for more than two decades to study photosynthesis in plants, algae and bacteria. In Croatia, the pioneering work in ChlF research began in the Department of Biology of the Josip Juraj Strossmayer University of Osijek. Since then, many scientific and agronomic laboratories and institutes in Croatia have used this technique in regular research and applied it in practise. This method has become the basis for many collaborative ventures among scientists in Croatia and abroad. Thanks to this cooperation, many young researchers were given the opportunity to learn the ChlF method and apply it in their research. This led to 14 doctoral theses, one master's thesis and numerous bachelor's theses. This monograph, published in English, is thus a tribute to twenty years of the application of ChlF methods in biology and agronomy in the Republic of Croatia.

The monograph begins with a historical overview of the use of ChlF in scientific research and continues with the application of the ChlF method in fundamental research in plant biology and agronomy. In the era of climate change, photosynthesis has proven to be a reliable marker for the degree of adaptation and tolerance of plants. The four chapters present examples of the application of the ChlF method in research and in the understanding of the responses of plants to various abiotic stress factors. The next six chapters present the practical application of the ChlF method in agronomic research, especially in elucidating the stress responses of plants under adverse environmental conditions and in screening for tolerant genotypes, which are of great importance in breeding programs for various crops. The penultimate chapter presents a case study and gives a detailed insight into the scope and effectiveness of the ChlF method in phenotyping plant material. The last chapter deals with the recent technological advances in the ChlF method and its future perspectives. As Dr. Šimić and Assoc. Prof. Dr. Mlinarić state in the final chapter of this monograph, "...chlorophyll fluorescence remains an important tool for understanding photosynthesis and its response to environmental stress, the health of plants and ecosystems. Continued advancements in technology and analysis will further improve its usefulness. Its future prospects are bright, with many potential applications in agriculture, urban farming, forestry, aquatic research, climate change, and bioenergy research."

The monography was presented by the editors Dr. Marija Viljevac Vuletić, senior research scientist from the Agricultural Institute Osijek, and Prof. Dr. Hrvoje Lepeduš from the Faculty of Humanities and Social Sciences, Josip Juraj Strossmayer University of Osijek, at a meeting of the Croatian Society of Plant Biologists in February 2024 in Zagreb (Fig. 1).

Fig. 1. Presentation of the monograph *CHLOROPHYLL a FLUORESCENCE MEASUREMENTS IN CROATIA • FIRST TWENTY YEARS*, ed. Dr. Marija Viljevac Vuletić and Prof. Dr. Hrvoje Lepeduš (A) at a meeting of the Croatian Society of Plant Biologists (B).

Dr. Branka Salopek Sondi
Ruđer Bošković Institute, Zagreb

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Broadwater, S. T., Scott, J., 1994: Ultrastructure of unicellular red algae. In: Seckbach, J. (ed.), Evolutionary pathways and enigmatic algae: *Cyanidium caldarium* (Rhodophyta) and related cells, 215–230. Kluwer Academic Publishers, Amsterdam.

On-line sources: Author, year, title, source. Retrieved October 15, 2015 from http://www....

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