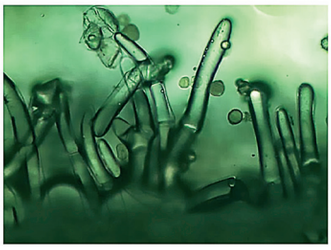
On the cover:

Non-glandular hairs covering the lower part of the filament of *Orobanche crenata* reported here by Mohamed et al., pp. 32–42



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Clarifying genetic and taxonomic relationships among *Pistacia* taxa (Anacardiaceae) in Croatia

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Abstract – In the eastern Adriatic flora the genus *Pistacia* L. (Anacardiaceae) is represented with *P. lentiscus* L., *P. terebinthus* L., their hybrid *P.* × *saportae* Burnat and the cultivated *P. vera* L. In addition, an endemic putative taxon from Mt. Biokovo was described in 1985 as *P. calcivora* Radić. Our aim was to resolve relationships between the four putative indigenous taxa of this genus in Croatia (*P. lentiscus*, *P. terebinthus*, *P.* × *saportae* and *P. calcivora*) based on genetic (AFLP) and morphological data. Specifically, we aimed to determine the presence of the hybrid taxon *P.* × *saportae* and to validate the controversial taxonomic status of *P. calcivora* at the molecular and morphological levels. Our combined results indicate the presence of two well separated groups of populations. The first group included all individuals of *P. terebinthus* and the individuals initially assigned to the potential taxon *P. calcivora* based on leaf morphology, suggesting that there is no support for the described taxon *P. calcivora* and that it should be considered as a synonym for *P. terebinthus*. The second group corresponded to *P. lentiscus* and included the majority of the presumed hybrid individuals of *P.* × *saportae*. However, four hybrid individuals were confirmed at the molecular level and were placed between the two parental taxa in the phylogenetic tree, confirming the presence of *P.* × *saportae* in Croatia. Although confirmed *P.* × *saportae* individuals were genetically closer to *P. lentiscus*, they were morphologically more similar to *P. terebinthus*, hindering their correct identification in the field.

Keywords: AFLP, eastern Adriatic, genetic structure, hybrid, morphology, *Pistacia*

Introduction

The genus *Pistacia* L. belongs to the Anacardiaceae family originating from Central Asia (Parfitt and Badenes 1997). It comprises eleven accepted species, two subspecies and one hybrid taxon, according to Plants of the World Online (POWO 2023). The genus has a wide but disjunct native distribution including Central to North America, the Mediterranean to Central Asia and northeast to east Africa (AL-Saghir and Porter 2012, Xie et al. 2014, POWO 2023). Representatives of the genus are dioecious, deciduous trees or evergreen shrubs, considered to be mainly xerophytes growing in arid and/or semi-arid habitats. Although the genus does not comprise many species, some of them are well known and have been used by mankind since ancient times due to their valuable products (Golan-Goldhirsh 2009). For example, *P. vera* L. (the pistachio tree) is a highly economically important species due to its edible seeds and for this reason it has been commercially cultivated and used for centuries in many parts of the world (Whitehouse 1957, Zeng et al. 2019, Kafkas et al. 2023). Other examples include the age-old use of the mastic gum and resins of *Pistacia* trees for various purposes such as medicine, food, aroma and cosmetics (Golan-Goldhirsh 2009).

The Mediterranean is considered to be the contemporary diversification centre of *Pistacia* (Xie et al. 2014). Six species of the genus are listed for the Euro-Mediterranean area (*P. atlantica* Desf., *P. eurycarpa* Yalt., *P. khinjuk* Stocks,

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P. lentiscus L., *P. terebinthus* L. and *P. vera*), with only two native species currently present in the eastern Adriatic according to Euro+Med PlantBase (Euro+Med 2006-2023): *P. lentiscus* and *P. terebinthus*. Interestingly, although the hybrid taxon *P.* × *saportae* Burnat between parental species *P. lentiscus* and *P. terebinthus* is listed as native for the area of western Mediterranean, Morocco, Palestine, Algeria, Cyprus and Turkey according to POWO (2023), it is not currently recorded in the Euro+Med PlantBase. *Pistacia* × *saportae* was described by Burnat in 1896 as a probable species from the Maritime Alps. Later, it was suggested and confirmed to be an interspecific hybrid taxon (*P. lentiscus* × *P. terebinthus*) in view of its morphological, wood anatomy and genetic data (Zohary 1972, Grundwag and Werker 1976, Werner et al. 2001, Yi et al. 2008, AL-Saghir and Porter 2012), reported in many Mediterranean countries so far. According to the Flora Croatica Database – FCD (Nikolić 2023), in the Croatian flora (eastern Adriatic) the genus *Pistacia* is represented by four taxa: *P. lentiscus*, *P. terebinthus*, their hybrid *P.* × *saportae* and sporadically cultivated *P. vera*. In addition, Radić (1985) described *P. calcivora* Radić as a new endemic taxon from Mt. Biokovo based on morphology (On-line Suppl. Fig. 1). Since the taxonomic status of this taxon is however doubtful and currently unresolved, it is not listed in the national flora i.e. FCD. *Pistacia* × *saportae* was reported and inserted in FCD for the first time in the Croatian flora by Jeričević et al. (2014) from the island of Korčula, the identification being based on morphology, although it was mentioned earlier for the area of Mt. Biokovo by Radić (1985) (On-line Suppl. Fig. 1).

The taxonomy and phylogeny of the genus *Pistacia* has been assessed on several occasions on the basis of morphological and genetic (RAPD, AFLP, RFLP, nuclear and plastid sequences) data (Parfitt and Badenes 1997, Golan-Goldhirsh et al. 2004, Kafkas 2006, Yi et al. 2008, AL-Saghir and Porter 2012, Xie et al. 2014), however there are still unresolved issues about some taxa, taxa number and species boundaries due to high morphological variation both among and within taxa (Golan-Goldhirsh 2009). This is further complicated by common hybridization between some of the representatives, often forming interspecific hybrids (Zohary 1952, Morgan et al. 1992, Yi et al. 2008, Aznarte-Mellado et al. 2014). There are several different taxonomic classifications of the genus. The first and probably most comprehensive one based on morphology was carried out by Zohary (1952) who recognized 11 species belonging to four sections: Sect. *Lentiscella* Zoh. (*P. mexicana* HBK, *P. texana* Swingle), Sect. *Eu Lentiscus* Zoh. (*P. lentiscus*, *P. saportae*, *P. weinmannifolia* Poiss. ex Franch.), Sect. *Butmela* Zoh. (*P. atlantica*) and Sect. *Eu Terebinthus* Zoh. (*P. terebinthus*, *P. palaestina* Boiss. *P. khinjuk*, *P. vera*, *P. chinensis* Bunge). Later, Parfitt and Badenes (1997) carried out the first molecular phylogeny of the genus (based on plastid DNA) including ten *Pistacia* species and proposed the division of the genus into only two sections: Sect. *Lentiscus* (including all evergreen species with paripinnate leaflets) and Sect. *Terebinthus* (deciduous species with imparipinnate leaflets). Yi et al. (2008) provided a more recent, updated molecular phylogeny of the whole genus based on five different data sets (sequences of two nuclear and three plastid regions) and including 11 species and one putative hybrid taxon *P.* × *saportae*. They confirmed that the genus is monophyletic, as well as the hybrid origin of *P.* × *saportae* suggesting that *P. lentiscus* is the maternal and *P. terebinthus* paternal taxon. Their phylogeny is largely consistent with the most recent taxonomic revision of the genus based on morphology (up to our knowledge) carried out by AL-Saghir and Porter (2012), which recognized a total of 13 taxa divided into two sections (sect. *Pistacia* Zoh. and sect. *Lentiscella*): nine species (*P. atlantica*, *P. chinensis*, *P. eurycarpa*, *P. khinjuk*, *P. terebinthus*, *P. vera*, *P. lentiscus*, *P. mexicana*, *P. weinmannifolia*), five subspecies (*P. chinensis* subsp. *chinensis*, *P. chinensis* subsp. *falcata* (Becc. ex Martelli) Rech. f., *P. chinensis* subsp. *integerrima* (J.L. Stew. ex Brandis) Rech. f., *P. lentiscus* subsp. *lentiscus*, *P. lentiscus* subsp. *emarginata* (Engl.) AL-Saghir) and one hybrid taxon (*P.* × *saportae*). Finally, the most recent biogeographic history and diversification of *Pistacia* genus was carried out by Xie et al. (2014), using an expanded set of sequences of multiple nuclear and plastid loci. The latter study better resolved the relationships within the genus compared to Yi et al. (2008) and did not support previous divisions of the genus into four (Zohary 1952) or two sections (Parfitt and Badenes 1997, AL-Saghir and Porter 2012). The authors estimated that the genus has diverged at 37.60 mya, which is much later than some previous estimations (Parfitt and Badenes 1997).

Over the past decades, the genetic relationships among some specific Mediterranean *Pistacia* taxa at the molecular level were assessed across different geographic areas of interest using various molecular markers such as RFLP (Parfitt and Badenes 1998), RAPD and/or AFLP (Kafkas and Perl-Treves 2002, Katsiotis et al. 2003, Golan-Goldhirsh et al. 2004, Karimi et al. 2009, Shanjani et al. 2009, Iranjo et al. 2016), ITS (Labdelli et al. 2022), SSR (Vendramin et al. 2009), and SAMPL (Karimi and Kafkas 2011) or in specific countries like Syria (Basha et al. 2007), Turkey (Kafkas and Perl-Treves 2001, Guney et al. 2021), Afghanistan (Kafkas et al. 2006), Iran (Mirzaei et al. 2006) etc. However, no such comparative study exists for the native *Pistacia* taxa in the eastern Adriatic region. Thus, building on this rich legacy to contribute to ongoing discussions about genetic relationships among *Pistacia* taxa, the aim of our research was to determine relationships among four putative indigenous taxa of the genus *Pistacia* in the eastern Adriatic (*P. lentiscus*, *P. terebinthus*, *P.* × *saportae* and *P. calcivora*) using AFLP molecular markers and leaf morphology. Specifically, we aimed to determine the presence of the hybrid taxon *P.* × *saportae* and to validate the taxonomic status of the putative taxon *P. calcivora* in the eastern Adriatic at the molecular and morphological levels. We chose AFLP markers because they proved to be efficient in identifying interspecific hybrids between closely related species (Oberprieler et al. 2022, Zalewska-Gałosz et al. 2023) and have been previously used to address genetic relationship between selected

Pistacia species (Karimi et al. 2009). In addition, relationships between our taxa of interest were evaluated based on leaf morphology to complement the genetic data and to provide the robustness of the taxonomic treatment because leaf characteristics have been traditionally used as the main taxonomic characters for *Pistacia* identification (Parfitt and Badenes 1997).

Material and methods

Plant material and study area

We collected plant material from a total of 13 natural *Pistacia* populations (116 individuals) corresponding to four putative indigenous taxa of the genus *Pistacia* present in the eastern Adriatic: *P. lentiscus*, *P. terebinthus*, *P.* × *saportae* and *P. calcivora* (Tab. 1). Our study area belongs to the Mediterranean phytogeographic region characterized by the typical Mediterranean climate with hot dry summers and mild wet winters. As an essential part of the Mediterranean vegetation, *Pistacia* taxa in the eastern Adriatic thrive in dry, warm, sunny, rocky habitats within the Adriatic coastal and/or coastal-montane belt where they are frequently exposed to prolonged droughts and high temperatures.

We collected three putative hybrid populations of *P.* × *saportae* (a total of 21 individuals) from the localities where the hybrids were previously reported or assumed based on their morphology: islands of Šolta, Korčula and Vis (Fig. 1). We also collected three populations (a total of 30 individuals) of the putative taxon *P. calcivora* from three different localities from the type locality on Mt. Biokovo (Tab. 1, On-line Suppl. Fig. 2). Both of these putative taxa were identified in the first place in the field based on leaf morphology by an expert botanist. In addition, at each sampling site of *P.* × *saportae* we collected individuals of both parental species: a total of 32 individuals of *P. lentiscus* and 30 individuals of *P. terebinthus* (Tab. 1, On-line Suppl. Fig. 2). One additional population of *P. lentiscus* was also collected from Mt. Biokovo for comparison with the putative *P. calcivora* (Tab. 1). Collected plant material for the DNA extraction was immediately stored in silica-gel. Finally, in each studied population we collected leaves for the morphological analyses from the same individuals sampled for the genetic analyses. From each individual tree or shrub we collected 10 leaves from short shoots which were herbarised in the field until the morphometric measurements. However, some of the collected herbarised plant material decayed prior to the measurements, thus a few individuals had to be excluded from the morphological analyses (Tab. 1). Herbarium specimens are deposited in ZAGR herbarium.

DNA extraction and AFLP fingerprinting

Total genomic DNA was extracted from 20 mg of silica-gel dried leaf tissue of each individual in the study using the DNAeasy plant mini kit (QIAGEN). The manufacturer's protocol was followed and slightly modified by adding 2-mercaptoethanol and 1% PVP (polyvinylpyrrolidone) to increase quality and concentration of DNA extract. DNA quality and concentration of 116 samples was verified using Nanophotometer P330 (Implen®, Munich, Germany). For AFLP protocol we followed Vos et al. (1995) with modifications described in Carović-Stanko et al. (2011).

We used the EcoRI and MseI (= Tru1I) restriction enzymes and five primer combinations for the selective amplification: FAM-EcoRI-ACA, NED-EcoRI-AGA, VIC-EcoRI-ACG, PET-EcoRI-ACC and Tru1I-CGA. For preselective amplification the PCR program was as follows: 2 min at 94 °C followed by 20 cycles: at 94 °C for 20 s, 56 °C for 30 s, 7 °C for 2 min, and 60 °C for 30 min. For selective amplification we

Tab. 1. Sampled taxa and populations of the genus *Pistacia* in the eastern Adriatic coast. Abbreviations: n – number of individuals for genetic analyses, n (M) – number of individuals for morphological analyses, x and y – geographical coordinates.

Population	Taxon	Locality	n	n (M)	x (°)	y (°)
P1	*P. lentiscus*	Šolta, Grohote	10	10	16.2827	43.39435
P2	*P.* × *saportae*	Šolta, Grohote	11	11	16.2827	43.39435
P3	*P. terebinthus*	Šolta, Grohote	10	10	16.2827	43.39435
P4	*P. lentiscus*	Korčula	12	12	17.10212	42.91128
P5	*P.* × *saportae*	Korčula	2	2	17.10212	42.91128
P6	*P. terebinthus*	Korčula	10	10	17.10212	42.91128
P7	*P.* × *saportae*	Vis, Komiža	8	8	16.10715	43.04736
P8	*P. lentiscus*	Vis, Komiža	10	10	16.10715	43.04736
P9	*P. terebinthus*	Vis, Komiža	10	10	16.10715	43.04736
P10	*P. calcivora*	Biokovo, Makar	10	10	17.03287	43.30384
P11	*P. calcivora*	Biokovo, Vrutak	11	11	17.03515	43.29695
P12	*P. calcivora*	Biokovo, Kotišina	9	9	17.0463	43.29023
P13	*P. lentiscus*	Biokovo, Kotišina	3	3	17.0463	43.29023
Total			116	109		

Fig. 1. Leaf variability of *Pistacia terebinthus* (left), *P.* × *saportae* (middle) and *P. lentiscus* (right) from the island of Vis in the eastern Adriatic (Photo: S. Bogdanović).

used the PCR program from Carović-Stanko et al. (2011). Amplified PCR fragments were separated by capillary electrophoresis on an ABI3730 XL automated sequencer (Applied Biosystems) by Macrogen Europe (Amsterdam, The Netherlands) using GeneScan-500 LIZ size standard (Applied Biosystems) for size calibration. We identified the AFLP alleles using GeneMapper® 5.0 software (Applied Biosystems) within the size range of 50–600 base pairs (bp) which were then exported as binary matrix for subsequent analyses with fragments scored as present (1) or absent (0). In total, we obtained 1502 AFLP alleles for the 116 individuals. Based on the DNA quality and quality of chromatograms in GeneMapper, three individuals were excluded from the further analyses. We then estimated the error rate based on eight replicated samples and selected the final set of AFLP alleles for the subsequent analyses using ScanAFLP ver. 1.2 R script following Herrmann et al. (2010). This resulted in a dataset of 584 AFLP loci for a total of 113 individuals used for all the subsequent statistical analyses, with a mean error rate of 1.79%.

AFLP analyses

We first estimated within-population genetic diversity for each investigated population by calculating the proportion of polymorphic markers (%P), Nei's gene diversity ($H_{E,}$ Nei 1987) and the frequency down-weighted marker values (DW; Schönswetter and Tribsch 2005) which indicate the genetic rarity of a population using AFLPdat R scripts (Ehrich 2006) in R version 4.1.3. In addition, we used AFLPdat to prepare the input file for the Bayesian model-based clustering method implemented in the STRUCTURE software 2.3.4 (Pritchard et al. 2000), which was used to assess the genetic structure of the investigated populations. We used the following parameters for the STRUCTURE analysis: number of genetic clusters (K) was set from 1 to 10, with 10 replicate runs per each K, each run consisting of a burn-in period of 10,000 Markov Chain Monte Carlo (MCMC) iterations followed by 90,000 MCMC iterations. We used an admixture model of individual ancestry and correlated allele frequencies. To determine the optimal number of K we used the ΔK method of Evanno et al. (2005). The STRUCTURE results were subsequently processed (averaging replicates), summarized and visualized using the STRUCTURE HARVESTER 0.6.94 software (Earl and von Holdt 2012) and CLUMPAK (Kopelman et al. 2015). Hybrid individuals with putative mixed ancestry were defined as those with an assignment probability to any of the genetic clusters below 0.7 (Q ≤ 70%). Next, we calculated pairwise genetic distances between all individuals based on Dice coefficient (Dice 1945; Nei and Li 1979) and conducted a principal coordinate analysis (PCoA) based on the resulting distance matrix using PAST 3.22 (Hammer et al. 2001). Finally, to investigate the phylogenetic relationships between individuals and putative taxa under study, we constructed a NeighborNet diagram of individuals based on Dice coefficient transformed into distance matrix using SplitsTree 4 (Huson and Bryant 2006). The reliability of the phylogenetic network was assessed by constructing a neighbor-joining (NJ) tree based on the same distance matrix using 1000 bootstrap (BS) repetitions, both implemented in PAST.

Morphological analyses

We carried out morphological analyses on a total of 13 *Pistacia* populations and 109 individuals (Tab. 1). After being herbarised and fully dried, leaves were scanned, using an A3-format scanner (MICROTEK ScanMaker 9800XL),

at resolution of 600 dpi (.TIF). Obtained image files were further analysed in the software package WinFolia PRO (Regent Instruments Inc., QC, Canada). Using the interactive measurement option, the length of rachis (RL) and leaf petiole (PL) were measured on each leaf. Afterwards, on each leaf one lateral leaflet in the middle part of the leaf was selected and measured using the leaf morphology option. In total, eight leaflet morphometric traits were measured, with accuracy of 0.1 mm: leaflet area (LA); leaflet length (LL); maximum leaflet width (MLW); leaflet length, measured from the leaflet base to the point of maximum leaflet width (PMLW); leaflet width at 90% of leaflet length (LW90); angle enclosed by the main leaflet vein (the centre of leaflet blade) and the line connecting the leaflet base to a set point on the leaflet margin, at 10% (LA10) of total leaflet length; and form coefficient (FC). In addition, the total number of leaflets per each leaf (NL) was counted.

To assess the possibility of conducting parametric tests, the symmetry and homoscedasticity of data were verified (Sokal and Rohlf 2012). Assumptions of normality were checked using the Shapiro–Wilk test, and the assumption of homogeneity of variance was checked using Levene's test. Arithmetic mean (M), standard deviation (SD) and coefficient of variation (CV/%) were calculated for the particular trait for each putative taxon, population and obtained genetic group (see results). Differences in leaf morphology between the studied taxa and populations were determined by Kruskal-Wallis test. In addition, statistically significant differences between all pairs of putative taxa were identified using Wilcoxon rank sum test, at $P \leq 0.05$. Descriptive statistics and nonparametric tests were carried out using the Statistica software package ver. 13 (StatSoft Inc., Tulsa, OK, USA).

We used two multivariate analyses to examine the morphological variability of the studied taxa. First, we used principal component analysis (PCA) to calculate the principal components across all individuals, and all studied morphological traits. PCA biplot was constructed based on the first two principal components showing all the analysed individuals and traits without any predefined groups. In addition, we performed discriminant analysis (DA) to evaluate the utility and importance of the studied morphological traits by determining which traits were most useful for maximizing differentiation between the identified genetic groups (see results). Individual assignment to the specific genetic group was based on the results of the STRUCTURE analysis. We then used classification discriminant analysis to determine the proportion of individuals that were correctly classified into the identified genetic groups. All multivariate statistical analyses were carried out using the "MorphoTools" R scripts in R v.3.4.3 (R Core Team 2017) by following the manual of Koutecký (2015).

Results

Population genetic diversity

Different genetic diversity parameters of each population showed a similar trend. The proportion of polymorphic markers (%P) per population ranged from 1.37% (population P5) to 26.71% (population P6), with an average of 18.81% (Tab. 2). Population P6 had the highest H_E, and population P5 the lowest. The average DW value was 566.72, with the highest value detected in population P7 and the lowest once again in population P5 (Tab. 2). Overall, populations P6 (*P. terebinthus*, Korčula), P9 (*P. terebinthus*, Vis), and P3 (*P. terebinthus*, Šolta) had the highest genetic diversity, while populations P7 (putative hybrid population from Vis), P13 (*P. lentiscus*, Biokovo), and P2 (putative hybrid population from Šolta) had the highest frequency of rare alleles (DW). Overall, the population with the lowest genetic diversity was P5 (*P.* × *saportae*, Korčula). However, in the case of P5 and P13, genetic diversity values should be treated with caution due to the low sample size of these populations.

Tab. 2. Genetic diversity parameters of the investigated *Pistacia* populations based on AFLP markers. %P – Proportion of polymorphic markers, H_E – Nei's gene diversity (Nei 1987), DW – frequency down-weighted marker values (rarity index). Genetic group – population assignment to each of the two genetic clusters identified by STRUCTURE analysis.

Population	%P	H_E	DW	Genetic group
P1	17.47	0.065	535.859	*P. lentiscus*
P2	21.58	0.059	697.055	*P. lentiscus*
P3	23.97	0.076	540.638	*P. terebinthus*
P4	19.52	0.060	527.211	*P. lentiscus*
P5	1.37	0.014	335.780	Hybrid
P6	26.71	0.085	622.109	*P. terebinthus*
P7	22.60	0.075	773.008	Hybrid
P8	15.41	0.049	546.442	*P. lentiscus*
P9	25.68	0.083	688.819	*P. terebinthus*
P10	21.40	0.071	454.733	*P. terebinthus*
P11	20.55	0.072	453.376	*P. terebinthus*
P12	19.52	0.071	469.006	*P. terebinthus*
P13	8.73	0.058	723.293	*P. lentiscus*
Average	18.81	0.064	566.72	
P. lentiscus group	16.54	0.058	605.972	
P. terebinthus group	22.97	0.076	538.114	

Genetic structure and phylogenetic relationships

The results of the PCoA analysis based on the Dice distance matrix between all individuals revealed two distinctly separated groups of individuals (Fig. 2a). One group consisted of *P. terebinthus* individuals and all individuals presumed to belong to the *P. calcivora*. The other group consisted of *P. lentiscus* individuals and most of the individuals initially identified based on morphology as *P.* × *saportae*. Closer to the *P. lentiscus* group, four potentially hybrid individuals (two from population P5 and two from P7) were

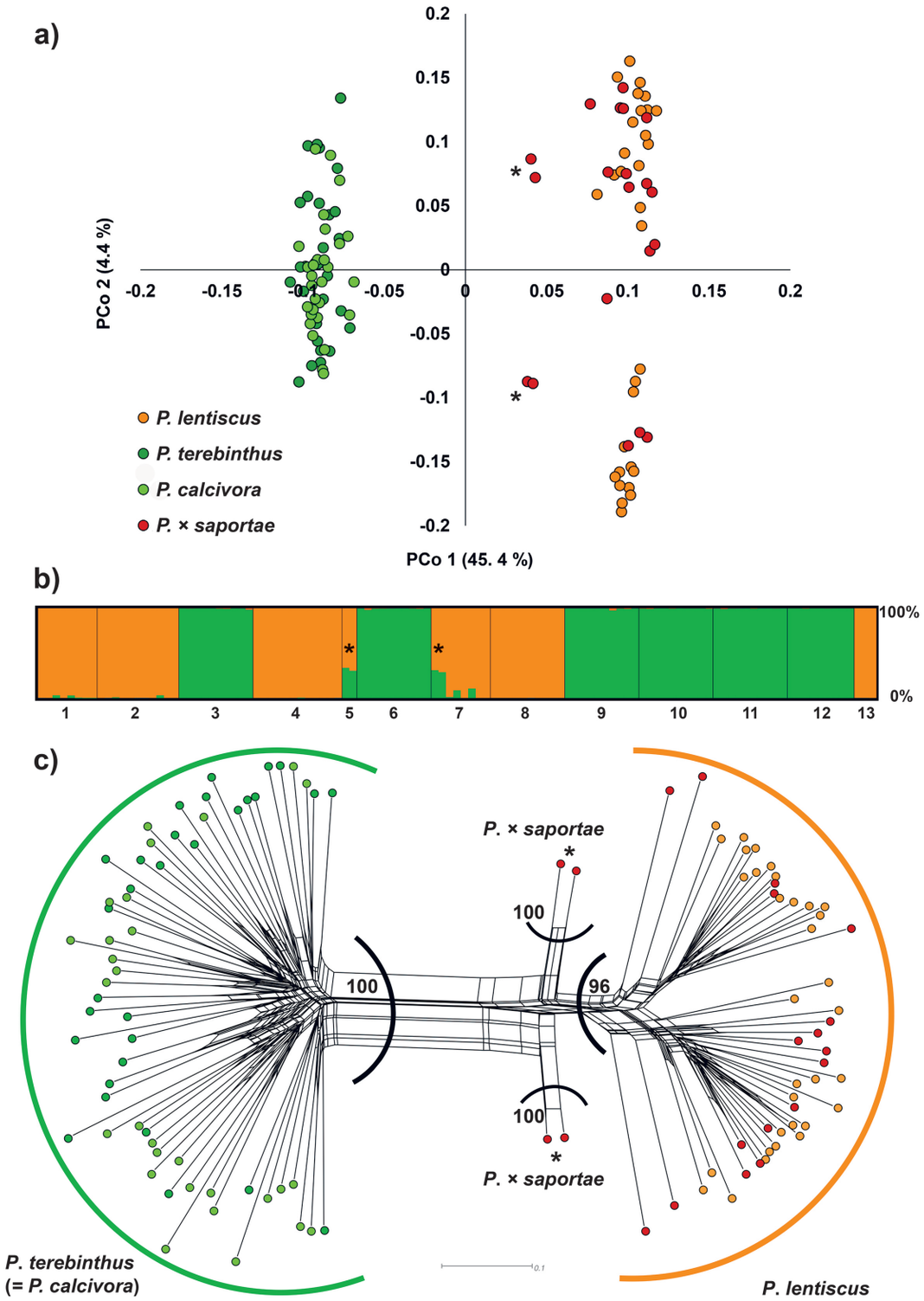

Fig. 2. Genetic relationships and genetic structure of the investigated *Pistacia* taxa (13 populations and 113 individuals) in the eastern Adriatic based on AFLP data. Hybrid individuals corresponding to *P.* × *saportae* are indicated with an asterisk. a – Principal coordinate analysis (PCoA) scatter-plot based on Dice distance matrix visualizing the genetic relationships among investigated *Pistacia* individuals and taxa. Individual assignment to the specific taxon is based on the initial *a priori* identification based on leaf morphology. b – population genetic structure based on the Bayesian STRUCTURE analysis at optimal K = 2. Each column represents an individual, and the membership coefficient (Q) indicating the assignment probabilities to each of the two genetic clusters is depicted on the scale from 0 – 100%. Dark green corresponds to genetic cluster of *P. terebinthus,* orange to the genetic cluster of *P. lentiscus*. Population numbers correspond to Tab. 1 and Tab. 2. c – neighbour-net diagram based on Dice distance matrix depicting the phylogenetic relationships among individuals. Colours of the points are as in a). Thick outlines correspond to the two genetic clusters inferred by STRUCTURE analysis in panel b). Dark green – genetic cluster of *P. terebinthus*, orange – genetic cluster of *P. lentiscus*. Indicated are only bootstrap values for the main groups, as obtained by neighbour-joining tree analysis.

clearly distinguished (Fig. 2a). The first two PCo axes explained 49.8 percentage of variation (Fig. 2a).

Based on the Bayesian clustering method implemented in STRUCTURE, the optimal number of genetic clusters (source populations) that can explain the genetic structure of the investigated populations was at K = 2 following ΔK (Evanno et al. 2005). The first genetic cluster included all individuals of *P. terebinthus* and the individuals that were originally assigned to the potential taxon *P. calcivora* based on leaf morphology. The second cluster corresponded to all individuals of *P. lentiscus* and included the majority of the putative hybrid individuals initially identified as *P.* × *saportae* based on leaf morphology. However, the same four hybrid individuals originating from the islands of Korčula and Vis were clearly identified based on their assignment probabilities to the two parental genetic clusters (Q = 0.66 – 0.71 to the *P. lentiscus* cluster), confirming the presence of *P.* × *saportae* at the molecular level (Fig. 2b).

Finally, the phylogenetic network based on Dice distance matrix among all individuals was largely congruent with the STRUCTURE and PCoA results and confirmed the presence of two main distinctly separated groups of individuals: one group corresponding to *P. lentiscus* and the other to *P. terebinthus* which also included all putative *P. calcivora* individuals. The above-mentioned hybrid individuals of *P.* × *saportae* were placed between the two parental taxa and positioned themselves closer to the *P. lentiscus* (Fig. 2c). The two major groups were also supported with high BS values (BS = 96 and 100), as well as hybrid individuals of *P.* × *saportae* which were clearly separated from both parental taxa (BS = 100).

Morphology

The results of the descriptive statistical analysis are shown for all the investigated *Pistacia* taxa in Tab. 3, and individually for each studied population in On-line Suppl. Tab. 1. Descriptive statistics for analysed leaf morphological traits for the inferred genetic groups are shown in Tab. 4. Based on the Kruskal–Wallis test, statistically significant differences (P < 0.0001) among the studied taxa were confirmed for all studied morphological traits. However, multiple comparison analysis indicated that putative *P. calcivora* and *P. terebinthus* differ only in two traits (LW90 and NL), while a higher number of differences were found between the other pairs of taxa, particularly between the putative *P. calcivora* and *P. lentiscus*, which differed in all studied morphological traits (On-line Suppl. Tab. 2). This was further confirmed with descriptive statistical analysis, which pointed out the differences, as well as the similarities between the studied *Pistacia* taxa. In particular, *P. terebinthus* and *P. calcivora* exhibited a high degree of morphological similarity, as the two taxa displayed the largest leaflets and the longest petiole and rachis (Tab. 3). However, significantly lower NL was observed in *P. calcivora* (6.91), in comparison with *P. terebinthus* (7.75). Furthermore, *P. lentiscus* differed the most in its morphology. It had the lowest values in almost all of the studied morphological traits, with the average leaflet area of 1.44 cm^2 and petiole length of 3.23 cm (Tab. 3). The only exception was NL, which was the highest among the studied taxa (7.94) in *P. lentiscus*. As expected, the presumed hybrid individuals between parental *P. terebinthus* and *P. lentiscus*, *P.* × *saportae*, showed intermediate values in all of the studied morphological traits, except for the NL

Tab. 3. Descriptive statistics for analysed leaf morphological traits of the studied *Pistacia* taxa. M – arithmetic mean, SD – standard deviation, CV – coefficient of variation (%). Analysed traits: LA – leaflet area; LL – leaflet length; MLW – maximal leaflet width; PMLW – leaflet length measured from the base to the point of maximum leaflet width; LW90 – leaflet width at 90% of leaflet length; FC – form coefficient; LA10 – angle enclosed by the main leaflet vein (the centre of leaflet blade) and the line connecting the leaflet base to a set point on the leaflet margin, at 10% of total leaflet length; RL – rachis length; PL – petiole length; NL – number of leaflets.

Trait	Descriptive parameter	*P. calcivora*	*P. lentiscus*	*P. terebinthus*	*P.* × *saportae*
LA (cm^2)	M	6.29	1.44	5.90	2.68
	SD	2.80	0.57	2.30	1.54
	CV (%)	44.53	39.55	39.02	57.44
LL (cm)	M	4.10	2.45	4.11	2.78
	SD	0.89	0.52	0.90	0.91
	CV (%)	21.79	21.09	21.81	32.89
MLW (cm)	M	1.97	0.81	1.90	1.28
	SD	0.54	0.20	0.42	0.33
	CV (%)	27.31	24.06	21.93	26.05
PMLW (cm)	M	1.99	1.15	1.88	1.34
	SD	0.52	0.27	0.46	0.40
	CV (%)	26.26	23.05	24.26	29.72
LW90 (cm)	M	1.07	0.40	0.91	0.64
	SD	0.40	0.12	0.32	0.19
	CV (%)	37.37	30.21	34.93	29.39
FC	M	0.69	0.53	0.69	0.67
	SD	0.08	0.08	0.09	0.09
	CV (%)	11.83	16.10	13.35	13.70
LA10 (°)	M	44.85	31.93	46.23	39.63
	SD	7.66	5.77	7.47	7.89
	CV (%)	17.08	18.07	16.15	19.90
RL (cm)	M	8.45	5.66	8.26	5.78
	SD	1.93	1.34	1.95	1.79
	CV (%)	22.82	23.75	35.80	30.97
PL (cm)	M	6.74	3.23	6.63	4.12
	SD	2.71	1.47	2.37	1.59
	CV (%)	40.19	45.73	35.80	38.66
NL	M	6.91	7.94	7.75	7.16
	SD	1.81	1.90	1.42	1.61
	CV (%)	26.14	23.98	18.32	22.55

(Tab. 3). However, when only those individuals confirmed as hybrid *P.* × *saportae* by genetic analysis were considered, they were of intermediate character for most traits (except for RL and NL) but were more similar to *P. terebinthus* than to *P. lentiscus*, when size of leaflets and rachis was considered (Fig. 1, Tab. 4). Furthermore, excluding the presumed hybrids of *P.* × *saportae*, putative *P. calcivora* stands out with the highest variability in leaf morphology (Tab. 3). In general, of the studied morphological traits, LA showed the highest variability, while parameters related to the leaflet shape (FC and LA10) displayed the lowest variability.

At the population level, statistically significant differences ($P < 0.0001$) were found among the studied populations. The locality Grohote (island of Šolta), containing populations P1, P2 and P3, seems to favour overall smaller but very variable leaves. On the other hand, the island of Korčula, with populations P4, P5 and P6, supported larger and less variable leaves, pronounced the most in *P.* × *saportae* (P5). Island of Vis, with populations P7, P8 and P9, displayed predominantly intermediate values in leaf morphology and variability, with the exception of *P. terebinthus* (P9) which had the largest leaflets at this locality. The locality Mt. Biokovo, containing three populations of *P. calcivora* and one population of *P. lentiscus*, was characterised with the largest (P12, *P. calcivora*) and smallest (P13, *P. lentiscus*) leaflets in this study.

PCA revealed that the first two PC axes had Eigen-values greater than one (>1). In addition, the cumulative variability explained by the first two PC axes was 84.3%, with the expected dominant percentage of 67.9% ascribed to the first PC axis (Fig. 3). The second PC axes contributed less to the overall variability, with 16.5%. The contribution of the individual measured traits to each of the PC axes can be found in On-line Suppl. Tab. 3. The first PC axis was highly negatively correlated with seven traits (MLW, LA, LW90, LL, PMLW, PL and RL), whereas the second PC axis was highly positively correlated with NL (Fig. 3). In the PCA biplot, a grouping of *P. terebinthus* and *P. calcivora* individuals, characterised by larger leaflet blades, is noticeable on the left side, unlike the right side, where *P. lentiscus* and presumed *P.* × *saportae*, with small leaflet blades, have grouped (Fig. 3). Identified hybrid individuals of *P.* × *saportae*, as confirmed by AFLP, grouped together with individuals of *P. terebinthus* and *P. calcivora,* whereas the remaining putative hybrid individuals showed partial overlap with *P. lentiscus*, although their intermediate character is clearly visible (Fig. 3).

Finally, DA for the three genetic groups of *Pistacia* individuals was performed. The first large group consisted of all *P. terebinthus* individuals, as well as all putative *P. calcivora* individuals. The second large group included *P. lentiscus* individuals, together with those individuals which were initially classified as *P.* × *saportae* in the field but were later identified as *P. lentiscus* by genetic analysis. The third group included only four hybrid individuals of *P.* × *saportae* confirmed by genetic analysis. DA revealed that traits LA10, LA and LL had the strongest discriminative power between the identified genetic groups (On-line Suppl. Tab. 4). Furthermore, classification analysis revealed that *P. lentiscus* individuals were correctly classified in 100% of cases, *P. terebinthus* individuals in 96.6% of cases, whereas only 50% of the confirmed hybrid individuals were classified correctly. Two confirmed hybrid individuals were correctly classified as *P.* × *saportae*, and two were classified as *P. terebinthus*.

Tab. 4. Descriptive statistics for analysed leaf morphological traits for the three identified genetic groups of *Pistacia* taxa. M – arithmetic mean, SD – standard deviation, CV – coefficient of variation (%). Analysed traits: LA – leaflet area, LL – leaflet length, MLW – maximal leaflet width, PMLW – leaflet length measured from the base to the point of maximum leaflet width, LW90 – leaflet width at 90% of leaflet length, FC – form coefficient, LA10 – angle enclosed by the main leaflet vein (the centre of leaflet blade) and the line connecting the leaflet base to a set point on the leaflet margin, at 10% of total leaflet length, RL – rachis length, PL – petiole length, NL – number of leaflets.

Trait	Descriptive parameter	*P. lentiscus*	*P. terebinthus*	*P.* × *saportae*
LA (cm^2)	M	1.61	6.09	4.78
	SD	0.69	2.56	1.55
	CV (%)	42.77	42.10	32.46
LL (cm)	M	2.43	4.11	4.05
	SD	0.52	0.89	0.74
	CV (%)	21.34	21.79	18.35
MLW (cm)	M	0.91	1.94	1.66
	SD	0.26	0.48	0.28
	CV (%)	29.06	24.89	16.63
PMLW (cm)	M	1.17	1.93	1.75
	SD	0.28	0.49	0.40
	CV (%)	23.77	25.45	22.85
LW90 (cm)	M	0.47	0.99	0.66
	SD	0.18	0.37	0.23
	CV (%)	37.49	37.32	34.67
FC	M	0.57	0.69	0.62
	SD	0.11	0.09	0.05
	CV (%)	20.06	12.62	7.54
LA10 (°)	M	34.08	45.55	39.85
	SD	7.31	7.59	7.73
	CV (%)	21.46	16.66	19.40
RL (cm)	M	5.47	8.35	8.35
	SD	1.31	1.94	1.16
	CV (%)	23.85	23.22	13.95
PL (cm)	M	3.35	6.69	5.64
	SD	1.41	2.54	1.83
	CV (%)	42.09	38.04	32.43
NL	M	7.65	7.34	7.95
	SD	1.89	1.67	1.20
	CV (%)	24.73	22.82	15.06

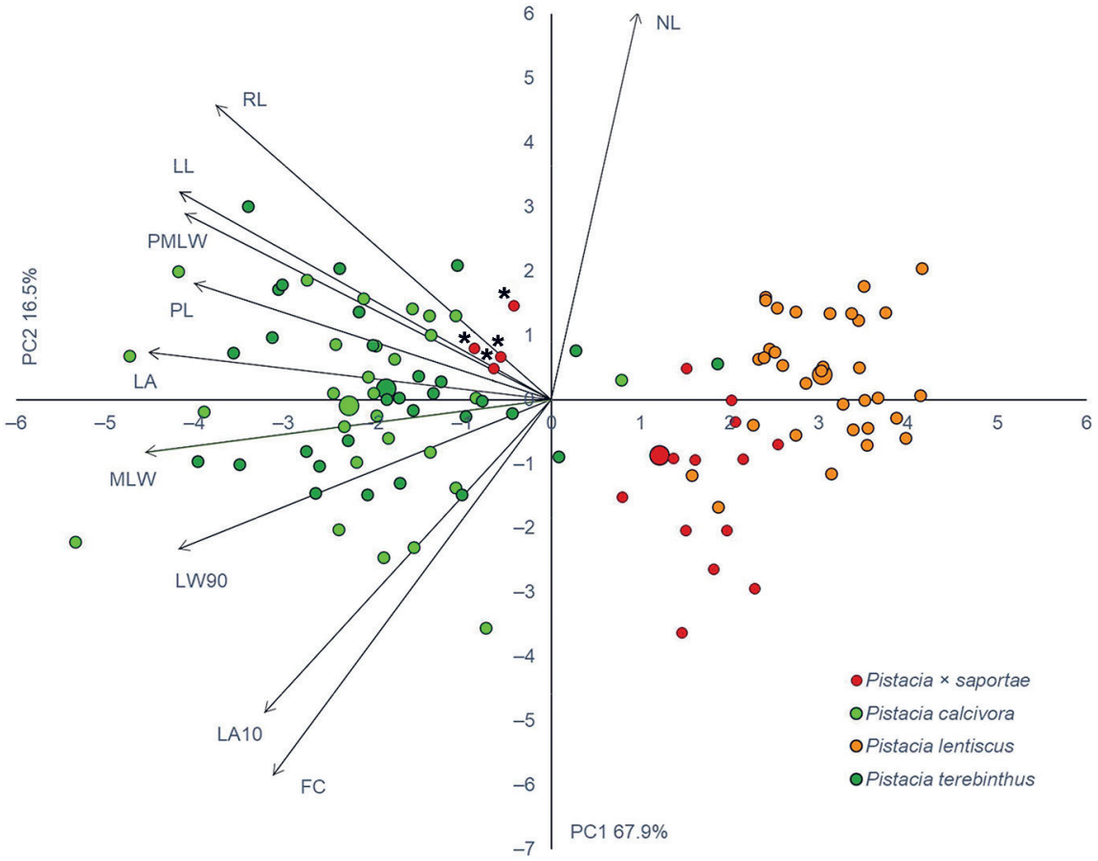

Fig. 3. Principal component analysis (PCA) of the investigated *Pistacia* taxa (109 individuals) in the eastern Adriatic based on analysed leaf morphological traits. Each individual shrub/tree is indicated by a small sign, while the taxa barycentres are represented by larger ones. Individual assignment to the specific taxon is based on the initial *a priori* identification based on leaf morphology. Hybrid individuals corresponding to the confirmed *P.* × *saportae* are indicated with an asterisk.

Discussion

In our study, we aimed to resolve genetic relationships and taxonomy of four putative indigenous *Pistacia* taxa growing in the eastern Adriatic (*P. lentiscus*, *P. terebinthus*, *P.* × *saportae* and *P. calcivora*) using molecular AFLP markers and morphology. Our results based on the genetic data have shown the presence of two well distinct genetic groups; the first group included all individuals of *P. terebinthus* and individuals initially identified based on morphology as an endemic taxon *P. calcivora*. The second group corresponded to *P. lentiscus* and included the majority of sampled individuals of the potential hybrid taxon *P.* × *saportae*. However, four individuals of *P.* × *saportae* showed a clear hybrid character at the molecular level and were placed between the two parental species in the phylogenetic tree (Fig. 2). Leaf morphology analyses have shown statistically significant differences between the identified genetic groups where LA10, LA and LL showed the highest discriminatory power. Moreover, we have demonstrated an overlap between individuals of *P. terebinthus* and putative *P. calcivora* based on the measured morphological traits, while putative individuals of *P.* × *saportae* exhibited intermediate morphological characteristics between the two parental species (*P. terebinthus* and *P. lentiscus*) (Fig. 3). Nevertheless, confirmed *P.* × *saportae* hybrids were morphologically more similar to *P. terebinthus*. Overall, based on the combined obtained results, our study clearly confirms the presence of the hybrid taxon *P.* × *saportae* in the eastern Adriatic, while we found no support for the described endemic taxon *P. calcivora*.

Despite numerous previous studies investigating genetic relationships among different wild *Pistacia* species, particularly in the Mediterranean (Kafkas and Perl-Treves 2002, Golan-Goldhirsh et al. 2004, Kafkas 2006, Yi et al. 2008, Vendramin et al. 2009, Xie et al. 2014), to the best of our knowledge this is the first study of genetic relationships of indigenous *Pistacia* taxa in the eastern Adriatic. For comparison, Katsiotis et al. (2003) investigated the genetic relationships among selected *Pistacia* species and cultivars in Greece using RAPD and AFLP markers, including *P. lentiscus* and *P. terebinthus* from the native species in this region. However, the samples of these two taxa were collected only from the Botanical Garden in Athens and the study has primarily focused on introduced species and cultivars present in Greece. Moreover, the vast majority of studies investigating genetic variation and phylogeny in *Pistacia* have been focused on numerous cultivars and/or species of commercial interest involved in fruit and resin production, among other purposes (Shanjani et al. 2009, Ziya Motalebipour et al. 2016).

On average, we detected higher genetic diversity (H_E and %P) in the *P. terebinthus* genetic group (including all individuals initially identified as putative *P. calcivora*), while lower genetic diversity was identified in the *P. lentiscus* group (Tab. 2). However, *P. lentiscus* exhibited higher frequency of rare alleles (DW). Although other more recent studies on the genetic diversity of wild *Pistacia* species mainly rely on SSR markers (Vendramin et al. 2009, Ziya Motalebipour et al. 2016, Karcı et al. 2020, Guney et al. 2021), making direct comparisons in terms of absolute values of genetic diversity parameters impossible, Ziya Motalebipour et al. (2016) and Karcı et al. (2020) also found higher genetic diversity in *P. terebinthus* compared to *P. lentiscus*, which is in relative terms consistent with our results. Higher genetic diversity of *P. terebinthus*, also in comparison to other species of the genus (Ziya Motalebipour et al. 2016, Karcı et al. 2020), may be due to the fact that *P. terebinthus* is likely one of the most recently evolved species of the *Pistacia* genus (Parfitt and Badenes 1997, Vendramin et al. 2009). On the other hand, *P. lentiscus* is considered the most distant species from the most ancestral *P. vera* (Parfitt and Badenes 1997, Kafkas 2006, Ziya Motalebipour et al. 2016, Karcı et al. 2020), which may explain higher genetic rarity observed for this taxon.

Pistacia calcivora, a taxon presumed endemic to the eastern Adriatic, was initially described by Radić (1985: 40) from the Biokovo Mountain based only on morphological characteristics (On-line Suppl. Fig. 1). Moreover, in his work Radić described two varieties and 14 forms of *P. calcivora* based on morphology, indicating very high intraspecific morphological variability of this putative taxon, which may be influenced by specific microhabitat and/or soil conditions in the Biokovo mountain range known for its limestone bedrock. *Pistacia calcivora* was described as occurring on rocky limestone slopes and cliffs, being exposed to harsh environmental conditions and strong winds, occurring mainly in the transitional area between the eu-Mmediterranean and sub-Mediterranean zones along the Biokovo mountain range. Indeed, we have confirmed that the putative taxon *P. calcivora* has very high variability of leaf morphology compared to other investigated taxa and has significantly lower number of leaflets than *P. terebinthus*. Nevertheless, *P. terebinthus* and *P. calcivora* had overall very similar and overlapping leaf morphology (Fig. 3). Moreover, in the genetic clustering and phylogenetic analyses, all presumed *P. calcivora* individuals clustered in the *P. terebinthus* genetic group, confirming that taken together there is no difference at the molecular or morphological level between *P. calcivora* and *P. terebinthus*. Thus, they are the same species and therefore the name *P. calcivora* can be only considered as a synonym of *P. terebinthus*. We can only assume that the description of *P. calcivora* is a result of distinct morphological variability and genetic diversity within the *P. terebinthus*.

In addition to consideration of the putative taxon *P. calcivora*, the emphasis of our research was placed on the potential hybrid taxon *P.* × *saportae* in the eastern Adriatic. *Pistacia* × *saportae* has been recorded widely in the Mediterranean, however, to date it was not recorded for the area of the eastern Adriatic in the relevant global databases (Euro+Med 2006-2023, POWO 2023). *Pistacia* × *saportae* was officially recorded in Croatia for the first time on the island of Korčula (Jeričević et al. 2014) based on morphological characteristics, although we found an older record, specimens collected by Radić from 1985, in the herbarium MAKAR from the Biokovo area (On-line Suppl. Fig. 1). There are also few additional recent records in FCD of the putative *P.* × *saportae* along the eastern Adriatic on the islands Šolta, Vis and Krk (Nikolić 2023). In Spain, it was investigated and described by Werner et al. (2001) using RAPD molecular markers and morphological characteristics, which was also the first molecular study of this taxon. The authors confirmed the hybrid origin of *P.* × *saportae* and the proposed parental species. This was later further supported by a more detailed phylogenetic study of the whole genus by Yi et al. (2008) who confirmed the taxonomic status of *P.* × *saportae* using two nuclear (ITS and NIA-i3) and three plastid (cpDNA) regions (ndhF, trnC-trnD, and trnL-F). The latter study also identified *P. terebinthus* as paternal and *P. lentiscus* as maternal taxon. In our study, identified hybrid *P.* × *saportae* individuals had a higher proportion of *P. lentiscus* gene pool and positioned themselves closer to *P. lentiscus* in the phylogenetic tree (Fig. 2). However, based on our results we cannot rule out the gender of *P.* × *saportae* parental taxa.

There is not much available literature data regarding the morphology of the hybrid *P.* × *saportae*. AL-Saghir and Porter (2012) in their latest taxonomic revision of the genus describe the leaves of this species as being evergreen, up to 10 cm long, with seven to nine leaflets, which are on average 5.8 cm long and 2.2 cm wide. Their description was based on the examined material from Cyprus, Palestine, and Spain. Provided dimensions are slightly larger than those obtained in our study (4.05 × 1.66 cm). Werner et al. (2001) described morphological characteristics based only on material from the Iberian Peninsula, where they mention five to 11 leaflets. The authors already indicated that *P.* × *saportae* shows intermediate characteristics between the parental species, particularly regarding leaf shape, and displays high morphological heterogeneity. This pattern was largely confirmed in our study where putative *P.* × *saportae* individuals, identified *a priori* solely on morphology, were highly variable in their traits and were located between the parental taxa showing intermediate morphological characteristics (Tab. 3, Fig. 3). However, only four individuals (19%) were confirmed as *P.* × *saportae* hybrids at the molecular level, which is much lower compared to identification based on morphology. For example, the population from the island of Šolta, which was according to the initial identification based on leaf morphology identified as *P.* × *saportae*, was shown to be within the *P. lentiscus* genetic group (Fig. 2). On the other hand, four hybrid individuals of *P.* × *saportae* confirmed at the molecular level grouped together with individuals of *P. terebinthus* based on morphology (Fig. 3).

Thus, our results emphasise previous observations that the correct identification of *P.* × *saportae* exclusively based on morphology is challenging and highly uncertain (Werner et al. 2001).

In conclusion, our results provide no support for treating the described taxon *P. calcivora* as a separate species; instead, it should be considered as a synonym of *P. terebinthus*. Furthermore, we have confirmed for the first time the presence of the hybrid taxon *P.* × *saportae* in the eastern Adriatic at the molecular level on the islands of Korčula and Vis. Given the wide natural distribution of the hybrid parental taxa *P. terebinthus* and *P. lentiscus* across the eastern Adriatic and eastern Mediterranean, we can expect *P.* × *saportae* to be also present in Montenegro, Albania, and Greece, although it may not been officially confirmed in those countries yet. Thus, its distribution along the eastern Adriatic should be further investigated.

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Secondary sexual dimorphism and morphological diversity in two allopatric juniper species: *Juniperus oxycedrus* and *J. deltoides*

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Abstract – *Juniperus* L. is a very diverse genus of dioecious or monoecious conifers distributed throughout the Mediterranean region. In addition to the complex taxonomic characteristics of the genus, sexual dimorphism leads to sex-specific differences in the morphology of juniper species, which further complicates the delimitation of species. Two of these species, *Juniperus oxycedrus* L., which occurs in the western part of the Mediterranean, and *J. deltoides* R.P.Adams, which occurs in the eastern part, have only recently been delimited as separate species. To further support the delimitation of the species, we examined the phenotypic traits of the cones and needles of both species for both sexes. Three populations from the western and three from the eastern part of the Mediterranean region were sampled and a total of 2400 needles, 1200 cones and 1200 seeds were measured and analyzed. Both needles and cones of *J. oxycedrus* were slightly larger, longer and wider than those of *J. deltoides* and also less variable. Sexual dimorphism was observed in most of the needle traits measured, with the majority of traits in *J. oxycedrus* being larger in females than in males. Although sexual dimorphism was confirmed based on needle morphology, no consistent pattern of diversity was observed between the two species. In addition, variability among populations of *J. oxycedrus* was higher than that of *J. deltoides*. Our results confirm the previous species delimitation and open the possibility for further exploration of sex-specific differences in adaptability, as well as the potential implications for differential management and conservation of individuals of both sexes.

Keywords: dioecy, *Juniperus*, Mediterranean, needle and cone morphology, morphometric analysis, population variability, sex-specific adaptations, taxonomy

Introduction

Genus *Juniperus* L. is the second most diverse genus of coniferous plants, as well as the most numerous genus of the Cupressaceae family, with 53 species distributed across broad regions of Northern Hemisphere (Farjon 2010). The initial diversification, which occurred during the Eocene in Europe (Allen and Armstrong 2008), continues today in three separate, though climatologically similar, arid areas of the Mediterranean, northern Mexico and southwestern USA, as well as in central Asia (Mazur 2021). The genus is characterized by high demands for light and pioneering character, often enabling juniper species to thrive in a climate where few woody plants survive (Sanchez-Salguero and Camarero 2020). Seeds of this wind-pollinated genus are dispersed by frugivores, mostly birds and small mammals (Santos et al. 1999). Taxonomically complex (Mazur 2021), the genus is easily recognized by fleshy cones with merged seed scales, as well as the diversity of leaf forms, with species having either needles or scaly leaves, or even both, depending on the age of the plant (Adams 2014a). In addition, the majority of junipers are characterized by sex-

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ual dimorphism, i.e., the presence of exclusively male or female cones on an individual plant (Nuñez et al. 2015).

Dioecy, the characteristic of a species that has distinct male and female individuals, even though relatively rare among plants, is present in 65% of contemporary gymnosperm species, and is a dominant sexual system in eight families (Walas et al. 2018). Individuals of dioecious plant species often differ in a range of ecological, morphological, and physiological traits (Hultine et al. 2016). This is most commonly expressed through the larger floral displays of male individuals, as well as through larger or more abundant flowers, or cones when gymnosperms are considered. Additionally, secondary sexual traits unrelated to sexual organs can also be used to differentiate the sexes, including shoot structuring, leaf morphology and physiology, water use efficiency and susceptibility to pests and herbivores (Moore and Pannell 2011). These changes in secondary traits reflect sex-specific adaptations to different resource needs, related to the timing and cost of male and female reproduction (Hultine et al. 2016). Additionally, spatial segregation of the sexes may cause physiological and morphological adaptations to different environmental conditions, further emphasizing dimorphic characters (Garbarino et al. 2015, Hultine et al. 2016).

In addition to extreme variability across environmental gradients, such as light availability (Coble and Cavaleri 2014), precipitation (Meier and Leuschner 2008) or temperature (Royer et al. 2009), leaf phenotypic traits have shown to be very variable between male and female individuals of dioecious plants as well (Li et al. 2007, Midgley 2010, Rabska et al. 2020). For many dioecious species, there is a general assumption that females are faced with an increased demand for higher reproductive investment, due to both flowering and fruit/cone bearing, fewer resources being allocated to other processes, such as growth or defense (Nicotra et al. 2003, Korgiopoulou et al. 2019). Therefore, differences in resource trade-offs and eco-physiological optima between sexes may directly influence vegetative growth (Moore and Pannell 2011) and leaf morphology (Ashman 2005, Midgley 2010, Rabska et al. 2020). Sex-specific adaptations to resource uptake, allocation and utilization, designed to meet the costs of reproduction, may be greatly influenced by increasingly pronounced climate change (Hultine et al. 2016), especially in the Mediterranean region.

Junipers, as species tolerant of drought and xeric conditions (Cano Ortiz et al. 2021), are common members of the Mediterranean landscape. Two of those species, commonly found throughout the Mediterranean basin, have until recently been considered synonymous: *J. oxycedrus* L. and *J. deltoides* R.P.Adams. The genetic separation of the two species was revealed using nrDNA sequencing, RAPDs (Adams 2004, Adams et al. 2005), simple sequence repeats, SSRs (Boratyński et al. 2014) and further confirmed by the analysis of needle terpenoids and morphology (Rajčević et al. 2013). When observing both species, differences in needle morphology and overall shape can be noted. Apart from having longer and narrower needles, *J. oxycedrus* is characterized by a tapered needle base. On the other hand, needle sides in *J. deltoides* are parallel or obtuse, giving them a triangular or deltoid appearance (Adams 2014b). An additional distinguishing character between the two species is a rounded crown shape in *J. oxycedrus*, as opposed to a pyramidal crown shape in *J. deltoides*. In addition, the cone scales of *J. oxycedrus* are smooth and do not protrude, whereas the cones of *J. deltoides* have protruding scales (Adams 2014b). Although both are widely distributed throughout their natural range, each covers a particular part of the Mediterranean basin, *J. oxycedrus* the western (from Northern Africa through Portugal to Italy), and *J. deltoides* the eastern (from Italy to Türkiye). Due to its central position in the Mediterranean region, the Apennine distribution of species is not entirely clear as it is likely that they are sympatric in some regions (Adams 2014b). Growing in the same, unfavorable xeric environments, on inaccessible limestone or siliceous terrain (Cano Ortiz et al. 2021) and commonly affected by wildfires, both of these species are under intensive anthropological influences (Rupprecht et al. 2011). With climate change affecting the Mediterranean basin more severely (Lange 2020), it is likely that some populations of both species will be under severe pressure, making it necessary to further explore their diversity, and resulting adaptability.

Although extensive previous research has been conducted on morphology (Klimko et al. 2004, 2007, Adams 2014b, Brus et al. 2011, 2016, Roma-Marzio et al. 2017), ecology (Muñoz-Reinoso 2003, Cano Ortiz et al. 2021), genetics (Boratyński et al. 2014, Adams et al. 2015), distribution models (Rupprecht et al. 2011) and studies on essential oils (Rajčević et al. 2013, Roma-Marzio et al. 2017, Semerdjieva et al. 2019), intersexual morphological differences between these two species are less well-known (Brus et al. 2011, 2016, Semerdjieva et al. 2019) but could provide insight into the adaptability of the species.

Therefore, in this research, morphometric analysis was performed on selected populations of *J. oxycedrus* from the western, and on populations of *J. deltoides* from the eastern Mediterranean, to test the degree of morphological distinctiveness among the taxa and sexes. Furthermore, phenotypic variation within and among populations was analyzed.

Materials and methods

Material and sampling

Plant material used in this study was derived from 60 individuals from three natural populations of *J. oxycedrus* originating from the Provence-Alpes-Côte d'Azur region in southern France and 60 individuals from three natural populations of *J. deltoides* from the north-eastern coast of the Adriatic Sea. Each population included samples from 10 male and 10 female individuals. Both studied regions are characterized by a Mediterranean climate, with mild winters, dry and hot summers, and high insolation rates

(Lionello et al. 2006). *Juniperus oxycedrus* populations are, compared to *J. deltoides* populations, located further inland and are found in areas of lower precipitation. In addition, the two study sites share very similar annual temperature values. Western Mediterranean populations representing *J. oxycedrus* included populations La Beaucet (P1), Grand Vallat (P2) and Les Plantades (P3) located in France. *Juniperus deltoides* populations in the Eastern Mediterranean included the island populations Cres (P4) and Krk (P5), as well as the coastal population Pula (P6), located in the Northern Adriatic region (Tab. 1, On-line Suppl. Fig. 1).

Needle and cone samples for morphometric analyses were collected during September and October of 2021. Great attention was paid to sampling only sexually mature individuals, in order to determine accurately the sex of the plant by the presence of cones or male strobili, or their residue on the branches (Brus et al. 2016). Several short shoots were cut from each individual on the sunlit side of the canopy, from which 20 random, fully developed and undamaged, one-year-old needles were selected from the central part of the shoot. Needles were first pressed and then glued to white paper, revealing the underside of the needles. Thus prepared, needles were measured and stored in the herbarium at the Faculty of Forestry and Wood Technology of the University of Zagreb (DEND). In addition, 20 mature cones were collected from female individuals. The number of scales was determined, and the cones were measured. The cones were then carefully opened, and seeds were extracted and counted. Out of the total seed count, 20 seeds per individual were randomly selected and measured. In total, 120 individuals, 2400 needles, 1200 cones and 1200 seeds were analyzed.

Studied phenotypic traits

Needle phenotypic traits were measured using the WinFolia program (WinFolia™ 2001) with an accuracy of 0.1 mm. Measurement involved six traits related to needle size: needle area (NA); needle length (NL); maximum needle width (MNW); needle length, measured from the needle base to the point of maximum needle width (PMNW); and needle width at 50% (NW50) and 90% (NW90) of needle length; and two angles describing needle base NA10 and NA25, representing angles closed by the main needle vein and the line connecting the needle base to a set point on the needle margin, at 10% and 25% of total needle length, respectively.

Cone length (CL), cone width (CW) and seed thickness (ST) were measured using digital caliper (Alpha Tools®, Bahag AG, Germany), with an accuracy of 0.01 mm. The number of cone scales (NCS) and the number of seeds (NS) were counted manually, while seed length (SL) and seed width (SW) were determined using the WinSeedle program (WinSeedle™ 2011).

Statistical analysis

Pearson correlation coefficients were calculated among all needle and cone traits including all studied individuals using the CORR procedure in SAS v9.3 (SAS Institute 2011).

The phenotypic diversity of needles and cones was analyzed using descriptive statistics parameters, including arithmetic mean, standard deviation, minimum and maximum values, and coefficient of variation (%).

Six traits related to needle morphology and seven traits related to cones were subjected to analysis of variance using the MIXED procedure in SAS (SAS Institute 2011). For needle-related traits, the model included the effect of taxon (T), sex (S) and their interaction (T × S), population within taxon (P(T)) and population × sex interaction within taxon (P × S(T)) as fixed effects and individual (I) within taxon, population and sex (I(T × P × S)) as a random effect. For cone-related traits, the model included the effect of taxon (T) and population within taxon (P(T)) as fixed effects and individual (I) within taxon and population (I(T × P)) as a random effect. Partitioned F-tests (SLICE option) were conducted to examine the significance of the population × sex interaction within each taxon and each population (only in the case of needle-related traits) and of populations within each taxon (in the case of all traits).

Principal component analysis (PCA) was performed using the PRINCOMP procedure in SAS (SAS Institute 2011). The biplot was constructed by two principal components showing analyzed individuals and traits.

Morphological differentiation was assessed by calculating the Euclidean distances between all pairs of individuals based on the scores of the first two principal components

Tab. 1. Sampling sites, geographic coordinates, and multivariate diversity index (MDI) for six studied *Juniperus oxycedrus* and *J. deltoides* populations. Values followed by the same letters are not significantly different at P > 0.05 according to Wilcoxon Rank Sum test.

Population	Sampling site	Taxon	Longitude (E)	Latitude (N)	Needle Multivariate diversity index (MDI)	Cone Multivariate diversity index (MDI)
P1	Le Beaucet	*J. oxycedrus*	5.127109	43.981774	1.373^{b}	1.373^{a}
P2	Grand Vallat	*J. oxycedrus*	5.020115	44.130109	1.551^{a}	1.070^{b}
P3	Les Plantades	*J. oxycedrus*	5.016226	44.110015	1.172^{c}	1.501^{a}
P4	Cres	*J. deltoides*	14.36315	45.05983	1.200^{c}	0.909^{b}
P5	Krk	*J. deltoides*	14.53893	45.09356	1.447^{b}	0.959^{b}
P6	Pula	*J. deltoides*	13.90052	44.03041	1.288^{bc}	1.236^{ab}
		J. oxycedrus – total			1.506	1.772
		J. deltoides – total			1.385	1.110

(PCs) considering needle and cone traits. The average Euclidean distances were calculated for each population and taxon and used as a multivariate diversity index (MDI) of a population and taxon. The Kruskal-Wallis test (among all populations) and the Wilcoxon rank sum test (between all possible population pairs) were performed using the NPAR-1WAY procedure in SAS (SAS Institute 2011), as was the Kruskal-Wallis test between studied taxa.

Discriminant analysis (DA) was performed to determine which of the needle and cone traits were most useful in maximizing discrimination between the studied taxa. The STEPDISC, DISCRIM and CANDISC procedures in SAS (SAS Institute 2011) were used for this purpose.

Results

Correlations

In order to determine how specific needle and cone variables correlated with each other, Pearson's correlation coefficients were calculated. When observing correlations among needle traits (On-line Suppl. Tab. 1), statistically significant correlations ($P < 0.05$) were determined among all of the studied traits except NA10 and MNW and NW50, as well as between NA25 and MNW, and NW50 and NW90. Of those, negative correlations were found only between traits related to needle base angles (NA10 and NA25) and NA, and NL and PMNW. Pearson's correlation coefficient value (r) was above 0.7 in nine cases, and below -0.7 in two cases. Among analyzed cone traits, statistically significant correlations were found between all traits, except NCS and NS, and SW and ST, in addition to NS, which was correlated only with ST (On-line Suppl. Tab. 2). Eight pairs had the r value above 0.7. Furthermore, positive correlations were also found between all of the needle traits related to its size and all of the cone traits except NS, which was only correlated to NW90 (On-line Suppl. Tab. 3). Needle traits related to its shape were either not significantly or negatively correlated with cone traits. The trait NA10 was negatively correlated with CL, CW, SL, SW and ST, while NA25 was correlated only with ST.

Needle phenotypic traits

The results of morphological analyses of needles can be observed at the species, sex, and population levels. Observing the differences in needle dimensions and shape between the species, it is clear that *J. oxycedrus* has, on average, larger (0.19 mm^2), longer (1.56 mm) and wider (0.17 mm) needles, compared to the smaller (0.12 mm^2), shorter (1.17 mm) and narrower (0.14 mm) needles in *J. deltioides* (Tab. 2). In both species the most variable needle phenotypic traits were PMNW (CV = 49.18 and 76.02%, respectively) and NW90 (CV = 33.88 and 44.87%), while the least variable were MNW (CV = 15.51 and 19.03%) and NW50 (CV = 18.81 and 17.78%). In general, *J. deltoides* showed greater variability, with CVs ranging from 17.78 to 76.02%, than *J. oxycedrus* with a range from 15.51 to 49.18%.

Tab. 2. Descriptive statistics for analyzed needle traits for *Juniperus oxycedrus* and *J. deltoides*. $\bar{X}$ – arithmetic mean; CV – coefficient of variation (%). Needle morphometric traits acronyms: NA – needle area; NL – needle length; MNW – maximum needle width; PMNW – needle length, measured from the needle base to the point of maximum needle width; NW50 – needle width at 50% of needle length; NW90 – needle width at 90% of needle length; NA10 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 10% of total needle length; NA25 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 25% of total needle length.

Trait	Descriptive parameter	*J. oxycedrus*			*J. deltoides*		
		Total	Male	Female	Total	Male	Female
NA (cm^2)	$\bar{X}$	0.19	0.18	0.19	0.12	0.11	0.13
	CV	24.40	21.84	26.12	28.79	30.92	24.58
NL (cm)	$\bar{X}$	1.56	1.56	1.57	1.17	1.08	1.27
	CV	20.23	20.24	20.23	22.71	23.46	19.21
MNW (cm)	$\bar{X}$	0.17	0.16	0.17	0.14	0.14	0.14
	CV	15.51	15.67	15.36	19.03	19.84	18.11
PMNW (cm)	$\bar{X}$	0.38	0.36	0.41	0.19	0.16	0.23
	CV	49.18	49.86	47.71	76.02	75.62	71.47
NW50 (cm)	$\bar{X}$	0.14	0.13	0.14	0.12	0.11	0.12
	CV	18.81	19.99	17.30	17.78	17.62	17.86
NW90 (cm)	$\bar{X}$	0.05	0.05	0.06	0.03	0.03	0.03
	CV	33.88	38.52	28.59	44.87	42.68	44.76
NA10 (°)	$\bar{X}$	23.88	23.99	23.76	28.77	31.27	26.28
	CV	22.75	23.48	22.00	27.22	25.64	25.83
NA25 (°)	$\bar{X}$	10.93	10.86	10.99	12.60	13.82	11.39
	CV	26.90	28.11	25.67	32.58	30.88	31.15

Sexual dimorphism was detected for the majority of measured needle traits, particularly in *J. oxycedrus*, where needles from female individuals displayed larger values for seven out of eight phenotypic traits (all traits except NA10). The distinction between the sexes was less pronounced in *J. deltoides*, where females had higher values in four of the measured phenotypic traits (NA, NL, PMNW and NW50), males in two (NA10 and NA25), while no difference was noted between two traits (MNW and NW90). In addition, male individuals of *J. oxycedrus* proved to be noticeably more variable than the females, having greater CV values of seven out of eight measured needle traits (all except NA). On the other hand, the degree of variability in *J. deltoides* was evenly distributed between males and females (Tab. 2).

Observing phenotypic characteristics of *J. deltoides* on a population level, it is evident that all three studied populations have the same mean value for NA (0.12 mm^2) (On-line Suppl. Tab. 4). However, there are differences according to other measured needle traits. For example, the population Pula (P6) had the highest mean values for the five measured needle traits, mainly those related to the needle width (MNW, NW50 and NW90) and the shape of the base (NA10 and NA25). On the other hand, the population with the majority of the lowest values was Krk (P5) with six minimum mean values (MNW, PMNW, NW50, NW90, NA10, NA25). However, the latter population had the longest needles. According to the obtained CV values, the most variable population in terms of needle phenotypic traits was Krk (P5), with five maximal CV values (MNW, PMNW, NW90, NA10, NA25), while NA and NL were the most variable in population Pula (P6). The lowest variability was detected equally frequently in populations Cres (P4) (NA, NL, NA10, NA25) and Pula (P6) (MNW, PMNW, NW50, NW90). Coefficients of variability among sexes within studied populations of *J. deltoides* were greatest in population Krk (P5), where both male and female individuals had the majority of the maximum CV values for five and eight traits, respectively. On the other hand, the least variable males and females, needle-morphology-wise, were found in population Pula (P6), with the lowest values for six and four traits, respectively.

In *J. oxycedrus*, the biggest and the widest needles were characteristic of the population Les Plantades (P3), which had the highest mean values for NA, MNW, PMNW, NW50 and NW90. Needles of *J. oxycedrus* were the smallest and the shortest in population Grand Vallat (P2) and the narrowest in Le Beaucet (P1), while these two populations shared the lowest values for MNW, NW50 and NW90. The longest needles were typical of the population Le Beaucet (P1), while needle base angles were largest in the population Grand Vallat (P2). When traits' variability is considered, the most variable population according to the obtained CV values was Grand Vallat (P2), with five maximum values (NA, NL, MNW, PMNW, NA10), followed by population Le Beaucet (P1) with three (NW50, NW90, NA25). According to coefficients of variability, the least variable population was Les Plantades (P3) with six minimal CV values (MNW, PMNW, NW50, NW90, NA10, NA25). Females had the largest needles in the population Les Plantades (P3) and the smallest in Grand Vallat (P2). Needles of male individuals were the longest and largest in the population Le Beaucet (P1), as opposed to the smallest and the shortest needles found in Les Plantades (P3), albeit the needles in P3 were the widest.

Cone phenotypic traits

Generally, when *J. oxycedrus*, is compared to *J. deltoides*, it excels in all studied characteristics of cones, from their dimensions, through the number of scales, to the dimensions and number of seeds (Tab. 3). On average, cones of *J. oxycedrus* were 2.09 mm longer and 1.13 mm wider, while seeds were 1.21 mm longer, 1.30 mm wider and 0.75 mm thicker than those of *J. deltoides*. In addition, dimensions of cones and seeds were less variable in *J. oxycedrus*, with coefficient of variation ranging from 11.21 to 11.79% and from 10.54 to 18.67%, respectively, compared to those of *J. deltoides* with CV values ranging from 13.66 to 14.37% for cone, and from 14.28 to 19.45% for seed dimensions. On the other hand, NCS and NS were less variable in *J. deltoides*, with CV values of 14.52 and 20.46%, respectively. Overall, the most variable cone trait in both species was NS, with CV values of 20.46 in *J. deltoides* and 30.88% in *J. oxycedrus*.

Observing individual populations of *J. deltoides* (On-line Suppl. Tab. 5), the largest cones and seeds, as well as the highest number of seeds, was present in population Cres (P4). Contrarily, the smallest cones were present in population Pula (P6), while the lowest number of seeds was detected in population Krk (P5). In addition, the lowest number of cone scales was detected in population Krk (P5), and the highest in population Pula (P6). The most variable population, according to cone traits, was Pula (P6), with five out of seven maximum CV values (CL, CW, NCS, SL, SW), while the least variable was population Cres (P4), with four of such traits (CW, NS, SW, ST). All of the measured cones in pop-

Tab. 3. Descriptive statistics for analyzed cone traits for *Juniperus oxycedrus* and *J. deltoides*. $\bar{X}$ – arithmetic mean; CV – coefficient of variation (%). Cone morphometric traits acronyms: CL – cone length; CW – cone width; NCS – number of cone scales; NS – number of seeds; SL – seed length; SW – seed width; ST – seed thickness.

Trait	Descriptive parameter	*J. oxycedrus*	*J. deltoides*
CL (mm)	$\bar{X}$	10.35	8.26
	CV	11.21	14.37
CW (mm)	$\bar{X}$	10.44	9.31
	CV	11.79	13.66
NCS	$\bar{X}$	3.36	3.08
	CV	22.11	14.52
NS	$\bar{X}$	2.83	2.73
	CV	30.88	20.46
SL (mm)	$\bar{X}$	7.37	6.16
	CV	10.54	14.28
SW (mm)	$\bar{X}$	4.77	3.47
	CV	18.27	19.45
ST (mm)	$\bar{X}$	3.63	2.88
	CV	18.67	18.80

Tab. 4. Results of the General Linear Model (GLM) for needle traits. Needle morphometric traits acronyms: NA – needle area; NL – needle length; MNW – maximum needle width; PMNW – needle length, measured from the needle base to the point of maximum needle width; NW50 – needle width at 50% of needle length; NW90 – needle width at 90% of needle length; NA10 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 10% of total needle length; NA25 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 25% of total needle length. df – degrees of freedom; F – F-test in GLM; P – significance of GLM's F-test.

Trait	Components of the variance	df	F	P-value
NA	Taxon	1	171.01	< 0.05
	Sex	1	9.73	< 0.05
	Taxon × Sex	1	0.81	0.370
	Population (Taxon)	4	2.07	0.090
	Population × Sex (Taxon)	4	1.89	0.118
NL	Taxon	1	103.53	< 0.05
	Sex	1	7.33	< 0.05
	Taxon × Sex	1	5.77	< 0.05
	Population (Taxon)	4	3.84	< 0.05
	Population × Sex (Taxon)	4	2.43	0.052
MNW	Taxon	1	72.42	< 0.05
	Sex	1	0.05	0.822
	Taxon × Sex	1	0.72	0.398
	Population (Taxon)	4	2.88	< 0.05
	Population × Sex (Taxon)	4	4.36	< 0.05
PMNW	Taxon	1	211.96	< 0.05
	Sex	1	21.42	< 0.05
	Taxon × Sex	1	0.53	0.47
	Population (Taxon)	4	6.47	< 0.05
	Population × Sex (Taxon)	4	0.82	0.513
NW50	Taxon	1	103.55	< 0.05
	Sex	1	5.44	< 0.05
	Taxon × Sex	1	1.18	0.279
	Population (Taxon)	4	8.51	< 0.05
	Population × Sex (Taxon)	4	4.34	< 0.05
NW90	Taxon	1	154.78	<0.05
	Sex	1	10.41	<0.05
	Taxon × Sex	1	0.04	0.033
	Population (Taxon)	4	10.62	<0.05
	Population × Sex (Taxon)	4	4.43	<0.05
NA10	Taxon	1	52.38	<0.05
	Sex	1	14.92	<0.05
	Taxon × Sex	1	12.36	<0.05
	Population (Taxon)	4	5.44	<0.05
	Population × Sex (Taxon)	4	2.26	0.067
NA25	Taxon	1	21.48	<0.05
	Sex	1	10.11	<0.05
	Taxon × Sex	1	12.59	<0.05
	Population (Taxon)	4	7.05	<0.05
	Population × Sex (Taxon)	4	2.43	0.052

ulation Krk (P5) had three cone scales, resulting in a CV value of 0.00%.

In *J. oxycedrus* populations (On-line Suppl. Tab. 5), Les Plantades (P3) had the largest cones with the most cone scales and seeds, as well as the longest seeds. On the other hand, the smallest cone and seed dimensions were characteristic of the population Grand Vallat (P2). The highest number of cone scales was present in the population Les Plantades (P3), and the lowest one in the population Le Beaucet (P1). In general, the most variable was the population Le Beaucet (P1), with five maximum CV values (CW, NS, SL, SW and ST), as opposed to the least variable population of Les Plantades (P3), with four minimum CV values (CW, NS, SL, ST). The number of cone scales was the same for all of the measured cones in the population Le Beaucet (P1), generating a CV value of 0.00%.

Analysis of variance

As expected, the analysis conducted showed statistically significant differences in needle morphology between the two species, according to all of the measured needle traits (Tab. 4). Furthermore, significant differences in all traits, except MNW, were found between the sexes. However, sex differentiation was far less pronounced on the within-taxon level, with only three statistically significant differences (NL, NA10, NA25). Significant differentiation was also revealed on the within-population level, for all measured needle traits, except NA. Additionally, differences between populations and sexes within each taxon were found to be statistically significant only in MNW, NW50 and NW90.

Results clearly indicate a different number of distinguishable traits between the two sexes of both species, with sexes in *J. oxycedrus* distinguished by all of the measured needle traits on a statistically significant level, as opposed

Tab. 5. Results of the General Linear Model (GLM) for cone traits. Cone morphometric traits acronyms: CL – cone length; CW – cone width; NCS – number of cone scales; NS – number of seeds; SL – seed length; SW – seed width; ST – seed thickness. df – degrees of freedom; F – F-test in GLM; P – significance of GLM's F-test.

Trait	Components of the variance	df	F	P-value
CL	Taxon	1	74.43	< 0.05
	Population (Taxon)	4	2.45	0.058
CW	Taxon	1	19.05	< 0.05
	Population (Taxon)	4	1.72	0.16
NCS	Taxon	1	16.69	< 0.05
	Population (Taxon)	4	13.54	< 0.05
NS	Taxon	1	0.73	0.397
	Population (Taxon)	4	0.59	0.674
SL	Taxon	1	70.3	< 0.05
	Population (Taxon)	4	4.02	< 0.05
SW	Taxon	1	103.79	< 0.05
	Population (Taxon)	4	4.97	< 0.05
ST	Taxon	1	36.54	< 0.05
	Population (Taxon)	4	2.37	0.064

to sexes in *J. deltoides*, which were only differentiated by NL, PMNW, NA10 and NA25 (On-line Suppl. Tab. 6).

The population with the most significant differences between the sexes was P6 (NA, NL, PMNW, NW90, NA10, NA25), followed by P1 (MNW, NW50, NW90, NA25). Furthermore, sexes in populations P2 and P3 were distinguished by the lowest number of traits, MNW and NA and NL, respectively. Considering differences between populations within each taxon, it is clear that differentiation is more intensely pronounced in *J. oxycedrus*, with populations differing in all of the measured needle traits. On the other hand, populations of *J. deltoides* differ only in NA10 and NA25 (On-line Suppl. Tab. 6).

Cones of *J. oxycedrus* and *J. deltoides* differed significantly in all of the analyzed cone traits, except NS (Tab. 5). However, populations within each taxon were significantly differentiated only by traits NCS, SL and SW. Interestingly, cones of *J. deltoides* showed statistically significant differences only in SL, while cones of *J. oxycedrus* had four such traits (CL, CW, NCS, SW) (On-line Suppl. Tab. 7). Number of seeds demonstrated no significant variation, neither among taxa and populations nor within taxa.

Principal component analysis

Principal component analysis (PCA) based on eight needle phenotypic traits in two juniper taxa populations revealed that the first two principal components had an eigenvalue greater than one and accounted for 89.59% of the total variation (Fig. 1A). Strong positive correlations (r > 0.70)

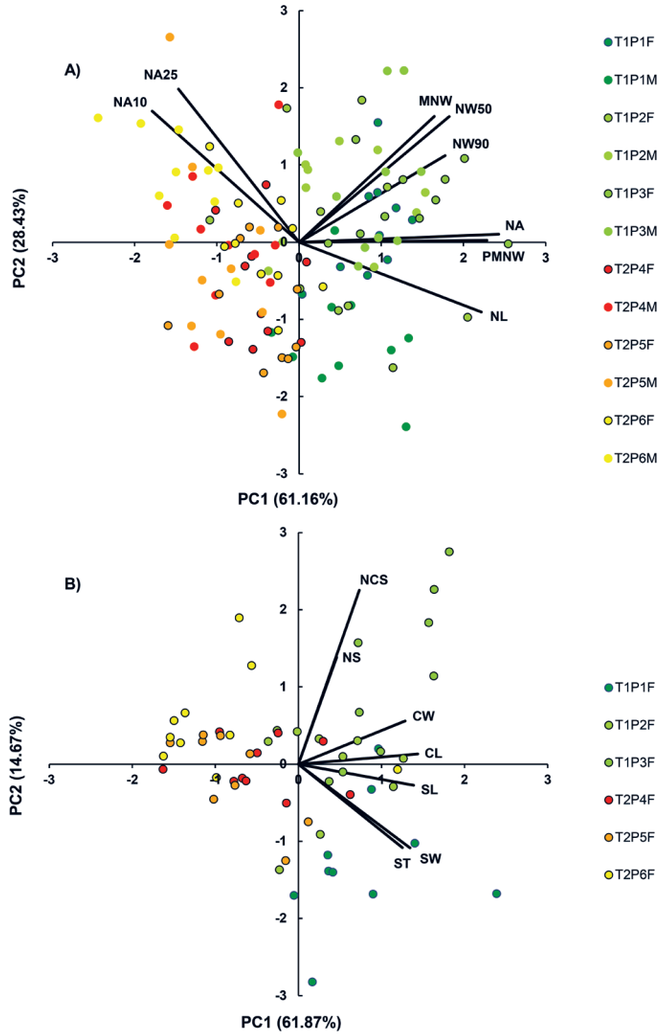

Fig. 1. Results of the principal component (PC) analysis based on (A) eight needle and (B) seven cone morphometric traits in studied *Juniperus oxycedrus* and *J. deltoides* populations. Needle morphometric traits acronyms: NA – needle area; NL – needle length; MNW – maximum needle width; PMNW – needle length, measured from the needle base to the point of maximum needle width; NW50 – needle width at 50% of needle length; NW90 – needle width at 90% of needle length; NA10 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 10% of total needle length; NA25 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 25% of total needle length. Cone morphometric traits acronyms: CL – cone length; CW – cone width; NCS – number of cone scales; NS – number of seeds; SL – seed length; SW – seed width; ST – seed thickness. T1 – *J. oxycedrus*; T2 – *J. deltoides*. F – female; M – male. Populations: P1 – Le Beaucet; P2 – Grand Vallat; P3 – Les Plantades; P4 – Cres; P5 – Krk; P6 – Pula.

were observed between the first principal component (PC1) and the needle area (NA), position of maximal needle length (PMNW), needle length (NL), needle width at 50% (NW50) and 90% (NW90) of needle length. For the same principal component, a strong negative correlation was observed only for one phenotypic trait – needle angle at 10% of needle length (NA10). The second principal component (PC2) was strongly positively correlated with the needle angle at 25% of needle length (NA25). Biplot revealed a clear grouping of the individuals according to their respective taxa (Fig. 1A). Within taxon, significant overlapping has been demonstrated. In addition, males and females of each population overlapped with one another, i.e., demonstrated the same grouping trends. The slight divergence between males and females is only noticeable in population P1, with males partially separating.

The second PCA included seven cone phenotypic traits of the juniper species studied. The first two principal components had an eigenvalue greater than one and described 76.54% of the total variation (Fig. 1B). The first principal component (PC1) was in a strong positive correlation with five out of seven measured cone phenotypic traits (CL, SL, SW, CW, ST), and explained 61.87% of the total variability. No negative correlations were determined between the first principal component and cone traits. On the other hand, the second principal component (PC2), which accounted for 14.67% of total variability, was in a strong negative correlation with seed thickness (ST) and seed width (SW). Pronounced positive correlations were found with the number of cone scales (NCS) and the number of seeds (NS). The constructed biplot demonstrates clear clustering of individuals by their respective taxa (Fig. 1B). Interestingly, some

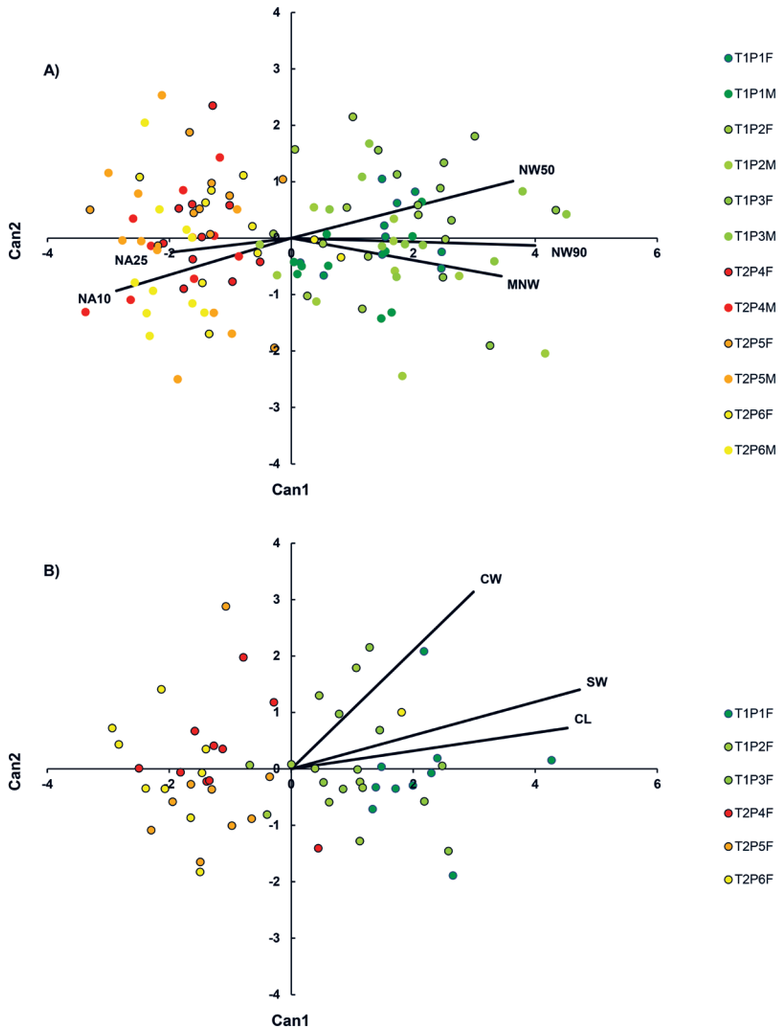

Fig. 2. The first two canonical variates of the canonical discriminant analysis (Can1 and Can2) of three *Juniperus oxycedrus* and three *J. deltoides* populations based on (A) five needle and (B) three cone morphometric traits. Each individual shrub is indicated by a small circle. Needle morphometric traits acronyms: MNW – maximum needle width; NW50 – needle width at 50% of needle length; NW90 – needle width at 90% of needle length; NA10 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 10% of total needle length; NA25 – angle closed by the main needle vein and the line connecting the needle base to a set point on the needle margin at 25% of total needle length. Cone morphometric traits acronyms: CL – cone length; CW – cone width; SW – seed width. T1 – *J. oxycedrus*; T2 – *J. deltoides*. F – female; M – male. Populations: P1 – Le Beaucet; P2 – Grand Vallat; P3 – Les Plantades; P4 – Cres; P5 – Krk; P6 – Pula.

individuals of *J. deltoides* have grouped with *J. oxycedrus*, mainly from populations P4 and P6. On a population level, a greater level of divergence was shown, compared to needles, especially in P1 and P3.

Multivariate diversity index

To explore the diversity of individuals within the studied populations and taxa multivariate diversity index (MDI) was used. Statistically significant differences were confirmed for both the needles and the cones, on both the intrapopulation and the intrataxon levels. When considering the diversity of individuals within each taxon, based on the morphology of both needles and cones, *J. oxycedrus* showed to be more diverse, demonstrating higher MDI values than those of *J. deltoides*, 1.506 and 1.772, as opposed to 1.385 and 1.110, respectively. On the population level, both *J. oxycedrus* and *J. deltoides* demonstrated MDI values within a similar range of data, i.e., populations of both species were similarly diverse. For needles, MDI values in *J. oxycedrus* populations ranged from 1.172 to 1.551, whereas *J. deltoides* ranged from 1.200 to 1.447. Similar results were obtained for cones as well, with respective species' populations ranges of 1.070 to 1.501, and 0.909 to 1.236 (Tab. 1).

Discriminant analysis (DA)

According to conducted discriminant analysis (DA), the needle traits that separated the two species the best were MNW, NW90, NW50, NA10 and NA25. Individuals of *J. oxycedrus* were characterized by wider needles, while individuals of *J. deltoides* had a wider needle base. Canonical discriminant analysis based on five traits showed that the first CV explained 100% of the variation between taxa. This is demonstrated also in the diagram (Fig. 2A), where a clear distinction between the two species along the first discriminant axis is visible. Significant overlap was detected on both population and sex levels, with no clear structuring. After the cross-validation, the discriminant function correctly classified 94.17% plants into their respective taxon. Individuals of *J. oxycedrus* were correctly classified in 91.67% cases, while *J. deltoides* showed even greater correct classification of 96.67%.

Cone traits that best distinguished species according to discriminant analysis were CL, SW and CW. All of the three mentioned traits showed greater values in *J. oxycedrus*. Canonical discriminant analysis based on three traits showed that the first CV explained 100% of the variation between taxa. As shown in the diagram (Fig. 2B), the two species were clearly separated by cone phenotypic traits as well, with negligible overlapping. However, no structuring was detected on the population level. Discriminant function correctly classified 93.10% plants into their respective taxon.

Discussion

As expected, morphological differentiation between *J. oxycedrus* and *J. deltoides* was evident in all of the studied needle and cone traits, which further confirms the existence of the two separate species, first described by Adams (2004). Compared to *J. oxycedrus*, *J. deltoides* tend to have shorter and wider needles, with deltoid base shape, as well as visible cone scales with protruded tips and glaucous powder cover (Adams 2014b). Although the morphological differences between these two allopatric, cryptic species are very small and seemingly imperceptible, their differentiation is widely accepted and has been confirmed by genetics (Adams et al. 2003, 2005, Mao et al. 2010, Boratyński et al. 2014, Adams et al. 2015) and essential oil research (Adams et al. 2003, 2005, Rajčević et al. 2013). The divergence time between the two species has been estimated at approximately 8–10 Mya, which suggests a separate colonization of the western and eastern parts of the Mediterranean (Mao et al. 2010). Namely, during the late Miocene, the formation of the Alps occurred, causing the ancestral taxa to separate into two groups.

Average values of the needle and cone dimensions of *J. deltoides*, even though slightly lower, did not differ significantly from the previously reported values (Klimko et al. 2007, Brus et al. 2011, Adams 2014b, Brus et al. 2016, Roma-Marzio et al. 2017). However, contrary to mentioned previous reports, we determined narrower needles in *J. deltoides*. On the other hand, the opposite has been determined for *J. oxycedrus*, where obtained needle and cone dimensions slightly positively deviate from prior reported values (Adams 2014b, Roma-Marzio et al. 2017). In both species, seed dimensions were somewhat larger than those reported by Roma-Marzio et al. (2017).

Overall, needles and cones of *J. deltoides* were shown to have higher coefficients of variability than those of *J. oxycedrus*. Similar results were obtained by Klimko et al. (2007), who investigated the variability of *J. oxycedrus* s.l. throughout the Mediterranean. Although their study included a larger number of populations, they did not cover specific regions of southeastern France and northern Adriatic, unlike in this research. However, since the results are congruent, it is reasonable to assume the same trend would be present throughout the entire distribution range of the two species. Nevertheless, Boratyński et al. (2014) reported greater variability in *J. oxycedrus*, supported by a higher level of genetic diversity of *Juniperus* species in the western Mediterranean, as opposed to the eastern Mediterranean. This result is in accordance with the MDI values obtained in our research, which indicate higher overall variability of *J. oxycedrus* as well. The loss of genetic diversity in the east after the emergence of the Alps could be due to processes like the founder effects resulting from repeated climatic oscillations (Zhang et al. 2005), microevolutionary changes arising from different environmental pressures and limited gene flow caused by range fragmentation (Boratyński et al. 2014).

In this study, significant sex-based differences in needle morphometric traits were observed in all of the measured traits except maximal needle width. In both species, greater needle area and length were characteristic of the female individuals. The same pattern was previously confirmed in another dioecious conifer species, *Taxus baccata* L. (Iszkuło

et al. 2009, Nowak-Dyjeta et al. 2017, Stefanović et al. 2017), as well as in some angiosperm species like *Simmondsia chinensis* (Link) C.K.Schneid. (Kohorn 1994). Larger needles in females are commonly believed to be the result of a need for greater assimilation area, required to support the reproductive efforts of females (Nowak-Dyjeta et al. 2017), as it is known that more resources are required to successfully mature fruit/cone than to produce pollen (Ashman 2005). In addition, Garbarino et al. (2015) proposed that growth in females could be favored by their occupation of more productive microhabitats, as opposed to not so fastidious male individuals. This could be further emphasized by improved soil properties under the female individuals, due to input of organic matter from the littered cones, as they are two to three times richer in nitrogen and potassium than needles and twigs (Gauquelin et al. 2002). Moreover, as wind-pollinated species, male trees of junipers are likely to be investing more resources into reproduction, due to the higher quantity and quality of reproductive material needed (Popp and Reinartz 1988, Gauquelin et al. 2002), therefore leaving fewer nutrients and less energy for foliar growth and ultimately resulting in smaller leaves (Harris and Pannell 2008). However, sexual dimorphism in this study was not detected uniformly in all of the populations. On the one hand, populations Le Beaucet (P1) and Pula (P6) displayed a significant number of gender-based differences in needle morphology, while other populations showed rather few differences. Diverse results between populations could be based on variable reproductive investment intensity of individuals of dioecious species, depending on many factors, like environmental and habitat conditions (Montesinos et al. 2011), plant size (Shibata and Kudo 2016), resource availability and pollination success (Rodríguez-García et al. 2018) and flowering and fruiting abundance (Montesinos et al. 2012). Therefore, secondary sex differences in dioecious plants are associated with trade-offs between the cost of reproduction and other plant functions, like growth, metabolism processes and defense (Maldonado-López et al. 2014, Liu et al. 2021).

In addition to significant phenotypic variability between both species and sexes, populations of the researched species demonstrated high levels of interpopulation diversity. All of the studied within-taxa populations differed significantly, according to all of the measured needle traits except NA, and three of the seven cone traits (NCS, SL, SW). Interpopulation diversity, however, is not uniform in the two taxa. Populations of *J. oxycedrus* differ significantly from each other according to all of the studied morphological traits, while differentiation in *J. deltoides* populations is present only according to two of the studied traits. This is likely the result of diminished gene flow or, alternatively, significant adaptations to specific climatic and edaphic conditions of each microhabitat (Kawecki and Ebert 2004). The lower levels of diversity noted in *J. deltoides* populations could be the result of their location, in the marginal, northernmost part of the species' distribution (Vilar et al. 2016). In such conditions, lower levels of variability are to be expected (Brus et al. 2011) due to additional selective pressures during which various events, such as the bottleneck effect, might have arisen (Abeli et al 2014), therefore impacting the allelic richness and subsequent variability of phenotype. Higher variability in morphological traits detected among *J. oxycedrus* populations could be explained by a higher level of overall genetic diversity of *Juniperus* species in the western Mediterranean than in the eastern (Boratyński et al. 2014), combined with processes like local adaptation (Savolainen et al. 2013) and phenotypic plasticity (Radersma et al. 2020). Although the results indicate higher variability in *J. oxycedrus* in relation to *J. deltoides*, no uniform trend was observed at the intrapopulation level, since some populations within both taxa were more or less variable, differing also between needle and cone traits.

Taxonomical complexity of junipers in these regions is further complicated by various other *Juniperus* taxa within the *Juniperus* (syn. *Oxycedrus* Spach) section that are widely distributed across Mediterranean region. According to Klimko et al. (2007) there are four subspecies within the *J. oxycedrus*: subsp. *oxycedrus*, subsp. *badia* (H.Gay) Debeaux; subsp. *transtagana* Franco and subsp. *macrocarpa* (Sm.) Ball. Nowadays, however, the latter two subspecies are recognized at species level, *J. navicularis* Gand and *J. macrocarpa* Sm., while former two subspecies had their status changed to varieties (WFO 2023). Furthermore, on the north-eastern coast of Adriatic Sea, sporadic reports of *J. macrocarpa* have been made (Topić and Šegulja 2000). However, none of these reports were confirmed at a later date during the research of Brus et al. (2016) and Roma-Marzio et al. (2017). As a result, there is a lack of conclusive proof of *J. macrocarpa* presence as far north as the north-eastern Adriatic coast. On the other hand, only the typical variety of *J. oxycedrus* has been reported in France (Boratyński et al. 2014, Cano Ortiz et al. 2021), with the exception of reports from Corsica. Its taxonomical congruence is, therefore, under certain terms.

Conclusions

The differences between the *J. oxycedrus* and *J. deltoides* have been confirmed in this research, with *J. oxycedrus* having larger needles and cones, although less variable, than those of *J. deltoides*. Furthermore, sexual dimorphism in needle morphology was found in both species, but more so in *J. oxycedrus*. In general, female individuals demonstrated higher trait values than males in both species. In addition to significant phenotypic variability between both species and sexes, populations of the researched species demonstrated high levels of interpopulation variability, especially in *J. oxycedrus*, while intrapopulation variability showed no uniform pattern between the two species.

The results of this research support earlier delimitations of the two taxa, in addition to opening new questions about the possible influence of gender imbalance on the morphological diversity and variability within and between the different taxa. Additional research into genetic variability could be used to further discern the specifics of the intra- and interpopulation variability.

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Astragalus nallihanicus (sect. *Caprini*, Fabaceae), a new species from Türkiye

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Abstract – Some interesting *Astragalus* L. (Fabaceae) specimens that have short stems, long stipules and short yellow petals were collected from Nallıhan, Ankara. In the careful examination made, it was seen that the specimens resemble *Astragalus ovinus* Boiss., *A. fabaceus* M.Bieb. and *A. angustiflorus* K.Koch belonging to the section *Caprini*, but it was determined that there is a difference due especially to some generative characters. After comparison with the closest taxa, it was decided that it is new for science and was named *Astragalus nallihanicus*. It grows at altitudes between about 850–1050 meters a.s.l. in a pine forest clearing. The size of some morphological features such as stipules (lower ones 17–22 mm long), calyx teeth (8–9 mm long), standards (19–22 mm long), wings (16–18 mm long), legumes (8.3–10.5 mm wide) and the dimensions of the seeds (3.5–4.1 × 2.1–2.5 mm), allow *Astragalus nallihanicus* to be distinguished from the closest taxa. Here, a description of the new species, comparison with similar taxa, informative photographs, and some ecological preferences have been given.

Keywords: Ankara, *Astragalus*, new species, taxonomy, Türkiye

Introduction

Fabaceae is one of the largest families in the world and includes approximately 650 genera and 18,000 species (Ke 2010). *Astragalus* L. is the richest genus of the family and includes approximately 3,000 taxa (Xu and Podlech 2010). *Astragalus* is also the genus that includes the most taxa in Türkiye and has become mostly adapted to the steppe habitat, as represented by 490 taxa connected to 63 sections (Polhill 1981a, b). Approximately 43% of these taxa are endemic (Chamberlain and Matthews 1970, Davis et al. 1988, Aytaç 2000, Duman and Akan 2003, Taeb and Uzunhisarcıklı 2012, Dinç et al. 2013, Karaman Erkul and Aytaç 2013, Ekici et al. 2015, Çeçen et al. 2016, İlçim and Behçet 2016, Dönmez and Uğurlu Aydın 2018, Aytaç et al. 2020, Tunçkol et al. 2020, Hamzaoğlu 2020).

Some interesting *Astragalus* specimens were collected during a student observation trip in 2022 within the scope of the forest flora and fauna. The short stems, long stipules and short yellow petals are the attributes of the specimens that first draw the attention. Specimens of the plants with flowers and fruits, adapted to the dry, pine forest openings, were collected around the end of June. It was decided at the conclusion of the investigation conducted, taking into consideration the most recent revision studies and the *Flora of Turkey and the East Aegean Islands* (here section *Myobroma* (Steven) Bunge), that the specimens are a new species for the section *Caprini* DC. (Chamberlain and Matthews 1970, Aytaç 2000, Podlech and Zarre 2013).

Caprini (incl. sect. *Myobroma*) is the largest section of the genus including a total of 273 species, a majority of which are grown in South, Central and East Europe to Middle Asia, Siberia, Mongolia and China, North Africa, Türkiye, Near East to Pakistan and North India. The plant is usually herbaceous; basifixed hairy; stipules adnate to the petioles; imparipinnate leaves; inflorescence usually sessile or sometimes long-pedunculate, 1-manny-flowered; bracts are mostly whitish-membranous; almost never has bracteoles; the calyx is short to long tubular, symmetrical base, ruptured by the legume as it grows; the petals are glabrous or more rarely hairy, whitish to mostly yellow, rarely red to dark violet from the beginning; the style is glabrous, at least in the upper part; the legume is very variable, sessile or with a distinct stipe, unilocular or incompletely to completely bilocular, and the valves are thin to strongly coriaceous in the species included in the section. The section is represented in Türkiye by 26 species (Chamberlain and Matthews 1970, Podlech 1988, Aytaç 2000, Podlech and Zarre 2013). Together with the new species described here, the number of species of the section *Caprini* in Türkiye has risen to 27.

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Materials and methods

Specimens belonging to the new species were collected in June from the surroundings of Karacasu Village, in Nallıhan District of Ankara Province, in Türkiye. The related literature (Chamberlain and Matthews 1970, Podlech 1988, Aytaç 2000, Podlech and Zarre 2013,), specimens in the GAZI and ANK herbaria, and high-resolution photographs in the Conservatoire et Jardin Botaniques de la Ville de Genève (G), Royal Botanic Garden Kew (K), Royal Botanic Garden Edinburgh (E), Naturhistorisches Museum Wien (W), and Muséum National d'Histoire Naturelle Paris (P) herbaria were utilized in the identification and evaluation of the specimens (Thiers 2023). A Leica EZ4 stereo microscope and a Samsung A33 5G mobile telephone were used in the examination of the specimens and the taking of photographs, and a ruler with a sensitivity of 0.5 mm was used in the measurements.

Results

Taxonomic treatment

The first comprehensive information in Türkiye about the genus *Astragalus*, section *Caprini* (as section *Myobroma*) was included in Volume 3 of the work titled *Flora of Turkey and the East Aegean Islands* (Chamberlain and Matthews 1970). *Astragalus ovinus* Boiss., *A. fabaceus* M.Bieb. and *A. angustiflorus* K.Koch (incl. subsp. *angustiflorus* and subsp. *anatolicus* (Boiss.) D.F.Chamb.) are three of the 15 species of the section known from Türkiye. The validity of these species was also accepted in the later revision studies of the section *Caprini* and the genus *Astragalus*. These species are very similar to each other in habit because they are acaulescent or short caulescent, their petals are yellow and their calyces are tubular. On the other hand, these species differ from each other by the shape of their leaflets and the pubescence of their legumes. The distribution areas of these species are also very close to each other and apart from in Türkiye are also known from Iraq, Iran, Armenia, Georgia, Azerbaijan, Russia and Greece. According to the latest data, 26 species of the section *Caprini* grow in Türkiye (Podlech 1988, Podlech and Zarre 2013, Aytaç 2000).

Astragalus ovinus, *A. fabaceus* and *A. angustiflorus* differ slightly from each other (in, for example, leaflet shape, legume hairiness). However, *Astragalus nallihanicus* (Fig. 1.) can be distinguished from these species by more obvious differences. When the descriptions and herbaria specimens were examined, it was understood that *Astragalus nallihanicus*, especially with respect to flower characters, was different from *A. ovinus*. For example: calyx teeth 8–9 mm long (not 3–6 mm), standard 19–22 mm long (not 24–32 mm), and keel 15–17 mm long (not 19–25 mm). It is also similar to the new species *A. fabaceus*. However, the calyx tube is glabrous and its teeth are 8–9 mm long (not hairy and teeth 2–5 mm long), wings are 16–18 mm long, the auricle is 1.4–1.8 mm long, and the claw is 8–9 mm long (not 20–24(–29) mm long, auricle 2.5–3 mm long, and claw 11–16 mm long), the keel is 15–17 mm long and the claw is 8–9 mm long (not 18–21(–26) mm long and claw 11–16 mm long). It is also similar to *A. angustiflorus*, but lower stipules are 17–22 mm long (not 10–14 mm long), calyx teeth are 8–9 mm long (not 5–7 mm long), claw of standard is 6–7 mm long (not c. 2 mm long), blades of wings are 8–9 mm long (not 11–16 mm long); seed dimensions are 3.5–4.1 × 2.1–2.5 mm (not c. 5 × 4 mm) (Tab. 1, Fig. 1, Fig. 2, Fig. 3, Fig. 4).

Discussion

The works titled "Revision von *Astragalus* L. sect. *Caprini* DC. (Legunninosae)" and "A taxonomic revision of the

Fig. 1. *Astragalus nallihanicus* Hamzaoğlu. A – habitat, B – habit, C – inflorescence and flowers, D – inflorescence and legumes.

Tab. 1. Comparison of diagnostic characters of *Astragalus nallihanicus* and related species.

Diagnostic characters	*A. nallihanicus*	*A. ovinus*	*A. fabaceus*	*A. angustiflorus*
Stipules	Adnate to petiole for 2–3 mm, lower ones 17–22 mm long	Adnate to petiole for (3–)5–7 mm, lower ones 8–10 mm long	Adnate to petiole for 2–3 mm, lower ones up to 17 mm long	Adnate to petiole for (1–)2–5 mm, lower ones 10–14 mm long
Leaves	Entirely glabrous	Glabrous or only lower surface hairy	Only rachis and lower surface hairy	Entirely glabrous or hairy
Petioles	2–6 cm long	7–17(–24) cm long	(3–)5–6(–9) cm long	(5–)8–11(–15) cm long
Racemes	9–12-flowered	3–8-flowered	6–10-flowered	4–15-flowered
Calyx	Tube glabrous, teeth 8–9 mm long	Tube glabrous, teeth 3–6 mm long	Tube hairy, teeth 2–5 mm long	Tube glabrous or hairy, teeth 5–7 mm long
Standard	19–22 mm long, claw 6–7 mm long	24–32 mm long, claw 8–9 mm long	21–31 mm long, claw 8–10 mm long	18–32 mm long, claw c. 2 mm long
Wings	16–18 mm long, blade 8–9 mm long, auricle 1.4–1.8 mm long, claw 8–9 mm long	21–27(–29) mm long, blade 11–13 mm long, auricle c. 1 mm long, claw 15–17 mm long	20–24(–29) mm long, blade 8–15 mm long, auricle 2.5–3 mm long, claw 12–16 mm long	16–22(–25) mm long, blade 11–16 mm long, auricle 1–1.5 mm long, claw 7–10 mm long
Keel	15–17 mm long, claw 8–9 mm long	19–25 mm, claw c. 15 mm long	18–21(–26) mm long, claw 11–16 mm long	14–18(20) mm long, claw 7–10 mm long
Style	Sparsely hairy at apex	Glabrous	Glabrous	Glabrous or hairy in basal part
Legumes	Hairy, 25–35 × 8.3–10.5 mm	Glabrous or rarely hairy, 25–45(–55) × 12–15(–20) mm	Glabrous or rarely hairy, (22–)25–40 × 10–12 mm	Hairy or rarely glabrous, 15–23(–35) × 6–10 mm
Seeds	3.5–4.1 × 2.1–2.5 mm	5–6.5 × 3–4 mm	5–6 × 4 mm	c. 5 × 3 mm

genus *Astragalus* L. (Leguminosae) in the Old World" prepared by Podlech (1988) and Podlech and Zarre (2013), are broadly based studies that include all the species of the section *Caprini*. In these studies, a total of 273 species belonging to the section were given. In addition, many sections are synonymous, including *Myobroma*. The moment *Astragalus nallihanicus* was seen for the first time, the most interesting aspect was that it was short-stemmed, had long stipules and short yellow petals. When compared with *A. ovinus*, *A. fabaceus* and *A. angustiflorus* specimens, *A. nallihanicus* was still most strikingly different from these species in the characters mentioned above (Tab. 1, Fig. 1, Fig. 2, Fig. 3, Fig. 4).

Fig. 2. Flower parts of *Astragalus nallihanicus*. A – calyx, B – standard, C – wings, D – keel, E – stamens, F – pistil. Scale bars: 3 mm.

Fig. 3. Fruit and seed of *Astragalus nallihanicus*. A – long hairs on immature fruit, B – mature fruit, C – inside of mature fruit and septum, D – seed. Scale bars: B and C – 10 mm, D – 2 mm.

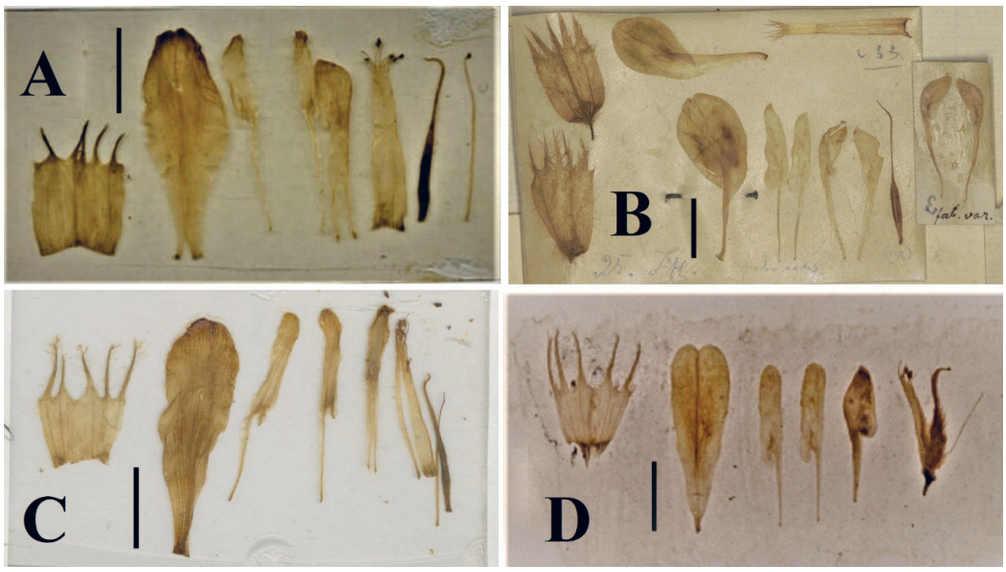

Fig. 4. Flower parts. A – *Astragalus ovinus* (*Aucher-Eloy 1316*, G00386166), B – *Astragalus fabaceus* (*Kolenati 2342*, P02915711), C – *Astragalus angustiflorus* subsp. *angustiflorus* (*Aucher-Eloy 4467*, P00609624), D – *Astragalus angustiflorus* subsp. *anatolicus* (*Aucher-Eloy 1320*, K000911370). Scale bars: 10 mm (The names of the flower parts are as given in Fig. 2).

***Astragalus nallihanicus* Hamzaoğlu, sp. nov.** (Fig. 1, Fig. 2, Fig. 3)

Type. Türkiye, Ankara, Nallıhan, south of Karacasu village, the road to Uyuzsuyu Waterfall, 950 m a.s.l., pine forest clearing, 24 June 2022, *Hamzaoğlu 7981* (**holotype** GAZI!, **isotypes** GAZI!, ANK!, HUB!).

Diagnosis. *Astragalus nallihanicus* is related to *A. ovinus*, *A. fabaceus*, and *A. angustiflorus*. It differs from *A. ovinus*, mainly by stipules adnate to petiole for 2–3 mm long (not adnate to petiole for (3–)5–7 mm), and lower ones 17–22 mm long (not 8–10 mm long); calyx teeth 8–9 mm long (not 3–6 mm long); standard 19–22 mm long (not 24–32 mm long); wings 16–18 mm long and claw 8–9 mm long (not 21–27(–29) mm long and claw 15–17 mm long); keel 15–17 mm long and claw 8–9 mm long (not 19–25 mm long and claw c. 15 mm long); legumes 8.3–10.5 mm wide (not 12–15(–20) mm wide). Also, it differs from *A. fabaceus*, mainly by calyx tube glabrous and teeth 8–9 mm long (not hairy and teeth 2–5 mm long); wings 16–18 mm long, auricle 1.4–1.8 mm long, and claw 8–9 mm long (not 20–24(–29) mm long, auricle 2.5–3 mm long, and claw 11–16 mm long); keel 15–17 mm long and claw 8–9 mm long (not 18–21(–26) mm long and claw 11–16 mm long). And also, it differs from *A. angustiflorus*, mainly by lower stipules 17–22 mm long (not 10–14 mm long); calyx teeth 8–9 mm long (not 5–7 mm long); claw of standard 6–7 mm long (not c. 2 mm long); blade of wings 8–9 mm long (not 11–16 mm long); seeds 3.5–4.1 × 2.1–2.5 mm (not c. 5 × 4 mm).

Description. Plants 15–35 cm tall, short caulescent. Rootstock up to 15 mm thick, slightly branched, subterranean stolons sometimes furnished with 4–6 mm, highly up connate stipules without leaves. Stems 5–12 cm, angular-sulcate, glabrous. Stipules whitish-membranous, adnate to petiole for 2–3 mm, lower ones 17–22 mm, ovate-triangular, acute, upper ones 15–20 mm, narrowly triangular, glabrous. Leaves (10–)15–25(–32) cm, glabrous; petiole 2–6 cm, like rachis straw-colored, up to 3 mm thick. Leaflets in (8–)10–13(–15) pairs, green, remote, elliptic, (10–)13–26(–32) × (4–)7–12(–15) mm, rounded, minutely mucronulate, at base rounded, glabrous. Racemes with a peduncle 2.5–8.5 cm, 9–12-flowered, prostrate to ascending. Bracts white-membranous, 8–13(–16) × 2–3 mm, linear-triangular, at margins and apex with sparsely spreading 1–1.5 mm long simple hairy. Pedicels 3–5 mm, glabrous, sometimes slightly thickened in fruiting. Bracteoles absent. Calyx 15–17 mm, yellowish-green, glabrous, tubular; tube 7–8 mm long; teeth linear-subulate, 8–9 mm. Petals glabrous, yellow. Standard 19–22 mm; blade 13–15 × 8–10 mm, obovate, emarginate, gradually narrowed into claw; claw 6–7 mm long. Wings 16–18 mm long; blade oblong, rounded at apex, 8–9 × 2.7–3.2 mm; auricle ovate-orbicular, 1.4–1.8 mm long; claw 8–9 × 0.7–1.1 mm. Keel 15–17 mm long; blade oblique-triangular, obtuse, 7–8 × 3.7–4.3 mm; auricle 0.7–0.9 mm long; claw 8–9 × 1.1–1.6 mm. Stamens 15–16 mm long; tube 13–14 mm long, obliquely cut at mouth. Pistil 16–20 mm long; ovary sessile, 8–11 mm long, ellipsoid, loosely white, long, hairy; style 8–9 mm long, sparsely long hairy at apex. Legume sessile, oblong-cylindrical, 25–35 × 8.3–10.5 mm, keeled, with indistinct nerve ventrally, widely rounded to flattened, more rarely shallowly widely grooved dorsally, with a rigid, straight beak 4–6 mm long, incompletely bilocular (septum up to 2/3(–3/4) of fruit's height); valves coriaceous, rugulose to rugose, pale brownish, with loosely 2–3 mm long spreading white hairs. Seeds 3.5–4.1 × 2.1–2.5 mm, pale brownish.

Etymology. The type locality of the species, which is from Nallıhan (Ankara, Türkiye), inspired the name of the new species.

Proposed vernacular name. Nallıhan Geveni (Turkish), Nallıhan Milkvetch (English).

Flowering time. June to July.

Distribution and habitat. Specimens of *Astragalus nallihanicus* were collected from the surroundings of south of Karacasu village. It is estimated that the species grows in pine forest openings of northwest of Nallıhan, approximately between 850 and 1050 m a.s.l. It is probable that the species also grows in Göynük, Mudurnu, and Seben Districts (Bolu Province, Türkiye), but there are still no data about this. Consequently, at present, the species is an endemic of Türkiye and when the area of distribution is considered, it is an element of the Euro-Siberian phytogeographic region.

IUCN Conservation assessment. According to the existing data, *Astragalus nallihanicus* is a species only known from the type locality. Approximately 50 individuals having independent roots were counted in the type locality. The species grows in pine forest openings south of Karacasu village (Nallıhan, Ankara). There are settlements and small agricultural areas in the close surroundings of the individuals belonging to the species. On the other hand, the locality is formed of rather dense pine forest and there is a very low probability of these areas becoming completely settled or devoted to agriculture in the future. There is also only a rather slight probability that domestic animals will be put out to pasture in the area due to the density of the pine forest. When the areas where the species could be grown are considered, it is estimated that *A. nallihanicus* showed a distribution on an area smaller than 100 km^2. When the existing or envisaged threats were evaluated together, with the species being known from only one location at present (area of life less than 10 km^2) and the breadth of the area of distribution was calculated (less than 100 km^2), it was decided that it would be suitable to propose the classification Vulnerable [VU: D2] for the extinction risk of the species (IUCN Standards and Petitions Committee 2019).

Additional specimens examined

Astragalus ovinus. **Türkiye**. In Mte. Taurus, *Aucher-Eloy 1916* (K, K000911383!); In Mte Tauro, Reçu en 1837, *Aucher-Eloy 1316* (G, G00386165!); [Elâziğ], Bakker Maden, Majo in alpibus, 1852, *Noë 859* (G-BOIS, G00792962!); Hakkari: Zap gorge, 9 km from Hakkari to Van, 1200 m, 14.06.1966, *Davis 44932* (E, E00347578!); Adana: Saimbeyli, c. 1450 m a.s.l., black pine forest, 11.06.1976, *Akman 6104* (ANK!; E, E00347571!); Prov. Van: Dist. Gevas, Artos Dağ, 3150 m, 16.07.1954, *Davis 22776* (E, E00347577!); Kahramanmaraş: Engizek Mount, around Engizek neighbourhood, 1400–1500 m a.s.l., 15.06.1987, *Duman 3336* (GAZI!); Kahramanmaraş: Öksüz Mount, 1400–1500 m a.s.l., 14.07.1987, *Duman 3279* (GAZI!). **Iran.** Prov. Hamadan: Faghire prope Hamadan, *Sabeti 627* (W, W19730012154!); Kuh-e-Hamzeh arab, between Bijar and Hamadan, 01.07.1971, *Lamond 4347* (E, E00340415!); In arivis schistofis Mesgivon, 2400 ped. [732 m a.s.l.], 18.06.1862, *Kotschy 295* (W, W0025302!); prope Ssof et inter Ssof et Kohrud, 05.1859, *Bunge s.n.* (P, P00607781!); Perse australe, in argillosis m. Kuh-Daëna, 14.07.1842, *Kotschy 643* (P, P00607766!); Seidabad, 10.06.1859, *Bunge s.n.* (P, P00607773!); Prov. Khamseh: Manjil to Zanjan, Tarom pass, c. 33 km from Tashvir, 02.06.1971, *Lamond 3598* (E, E00340416!); 48 km W.N.W. of Sanandaj towards Marivan, 17.05.1966, *Archibald 1960* (E, E00347572!). **Iraq.** Pl. allep. Kurd. moss; m. Gara [Kursst.], 07.1841, *Kotschy 417-A* (K, K000911410!); In summo jugo m. Gara Kurdist., 07.1841, *Kotschy 417-B* (P, P00607778!).

Astragalus fabaceus. **Türkiye**. Malatya: Darende, Akçatoprak, 1000 m a.s.l., 26.05.2006, *Uzunhisarcıklı & Bilgili 2143* (GAZI!); Kars: 3–5 km E of Aralık, Aras valley, 850 m a.s.l., 26.05.1966, *Davis 43673* (E, E00347491!); Van: 2 km E of Hoşap, 2100 m a.s.l., 09.06.1966, *Davis 44562* (E, E00347488!); **Georgia**. Tiflis, *Kolenati 2342* (P, P02915711!); In montosis Georg. Cauc. Unio itiner., 1838, *Hohenacker s.n.* (G, G00792970!); **Iran**. Azerbaijan Garbi: ad urbem Khoi ad pagum Seichadzi, 06.1828, *Szovits 26* (P, P02915707!; G, G00792971!); Azerbaijan Sharoi: Moghan, Eyvaz village, c. 3 km SE Aslanduz, c. 250 m a.s.l., 22.05.1971, *Lamond 3204* (E, E00347492!); Moghan, 25 km S Alireza-Abad on road to Sarband, 200?250 m, 23.05.1971, *Lamond 3208* (E, E00347489!).

Astragalus angustiflorus subsp. ***angustiflorus***. **Türkiye**. Ağrı: NE slope of Ağrı Dağı, below Serdar Bulak, 1500 m a.s.l., 27.05.1966, *Davis 43695* (E, E00347417!); Bitlis: Ahlat, 1750 m a.s.l., 21.05.1966, *Davis 43391* (E, E00347416!); Hakkari: Nehil Çayı, 48?55 km from Hakkari to Yüksekova, 1600?1700 m a.s.l., 14.06.1966, *Davis 44907* (E, E00347411!); Mardin: Mardin, 09.06.1888, *Sintenis 976* (E, E00347406!); Mardin castle, 1200 m a.s.l., 20.05.1957, *Davis 28343 & Hedge* (E, E00347412!); Van: 5 km S of Bendimahi (Erciş-Van), 1750 m a.s.l., 03.06.1966, *Davis 44210* (E, E00347410!); **Azerbaijan**. In Armenia et Aderbidjan, *Aucher-Eloy 4412* (P, P00584412!); **Iran**. Prov. Bakhtiari: Bakhtiari, c. 2130 m a.s.l., 21.05.1890, *Sawyer 13161* (E, E00347415!); Prov. Khamseh: Manjil to Zanjan, south side of Tarom pass, c. 42 km from Tashvir, 02.06.1971, *Lamond 3615* (E, E00347399!); Esfahan: Ispahan, *Aucher-Eloy 4467* (P, P00609624!); Kordestan: c. 108 km from Zanjan on road to Bijar, 1700 m a.s.l., *Lamond 4319* (E, E00347403!); 16 km N of Husianabad between Sanandaj and Saqez, 2340 m a.s.l., 20.05.1966, *Archibald 2094* (E, E00347419!). ***Astragalus angustiflorus*** subsp. ***anatolicus***. **Türkiye**. [Çanakkale]: Iter trojanum, Renkoei [Erenköy], prope Dumbrek (Dümrek), 22.04.1883, *Sintenis 91* (E, E00347390!); Gallipoli [Gelibolu], Angadere, Biyick Yakajik Tepe Büyük Yakacık Tepe], 250 m a.s.l., 29.04.1923, *Ingoldby 29* (K, K000895539!); ibid., *Ingoldby 119* (K, K000895540!); Angadere, 22.–24.7.1923, *Ingoldby 443* (K, K000895541!); Gallipoli [Gelibolu], Kilia, 24.04.1924, *Durham 101* (K, K000895542!); İçel [Mersin]: Akardja bei Mersina, 750 m a.s.l., 15.05.1912, *Siehe 389* (E, E00347396!); Manisa: Mte. Sypilo Manisa Dağı], *Aucher-Eloy 1320* (P, P00584401!; G, G00386971!); In montibus Cariae, *Aucher-Eloy 1319* (G, G00386969!).

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New hosts and diagnostic characteristics of *Orobanche crenata* (Orobanchaceae) in Egypt

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Abstract – The holo-parasitic weed *Orobanche crenata* Forrsk. is a threat to economically important legumes and vegetables in Mediterranean countries, including Egypt. The crenate broomrape attacks several wild and cultivated plant species, and documentation of new hosts of the parasite is always required. To the best of our knowledge, this study is the first report of parasitism of the crenate broomrape on two ornamental species, *Arctotis fastuosa* Jacq. and *Callistephus chinensis* (L.) Nees. (Asteraceae). We also recorded for the first time its parasitism on the wild weeds (*Ammi majus* L., *Lactuca serriola* L., and *Melilotus indicus* (L.) All.) and the cultivated plant species (*Carthamus tinctorius* L. and *Tropaeolum majus* L.) from Egypt. The occurrence of *O. crenata* parasitism was confirmed by the attachment of its haustoria to the roots of host plants. The incidence of crenate broomrape disease was estimated for the seven species. The study also provides a morphological description of the polymorphic *O. crenata* on the samples from Egypt and determines the most useful characteristics for its easier identification in the field.

Keywords: *Arctotis fastuosa*, *Callistephus chinensis*, crenate broomrape, disease incidence, holo-parasitic, morphological features, taxonomy

Introduction

The family Orobanchaceae includes the largest number of parasitic species (more than 2000 species from 100 genera) of facultative and obligate hemi- and holo-parasites (Mutuku et al. 2021). The genus *Orobanche* L. is one of the most harmful weeds in the world, along with the genera *Striga* Lour. and *Phelipanche* Pomel from the same family (Mutuku et al. 2021) and the genus *Cuscuta* L. of the family Convolvulaceae (Mousavi et al. 2018). It is the most species-rich genus in the family with about 150-200 species of obligate root holo-parasites (Piwowarczyk et al. 2019), although only a few of them are capable of infecting crops. The *Orobanche* species are widespread in the Mediterranean basin, Central and Eastern Europe, Southern and Western Asia, the United States, and Australia (Parker 2009). They were recorded to parasitize more than 87 dicotyledonous host plants (field crops, fruit trees, and forage plants) belonging to 24 families (Pedraja et al. 2007, Qasem and Foy 2007, Qasem 2009, 2011, Akhter et al. 2020). In particular, nine broomrape species (*Orobanche aegyptiaca* Pers., *O. cernua* Loefl., *O. crenata* Forrsk., *O. cumana* Wallr., *O. foetida* Poir., *O. minor* Sm., *O. palaestina* Reut., *O. ramosa* L., and *O. schultzii* Mutel) are known to threaten a wide array of wild and cultivated plants in the agricultural systems of temperate and semi-arid biogeographical regions (Qasem 2009, 2011, Samejima and Sugimoto 2018).

Orobanche crenata, crenate broomrape is one of the most common and most harmful broomrape species that parasite important legume and oil crops, vegetables, and ornamental plants of Fabaceae, Solanaceae, Apiaceae, and Asteraceae families grown in arable lands (mainly in Mediterranean countries) (Schaffer et al. 1991, Qasem 2009, Rubiales et al. 2009). In Egypt and other Mediterranean countries, *O. crenata* threatens *Vicia faba* L., *Daucus carota* L., and *Lupinus albus* L. (Korashi et al. 1996, Ghalwash et al. 2014). Furthermore, it attacks several annual weeds such as *Ammi majus* L., *Melilotus indicus* (L.) All., and *Rhagadiolus stellatus* (L.) Gaertn. in Jordan and *Matricaria chamomilla* L., *Polygonum* sp., and *Sonchus oleraceus* L. in Egypt (Korashi et al. 1996, Qasem 2009). *Orobanche crenata* also parasites on *Carthamus tinctorius* L., which is cultivated as an important source of a relatively healthy oil, a natural food dye, and fabrics (Qasem 2009, Khalil et al. 2013, Taha and

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Matthäus 2018). Oliveira-Velloso (1990) reported that *Lactuca serriola* L. was among 34 non-crop species that were alternative hosts for *O. crenata* in Spain.

Taxonomically, *Orobanche* is considered to be one of the most problematic genera. This is mainly due to the slight morphological variations among the species, the phenotypic variability among the individuals of each species, the limited number of potential taxonomically relevant traits (i.e., strongly reduced vegetative parts), and the loss of color during preservation of herbarium specimens (Mohamed and Musselman 2008, Pujadas-Salva and Munoz Garmendia 2010). Therefore, information on the host plant may facilitate, to some extent, the identification of the *Orobanche* species. The correct identification of the broomrape species is the first step in developing a meaningful control strategy. In this regard and to help to identify *O. crenata* plants accurately, we record information on its parasitism on seven new host plant species (*Ammi majus*, *Arctotis fastuosa*, *Callistephus chinensis*, *Carthamus tinctorius*, *Lactuca serriola*, *Melilotus indicus*, and *Tropaeolum majus* L.), and provide a full description of morphological characteristics diagnostic to the species, supported by vouchers and images for verification.

Materials and methods

Plant material

Orobanche crenata samples infecting *Ammi majus*, *Arctotis fastuosa*, *Callistephus chinensis*, *Lactuca serriola*, *Melilotus indicus*, and *Carthamus tinctorius* plants were collected from the experimental farms of the Faculty of Agriculture (27 11 05 N, 31 09 21 E, 52 m a.s.l. and the Faculty of Science (27 11 27 N, 31 10 17 E, 52 m a.s.l.), Assiut University, Assiut, Egypt during February to April 2021 and 2022. *Ammi majus*, *L. serriola*, and *M. indicus* were grown naturally (wild species) while the remaining species were cultivated as scientific samples for students or as ornamentals. *Orobanche crenata* samples parasitizing *Tropaeolum majus* plants (grown as ornamentals) were collected from another location (27 11 22 N, 31 10 09 E, 55 m a.s.l.) from Assiut University during the same period.

Samples and techniques

The inflorescence shoots of crenate broomrape and their host plants were photographed in the field before and after boring of the soil. The attachments between the parasitic weed haustoria and infected host roots were observed and documented. Samples of broomrape and host plants were carefully collected from the field and sent for identification to Assiut University Herbarium (ASTU). For the determination of broomrape species, we used the descriptions and taxonomic keys of Rumsey and Jury (1991), Boulos (2002), Joel et al. (2007), Mohamed and Musselman (2008), and Parker (2013). Voucher specimens of *O. crenata* attached to different hosts were deposited at ASTU Herbarium, Assiut, Egypt.

Morphological measurements and dissection of crenate broomrape flowers were done using an Olympus SZ61 stereo-microscope and photographed with an Olympus SC100 digital camera.

For light microscopy, pollen grains were treated with 10% potassium hydroxide solution, stained with Safranin (1% Safranin solution in 50% ethanol), and mounted in glycerol before observations. Pollen grains and glandular and non-glandular hairs were photographed by an Olympus SC100 digital camera coupled to an Olympus CX41 microscope. For scanning electron microscopy, hairs of different floral parts, seeds, and pollen grains were examined without coating using a TM3000 miniscope SEM (Hitachi High-Tech).

Morphological characteristics of crenate broomrape such as the height of the plant, the length of the inflorescence, and dimensions of the stem, scale, bract, calyx, corolla, androecium, gynoecium, seed, and pollen grains were recorded, and were based on at least 40 observations.

The incidence of *O. crenata* was estimated in each wild or crop species and calculated using the following formula:

$$\text{Incidence} = \frac{\text{number of infected host plants per m}^{-2}}{\text{total number of host plants per m}^{-2}} \times 100$$

The incidence of the disease was classified into three categories as described by Kroschel (2002) and Mengistu et al. (2017): low incidence (< 20% infestation), medium incidence (20–50% infestation), and high incidence (> 50% infestation).

Results

Orobanche crenata was reported to parasitize certain crops and weeds of the families Fabaceae (*Vica faba*, *Pisum sativum* L., *Lupinus albus*, and *Cicer arietinum* L.), Apiaceae (*Daucus carota*, *Anethum graveolens* L., *Carum carvi* L., and *Cuminum cyminum* L.), and Asteraceae (*Matricaria chamomilla* L. and *Sonchus oleraceus*) (Tab. 1). In addition, a few members of the families Amaranthaceae (*Beta vulgaris* L.) and Polygonaceae (*Polygonum* sp.) were attacked by this parasite (Tab. 1). We recorded crenate broomrape parasitism of two ornamentals (*Arctotis fastuosa*, and *Callistephus chinensis*), three wild (*Ammi majus*, *Lactuca serriola*, and *Melilotus indicus*), and two cultivated (*Carthamus tinctorius* and *Tropaeolum majus*) plant species from Egypt (Fig. 1, Fig. 2, Tab. 1).

A morphological comparison of *O. crenata* specimens infecting the seven different hosts revealed the main diagnostic morphological characteristics that can be used for easier identification.

In general, morphological observations of whole individuals, inflorescence, scales, and floral parts of specimens indicated that crenate broomrape was erect, stout, unbranched, and 14 to 83 cm in height and 0.4 to 1.5 cm in width (Fig. 1, Fig. 2, Tab. 2). The inflorescence was a dense spike that occupies about 31–72% of the emerged

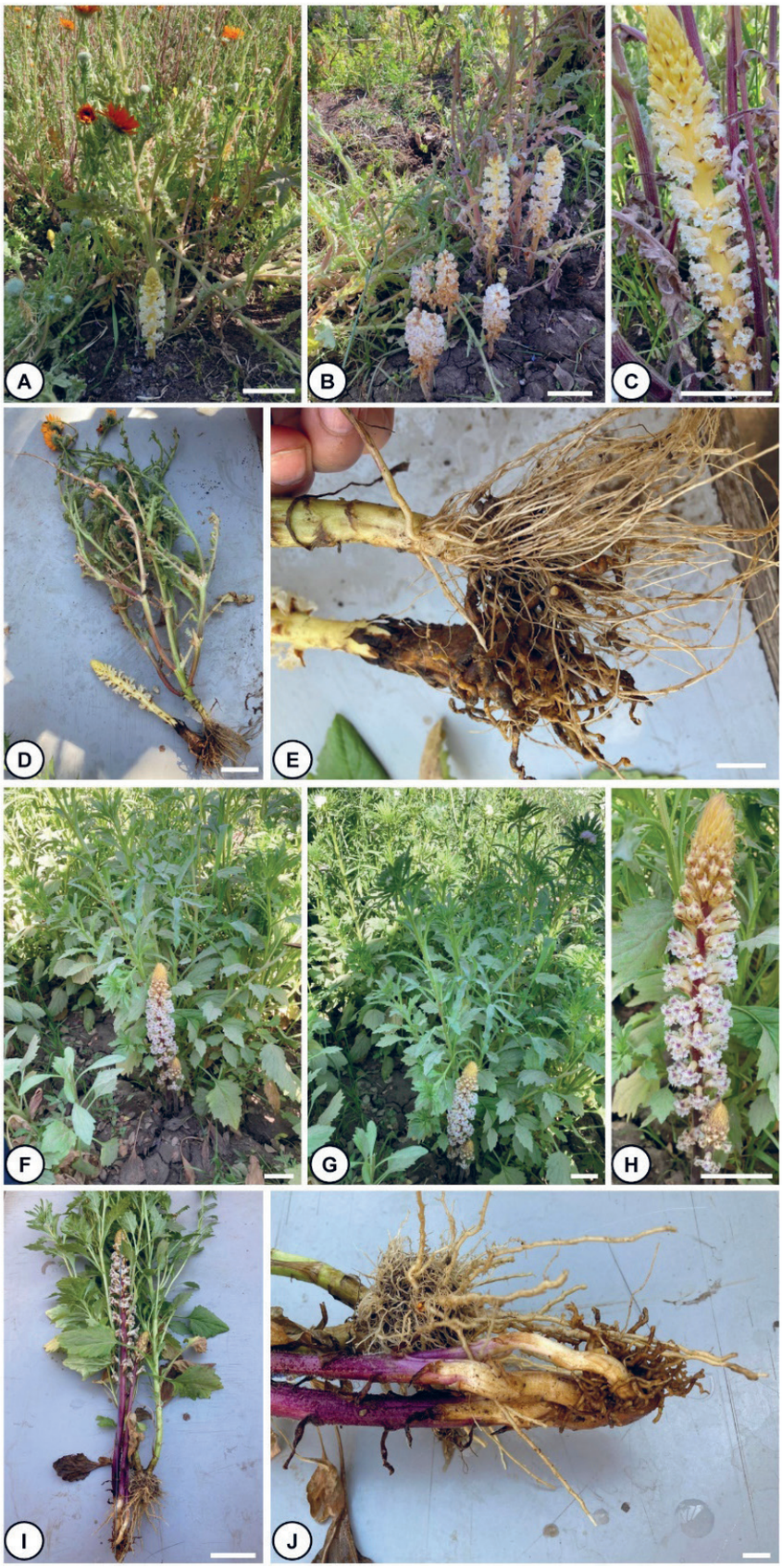

Fig. 1. *Orobanche crenata* parasitizing *Arctotis fastuosa* and *Callistephus chinensis*. A-E – *Arctotis fastuosa*, F-J – *Callistephus chinensis*. A, B, F, G – general habit of *O. crenata* with the host plant in the field. C, H – *Orobanche* inflorescence. D, I – *O. crenata* attached to the root of the host plants. E, J – attachment area of *O. crenata* haustoria on the root of the host plants. Scale bars: 10 cm in (A, B, C, D, F, G, H, I) and 2 cm in (E, J).

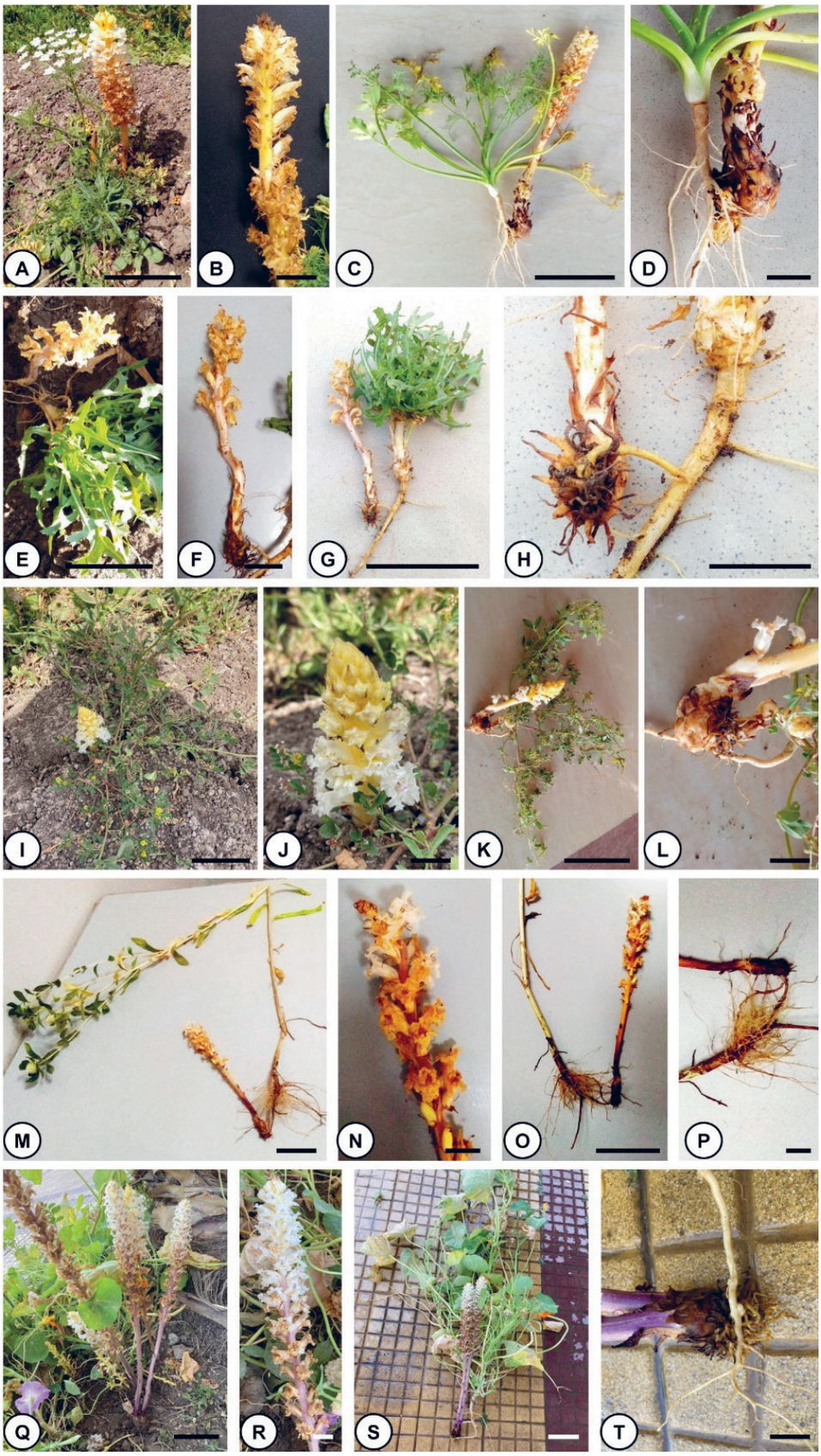

Fig. 2. *Orobanche crenata* parasitizing different host species in Egypt. A-D – *Ammi majus*, E-H – *Lactuca serriola*, I-L – *Melilotus indicus*, M-P – *Carthamus tinctorius*, and Q-T –*Tropaeolum majus*. A, E, I, M, Q – general habit of *O. crenata* with the host plant. B, F, J, N, R – *Orobanche* inflorescence. C, G, K, O, S – *O. crenata* attached to the root of the host plants. D, H, L, P, T – attachment area of *O. crenata* haustoria on the root of the host plants. Scale bars: 10 cm in (A, C, E, G, I, K, M, O, Q, S) and 2 cm in (B, D, F, H, J, L, N, P, R, T).

Tab. 1. A list of host species infested by *Orobanche crenata* Forssk. in Egypt including seven new records in this study.

Family	Host species	Reference
Amaranthaceae	*Beta vulgaris* L. (Beet)	Korashi et al. (1996)
Apiaceae	*Ammi majus* L. (Bishop's weed)	This study
	Anethum graveolens L. (Dill)	Korashi et al. (1996)
	Carum carvi L. (Caraway)	Korashi et al. (1996)
	Cuminum cyminum L. (Cumin)	Korashi et al. (1996)
	Daucus carota L. (Carrot)	Korashi et al. (1996); Ghalwash et al. (2014)
Asteraceae	*Arctotis fastuosa* Jacq. (Cape daisy)	This study
	Callistephus chinensis (L.) Nees (China Aster)	This study
	Carthamus tinctorius L. (Safflower)	This study
	Lactuca serriola L. (Prickly lettuce)	This study
	Matricaria chamomilla L. (Chamomile)	Korashi et al. (1996)
	Sonchus oleraceus L. (Sow thistle)	Korashi et al. (1996)
Fabaceae	*Cicer arietinum* L. (Chickpea)	Korashi et al. (1996)
	Lupinus albus L. (White Lupine)	Al-Menoufi et al. (1996); Fernández-Aparicio et al. (2009)
	Melilotus indicus (L.) All. (Sweet clover)	This study
	Pisum sativum L. (Pea)	Hassan (1998); Abdel-Kader and El-Mougy (2001)
	Vica faba L. (Broad bean)	Shabetai (1933); Zahran (1982); El Ghamrawy et al. (1990)
Polygonaceae	*Polygonum* sp.	Korashi et al. (1996)
Tropaeolaceae	*Tropaeolum majus* L. (Garden Nasturtium)	This study

Tab. 2. Morphological characteristics of *Orobanche crenata* infecting wild (*Ammi majus*, *Lactuca serriola*, and *Melilotus indicus*) or cultivated (*Arctotis fastuosa*, *Callistephus chinensis*, *Carthamus tinctorius*, and *Tropaeolum majus*) plants. Values represent means ± SD of replicates based on 40 measurements per character (n = 40).

Character (cm)	Minimum	Maximum	Mean ± SD
Plant height	14	83	41.7 ± 18.9
Inflorescence length	7	53	23.6 ± 12.9
Inflorescence occupation (%)	31	72	54 ± 1
Stem length	5	30	17.6 ± 6.4
Stem width	0.4	1.5	0.85 ± 0.31
Scale length	1.1	2.3	1.67 ± 0.36
Scale width	0.3	0.7	0.47 ± 0.11
Bract length	0.8	2	1.27 ± 0.26
Bract width	0.25	0.6	0.40 ± 0.77
Calyx length	0.7	1.5	1.12 ± 0.21
Calyx width	0.2	0.6	0.36 ± 0.1
Calyx teeth	0.5	1	0.67 ± 0.13
Calyx teeth/calyx length (%)	50	75	59 ± 6
Corolla length	1.4	2.4	1.93 ± 0.26
Corolla width	1	2	1.47 ± 0.28
Stamens length	0.8	1.3	1.03 ± 0. 12
Filament insertion on corolla	0.2	0.4	0.28 ± 0.06
Lower part of filament with non-glandular hairs/filament length (%)	23.3	40	33.4 ± 5.1
Ovary length	0.5	1.5	1 ± 0.26
Ovary width	0.3	0.8	0.46 ± 0.12
Style length	0.6	1.1	0.87 ± 0.14
Stigma width	0.2	0.4	0.28 ± 0.05
Pollen dimensions (length x width; μm)	20.9 x 20.4	33.1 x 31.2	27.3 ± 2.4 x 26.2 ± 2.5
Seed dimensions (length x width; mm)	0.26 x 0.17	0.5 x 0.31	0.4 ± 0.04 x 0.25 ± 0.03

shoot (Fig. 1, Fig. 2, Tab. 2). The scales (leaves) were lanceolate with acute or acuminate tips, 1.1-2.3 cm in length, and 0.3-0.7 cm in width (Fig. 1, Fig. 2, Tab. 2). The flowers were strongly fragrant when fresh and subtended with one bract and no bracteoles (Fig. 1, Fig. 2, Fig. 3). The bracts were narrowly lanceolate, glandular-hairy, yellowish to brownish, 0.8-2 cm in length, and 0.25-0.6 cm in width (Fig. 3, Fig. 4, Tab. 2). The calyx was glandular-hairy, unequally bifid, and

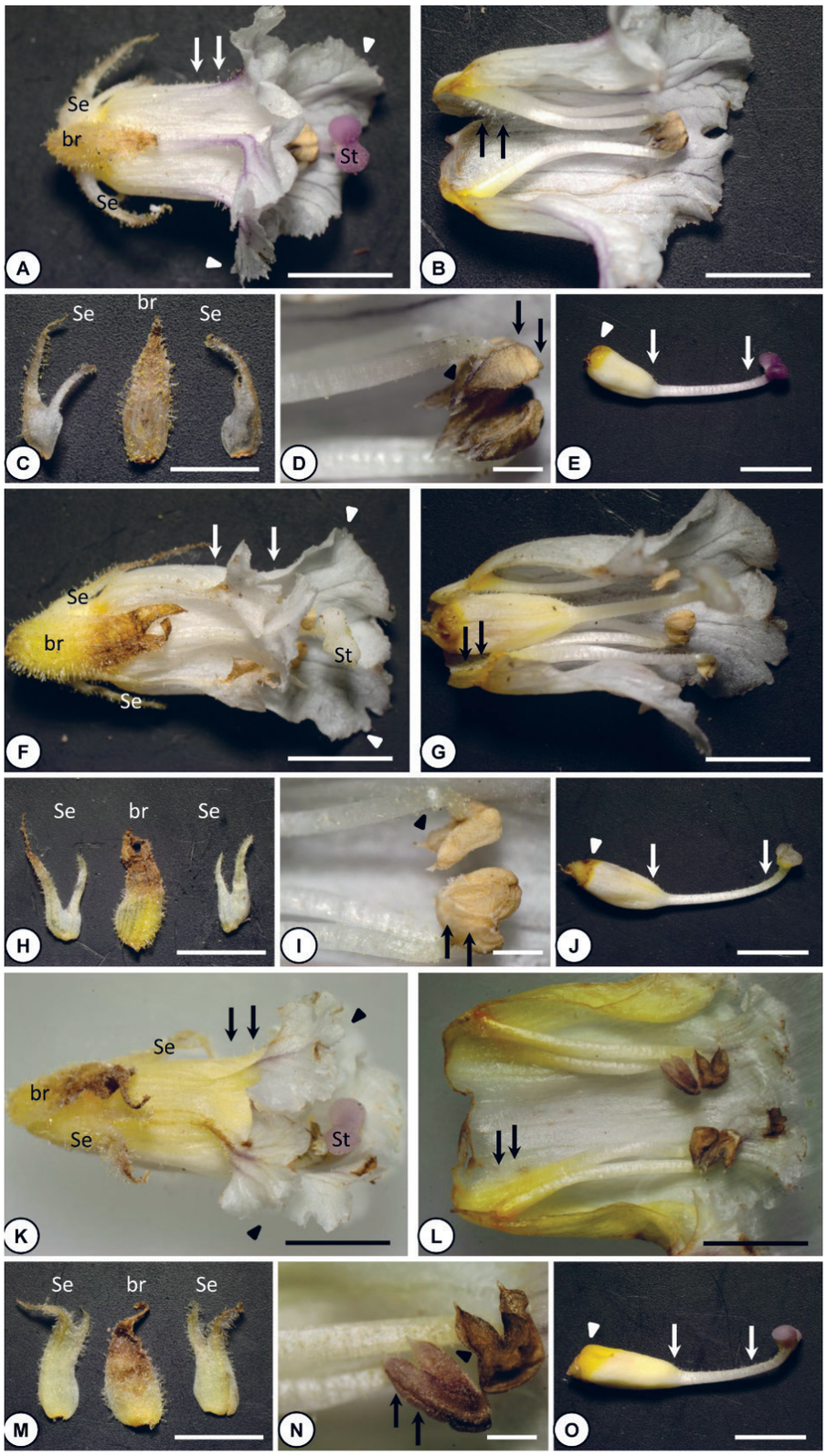

Fig. 3. Morphological characteristics of *Orobanche crenata* flowers. A-J – *O. crenata* parasitizing on two individuals of *Tropaeolum majus* and K-O – *O. crenata* parasitizing on *Ammi majus*. A, F, K – a flower at the anthesis stage. Note purplish stigmas and purplish veins of petals in (A, K), while whitish stigmas and dull whitish veins of petals in (F). Also, note the glandular hairs visible on the abaxial side of petals (arrows) and the denticulate (irregularly notched) corolla (arrowheads). B, G, L – dissected flower showing the androecium (four stamens). Note non-glandular hairs visible on the lower part of stamens (arrows). C, H, M – a bract (middle) and sepals (right and left) covered by dense glandular hairs. D, I, N – dehiscent anthers. Note the glabrous surface of the anthers (arrows) and the glandular hairs on the upper part of the filaments (arrowheads). E, J, O – gynoecium. Note glandular hairs visible on the style and the upper part of the ovary (arrows) and nectary (arrowheads). br: bract, se: sepals, st: stigma. Scale bars: 0.5 mm, D, I, N: 0.1 mm.

deeply divided to more than halfway (50-75%) (Fig. 3, Fig. 4, Tab. 2). The calyx was 0.7-1.5 cm in length and each half of the calyx pair had a width of 0.2-0.6 cm (Tab. 2).

The corolla was whitish or yellowish with purplish veins, especially on the lips (Fig. 3). Few crenate broomrape individuals had flowers with no purplish but usually dull white veins (Fig. 3). The corolla was always yellowish at the base, especially at the position of stamen insertion in the inner side of the corolla (Fig. 3). The corolla was bilabiate with a long tube curved towards the lower lip, opening out to widely divergent lobes (1-2 cm in width), and denticulate (irregularly notched) (arrowheads in Fig. 3). The corolla tube is not constricted, slightly longer than the calyx, and with dispersed glandular hairs at the abaxial side (arrows in Fig. 3, Fig. 4). The length of the corolla was 1.4-2.4 cm (Tab. 2).

The stamens were 0.8-1.3 cm in length, provided with dense multi-cellular non-glandular hairs at the lower 23–40% part of the filaments (arrows in Fig. 3, Fig. 4, Tab. 2) and with dispersed short glandular hairs on the remaining upper part (arrowheads in Fig. 3, Fig. 4). The maximum density of the glandular hairs in the filament was at the re-

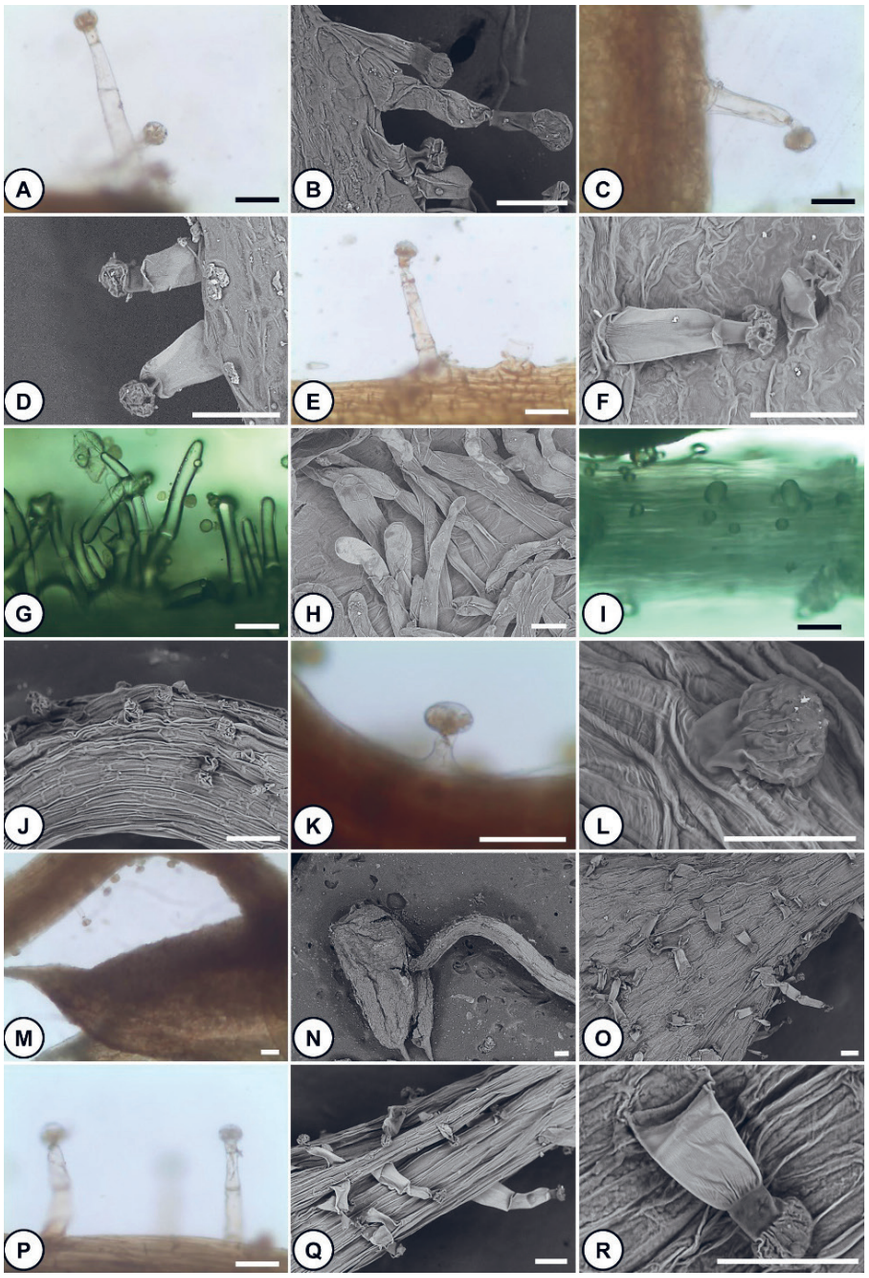

Fig. 4. Hairs of different floral parts of *Orobanche crenata* parasitizing *Tropaeolum majus*. A, B – glandular hairs covering the abaxial side of the bract. C, D – glandular hairs covering the abaxial side of the calyx. E, F – glandular hairs covering the abaxial side of the corolla. G, H – non-glandular hairs covering the lower part of the filament. I, J, K, L – glandular hairs covering the upper part of the filament. Note the short length of these hairs. M, N – glabrous surface of anther. Note the density of glandular hairs on filament increased towards the anther. O – glandular hairs covering the ovary. P, Q, R – glandular hairs covering the style. Scale bars: 100 μm in all, except L: 50 μm.

gion just below the anther. Filaments were inserted 2-4 mm from the base of the corolla tube (Fig. 3, Tab. 2). Anthers were glabrous, brownish-greyish, with a rounded tip and a pointed base at each anther lobe (arrows in Fig. 3, Fig. 4).

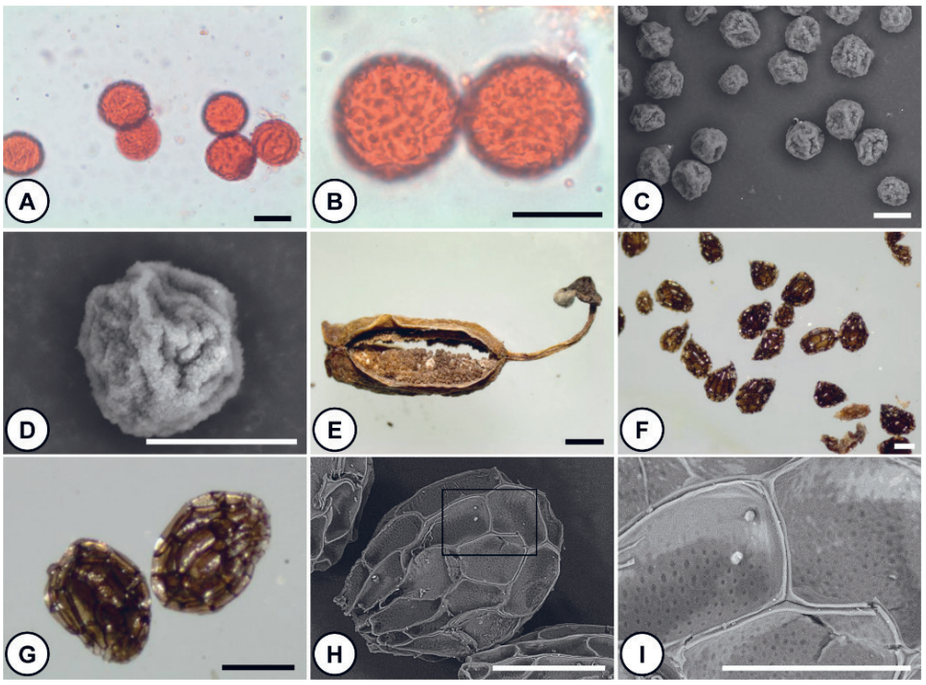

Fig. 5. Pollen grains, capsule, and seeds of *Orobanche crenata* flowers parasitizing *Tropaeolum majus*. A, B – light micrographs of inaperturate pollen grains. C, D – SEM micrographs of inaperturate pollen grains. Note the granules on the pollen surface. E – a dehiscent capsule with seeds. F, G – light micrographs of seeds at different magnifications. H – SEM micrograph of a single seed. I – a magnified view of the boxed area of (H). Scale bars: 20 µm in (A, B, C, D), 0.5 mm in (E), 0.2 mm in (F, G, H), 0.1 mm in (I).

The size of pollen grains varies from small to medium (20.9-33.1 µm long and 20.4-31.2 µm wide) (Fig. 5, Tab. 2). Pollen grains were inaperturate with a surface covered by many granules of irregular sizes interrupted with some grooves (Fig. 5).

The ovary was ovoid, 0.5-1.5 cm in length and 0.3-0.8 cm in width, covered with dispersed glandular hairs on its upper part, and lay on a yellow-orange nectary disc (arrowheads in Fig. 3, Fig. 4, Tab. 2). The style was 0.6-1.1 cm in length and covered with dispersed glandular hairs (arrows in Fig. 3, Fig. 4, Tab. 2). The stigma was bi-lobed, 2-4 mm in width, with a pleasant carnation scent, pinkish, pale purplish, or whitish (Fig. 3, Tab. 2).

The fruit was a two-split capsule, with several hundred seeds (Fig. 5). Seeds were very small, 0.26-0.55 mm long and 0.17-0.38 mm wide, with a terminal funicular attachment. Seeds were brown in color, ellipsoid in shape with a reticulated coat made of polygonal cells (Fig. 5).

The incidence of *O. crenata* varied within the wild and cultivated plants (Fig. 6). *Tropaeolum majus* and *Arctotis fastuosa* plants had the highest incidence of *O. crenata,* 100% and 90%, respectively. *Ammi majus* was also highly infected (about 75%). *Carthamus tinctorius* showed medium-level *O. crenata* parasitism (41.3%). However, the parasitism of *O. crenata* was also recorded on individuals of the wild plants *Lactuca serriola* and *Melilotus indicus*, although with the lowest incidence.

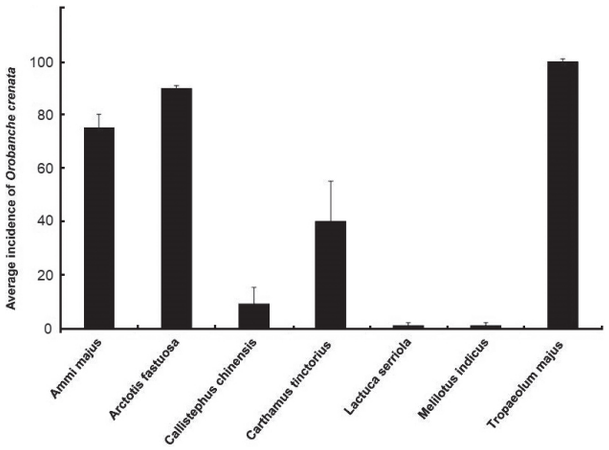

Fig. 6. Incidence of *Orobanche crenata* in wild (*Ammi majus*, *Lactuca serriola*, and *Melilotus indicus*) or cultivated (*Arctotis fastuosa*, *Callistephus chinensis*, *Carthamus tinctorius* and *Tropaeolum majus*) plants. Error bars indicate the standard error.

Discussion

To the best of our knowledge, this investigation is the first report on parasitism of the crenate broomrape on *Arctotis fastuosa* and *Callistephus chinensis*. We also recorded for the

first time its parasitism on three wild (*A. majus*, *L. serriola*, and *M. indicus*) and two cultivated (*C. tinctorius* and *T. majus*) species from Egypt. Results of our study on the vegetative and reproductive parts of *O. crenata* plants infecting the seven plant species were, in general, consistent with previous reports. Only a few differences in some measurements were recorded; they were mainly due to environmental factors or the characters of host plants.

The crenate broomrape plants exhibited some morphological characteristics that can be used in differentiating it from other broomrape species, and thus, are of great value in the identification and classification of the broomrape species. These diagnostic morphological characteristics include the form of the stem (stout, unbranched); the absence of bracteoles; the shape of the calyx; the size, scent, color, and shape of the corolla; the insertion position of stamens and the distribution of trichomes on stamens; the aperture number and surface ornamentation of pollen grains.

The stems of the crenate broomrape were erect, stout, and unbranched. We showed that the height of *O. crenata* plants ranged from 14 to 83 cm. Previous studies showed that the height of *O. crenata* varied from 38-52 cm (Akhter et al. 2020), 30-70 cm (Restuccia et al. 2009), and up to 100 cm (Rubiales et al. 2008). The branching of the stem is an important feature that distinguishes the sections *Orobanche* and *Trionychon*, two sections of the genus *Orobanche*. In contrast, the height of the stems provides no significant value in distinguishing the broomrape species.

Our results showed that the flowers had no bracteoles, the bracts were 0.8-2 cm in length and 0.25-0.6 cm in width, and the calyx was unequally bifid to more than halfway (50-75%), 0.7-1.5 cm in length and each half of the calyx pair had a width of 0.2-0.6 cm. Akhter et al. (2020) reported that the bracts were 0.38-0.72 cm long and 0.25-0.61 cm wide; and the calyx was divided at the base, 1.4-1.8 cm long, and 0.4-0.6 cm wide. Rubiales et al. (2008) described the calyx as bidentate, with segments free, and 1.3-1.8 cm long. The absence of bracteoles in the crenate broomrape and the calyx with two lateral halves is characteristic of species from the section *Orobanche*. In contrast, the presence of two extra bracteoles besides the bract and the entire campanulate calyx are characteristic of the section *Trionychon*. The size, shape, and depth of indentation of the calyx halves were previously used as important taxonomic features facilitating the classification of broomrape species (Kreutz 1995).

The color, scent, size, and shape of the corolla are among the most significant traits used for the identification of broomrape species. We showed that the corolla was bilabiate with a long tube curved towards the lower lip, strongly fragrant, whitish or yellowish with purplish veins, especially on the lips; however, few individuals had flowers with no purplish veins. The corolla was with widely divergent lobes, not constricted, denticulate (irregularly notched), and 1.4-2.4 cm long. Rubiales et al. (2008) and Akhter et al. (2020) reported that the corolla of *O. crenata* had divergent lips often whitish or yellowish with lilac veins, glandular-pubescent, and 1.8 to 2.8 cm long; whereas Restuccia et al. (2009) described the corolla as white, subglabrous, usually 2-3 cm long. Previous studies have reported that the bilabiate corolla with a long tube curved towards the lower lip is characteristic of section *Orobanche* (Restuccia et al. 2009, Zare and Dönmez 2016). The color of the corolla in broomrape species varied greatly from white to yellow, red, purple, and blue and this diversity allows it to be used in the identification of different species (Boulos 2002, Mohamed and Musselman 2008, Parker 2013). For example, *O. cernua* can be differentiated from *O. crenata* and *O. minor* by the color of the corolla (glossy white with blue limbs in *O. cernua*) and the constriction of the corolla above the ovary (constricted in *O. cernua*) (Boulos 2002, Mohamed and Musselman 2008, Parker 2013). Mohamed and Musselman (2008) separated *O. crenata* from *O. cernua* by the denticulate (irregularly notched) corolla in *O. crenata*, in contrast to the entire corolla of *O. cernua*. The scent, size, and divergence of the corolla are diagnostic of the crenate broomrape and are useful in its differentiation from the closely related *O. minor*. The corolla of crenate broomrape is larger in size (almost exceeding 1.5 cm), widely divergent, with a pleasant scent. In contrast, the corolla of *O. minor* rarely exceeds 1.5 cm, is not divergent, and is unscented or has a fetid scent (Rumsey and Jury 1991, Parker 2013).

Our observations confirmed that the stamens were 0.8-1.3 cm long, provided with dense non-glandular hairs at the lower part of the filaments and with dispersed short glandular hairs at the upper part. Anthers were glabrous and brownish-greyish and the filaments were inserted 2-4 mm from the base of the corolla tube. Akhter et al. (2020) showed that the stamens were hairy and 0.5 ± 0.08 cm in length. Rubiales et al. (2008) reported that the anthers were brown, glabrous, or subglabrous, and the filaments were inserted 2-3 mm above the base of the corolla. The insertion position of stamens and the distribution of trichomes on stamens were used by Rumsey and Jury (1991), Joel et al. (2007), and Parker (2013) for the classification of the broomrape species. The crenate broomrape can be differentiated from *O. cernua* by the insertion position of stamens (in the middle of the corolla in *O. cernua* vs. in the lower part of the corolla in *O. crenata*) and the distribution of trichomes on stamens (hairy below in *O. cernua* vs. hairy throughout the filament in *O. crenata*).

Pollen grains in our observations had small to medium sizes (20.9-33.1 μm long and 20.4-31.2 μm wide) and were inaperturate and granulate with some grooves. Coutinho et al. (2019) showed that pollen grains of *O. crenata* were granulate with small to medium sizes (24.7-30.5 μm long and 21.9-26.9 μm wide). Zare et al. (2014) described the surface of *O. crenata* pollen grains as verrucate, inaperturate, and pollen size as small ranging from 20.4-26.2 μm long and 20.4-26.3 μm wide, whereas Akhter et al. (2020) described much smaller pollen grains of *O. crenata* (7.8–10.9 μm long and 7.8–10.9 μm wide). Pollen morphological characteristics are of a high value for the classification of the broomrape species (Abu Sbaih et al. 1994, Zare et al. 2014, Coutinho et

al. 2019). For example, the aperture number and surface ornamentation of pollen grains suggest the separation of section *Orobanche* from section *Trionychon*. Pollen grains of section *Orobanche* are generally inaperturate with granulate or scabrate-verrucate ornamentation; whereas, those of section *Trionychon* are, in general, tri-colpate with microreticulate-scabrate ornamentation (Abu Sbaih et al. 1994, Zare et al. 2014).

The fruit in our observations was a two-split capsule, with several hundred very small seeds. Seeds were ellipsoid in shape with a reticulated coat made of polygonal cells, small (0.26-0.55 mm long and 0.17-0.38 mm wide). Zare and Dönmez (2016) showed that the fruit of *O. crenata* from Türkiye was a loculicidal capsule dehiscing with two slits, and the seeds were small (0.24-0.38 mm long and 0.16-0.24 mm wide) with reticulate ornamentation and ovoid, pear-shaped, to sub-globose shape. Aly et al. (2012) and Akhter et al. (2020) described seeds as small, dust-like, 0.2-0.4 mm in length and 0.1-0.3 mm in width, whereas Plaza et al. (2004) reported that seeds of *O. crenata* were ellipsoid in shape, 0.28-0.48 mm long, and 0.19-0.3 mm wide. Seed morphological features such as size, shape, and color showed little variation among species and were not useful for the classification of the broomrape species (Zare and Dönmez 2016).

According to Boulos (2002, 2009), there are nine *Orobanche* species in Egypt, *O. aegyptiaca*, *O. cernua*, *O. crenata*, *O. lavandulacea* Rchb., *O. minor*, *O. mutelii* F.W.Schultz, *O. nana* Noë ex Reut., *O. ramos*a L., and *O. schultzii*. Six of them (*O. aegyptiaca*, *O. lavandulacea*, *O. mutelii*, *O. nana*, *O. ramosa*, and *O. schultzii*), which represent the section *Trionychon*, have been transferred to the genus *Phelipanche* (Schneeweiss et al. 2004, Banfi et al. 2011). *O. crenata* can be easily distinguished from these six species by the absence of bracteoles, the single stout stem, the calyx with two lateral halves, and the inaperturate pollen grains. The remaining two species, *O. cernua* and *O. minor*, in addition to *O. crenata*, now represent the re-circumscribed *Orobanche* in Egypt. *O. cernua* can be distinguished from *O. crenata* and *O. minor* by the constriction of the corolla above the ovary, the color of the corolla veins, and the insertion of stamens in the corolla tube. On the other hand, *O. crenata* can be separated from *O. minor* by the pleasant scent of the whole plant, the large size of the corolla (> 1.5 cm), and the wide divergence of the corolla (Rumsey and Jury 1991, Parker 2013).

Orobanche crenata is a holo-parasitic species with a high rate of target crop parasitism (Qasem 2009, Restuccia et al. 2009, Qasem 2011). The severity of parasitism hinders the cultivation of many strategic crops in the Mediterranean area including legumes, and vegetables (Parker 2009, Mutuku et al. 2021). Our results revealed that the severity of incidence of *O. crenata* reached 90% and 100% in *Arctotis fastuosa* and *Tropaeolum majus* plants, respectively, 75% in *Ammi majus*, and 41.3% in *Carthamus tinctorius*. However, a low incidence was recorded in *Lactuca serriola* and *Melilotus indicus*. *Orobanche crenata* plants produce a high number of tiny seeds per plant. Seeds remain viable for several years in the soil and they have the ability to spread over long distances to infest new areas. Negligence, improper farming practices, and lack of knowledge about the host range of *O. crenata* are the main causes of the spread and the increase of infestation. Raising awareness about the biology of *Orobanche* is a key element in overcoming the spread of infestation.

Acknowledgments

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Forest communities of the relict Balkan endemic *Aesculus hippocastanum*

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Abstract – *Aesculus hippocastanum* L. (European Horse-chestnut) constitutes a biogeographical relict species of the Balkan Peninsula, occurring in isolated and topographically distinct localities in Albania, Bulgaria, Greece and North Macedonia. Despite its great botanical, ornamental and pharmaceutical value, a thorough investigation of *Ae. hippocastanum* habitat diversity in its native distribution range has not been conducted yet. The present study aims at the syntaxonomic classification and ecological features of plant communities dominated by this species across its overall native distribution range. On the basis of 55 phytosociological relevés, five ecologically, floristically, and spatially well differentiated clusters were identified, with the main revealed gradients of differentiation being geographic location (longitude, latitude), altitude, annual precipitation and precipitation seasonality. The distinct microhabitats with a special refugial character where these plant communities occur meet the species' requirement for relatively high air and soil humidity. They have allowed the preservation of *Ae. hippocastanum* through time highlighting their great conservational value. The last one could be useful for the implementation of some appropriate measures for effective conservation of these communities.

Keywords: Braun-Blanquet's approach, dispersal strategy, phytosociology, ravine forests, Tertiary relict

Introduction

The European Horse-chestnut (*Aesculus hippocastanum* L.) is the only native European species of the *Aesculus* genus, which counts 13 tree and shrub species living in mesophilous, temperate deciduous forests (Hardin 1960). It is an endemic species of the mountainous range of Western Balkans, forming small, isolated populations. Its natural distribution is restricted to the mountains of Greece, Albania and North Macedonia as well as to one remote locality in Bulgaria (Ravazzi and Caudullo 2016). While the genus *Aesculus* was widespread in Europe during the Neogene (Postigo Mijarra et al. 2008), fossil records of *Ae. hippocastanum* in mainland Europe date back at least 1 million years (Harris et al. 2009). Therefore, the species' occurrence in Europe, which is documented since the Early Pleistocene, accompanied by the subsequent shrinkage of the distribution of the species, like that of the *Aesculus* genus, makes *Ae. hippocastanum* a biogeographic relict (Postigo Mijarra et al. 2008). Decline of the species' populations may be related to climatic changes (Harris et al. 2009), low tolerance of seeds to desiccation as well as specific ecology and seed dispersal strategy (Walas et al. 2018, 2019, Thomas et al. 2019).

Despite its very restricted natural distribution, *Ae. hippocastanum* has been either introduced or cultivated across several European countries (Euro + Med PlantBase 2006-2022). Historically (Lack 2002), this extended reintroduction of the species in Europe has been attributed to the import of seeds of uncertain provenance in 1557 from Türkiye

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to Prague. After that, the cultivation of *Ae. hippocastanum* as an ornamental tree started throughout Europe and resulted in numerous horticultural varieties.

Aesculus hippocastanum is nowadays widespread in urban areas of temperate Europe, while it also constitutes an important medicinal plant (Ravazzi and Caudullo 2016). However, the species is assessed as "Vulnerable" within its natural habitat at the European level (Rivers 2019). Its natural populations are declining due to strong infections by *Cameraria ohridella* Deschka et Dimic (Lepidoptera), which feeds on *Ae. hippocastanum* leaves, causing midsummer defoliation and exhaustion of the affected individuals, leading to reduced reproductive success of natural populations (Barredo et al. 2015). The species is affected also by *Guignardia aesculi*, a fungus that causes leaf blotch disease. The combined negative effects of these two biotic factors lead to the large-scale periodical defoliation of *Ae. hippocastanum* stands (Walas et al. 2018). Despite the botanical, conservational, ornamental, and pharmaceutical significance of *Ae. hippocastanum*, the vegetation patterns in the species' natural habitats have never been thoroughly studied throughout its overall distribution range. There is some more recent information in the works of Thomas et al. (2019), who summarized already existing data from Greece (Tsiroukis 2008), Bulgaria (Gussev and Valchev 2015), etc. Vegetation studies, so far only at a local scale, of plant communities hosting *Ae. hippocastanum* have been made in Greece (Barbero and Quézel 1976, Raus 1980, Bergmeier 1990, Mastrogianni 2020), North Macedonia (Em 1957, Matvejeva and Nikolovski 1976, Em et al. 1985, Rizovski and Džekov 1990, etc.), Albania (Peçi et al. 2012) and Bulgaria (Adamović 1908, Gussev and Valchev 2015). Its habitat characteristics throughout this area, and more specifically the assemblages that it inhabits, remain relatively undescribed. This study aims at the identification of the different syntaxonomic units of the relict *Ae. hippocastanum* communities, throughout its natural distribution range. Additionally, the main ecological gradients of floristic differentiation of *Ae. hippocastanum* communities are inferred. Finally, based on the species composition of these communities, as well as on literature sources, we discuss the evolutionary history of the species and the putative refugial origin of its communities.

Material and methods

In total, 55 phytosociological relevés were used in the present study, 25 published (Barbero and Quézel 1976, Matvejeva and Nikolovski 1976, Raus 1980, Bergmeier 1990, Rizovski and Džekov 1990) and 30 unpublished from Bulgaria and Greece. All published and unpublished relevés were carried out according to the Braun-Blanquet approach (Westhoff and van der Maarel 1978) in forest types where *Ae. hippocastanum* dominates or co-dominates in cover. Vegetation data used in the present study cover most of the total natural distribution of *Ae. hippocastanum* (Fig. 1). More specifically, vegetation data from most of the localities with dominance or co-dominance of *Ae. hippocastanum* in Greece, Bulgaria and North Macedonia were included in our dataset, but not from Albania, from where no published relevés were found. Header data of all relevés used in this study are presented in On-line Suppl. Mat. (On-line Suppl. Tab. 1). Plant nomenclature follows the Euro + Med PlantBase (2006-2022) and the nomenclature of mosses – Hodgetts et al. (2020). The area of sampling plots varied between 100 to 1000 m^2, with most of the plots having an area of 400 m^2. Moss taxa were identified for relevés from Bulgaria and North Macedonia, but they were not used in the cluster analysis as they were not recorded in all plots. However, despite their exclusion from the cluster analyses, moss taxa were included in the vegetation table presented in Tab. 1 as well as in On-line Suppl. Tab. 2 to present their occurrence in the relevés where moss taxa were recorded. The different layers of species occurring in the vegetation strata (especially for trees and shrubs), were merged for the analysis. Cover/abundance of plant taxa was recorded as percentages or on the basis of the 7-degree or 9-degree Braun-Blanquet scale (Westhoff and van der Maarel 1978). For the last two cases, cover-abundances were subsequently transformed to percentages according to the default correspondence in Turboveg software (Hennekens and Schaminée 2001) in order to allow the application of numerical analyses.

Cluster analysis was performed using the vegan package (Oksanen et al. 2020) in R (R Core Team 2022). Bray-Curtis dissimilarity was used as distance measure, and flexible beta with β equal to -0.25 as linkage method. Cover data of taxa were square-root transformed prior to cluster analysis. Number of clusters was defined on the basis of fusion level values plot, as well as ecological interpretation of clusters. Fusion level values represent the dissimilarity values, where a fusion between two branches of a dendrogram occurs (Borcard et al. 2011). The function "hcoplot" was used to reorder the clusters in the dendrogram according to their similarity.

Diagnostic taxa of the distinguished clusters were determined with the help of the "indicspecies" package in R, by means of the point-biserial correlation coefficient calculated on abundance as well as presence-absence data and for equalized groups of plots. We have determined the diagnostic species for all possible group combinations (i.e. starting from combinations of two up to n-1, where n is the number of defined clusters), as this allows us to identify important diagnostic taxa that are shared between the groups (Tsiripidis et al. 2009, De Cáceres et al. 2010). The number of permutations was set to 999 and those taxa with $P \leq 0.01$ were considered as diagnostic. Finally, we chose to keep the results derived by using the presence-absence data, based on interpretation as well as on the fact that point-biserial correlation coefficient calculated in this way corresponds to the phi coefficient which is widely used in vegetation science (Chytrý et al. 2002). Few taxa were designated as characteristic or differential ones of certain vegetation units without following fully the results of numerical determination, based on

Fig. 1. Distribution of *Aesculus hippocastanum* including its main native range (green areas) and isolated populations (green crosses) and the identified syntaxonomical units: Cluster 1 = *Staphyleo pinnatae-Aesculetum hippocastani* ass. nova, Cluster 2 = *Rusco hypoglossi-Aesculetum hippocastani*, Cluster 3 = *Aesculus hippocastanum-Tilia platyphyllos* community, Cluster 4 = *Juglando-Aesculetum hippocastani*, Cluster 5 = *Aesculus hippocastanum-Abies borisii-regis* community. The map is based on the map published by Caudullo et al. (2018).

the bibliography (i.e., the diagnostic taxa combinations given for *Ae. hippocastanum* vegetation units in the original publications), their ecology and geography as well as their abundance in the relevés.

Direct and indirect ordination analysis was conducted to facilitate the interpretation of clustering and the exploring of floristic gradients. An initial running of a detrended correspondence analysis (DCA) resulted in a length of gradient higher than 3, allowing the application of unimodal methods. Canonical correspondence analysis was used to explore which of the examined explanatory variables explained relatively high and significant proportion of species data variance. This was tested by applying 999 unrestricted permutations in a forward selection of explanatory variables. As explanatory variables the following were used: altitude, ground inclination (slope), plots' geographical coordinates (latitude and longitude) as well as 19 bioclimatic variables (Karger et al. 2017). The explanatory variables that were found to explain a statistically significant portion of the variance at $P \leq 0.01$ based on the forward selection of the CCA were used as passive (supplementary) variables in a DCA analysis. In both ordination analyses species cover values were square root transformed and rare species were down weighted. Ordination analyses were performed with the use of CANOCO 5 software (Šmilauer and Lepš 2014).

Classification results were interpreted and discussed in the light of the most influential syntaxonomic interpretations of vegetation types of the Balkan Peninsula. The new syntaxa were named according to the rules of the 4th edition of the International of Phytosociological Nomenclature (Theurillat et al. 2021).

Results

Classification of *Aesculus hippocastanum*-dominated communities

The fusion levels graph, which is presented in the On-line Suppl. Mat. (On-line Suppl. Fig. 1) indicated that the distinction of four up to six clusters is reasonable. From an interpretation of these possible classification schemes we concluded with the distinction of five clusters (Fig. 2). The first cluster includes all relevés from Bulgaria, the second and third - most of the relevés from Greece, the fourth - the relevés from North Macedonia and the fifth cluster - the remaining relevés from Greece. In Tab. 1, a synoptic table of differential taxa of the distinguished clusters is presented, while in the On-line Suppl. Tab. 2 and Tab. 3 with all the complete relevés, the geographical distribution of these clusters within the study area is given. On one hand, clusters are differentiated positively as well as negatively by an adequate number of absolute differential taxa (Tab. 1) and thus the ecological and plant geographical differentiation of the vegetation units distinguished is highlighted. There are also enough taxa differentiating combinations of clusters and thus revealing the common ecological conditions but maybe also the common history that these vegetation units share.

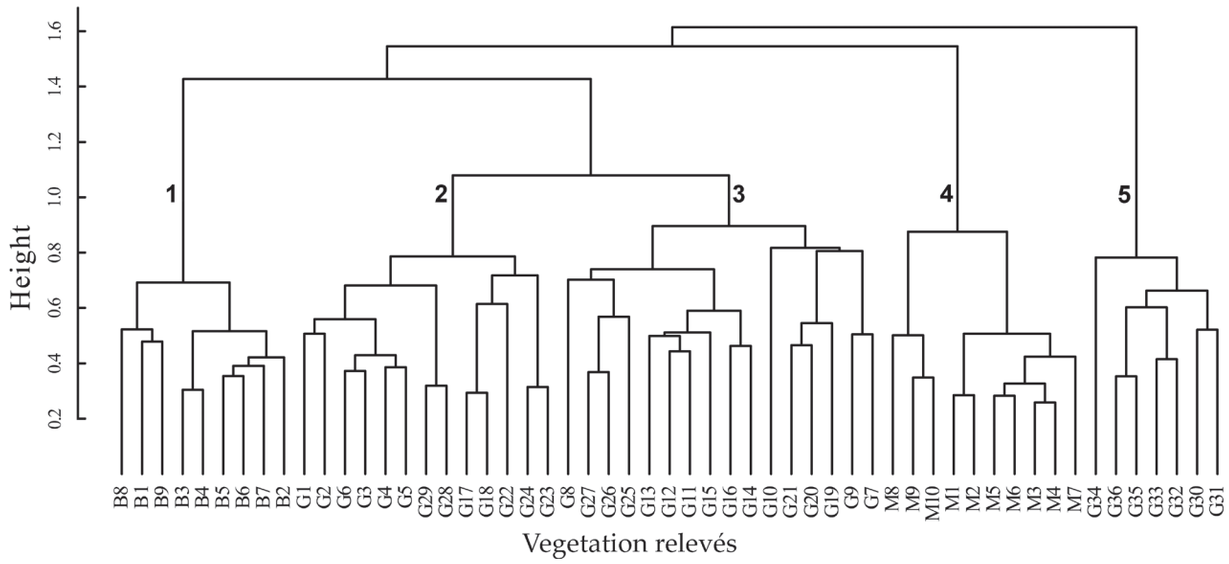

Fig. 2. Cluster dendrogram of the studied vegetation relevés from Bulgaria (B1-B9), Greece (G1-G36) and North Macedonia (M1-M10) and their classification in vegetation units. The numbers inserted in the dendrogram correspond to the following vegetation units: 1. *Staphyleo pinnatae-Aesculetum hippocastani* ass. nova – Bulgaria; 2. Ass. *Rusco hypoglossi-Aesculetum hippocastani* Raus et Bergmeier 1990 – Greece; 3. *Aesculus hippocastanum-Tilia platyphyllos* community – Greece; 4. Ass. *Juglando-Aesculetum hippocastani* Matvejeva & Nikolovski 1976 – N. Macedonia; 5. *Aesculus hippocastanum – Abies borisii-regis* community of Barbero & Quézel 1976 – Greece.

Ordination of *Aesculus hippocastanum*-dominated communities

Floristic differentiation between the five clusters and the two main groups (1 - 4 and 5) was confirmed also by the ordination diagram of the plots (Fig. 3a). Total variation in DCA was equal to 3.24715. The first DCA axis (eigenvalue equal to 0.3669; explained variation 11.30%) discriminates the 5th cluster from the rest of the clusters, supporting its significant floristic and ecological differentiation. Clusters 2 and 3, which include relevés from Greece, are positioned

Tab. 1. Synoptic table for the forest vegetation hosting *Aesculus hippocastanum*. Diagnostic taxa have their constancy values shaded in grey. In the second column, the taxa abbreviations used in Fig. 3 are given. Furthermore, Con.: relative constancy of taxa in the whole dataset; P.val: P values according to the permutation test for the determination of diagnostic taxa. Taxa with constancy values equal or lower than 20% in any cluster were omitted.

	Cluster number		**1**	**2**	**3**	**4**	**5**	Con.	P.val
	Number of relevés		9	13	16	10	7		
	Altitude (m; average)		358	681	912	965	1407		
	Slope (%; average)		23	35	60	40	24		
	Average species richness		23	44	50.5	52.5	26		
Ch	***Aesculus hippocastanum* L.**	*AescHipp*	**100**	**100**	**100**	**100**	**100**	100	-
	Association *Staphyleo pinnatae-Aesculetum hippocastani*								
Ch	*Asplenium scolopendrium* L.	*AsplScol*	100	23	19	0	0	27	0.001
Ch	*Staphylea pinnata* L.	*StapPinn*	67	0	0	0	0	11	0.001
Ch	*Mercurialis perennis* L.	*MercPere*	56	0	13	0	0	13	0.002
Ch	*Homalothecium lutescens* H. Robinson	*HomaLute*	78	0	0	0	0	13	-
Df	*Stachys sylvatica* L.	*StacSylv*	56	8	0	0	0	11	0.001
Df	*Viola hirta* L.	*ViolHirt*	56	0	0	10	0	11	0.001
Df	*Pulmonaria mollis* Hornem.	*PulmMoll*	33	0	0	0	0	5	0.005
Df	*Corylus colurna* L.	*CoryColu*	33	0	6	0	0	7	0.008
	Association *Rusco hypoglossi-Aesculetum hippocastani*								
Ch	*Abies borisii-regis* Mattf.	*AbieBori*	0	92	75	0	100	56	0.001
Ch	*Athyrium filix-femina* (L.) Roth	*AthyFili*	0	77	6	0	0	20	0.001

	Cluster number		**1**	**2**	**3**	**4**	**5**	Con.	P.val
Ch	*Taxus baccata* L.	*TaxuBacc*	0	69	19	0	0	22	0.001
Ch	*Ruscus hypoglossum* L.	*RuscHypo*	11	69	19	0	0	24	0.001
Ch	*Platanus orientalis* L.	*PlatOrie*	0	54	19	0	0	18	0.002
Ch	*Clinopodium grandiflorum* (L.) Kuntze	*ClinGran*	22	69	31	0	0	29	0.009
Ch	*Cyclamen hederifolium* Aiton	*CyclHede*	0	38	38	0	0	20	0.008
Ch	*Polystichum setiferum* (Forssk.) Woyn.	*PolySeti*	78	92	69	0	0	55	0.001
Df	*Galium odoratum* (L.) Scop.	*GaliOdor*	0	100	13	40	0	35	0.001
Df	*Drymochloa drymeja* (Mert. & W. D. J. Koch) Holub	*DrymDrym*	0	85	25	10	0	29	0.001
Df	*Milium effusum* L.	*MiliEffu*	0	69	13	10	0	22	0.001
Df	*Veronica urticifolia* Jacq.	*VeroUrti*	0	46	0	0	0	11	0.001
Df	*Melittis melissophyllum* L. subsp. *albida* (Guss.) P. W. Ball	*MeliMeli*	0	62	19	0	0	20	0.001
Df	*Ilex aquifolium* L.	*IlexAqui*	0	54	13	0	0	16	0.001
Df	*Luzula sylvatica* (Huds.) Gaudin	*LuzuSylv*	0	54	13	0	0	16	0.002
Df	*Prenanthes purpurea* L.	*PrenPurp*	0	38	0	0	0	9	0.001
Df	*Circaea lutetiana* L.	*CircLute*	0	38	0	0	0	9	0.002
Df	*Asplenium adiantum-nigrum* L.	*AsplAdia*	22	69	25	0	0	27	0.003
Df	*Cardamine pectinata* Pall. ex DC	*CardPect*	0	31	0	0	0	7	0.005
	***Tilia platyphyllos* - *Aesculus hippocastanum* community**								
Df	*Tilia platyphyllos* Scop.	*TiliPlat*	78	15	69	60	14	49	0.002
Df	*Dryopteris dilatata* (Hoffm.) A. Gray	*DryoDila*	0	0	50	0	0	15	0.003
Df	*Arabis alpina* L.	*ArabAlpi*	0	0	31	0	0	9	0.009
Df	*Viola odorata* L.	*ViolOdor*	0	0	75	0	0	22	0.001
Df	*Rosa canina* L.	*RosaCani*	0	0	31	0	0	9	0.008
	Association *Juglando regiae-Aesculetum hippocastani*								
Ch	*Chaerophyllum hirsutum* L.	*ChaeHirs*	0	0	0	80	0	15	0.001
Ch	*Fraxinus excelsior* L.	*FraxExce*	0	0	0	80	0	15	0.001
Ch	*Rhamnus alpina* L. subsp. *fallax* (Boiss.) Maire & Petitm.	*RhamAlpi*	0	0	0	70	0	13	0.001
Ch	*Juglans regia* L.	*JuglRegi*	33	0	0	90	0	22	0.001
Ch	*Lunaria rediviva* L.	*LunaRedi*	0	0	0	60	0	11	0.001
Ch	*Umbilicus rupestris* (Salisb.) Dandy	*UmbiRupe*	0	8	0	50	0	11	0.002
Ch	*Asperula taurina* L.	*AspeTaur*	0	0	0	30	0	5	0.007
Ch	*Mercurialis ovata* Sternb. & Hoppe	*MercOvat*	0	8	19	50	0	16	0.008
Ch	*Saxifraga rotundifolia* L.	*SaxiRotu*	0	62	56	90	29	51	0.003
Ch	*Geranium macrorrhizum* L.	*GeraMacr*	0	38	31	60	0	29	0.004
Ch	*Acer hyrcanum* Fisch. & C. A. Mey.	*AcerHyrc*	0	31	31	10	0	18	0.066
Ch	*Frangula rupestris* (Scop.) Schur	*FranRupe*	0	8	0	20	0	5	0.172
Ch	*Asarum europaeum* L.	*AsarEuro*	0	0	0	10	0	2	-
Ch	*Dryopteris carthusiana* (Vill.) H. P. Fuchs	*DryoCart*	0	0	0	10	0	2	-
Ch	*Galium pseudaristatum* Schur	*GaliPseu*	0	0	0	10	0	2	-
Df	*Euonymus verrucosus* Scop.	*EuonVerr*	0	0	25	100	0	25	0.001
Df	*Lapsana communis* L.	*LapsComm*	0	15	6	90	0	22	0.001
Df	*Clinopodium nepeta* (L.) Kuntze	*ClinNepe*	0	0	0	70	0	13	0.001
Df	*Lonicera xylosteum* L.	*LoniXylo*	0	0	0	70	0	13	0.001
Df	*Rhamnus cathartica* L.	*RhamCath*	0	0	0	60	0	11	0.001
Df	*Solanum dulcamara* L.	*SolaDulc*	0	0	0	60	0	11	0.001

	Cluster number		**1**	**2**	**3**	**4**	**5**	Con.	P.val
Df	*Dactylis glomerata* L.	*DactGlom*	11	0	38	90	0	29	0.001
Df	*Digitalis laevigata* Waldst. & Kit.	*DigiLaev*	0	0	19	70	0	18	0.001
Df	*Hylotelephium maximum* (L.) Holub	*HyloMaxi*	0	0	0	50	0	9	0.001
Df	*Knautia drymeia* Heuff.	*KnauDrym*	0	0	13	60	0	15	0.001
Df	*Polygonatum odoratum* (Mill.) Druce	*PolyOdor*	0	23	44	90	0	35	0.001
Df	*Geranium sylvaticum* L.	*GeraSylv*	0	0	0	40	0	7	0.001
Df	*Silene coronaria* (L.) Clairv.	*SileCoro*	0	0	0	40	0	7	0.001
Df	*Salix eleagnos* Scop.	*SaliElea*	0	0	0	40	0	7	0.002
Df	*Daphne mezereum* L.	*DaphMeze*	0	0	0	40	0	7	0.004
Df	*Veronica chamaedrys* L.	*VeroCham*	0	23	38	90	29	36	0.001
Df	*Chelidonium majus* L.	*ChelMaju*	22	8	0	60	0	16	0.003
Df	*Geranium phaeum* L.	*GeraPhae*	0	0	0	30	0	5	0.007
Df	*Amelanchier ovalis* Medik.	*AmelOval*	0	0	0	30	0	5	0.009
	***Abies borisii-regis* - *Aesculus hippocastanum* community**								
Ch	*Prunus mahaleb* L.	*PrunMaha*	0	0	13	0	71	13	0.001
Ch	*Sorbus graeca* (Spach) S. Schauer	*SorbGrae*	0	0	0	0	57	7	0.001
Ch	*Tanacetum parthenium* (L.) Sch. Bip.	*TanaPart*	0	0	0	0	57	7	0.001
Ch	*Cardamine graeca* L.	*CardGrae*	0	8	0	0	57	9	0.001
Df	*Astragalus glycyphyllos* L.	*AstrGlyc*	0	8	0	0	86	13	0.001
Df	*Acer monspessulanum* L.	*AcerMons*	0	8	38	10	86	25	0.001
Df	*Hippocrepis emerus* (L.) Lassen subsp. *emeroides* (Boiss. & Spruner) Lassen	*HippEmer*	0	0	25	10	71	18	0.001
Df	*Elymus panormitanus* (Parl.) Tzvelev	*ElymPano*	0	0	0	0	43	5	0.002
Df	*Fragaria vesca* L.	*FragVesc*	0	0	13	30	71	18	0.001
Df	*Doronicum orientale* Hoffm.	*DoroOrie*	0	0	6	0	43	7	0.005
Df	*Juniperus communis* L.	*JuniComm*	0	0	13	0	43	9	0.005
	Other differential vascular plant species								
Df	*Hedera helix* L.	*HedeHeli*	100	100	94	100	29	89	0.001
Df	*Fraxinus ornus* L.	*FraxOrnu*	89	92	94	100	14	84	0.001
Df	*Melica uniflora* Retz.	*MeliUnif*	44	85	88	70	0	65	0.001
Df	*Acer campestre* L.	*AcerCamp*	78	15	13	0	57	27	0.001
Df	*Acer platanoides* L.	*AcerPlat*	67	85	69	10	0	53	0.001
Df	*Viola reichenbachiana* Boreau	*ViolReic*	78	62	0	60	0	38	0.001
Df	*Fagus sylvatica* L.	*FaguSylv*	56	92	38	60	0	53	0.004
Df	*Carpinus betulus* L.	*CarpBetu*	78	8	38	40	0	33	0.005
Df	*Aegopodium podagraria* L.	*AegoPoda*	78	0	19	50	0	27	0.001
Df	*Lamium galeobdolon* (L.) Crantz	*LamiGale*	78	0	0	40	0	20	0.001
Df	*Salvia glutinosa* L.	*SalvGlut*	0	62	31	70	0	36	0.001
Df	*Rubus hirtus* aggr.	*RubuHirt*	0	77	50	20	0	36	0.001
Df	*Asplenium trichomanes* L.	*AsplTric*	22	62	69	30	0	44	0.004
Df	*Sambucus nigra* L.	*SambNigr*	22	77	56	30	0	44	0.007
Df	*Galium rotundifolium* L.	*GaliRotu*	0	46	6	0	57	20	0.001
Df	*Cephalanthera rubra* (L.) Rich.	*CephRubr*	0	31	6	0	57	16	0.002
Df	*Geranium robertianum* L.	*GeraRobe*	33	100	81	100	0	71	0.001
Df	*Campanula trachelium* L.	*CampTrac*	0	38	69	80	0	44	0.001
Df	*Cicerbita muralis* (L.) Wallr.	*Cicemura*	67	100	100	100	29	85	0.002

	Cluster number		**1**	**2**	**3**	**4**	**5**	Con.	P.val
Df	*Polypodium vulgare* L.	*PolyVulg*	11	69	44	90	0	47	0.001
Df	*Primula acaulis* (L.) L.	*PrimAcau*	0	38	63	60	14	40	0.008
Df	*Aremonia agrimonoides* (L.) DC.	*AremAgri*	11	62	75	30	86	55	0.002
Df	*Corylus avellana* L.	*CoryAvel*	22	0	69	90	0	40	0.001
Df	*Lamium maculatum* (L.) L.	*LamiMacu*	11	8	63	60	0	33	0.001
Df	*Sedum cepaea* L.	*SeduCepa*	0	15	38	70	0	27	0.002
Df	*Daphne laureola* L.	*DaphLaur*	0	31	56	0	71	33	0.002
Df	*Physospermum cornubiense* (L.) DC.	*PhysCorn*	0	0	31	0	29	13	0.009
Df	*Ostrya carpinifolia* Scop.	*OstrCarp*	0	69	63	80	71	58	0.001
Df	*Helleborus odorus* Willd. subsp. *cyclophyllus* (A. Braun) Maire & Petitm.	*HellOdor*	0	0	38	60	71	31	0.001
Df	*Clinopodium vulgare* L.	*ClinVulg*	0	8	0	40	43	15	0.008
	Other vascular plant species								
	Clematis vitalba L.	*ClemVita*	56	62	69	80	57	65	0.78
	Brachypodium sylvaticum (Huds.) P. Beauv.	*BracSylv*	56	54	44	60	86	56	0.399
	Acer pseudoplatanus L.	*AcerPseu*	44	15	69	40	57	45	0.242
	Poa nemoralis L.	*Poa Nemo*	22	69	38	50	43	45	0.437
	Dioscorea communis (L.) Caddick & Wilkin	*DiosComm*	11	69	38	50	14	40	0.065
	Sanicula europaea L.	*SaniEuro*	33	62	25	40	43	40	0.6
	Ulmus glabra Huds.	*UlmuGlab*	44	31	44	40	0	35	0.152
	Viola riviniana Rchb.	*ViolRivi*	0	38	50	0	29	27	0.011
	Pseudoturritis turrita (L.) Al-Shehbaz	*PseuTurr*	11	31	50	20	0	27	0.104
	Cornus mas L.	*CornMas*	44	0	38	30	29	27	0.203
	Dryopteris filix-mas (L.) Schott	*DryoFili*	0	62	19	30	0	25	0.012
	Urtica dioica L.	*UrtiDioi*	22	54	31	0	0	25	0.03
	Euphorbia amygdaloides L.	*EuphAmyg*	11	15	38	50	0	25	0.04
	Acer obtusatum Willd.	*AcerObtu*	0	8	38	50	14	24	0.02
	Cardamine bulbifera (L.) Crantz	*CardBulb*	0	23	31	50	0	24	0.031
	Geum urbanum L.	*GeumUrba*	22	15	6	50	43	24	0.116
	Potentilla micrantha DC.	*PoteMicr*	0	15	44	30	14	24	0.151
	Arum cylindraceum-et- *maculatum*	*ArumCyli*	0	23	19	60	0	22	0.015
	Scutellaria columnae All.	*ScutColu*	0	38	38	10	0	22	0.017
	Euonymus latifolius (L.) Mill.	*EuonLati*	0	15	31	10	57	22	0.02
	Alliaria petiolata (M. Bieb.) Cavara & Grande	*AlliPeti*	11	23	38	20	0	22	0.331
	Selinum silaifolium (Jacq.) Beck	*SeliSila*	0	23	25	0	57	20	0.018
	Prunus avium (L.) L.	*PrunAviu*	22	8	19	10	57	20	0.037
	Bromopsis benekenii (Lange) Holub	*BromBene*	11	15	38	0	29	20	0.169
	Asplenium ceterach L.	*AsplCete*	11	15	25	40	0	20	0.234
	Lathyrus laxiflorus (Desf.) Kuntze	*LathLaxi*	0	8	44	20	0	18	0.038
	Umbilicus luteus (Huds.) Webb & Berthel.	*UmbiLute*	0	15	31	30	0	18	0.113
	Moehringia trinervia (L.) Clairv.	*MoehTrin*	0	15	31	10	29	18	0.313
	Lamium garganicum L.	*LamiGarg*	0	31	13	0	43	16	0.022
	Anemone nemorosa L.	*AnemNemo*	0	46	6	20	0	16	0.031
	Ruscus aculeatus L.	*RuscAcul*	44	15	19	0	0	16	0.039
	Lathyrus venetus (Mill.) Wohlf.	*LathVene*	11	54	44	20	14	18	0.088
	Pteridium aquilinum (L.) Kuhn.	*PterAqui*	0	38	19	0	0	15	0.02

Cluster number		**1**	**2**	**3**	**4**	**5**	Con.	P.val
Carpinus orientalis Mill.	*CarpOrie*	22	0	25	0	29	15	0.124
Hieracium murorum L.	*HierMuro*	0	15	6	40	0	13	0.034
*Tilia tomentos*a Moench	*TiliTome*	0	15	31	0	0	13	0.061
Prunella vulgaris L.	*PrunVulg*	0	8	25	20	0	13	0.196
Quercus cerris L.	*QuerCerr*	22	0	19	20	0	13	0.223
Neottia nidus-avis (L.) Rich.	*NeotNidu*	0	15	19	0	29	13	0.226
Sorbus torminalis (L.) Crantz	*SorbTorm*	11	15	25	0	0	13	0.329
Symphytum tuberosum L.	*SympTube*	33	0	0	30	0	11	0.017
Scutellaria altissima L.	*ScutAlti*	11	0	6	40	0	11	0.019
Symphytum bulbosum K. F. Schimp.	*SympBulb*	0	15	6	0	43	11	0.021
Galium sylvaticum L. subsp. *laconicum* (Boiss. & Heldr.) Stoj. & Stef.	*GaliSylv*	0	8	31	0	0	11	0.029
Doronicum columnae Ten.	*DoroColu*	0	0	25	20	0	11	0.064
Rubus canescens DC.	*RubuCane*	0	15	25	0	0	11	0.106
Carex pendula Huds.	*CarePend*	0	31	6	0	0	9	0.023
Castanea sativa Mill.	*CastSati*	0	31	6	0	0	9	0.029
Laburnum alpinum (Mill.) Bercht. & J. Presl	*LabuAlpi*	0	0	19	0	29	9	0.057
Geranium versicolor L.	*GeraVers*	0	8	25	0	0	9	0.067
Primula veris L.	*PrimVeri*	0	8	25	0	0	9	0.083
Quercus dalechampii Ten.	*QuerDale*	0	8	25	0	0	9	0.083
Rosa arvensis Huds.	*RosaArve*	0	8	25	0	0	9	0.095
Dactylorhiza saccifera (Brongn.) Soó	*DactSacc*	0	23	13	0	0	9	0.149
Epilobium lanceolatum Sebast. & Mauri	*EpilLanc*	0	23	13	0	0	9	0.156
Cephalanthera longifolia (L.) R. M. Fritsch	*CephLong*	0	15	6	0	29	9	0.212
Allium ursinum L.	*AlliUrsi*	0	31	0	0	0	7	0.014
Anthriscus sylvestris (L.) Hoffm. subsp. *nemorosus* (M. Bieb.) Koso-Pol.	*AnthSylv*	22	8	0	0	0	5	0.142
Thalictrum minus L.	*ThalMinu*	0	0	0	0	29	4	0.011
Anemone blanda Schott & Kotschy	*AnemBlan*	0	0	0	0	29	4	0.019
Campanula abietina Griseb.	*CampAbie*	0	0	0	0	29	4	0.019
Solidago virgaurea L.	*SoliVirg*	0	0	0	0	29	4	0.019
Mosses								
Anomodon viticulosus (Hedw.) Hook. & Taylor	*AnomViti*	78	0	0	70	0	25	-
Alleniella besseri (Lobarz.) S.Olsson, Enroth & D.Quandt	*NeckBess*	89	0	0	0	0	15	-
Homomalium incurvatum Loesske	*HomoIncu*	0	0	0	60	0	11	-
Homalothecium sericeum W.P.Schimper	*HomaSeri*	0	0	0	50	0	9	-
Frullania dilatata (L.) Dumort.	*HygrDila*	0	0	0	20	0	4	-

more or less in the central part of the horizontal axis (first DCA axis), albeit closer to the right end of the axis, where the relevés from Bulgaria and N. Macedonia occur. From the above-mentioned distribution of relevés along the first DCA axis as well as from the ordination diagram of the explanatory variables passively projected onto the ordination space of the first two DCA axes (Fig. 3b), it seems that the first DCA axis represents a latitudinal gradient, which is also related to some bioclimatic variables, such as Bio12 (annual precipitation) and Bio15 (precipitation seasonality), as well as altitude. The latter 3 variables (Bio12, Bio15 and latitude), however, are also correlated with the second DCA axis, as they are directed diagonally in relation to the first two DCA axes. The bioclimatic variables Bio14 (precipitation of driest month), Bio17 (precipitation of wettest quarter) and Bio4 (temperature seasonality), which explain a small but significant proportion of unique variation of species data are also positively correlated with the first DCA

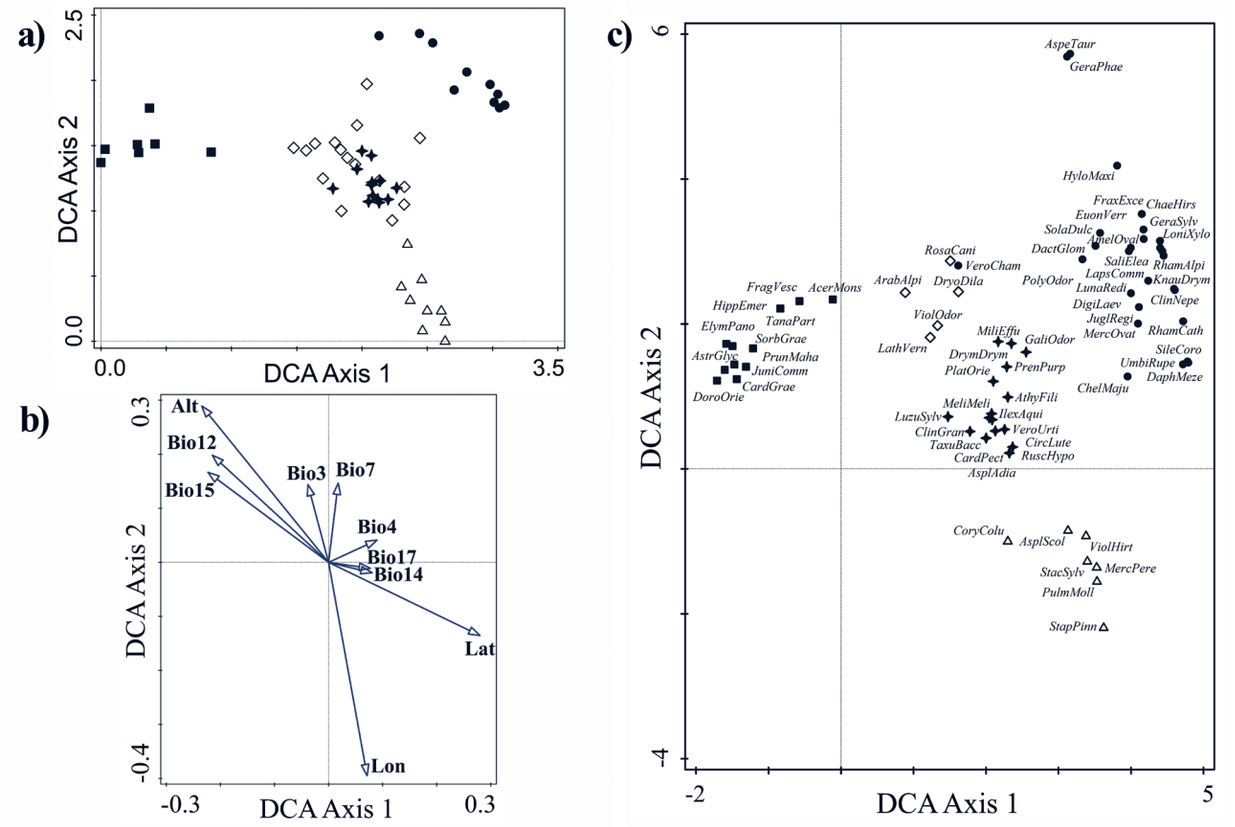

Fig. 3. Ordination diagrams of relevés (a), explanatory variables [altitude (Alt); latitude (Lat); longitude (Lon); isothermality (Bio3); temperature seasonality (Bio4); temperature annual range (Bio7); annual precipitation (Bio12); precipitation of driest month (Bio14); precipitation seasonality (Bio15), and precipitation of wettest quarter (Bio 17)] passively projected onto the ordination space (b) and absolute differential taxa (c) on the basis of the first two DCA axes. In the ordination diagrams of relevés (a) and differential taxa (c) the different symbols denote the clusters and vegetation units in which the relevés were classified or differentiated by the taxa, as follows: Δ (empty triangles): Cluster 1 = *Staphyleo pinnatae-Aesculetum hippocastani*, Bulgaria, ✦ (filled stars): Cluster 2 = *Rusco hypoglossi-Aesculetum hippocastani*, Greece, ◊ (empty rhombuses): Cluster 3 = *Aesculus hippocastanum-Tilia platyphyllos* community, Greece, ● (filled circles): Cluster 4 = *Juglando-Aesculetum hippocastani*, ■ (filled squares): Cluster 5 = *Aesculus hippocastanum-Abies borisii-regis* community, Greece.

axis, albeit weakly. The conditional effects of the explanatory variables are presented in the On-line Suppl. Tab. 3 and ten variables were found to significantly explain a unique proportion of species data variation.

The second DCA axis (eigenvalue equal to 0.1974; cumulative explained variation together with the first axis 17.38%) discriminates mainly the relevés sampled in Bulgaria from those sampled in N. Macedonia (Fig. 3a) and it is correlated to longitude. Furthermore, it also contributes to the discrimination between the two clusters from Greece (the 2nd and 3rd) but not so clearly. The second axis may represent a longitudinal gradient of transition from the drier climatic conditions of the western part of the Balkan Peninsula towards the moister ones of the eastern part. This is also revealed from the correlation of the second axis to temperature annual range (Bio7), isothermality (Bio3) and precipitation seasonality (Bio15). However, the second axis represents an even more complex gradient as it is partly correlated to altitude and annual precipitation (Bio12).

Figure 3c clearly depicts the strong floristic differentiation between most of the distinguished vegetation units, as their differential taxa are distributed at certain and distinct parts of the ordination diagram. It also complements the abovementioned results, which revealed that both ordination axes represent ecological and phytogeographical differentiation between the localities of *Ae. hippocastanum* vegetation in the different countries or geographic regions. This is depicted from the position at the left part of the first DCA axis of typical East Mediterranean incl. oro-Mediterranean species (e.g. *Doronicum orientale*, *Acer monspessulanum*, *Cardamine graeca*), while European (s. str.) and Euro-Siberian species (e.g. *Geranium sylvaticum*, *Hylotelephium maximum*, *Fraxinus excelsior*, *Daphne mezereum*) are at the right part of the axis. Interestingly, the more mesophilous species, which are typical of the habitat of *Ae. hippocastanum* (i.e. low mountain ravine forests with high air and soil humidity), such as *Ruscus hypoglossum*, *Taxus baccata*, *Athyrium filix-femina*, *Galium odoratum*, *Drymochloa drymeja*, *Ilex aquifolium*, *Luzula sylvatica*, are distributed at the center of the ordination space and are diagnostic of the 2nd cluster, demonstrating possibly the range of the ecologically favorable conditions for the refugial *Ae. hippocastanum* communities.

Association *Staphyleo pinnatae-Aesculetum hippocastani* ass. nova, Tzonev, Mastrogianni, Tsiripidis, Dimitrov, Gussev, Mandzhukovski et Pachedjieva

(Cluster 1), holotypus rel. № B4 hoc loco (Appendix 1)

Diagnostic species: *Asplenium scolopendrium* (Ch, Df), *Staphylea pinnata* (Ch, Df), *Stachys sylvatica* (Df), *Viola hirta* (Df), *Mercurialis perennis* (Ch, Df), *Pulmonaria mollis* (Df), *Corylus colurna* (Df) and *Homalothecium lutescens* (Ch).

The unique locality of *Aesculus hippocastanum* in Bulgaria is situated in the Preslavska Mountains of the East Balkan Range. It is so remote and isolated compared to the main range of this species that it was considered to be of non-native origin. However, firstly Adamović (1908) and subsequently many other authors (Gussev and Valchev 2015) proved that this is a relict locality of the species, emphasizing its Tertiary origin.

The communities in Bulgaria are located along Dervishka and Lazarska rivers, on the northern slopes of the low Preslavska Mountains. It is a part of the Forebalkan area – a great calcareous foothill of Balkan (Stara Planina) range with many plateaus and low mountains, mostly with altitude lower than 1000 m. Mt. Preslavska is also known as a remnant locality of *Cercis siliquastrum* in Bulgaria. The communities of *Ae. hippocastanum* occupy the steep slopes (25–60°) of these limestone valleys and form stands of variable density in the most humid parts (Gussev and Valchev 2015).

This is a newly described association, which is floristically and ecologically related mainly to the *Ae. hippocastanum* stands in Greece and especially those classified in the 2nd cluster but also with the species stands in N. Macedonia. Among the common differential taxa that this association shares with clusters 2 to 4, the taxa *Polystichum setiferum, Acer platanoides, Tilia platyphyllos* and *Carpinus betulus* indicate its mesic character and its high representativeness concerning the ravine forests of the southern Balkans. Probably because of the more mesic conditions and the higher latitude of its locality, it lacks many thermophilous species occurring in all other vegetation units distinguished here, such as *Ostrya carpinifolia* and *Helleborus odorus* subsp. *cyclophyllus*. The name-giving taxon is *Staphylea pinnata*, which forms a lower tree sub-layer in some parts of the stands. The species is mostly distributed in southeastern Europe, but not in the drier parts of the Mediterranean region, for example in Greece, where it occurs outside the range of the *Ae. hippocastanum* (Heiss et al. 2014).

Association *Rusco hypoglossi-Aesculetum hippocastani* Raus et Bergmeier 1990

(Cluster 2)

Diagnostic species: *Athyrium filix-femina* (Ch, Df), *Cyclamen hederifolium* (Ch), *Polystichum setiferum* (Ch), *Galium odoratum* (Df), *Drymochloa drymeja* (Df), *Taxus baccata* (Ch, Df), *Milium effusum* (Df), *Veronica urticifolia* (Df), *Ruscus hypoglossum* (Ch, Df), *Melittis melissophyllum* subsp. *albida* (Df), *Ilex aquifolium* (Df), *Luzula sylvatica* (Df), *Prenanthes purpurea* (Df), *Circaea lutetiana* (Df), *Platanus orientalis* (Ch, Df), *Asplenium adiantum-nigrum* (Df), *Clinopodium grandiflorum* (Df) and *Cardamine pectinata* (Df).

This association comprises the most representative habitat type of *Ae. hippocastanum* in its native distribution range. It comprises an azonal forest described from Mt. Ossa, 800 m a.s.l. near Karitsa village (Raus 1980). Raus (1980) classified the two relevés as a community named *Aesculus hippocastanum-Tilia platyhyllos*. Furthermore, the author noticed the absence of some taxa, such as *Juglans regia, Fraxinus exclesior, Acer pseudoplatanus, A. campestre* and *Tilia cordata*, usually present in forests hosting *Ae. hippocastanum* in Albania, N. Macedonia and Bulgaria. This may indicate that this community is a floristically impoverished form of the corresponding vegetation from the above-mentioned northern countries. Bergmeier (1990) found forest stands with similar floristic composition and ecological characteristics in Mt. Kato Olympos and described a new association, *Rusco hypoglossi-Aesculetum hippocastani*, in which he also classified the two relevés of Raus (1980) from Mt. Ossa.

From the high frequency and cover of mesic species in this association (e.g. *Athyrium filix-femina, Drymochloa drymeja, Ruscus hypoglossum, Circaea lutetiana, Taxus baccata*, etc.), many of which are also absolute differentials of this cluster, it can be inferred that *Ae. hippocastanum* finds in this vegetation type a habitat with increased air and soil moisture conditions. These highly moist conditions may be due to the topographic characteristics (they occur in deep ravines, usually on steep north-facing slopes and on colluvial soils) of the localities of this association, but also to the fact that the slopes of Mts. Ossa and Kato Olympos are facing the Aegean Sea and so receiving higher precipitation (Styllas and Kaskaoutis 2018).

Some of the above-mentioned species (e.g. *Ruscus hypoglossum, Taxus baccata,* etc.) are considered typical of the ravine forests of southern Balkans (Bergmeier 1990). Bergmeier (1990) classified this association in his newly proposed sub-alliance: *Aesculo-Tilienion tomentosae*, of the alliance *Tilio-Acerion*. However, some of the diagnostic species of the proposed sub-alliance (e.g. *Asplenium scolopendrium, Carpinus betulus, Euonymus latifolius, Tilia tomentosa*) do not present a high or a differentiating constancy in this association. The floristic and ecological distinction of this community type is further supported by the classification of Mastrogianni et al. (2019) and Mastrogianni (2020), where 3493 relevés from mountainous broadleaved and coniferous forests of Greece were used. In the dataset used by the former authors all relevés from this cluster (except one that was not used in the dataset) were included and classified in the ravine forests community types and specifically in the one characterized as "mesic mixed broadleaved ravine forests, occurring on soils with very good water and nutrient availability at mid-altitudes".

This association presents relatively high floristic similarity with the next vegetation unit (3rd cluster), as well as

with the vegetation units distributed in Bulgaria and N. Macedonia (1st and 4th clusters). Most of these common species are mesic or diagnostic taxa of the *Fagetalia sylvaticae* order.

Community *Aesculus hippocastanum-Tilia platyphyllos*

(Cluster 3)

Diagnostic species: *Viola odorata* (Df), *Dryopteris dilatata* (Df), *Rosa canina* (Df), *Arabis alpina* (Df).

This vegetation unit seems to represent a transitional type from ravine forests to mesic broadleaved forests. Floristically and ecologically it is close to the former association. During tests of classification analyses we conducted by applying different distance measures, linkage methods or species cover transformations, the 2nd and 3rd clusters were the only ones between which there was an exchange of relevés i.e., the two clusters almost always came up but with slightly different composition in relevés. Their floristic similarity is evident also in the ordination plot of relevés (Fig. 3a). The tree layer of this vegetation unit is comprised of *Ae. hippocastanum* accompanied by mesophilous broadleaved deciduous species such as *Tilia platyphyllos* (most often), *Carpinus betulus*, *Acer pseudoplatanus* or the more thermophilous *Tilia tomentosa*. Despite the high number of taxa occurring in the vegetation plots of this cluster, it had the lowest number of absolute diagnostic taxa, with none of them typical of ravine forests. It is characterized by the co-occurrence of mesic and thermophilous species of beech and oak forests (e.g. *Dryopteris dilatata*, *Hedera helix*, *Melica uniflora*, *Polystichum setiferum*, *Acer platanoides,* etc.). However, it hosts and is dominated by ravine forest species such as those described above comprising its tree layer. The floristic composition and ecology of this unit to a great extent resembles the association *Tilio-Castanetum* Dafis 1973 found from Mt. Kato Olympos by Bergmeier (1990). This author stressed the floristic similarity of this association to the *Rusco hypoglossi-Aesculetum hippocastani* from the same mountain, and commented that *Tilio-Castanetum* is ecologically as well as spatially positioned between the mesic forests of *Ostryo-Carpinion orientalis* Horvat 1959 (*Dryopterido pallidae–Ostryetum carpinifoliae* Bergmeier 1990) and the ravine forests of *Tilio-Acerion* Klika 1955 (*Rusco hypoglossi-Aesculetum hippocastani*). *Tilio-Castanetum* forests described by Raus (1980) for Mt. Ossa and by Dafis (1973) from other localities, are more xeric and thermophilous than the *Aesculus hippocastanum-Tilia platyphyllos* community described here and also than the *Tilio-Castanetum* Bergmeier (1990). In general, such forests described in Greece and characterized by the dominance of *Tilia tomentosa* (see Raus 1980; Bergmeier 1990, etc.) are more or less equally shared between the "thermophilous mixed broadleaved ravine forests of mid-altitudes" and the "mesic mid-altitude thermophilous, broadleaved mixed forests" or "*Quercus dalechampii* forests" according to the classification scheme of Mastrogianni et al. (2019) and Mastrogianni (2020).

Association *Juglando-Aesculetum hippocastani* Matvejeva et Nikolovski 1976

(Cluster 4)

Diagnostic species: *Chaerophyllum hirsutum* (Ch, Df), *Fraxinus excelsior* (Ch, Df), *Euonymus verrucosus* (Df), *Mercurialis ovata* (Ch, Df), *Saxifraga rotundifolia* (Ch), *Rhamnus alpina* subsp. *fallax* (Ch, Df), *Juglans regia* (Ch, Df), *Lunaria rediviva* (Ch, Df), *Geranium macrorrhizum* (Ch), *Acer hyrcanum* (Ch), *Frangula rupestris* (Ch), *Asarum europaeum* (Ch), *Dryopteris carthusiana* (Ch), *Galium pseudoaristatum* (Ch), *Lapsana communis* (Df), *Clinopodium nepeta* (Df), *Lonicera xylosteum* (Df), *Rhamnus cathartica* (Df), *Solanum dulcamara* (Df), *Dactylis glomerata* (Df), *Digitalis laevigata* (Df), *Hylotelephium maximum* (Df), *Knautia drymeia* (Df), *Polygonatum odoratum* (Df), *Umbilicus rupestris* (Ch, Df), *Geranium sylvaticum* (Df), *Silene coronaria* (Df), *Salix eleagnos* (Df), *Daphne mezereum* (Df), *Veronica chamaedrys* (Df), *Chelidonium majus* (Df), *Asperula taurina* (Ch, Df), *Geranium phaeum* (Df), *Amelanchier ovalis* (Df).

There are four localities of *Ae. hippocastanum* communities in the western part of N. Macedonia, especially in wet and calcareous valleys and ravines between 750 m and 1100 m a.s.l. As accompanying species Em (1957) determined *Fraxinus excelsior*, *Acer obtusatum*, *Corylus colurna*, *Ostrya carpinifolia*, *Rhamnus alpinus* subsp. *fallax*, etc. The associations described by Em (1957) were *Aesculi-Ostryetum* and *Aesculi-Fagetum*, which were subsequently accepted and described in greatest detail on Mt. Bistra by Rizovski and Džekov (1990). However, these associations are "nomina nuda" (see Art. 2b in Theurillat et al. 2021), because Em (1957) did not publish any phytocoenological relevé from them. Therefore, the only valid and priority published association is *Juglando-Aesculetum hippocastani* Matvejeva and Nikolovski 1976, which was also reported from Mt. Bistra. Cluster analysis did confirm the great floristic and ecological similarity between the phytocoenoses from all localities in N. Macedonia. Therefore, they all belong to this association. Several of the diagnostic species of this syntaxon, such as *Rhamnus alpina* subsp. *fallax*, *Lunaria rediviva*, *Fraxinus excelsior*, *Chaerophyllum hirsutum*, *Umbilicus rupestris*, etc., are mesic species mostly distributed across southern and central Europe.

The association from N. Macedonia occupies very similar habitats to those of the southern part of the species range in Greece. It is also distributed on steep slopes of ravines and gorges, on often exposed limestone bedrocks, with relatively poor soils, but with high soil moisture. Its floristic composition, however, is enriched by thermophilous and light demanding species of oak forests, possibly because of degradation caused by anthropogenic disturbances, such as logging. This thermophilous species group includes taxa such as *Fraxinus ornus, Lonicera xylosteum, Silene coronaria, Helleborus odorus, Galium pseudaristatum, Quercus pubescens, Dictamnus albus, Amelanchier ovalis, Aegonychon purpurocaueruleum, Colutea arborescens, Acer*

mospessulanum, etc. However, the main group of mesic and relict species, especially trees and shrubs, such as *Ulmus glabra, Tilia platyphyllos, Ostrya carpinifolia, Carpinus betulus*, as well as many typical fern species, occur with a high constancy in this association.

Community *Aesculus hippocastanum – Abies borisii-regis*

(Cluster 5)

Diagnostic species: *Astragalus glycyphyllos* (Df), *Prunus mahaleb* (Ch, Df), *Sorbus graeca* (Ch, Df), *Tanacetum parthenium* (Ch, Df), *Cardamine graeca* (Ch, Df), *Acer monspessulanum* (Ch, Df), *Hippocrepis emerus* subsp. *emeroides* (Df), *Elymus panormitanus* (Df), *Fragaria vesca* (Df), *Doronicum orientale* (Df), *Juniperus communis* (Df).

This 5th cluster was described as a distinct association by Barbero and Quézel (1976) with the name "Association à *Abies borisii regis* et *Aesculus hypocastanum*". It has a limited distribution in the central Pindus Mts., on calcareous outcrops (rocky formations of exposed limestone) with Cretaceous (Senonian) age. It is clearly differentiated from the other two clusters (2nd and 3rd) occurring in Greece, despite its spatial proximity to them. Barbero and Quézel (1976) emphasize the distinct floristic character and rare distribution occurrence of this community, and they attribute it to the special substrate of the area, characterized mostly by steep calcareous walls. The locality of relevés sampled by Barbero and Quézel (1976) was visited by Anna Mastrogianni in 2016. It was established that together with vegetation in which individuals of *Ae. hippocastanum* participate, they were still found to occur in open habitats, rich in thermophilous species. This fact supports the interpretation of Bergmeier (1990) concerning the peculiar structure and composition of this community by excluding other possible interpretations, such as disturbances or field sampling peculiarities. Because this vegetation type depends on local ecological conditions, we chose to distinguish it as a community.

Because of its vegetation this community is transitional to the sub-Mediterranean mountain oak forests and represents the driest habitats in which *Ae. hippocastanum* was found, constituting an ecological extreme for the species. This is further supported by its floristic composition, which includes, even among its diagnostic species, typical xerophytic and thermophilous species, such as *Prunus mahaleb, Acer monspessulanum, Hippocrepis emerus* subsp. *emeroides, Sorbus graeca*, etc. Although this community spatially occurs within a general area of high habitat suitability for *Ae. hippocastanum*, the extreme conditions, in terms of soil moisture, allow inferences regarding the niche breadth of the species, indicating the ability of the species to survive under less optimum environmental conditions. It should be noted however, that this community has the highest average altitude (1407 m) among the vegetation units of *Ae. hippocastanum*.

Aesculus hippocastanum vegetation in Albania

Communities from the most northwestern part of the *Ae. hippocastanum* natural distribution, located in Albania, are not represented in the present study due to the lack of published relevés. However, relatively detailed phytocoenological, phytoecological and population data are available in the work of Peçi et al. (2012). The species habitat in this area mostly includes scree and deforested terrains of altitudes between 700-1200 m, where *Ae. hippocastanum* participates in mesic forest vegetation, growing on steep and rocky slopes of ravines. The vegetation has been determined as belonging to *Fagion sylvaticae*, but it is more probable that the accompanying tree species (*Sorbus torminalis*, *Acer hyrcanum*, *Acer pseudoplatanus*, *Tilia cordata*, *Tilia platyphyllos*, *Viburnum lantana*, *Ilex aquifolium*, *Carpinus betulus*, *Ostrya carpinifolia*), as well as the description of the habitats, indicate that it is related to *Ostryo carpinifoliae-Tilion platyphylli*. The plant communities of *Ae. hippocastanum* there are very similar to those in North Macedonia and probably belong to the same (ass. *Juglando-Aesculetum*) or some other, but close synvicariant syntaxon.

Discussion

Syntaxonomic relationships of *Aesculus hippocastanum*-dominated communities

On the basis of the floristic and ecological affinities of the first four clusters distinguished in the classification we consider that all of them belong to the same alliance.

Bergmeier (1990) placed the *Rusco hypoglossi-Aesculetum hippocastani* in the *Tilio-Acerion* alliance. However, it was latterly considered by Mucina et al. (2016) as *sensu lato* and this alliance is divided into four others with more restricted distribution. Geographical position, diagnostic species (such as *Hedera helix, Asarum europaeum, Tilia platyphyllos, Aremonia agrimonoides, Juglans regia, Campanula rapunculoides, C. trachelium, Lilium martagon, Sanicula europaea, Arum maculatum, Sorbus torminalis, Helleborus odorus, Scutellaria altissima, Digitalis grandiflora, Calamintha grandiflora, Pseudoturritis turrita*), ecological peculiarities, etc., placed the most widespread communities of *Ae. hippocastanum* on the Balkans in the alliance *Ostryo carpinifoliae-Tilion platyphylli*. It also includes typical refugial forests in the ravines at lower altitudes, such as foothills and lower mountains, mostly from the Western Balkans. However, the 5th cluster is strongly differentiated floristically and ecologically from the rest of the vegetation units, indicating its relationship with different higher syntaxa. On the basis of the dominance of *Abies borisii-regis* in this community as well as its thermophilous character we propose classifying this community in *Abietion cephalonicae*, which includes supra-Mediterranean Hellenic fir forests. Below the syntaxonomic scheme of the distinguished vegetation units in this study is given.

Class *Carpino-Fagetea sylvaticae* Jakucs ex Passarge 1968
Order *Aceretalia pseudoplatani* Moor 1976
Alliance *Ostryo carpinifoliae-Tilion platyphylli* (Košir et al. 2008) Čarni in Willner et al. 2016
Association *Rusco hypoglossi-Aesculetum hippocastani* Raus et Bergmeier 1990
Association *Juglando-Aesculetum hippocastani* Matvejeva et Nikolovski 1976
Association *Staphyleo pinnatae-Aesculetum hippocastani* Tzonev et al. 2024 ass. nova
Aesculus hippocastanum - Tilia platyphyllos community (Tzonev et al. 2024)
Class *Quercetea pubescentis* Doing-Kraft ex Scamoni et Passarge 1959
Order *Quercetalia pubescenti-petraeae* Klika 1933
Alliance *Abietion cephalonicae* Horvat et al. 1974
Aesculus hippocastanum-Abies borisii-regis community (Barbero et Quézel 1976)

Historical dynamic and conservation value of *Aesculus hippocastanum* dominated communities

Aesculus hippocastanum constitutes a biogeographic relict species of the Balkan Peninsula, since it is the only surviving descendant of the once widespread genus of *Aesculus* in the Europe. The initial entry of the *Aesculus* genus in Europe from eastern Asia is considered to have taken place by the early Oligocene (Harris et al. 2009). Although studies on the paleo-distribution of *Aesculus* representatives have revealed a wide distribution of the genus in Europe as well as the Balkan Peninsula since the Miocene, it is estimated that the genus was more widely distributed across the whole European continent mainly during the Pliocene (Postigo Mijarra et al. 2008). This distribution shrank abruptly due to the subsequent unfavorable climatic conditions that occurred during the end of the Pliocene, like the distribution of several other woody taxa (Svenning 2003).

The habitat suitability of temperate and even northern Europe for *Ae. hippocastanum* is indicated by the species widespread current distribution throughout Europe as a cultivated species and its systematic use as an ornamental tree in many European cities. This raises questions concerning the causes of the species' inability to recolonize naturally central and northern parts of Europe. Based on the available knowledge it can be inferred that species biology rather than habitat suitability is mostly responsible for this distribution pattern (Thomas et al. 2019, Walas et al. 2019). Specifically, the species reproduction ecology seems to constitute a significant limiting factor for its spreading. On one hand, its barochoric (mean weight per seed ≈ 42 g) seeds, which spread mainly by gravity, limit the ability of the species to disperse over great distances (Tsiroukis 2008, Thomas et al. 2019). It is considered that *Ae. hippocastanum* seeds can travel only short distances by gravity (up to 14.5 m), similarly with its relative Japanese Horse-chestnut (*Aesculus turbinata*) (Hoshizaki et al. 1999). Nevertheless, seeds can be carried greater distances by rodents, although this is not particularly common (Thomas et al. 2019). On the other hand, its seeds are recalcitrant and intolerant of desiccation, limiting its ability to successfully establish new localities that lack the necessary conditions related with humidity (Tsiroukis 2008, Walas et al. 2019). Therefore, the species' inability to travel great distances, as well as its seeds' sensitivity to dry conditions during the early stages of any attempt by the species to establish itself in a new location, constitute the main known causal factors for its current native range. After the initial restriction of the species in refugial low and mid-altitude habitats, such as ravines and gorges, that allowed the species persistence by ensuring relative environmental stability through periods of climatic changes (Woolbright et al. 2014), it was unable to overcome topographical barriers and recolonize the rest of Europe.

The present study, based on vegetation data of communities of *Ae. hippocastanum* across its whole distribution range, led to the classification of its communities into five syntaxonomical units assigned to the *Carpino-Fagetea sylvaticae* and *Quercetea pubescentis* classes, thus dividing the vegetation of *Aesculus hippocastanum*, respectively, in mesic and xeric-thermophilous vegetation groups. Inferences regarding the historical communities of *Aesculus* in the central and southern Europe during Pleistocene also indicate the co-occurrence of the species with several thermophilous and mesic taxa, such as *Ulmus*, *Pterocarya*, *Carya*, *Platanus*, *Juglans*, *Fagus* and *Carpinus* (Renault-Miskovsky and Girard 1978). Overall, it has been proposed that temperate deciduous communities occurring in moist habitats constitute the main historical habitat of *Aesculus*, which was mainly established in environments such as valley bottoms, riverheads, mid-mountain riverine forests or forests on temporarily flooded soils (Postigo Mijarra et al. 2008). These habitats allowed the preservation of the species during the Pleistocene as well as during the current adverse environmental conditions by ensuring high air humidity and water availability for its populations, which has been revealed to constitute one of its main ecological requirements (Walas et al. 2018).

Further indications of the special floristic character of *Ae. hippocastanum* communities could be derived through other relict species that occur and even dominate in them, which have similar ecological preferences, reproductive and dispersal strategies. Such are the Persian walnut (*Juglans regia*) and the European bladdernut (*Staphylea pinnata*), which have been also objects of economic interest since Ancient times, humans having expanded their distribution significantly for various commercial needs (Heiss et al. 2014, Pollegioni et al. 2017).

The current distribution of the *Staphylea pinnata* is sporadic, but significantly wider than that of *Ae. hippocastanum*. It is also considered a Tertiary relict (from the Pliocene), and a representative element of the nemoral flora in the sub-Mediterranean region (Heiss et al. 2014). According to Meusel and Jäger (1989), forest plants with ranges in the eastern part of sub-Mediterranean area (mostly in the Balkans and

western Anatolian Peninsula) were named "*Staphylea*-type". This group includes *Quercus cerris*, *Q. frainetto*, *Ostrya carpinifolia*, and *Fraxinus ornus*, but species such as *Tilia tomentosa*, *Syringa vulgaris* and *Ae. hippocastanum* are confined to the center of the this "*Staphylea*-type" area. The range of *Staphylea pinnata*, like that of *Aesculus hippocastanum*, has been affected by human activity due to the exploitation of the species for a variety of economic uses, since Antiquity, leading to a significantly wider distribution and its subspontaneous establishment in many places in Central and Western Europe (Heiss et al. 2014).

Juglans regia is known from macrofossils from Western Bulgaria (Sofia Basin) from the late Pliocene, but the genus was distributed in the Balkans (*Juglans ungeri*) even earlier - since the Lower Oligocene (Palamarev 1993). According to Pollegioni et al. (2017), at least in the eastern Mediterranean, in two phytogeographically well-defined plant refugia - the Balkans and Turkey, *Juglans regia* existed during all the cold and dry periods of the Pleistocene. Sudden increases of its fossil pollen between 2500 and 1000 years BP, presumably are due to the increase of its cultivation from Greek and Roman times onwards. This range's "fluctuation" is also strongly comparable with the history of *Ae. hippocastanum* surviving in the Last Glacial Maximum in even more restricted refugia than *Juglans regia* and its secondary expansion by humans.

The evolutional history of *Staphylea pinnata* and *Juglans regia* is also good evidence that their important phytocoenological role in the recent communities of *Ae. hippocastanum* is a result of long coexistence together in these Tertiary refugia. However, in general, we can conclude that these species represent the most conservative relict component of the Tertiary flora and forest vegetation that survived in the Last Glacial Maximum (LGM), only in specific habitats and refugial plant communities. These communities have hosted more widespread species as well, such as representatives of the genera *Tilia*, *Ulmus*, *Quercus*, *Carpinus*, *Corylus*, *Ostrya*, *Acer*, etc. However, the more successful dispersal strategies (mostly anemochory) of the latter did help them to expand their ranges, especially in the temperate regions of the Mediterranean and southern temperate Europe. Therefore, we could demonstrate especially *Ae. hippocastanum*-dominated communities as a "model example" for the refugia of mesophilous forest vegetation during the Pleistocene and at least, during the LGM.

Currently, natural relict populations of the species are to be found in the Western Balkans, with the Bulgarian subpopulation constituting a location of special character, since the area is not included in the potential distribution of *Ae. hippocastanum*, even since the period of Mid Holocene (Walas et al. 2019). The diversity patterns among the syntaxa of *Ae. hippocastanum*, as they were identified by the present study, revealed both spatial (longitude and latitude) as well as ecological variables (meso-climate differentiation represented by altitude, but also microhabitat differentiation depicted by the relevés' floristic composition), as driving factors of *Ae. hippocastanum* habitat differentiation. These results, derived from vegetation data, are in accordance with those found by Walas et al. (2019), concerning the genetic diversity of *Ae. hippocastanum* in the main part of its distribution range (Greece). According to them, species genetic diversity is, to a great extent, the result of the lack of connectivity among its populations, leading to unique genetic signatures even among spatially adjacent populations, but also of ecological differentiation, thus potentially highlighting the importance of the concept of microhabitat differentiation.

The taxonomic, functional and phylogenetic diversity of all relevés (except one) included in the communities that represent the main current habitat of *Ae. hippocastanum* (2nd and 3rd cluster) has been previously investigated and compared to the rest of the forest community types (broad-leaved deciduous and coniferous) occurring within the main native distribution area of the species, namely central and northern Greece (Mastrogianni et al. 2019, Mastrogianni 2020). According to these studies, the ravine forest types to which the communities of *Ae. hippocastanum* investigated belong, had a particularly unique profile of species diversity, mainly characterized by the occurrence of a great number of woody taxa in the forest overstorey layer, in agreement with the observation also of Raus (1980). Moreover, these ravine forest communities were found to have a distinct functional signature, defined mostly by the presence of several species with particular seed (large, heavy and recalcitrant seeds) and dispersal-related characteristics, indicative of a potential refugial role of these habitats (Keppel et al. 2018). Finally, the ravine forests were found to preserve high levels of phylogenetic diversity, which has been attributed mostly to the persistence of species with evolutionary distinctiveness in these communities due to long-term environmental stability (Mastrogianni et al. 2019). The aforementioned characteristics of ravine forests constitute significant arguments for the great conservational value of the relict *Ae. hippocastanum* and its habitat, in addition to the already recognized significance due to their rarity and distinct evolutionary history. In particular, from their distinct patterns of diversity it can be inferred that these habitats have played a significant role as shelters for several species during past climatic variations, therefore constituting potentially crucial localities for biodiversity conservation under the forthcoming effects of current climate change (Hampe and Petit 2005).

Conclusions

Despite the relict and endemic character of *Ae. hippocastanum*, as well as its narrow distribution range and relatively rare occurrence throughout this range, the habitat diversity of the species has not been thoroughly studied. The present study contributes to the understanding of the habitat diversity of *Ae. hippocastanum*, by identifying five floristically and ecologically well-defined syntaxonomical units as the main habitats of the species. These units are the associations *Rusco hypoglossi-Aesculetum hippocastani*, *Juglando-Aesculetum hippocastani*, the newly defined association *Staphyleo pinnatae-Aesculetum hippocastani*, but there

are also two communities (*Aesculus hippocastanum-Tilia platyphyllos*, and *Aesculus hippocastanum-Abies borisii-regis*). The geographic distribution and isolation of these distinct habitats across its overall distribution range are in agreement with the paleo-distribution of *Ae. hippocastanum* and the historical events that led to its restriction and survival in only a few spatially restricted refugial areas. The biological characteristics of the species, such as its seed weight and storage behavior, as well as its ecological requirements for high levels of humidity and special soil characteristics, constitute the main attributes that explain the floristic diversity of *A. hippocastanum* communities, which, according to historical evidence, have included taxa such as *Ulmus, Platanus, Juglans, Fagus* and *Carpinus* since the Pleistocene. Preexisting knowledge regarding the taxonomic, functional and phylogenetic diversity of its communities has highlighted the great conservational value of *Ae. hippocastanum* and its habitats. Therefore, the contribution of the present study towards the understanding of *Ae. hippocastanum* habitat diversity, can be particularly useful for the development and prioritization of the necessary conservation actions.

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Appendix 1. Holotypus relevé of *Staphyleo pinnatae-Aesculetum hippocastanii* ass. nova.

Relevé number in On-line Suppl. Tab 2: B4; Relevé area: 400 sq. m; Altitude (m): 357 m; Aspect (degrees): 30; Exposition: north-east; Cover tree layer (%) – 85%; Cover shrub layer (%) – 30%; Cover herb layer (%) – 50%; Latitude: N 43.14997; Longitude: E26.74987; Locality: "Dervisha" Reserve; Data: 23.10.2020; Authors: R. Tzonev, M. Dimitrov, Ch. Gussev.

Tree layer: *Aesculus hippocastanum* (5); *Acer platanoides* (+); *Carpinus betulus* (+); *Fagus sylvatica* (+), *Fraxinus ornus* (+); *Prunus avium* (+); *Tilia platyphyllos* (+); *Ulmus glabra* (+).

Shrub layer: *Staphylea pinnata* (3).

Herbaceous layer: *Aegopodium podagraria* (2b); *Asplenium scolopendrium* (+); *Brachypodium sylvaticum* (r); *Cicerbita muralis* (+); *Clematis vitalba* (+); *Geranium robertianum* (+); *Hedera helix* (1); *Mercurialis perennis* (+); *Hedera helix* (2); *Polystichim setiferum* (+); *Stachys sylvatica* (+); *Vincetoxicum hirundinaria* (+); *Viola hirta* (+); *Viola reichenbachiana* (+).

Moss layer: *Anomodon viticulosus* (+); *Alleniella besseri* (+); *Bryopsida spp.* (+).

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Nitric oxide alleviates mercury toxicity by changing physiological and biochemical pathways in maize (*Zea mays* L.) seedlings

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Abstract – Like all life forms, plants suffer from high levels of mercury (Hg), known as one of the most harmful heavy metals in soil. The present study was performed to explore the effects of exogenous nitric oxide (NO) on Hg toxicity in maize (*Zea mays* L., cv. Arifiye-2) seedlings. Plants were grown in a hydroponic system containing 1/2 diluted Hoagland at 16 h day length, 25/20 °C (day/night) and 60% relative humidity. Eight day-old maize seedlings were first treated with NO (as 0.1 μM sodium nitroprusside) and then they were exposed to Hg toxicity (as 100 μM $HgCl_2$) after 24 h. The toxic Hg decreased seedling growth, chlorophyll content, proline content, calcium and manganese contents, non-enzymatic antioxidant contents, cell membrane viscosity, and antioxidant enzyme activities (superoxide dismutase, catalase, peroxidases, and glutathione reductase) while it increased the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and super oxide anion ($O_2^{\cdot-}$), and lipid peroxidation (as malondialdehyde, MDA) content and the amount of sodium ion (Na^+) in the seedlings. However, NO treatment markedly enhanced the growth parameters (dry and fresh weight, and plant height) and manganese and potassium contents as well as contents of antioxidants and chlorophyll thus alleviating the negative effects caused by the Hg stress. Also, it decreased the generation of ROS and lipid peroxidation level by activating the antioxidant enzymes. These results show that NO in maize seedlings under Hg toxicity may improve stress response and mitigate oxidative stress by stimulating the antioxidant system and modulating ion homeostasis.

Keywords: antioxidant, nitric oxide, heavy metal, mercury, oxidative stress

Introduction

Heavy metals are naturally occurring elements that have a higher atomic weight and density than water (Tchounwou et al. 2012, Wani et al. 2021). Heavy metal pollution is increasing due to anthropogenic activities, such as mining, smelting, industrial production and metal-containing compounds in household and agriculture (He et al. 2005, Wani et al. 2021). Increasing heavy metal pollution gradually adversely affects the ecosystem, with terrestrial plants being particularly susceptible compared to other organisms. Heavy metals are transported to various plant parts after root uptake and then eventually enter the food chain (Ding et al. 2019). An excess of heavy metals, one of which is mercury (Hg), leads to damage to cells, tissues, and enzymes in organisms.

Mercury is a non-essential element and widely accumulates in an ecosystem as an industrial pollutant. Anthropogenic sources for Hg accumulation in soil include mining, gold smelting, fuel combustion, and the industries involved in manufacturing items such as paint, disinfectants, pharmaceuticals, paper, and antimicrobial drugs (Gontia-Mishra et al. 2016). Hg is one of the most important threats to agricultural areas and causes serious damage to crops at various growth stages. Due to its transitional properties Hg is easily taken up by plants, where it inhibits the root growth and development, and affects the water balance and mineral nutrition (Chen et al. 2015). Hg toxicity leads to a reduction in chlorophyll and carotenoid contents due to the peroxidation of thylakoid membranes (Amooaghaie and Enteshari 2017). Also, it induces lipid peroxidation (LPO) and oxidative damage by generating excessive reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxide radical (OH^-) and superoxide anion radical ($O_2^{\cdot-}$)

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within plant subcellular structures (Zhou et al. 2007, Gao et al. 2010, Sahu et al. 2012).

Nitric oxide (NO), which is a bioactive gaseous molecule and acts as a signaling molecule, may enhance the plant's response to environmental stresses, including heavy metals, by regulating their physiological and biochemical processes (García-Mata and Lamattina 2013). Additionally, NO acts as an antioxidant, effectively preventing and scavenging ROS in cells, thus alleviating oxidative damage (Chen et al. 2018). There are some previous studies about the role of exogenous NO in plants under heavy metal stress. They reported that the application of NO donors not only increased chlorophyll content, biomass and root length but it also decreased lipid peroxidation and ROS production under heavy metal stress such as cadmium (Cd), copper (Cu), nickel (Ni), zinc (Zn), and arsenic (As) (Terrón-Camero et al. 2019). Furthermore, NO may decrease Cu, Cd, lead (Pb) and Pb/Cd-induced oxidative damage by restraining heavy metal uptake capacity of plant root system in ryegrass, peanut, rice and alfalfa (Mostofa et al. 2014, Bai et al. 2015, Dong et al. 2016, Fang et al. 2019). Moreover, NO could alleviate phytotoxicity by directly regulating accumulation and translocation of mercury in rice (Chen et al. 2015). The detoxification by NO might be related to the modulation of cell wall components, pectin and hemicelluloses (Chen et al. 2015). However, there is little information regarding the role of NO in regulating Hg-induced stress in maize.

Maize (*Zea mays* L.), one of the oldest field crops cultivated by humans, is a vital cereal plant from the *Poaceae* family, widely grown worldwide. Due to its rich nutritional content, maize is very valuable for both human and animal nutrition, with a wide range of uses. Starch, glucose and corn oil obtained from corn grain, which are of great importance for the sufficient and economic production of plant-based proteins in the world, are used as raw materials in the economy. The protection of maize under adverse conditions is important in terms of both food supply and human health.

Based on the above studies, we hypothesized that NO is quite crucial in helping plant tolerance under Hg stress. Therefore, our aim was to show that NO is able to alleviate Hg-induced toxicity by activating the antioxidant system and reducing oxidative stress in maize seedlings.

Materials and methods

Plant material and growing conditions

In this study we utilized maize (*Zea mays* L. cv. Arifiye-2) as plant material, sodium nitroprusside (SNP) as the source of nitric oxide (NO), and mercury chloride ($HgCl_2$) as the source of mercury (Hg). Maize seeds were subjected to a surface sterilization in 70% ethanol for 1 min, subsequently in 5% sodium hypochlorite for 5 min, and then rinsed 6 times with distilled water.

In preliminary experiments surface-sterilized maize seeds were sown on Petri dishes (15 cm diameter) with double-layer filter paper and treated with different $HgCl_2$ concentrations (0, 1, 5, 10, 20, 40, 50, 100, 200, 500 and 1000 μM) for 5 days in a growth chamber at 25 ± 2 °C. The 100 μM $HgCl_2$ was chosen for further experiments because it caused at least a 50% reduction in germination rate and root length. A similar study was conducted to determine the appropriate SNP concentration by sowing maize seeds on Petri dishes with 100 μM $HgCl_2$ and different SNP concentrations, and 0.1 μM SNP was chosen because it markedly alleviated negative growth effects of Hg.

For treatments with 100 μM $HgCl_2$ and 0.1 μM SNP, the plants were grown in a hydroponic system containing 1/2 diluted Hoagland solution (Hoagland and Arnon 1950) in conditions of 16 h day length, 25/20 °C (day/night), and 60% relative humidity. When seedlings were 8-days old, initial treatment with 0.1 μM SNP was applied to the hydroponic medium. Half-strength Hoagland's solution used as control. After 24 h, the seedlings were exposed to 100 μM $HgCl_2$ and then seedlings were harvested after 3 days of Hg treatment. These periods (24 h and 3 days) were determined according to a previous experiment and literature (Chen et al. 2015). Harvested seedlings were stored at –80 °C for subsequent experiments or immediately used for measurement of growth parameters. The purity of all chemicals was more than 98%.

Measurement of seedling growth and photosynthetic pigments content

Ten randomly selected seedlings were used for morphological measurements. Firstly, fresh weight (FW) and shoot length (SL) were measured using a balance and a scale. Then the seedlings were put in an oven for 72 h at 80 °C to detect dry weight (DW). The chlorophyll pigments content in the leaves was detected in acetone extracts by the spectrophotometric method according to Lichtenthaler (1987) and was expressed as mg g^{-1} fresh weight (FW).

Determination of reactive oxygen species, lipid peroxidation and electrolyte leakage

Hydrogen peroxide (H_2O_2) level was determined according to the method of Hu et al. (2005). Briefly, 0.5 g of the samples were homogenized in 10 mL of cold acetone and then centrifuged at 5000 × g for 15 min at + 4 °C. Then, 0.5 mL of the supernatant was combined with 0.15 mL of 5% $Ti(SO_4)_2$ and 0.3 mL of 19% NH_4OH. The mixture was centrifuged at 3000 × g for 10 min at + 4 °C. The pellet was washed twice with cold acetone and dissolved in 3 mL of 1 M H_2SO_4. After filtration, absorbance measurement of filtrate was measured at 415 nm using H_2SO_4 as a blank. The amount of H_2O_2 was calculated by a constructed standard curve and defined as μg g^{-1} fresh weight (FW).

The production of super oxide anion ($O_2^{\cdot-}$) was determined by Liu et al. (2007). Fresh tissue (0.5 g) was homogenized in 3 mL of 50 mM phosphate buffer (pH 7.8), and the homogenate was centrifuged at 10000 × g for 10 min. The supernatant (0.1 mL) was combined with 0.9 mL of 65 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine

hydrochloride. It was incubated at 25 °C for 15 min, and afterwards 1 mL of the mixture, 1 mL of 17 mM 1-naphthylamine and 1 mL of 17 mM anhydrous amino benzene sulfonic acid were combined and again incubated at 25 °C for 20 min. Butyl alcohol (3 mL) was supplemented to the mixture and the absorbance was measured at 530 nm. $NaNO_2$ was used for a standard curve to calculate the content of $O_2^{\cdot-}$ which was defined as μg g^{-1} fresh weight (FW).

The lipid peroxidation (LPO) level was determined by measuring the content of malondialdehyde (MDA). Briefly, 0.5 g of the samples were homogenized in 5 mL of 1% trichloroacetic acid (TCA) and then centrifuged at 12000 × g for 20 min. One mL of the supernatant was combined with 4 mL of 0.5% TBA (2-thiobarbituric acid) in 20% TCA. The reaction mixture was incubated for 30 min in a boiling water bath, and then the reaction was stopped in an ice bath. Then the reaction tubes were centrifuged again at 5000 × g for 10 min. The absorbance of the supernatant was measured at 532 nm and it was corrected by subtracting nonspecific absorbance at 600 nm. MDA level was calculated using extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} fresh weight (FW) (Heath and Packer 1968).

For electrolyte leakage (EL), the fresh leaves (0.1 g) washed with distilled water were placed in each of the test tubes including 4 mL distilled water and incubated for 4 hours at + 4 °C. Then, the amount of ions that passed into the pure water in the tubes was measured by an electrical conductivity meter (Griffith et al. 1992).

Activity of antioxidant enzymes and their isoenzyme profiles

The sample (0.2 g) was ground in 2 mL of extraction buffer (0.1 M KH_2PO_4, pH 7.0) including 0.2% polyvinylpyrrolidone and 1 mM ethylenediaminetetraacetic acid (EDTA), and the homogenate was centrifuged at 12000 × g for 15 min at + 4 °C. The supernatant was collected for enzyme activity and also used for protein determination by using bicinchoninic acid assay (BCA) reagent spectrophotometrically at 562 nm (Smith et al. 1985).

Activity of antioxidant enzymes was measured according to the method of Agarwal and Pandey (2004). Superoxide dismutase (SOD, EC 1.15.1.1) activity was described as one unit (U), the value of enzyme that inhibited 50% of the photoreduction of nitroblue tetrazolium chloride (NBT). Activity of SOD enzyme was recorded as U mg^{-1} protein. Peroxidase activity (POX, EC 1.11.1.7) was assayed by determining the absorbance increase at 470 nm caused by tetraguaiacol, which is a product of the reaction in which guaiacol and H_2O_2 are used as substrates. One unit of POX is identified as the value of enzyme that increases the absorbance at a rate of 0.01 within 1 min at 25 °C, and data are recorded as U mg^{-1} protein. Catalase (CAT, EC 1.11. 1.6) activity is based on the measurement of the decrease in absorbance at 240 nm when CAT provides the conversion of H_2O_2 to O_2 and H_2O. One unit of CAT is determined as the value of enzyme disrupting 1 mM H_2O_2 within 1 min at 25 °C, and data are expressed as U mg^{-1} protein. Glutathione reductase (GR, EC 1.6.4.2) activity was measured by monitoring glutathione dependent oxidation of NADPH at 340 nm. The reaction mixture included 0.2 mM NADPH, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione (GSSG), and 100 mM Tris-HCl (pH 7.8). Data were expressed as U mg^{-1} protein.

Native proteins were run on polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions as suggested by Laemmli (1970). For SOD isoenzymes, the gel was incubated in 0.05 M KH_2PO_4 (pH 7.8) containing 0.24 mM NBT, 33.2 μM riboflavin, 0.2% N,N,N',N' -tetramethylethylenediamine (TEMED), and 1 mM EDTA on a shaker in the dark for 30 min at 37 °C. Then, the gel was placed in 0.05 M potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and incubated under white fluorescent light for 10 – 30 min to determine isoenzymes (Beauchamp and Fridovich 1971). POX and CAT isoenzymes were monitored according to Weydert and Cullen (2010). For POX isoenzymes, the activity staining was realized after incubation for 30 min in 0.2 M sodium acetate buffer (pH 5.0) containing 30 mM H_2O_2 and 10 mM guaiacol. For CAT isoenzymes, the gel was incubated in 30 mM H_2O_2 for 10 min, it was stained with 2% $FeCl_3$ and 2% K_3FeCN_6 solutions. GR staining was carried out by incubation in a reaction solution including 50 mL of Tris-HCl (pH 7.5) containing 10 mg of 3-(4, 5-dimethylthiazol-24)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH. Duplicate gels were assayed for GR activity, one with and one without GSSG (Rao et al. 1996).

Determination of glutathione, ascorbic acid, and proline contents

Reduced glutathione (GSH) content was determined enzymatically using the method of Griffith (1980) with slight modification. Fresh tissue (0.2 g) was homogenized in 2 mL of 5% meta-phosphoric acid and centrifuged at 12000 × g for 20 min at 4 °C. The reaction mixture consisted of 150 μL of the supernatant and 1850 μL of KH_2PO_4 (50 mM, pH 7.5) including 2.5 mM EDTA, 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.3 U glutathione reductase, and 1 mM NADPH. The increase in absorbance at 412 nm was monitored for 3 min at 25 °C. The amount of GSH was calculated by a constructed standard curve and data were expressed as nmol g^{-1} fresh weight (FW).

The content of ascorbate (AsA) was determined as described by Okamura (1980). Fresh tissue (0.2 g) taken from the powder obtained from liquid nitrogen grinding was homogenized in 2 mL of 5 % TCA. The extract was centrifuged at 12000 × g for 10 min at 4 °C. For AsA content, reaction mixture consisted of 1 mL of supernatant and 1.5 mL of KH_2PO_4 (pH 7.4) including 10 mM dithiothreitol (DTT), 0.014% N-ethylmaleimide, 2.6% TCA, 11.7% H_3PO_4, 1% 2,2'-dipyridyl, and 0.3% $FeCl_3$. Samples were incubated for 60 min at 37 °C and the absorbance was recorded spectrophotometrically at 525 nm. The amount of AsA was calculated by a constructed standard curve and data were expressed as nmol g^{-1} fresh weight (FW).

Colorimetric detection of proline was determined based on proline's reaction with ninhydrin (Bates et al., 1973). The amount of proline was calculated by a constructed standard curve and data were expressed as μg g^{-1} fresh weight (FW).

Determination of nutrient element contents

The seedlings (0.5 g) were powdered in a mill and sieved (0.5 mm) and dried at 70 °C and digested with concentrated HNO_3 in a microwave system CEM, Mars 5 (CEM Corp., USA). In the seedlings digested, the concentrations of Na, Ca, Mn, and K were measured by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent-7800, Agilent Technologies, Tokyo, Japan).

Statistical analysis

Each experiment was repeated at least three times in three replicates. Analysis of variance (ANOVA) was conducted in a one-way ANOVA test using SPSS 17.0 for Microsoft Windows, and means were compared by Duncan test at the 0.05 level of confidence. The data were represented as the mean ± standard error from three experiments.

Results

Physiological growth parameters

The outcomes of the fresh weight (FW), the dry weight (DW), and the shoot length (SL) for the control groups (control and SNP) and mercury (Hg) treated groups (Hg and SNP + Hg) are presented in Tab. 1. Exposure to toxic Hg caused a significant inhibition ($P < 0.05$) by 36% in FW, 20% in DW, and 18.8% in SL compared to the control (Tab. 1). But, when the SNP (as a donor of NO) was applied to the seedlings exposed to Hg toxicity (SNP + Hg), the same parameters were increased by 24%, 20%, and 6%, respectively, in comparison to Hg application alone (Tab. 1).

Chlorophyll content

Hg exposure resulted in decreased chlorophyll content in maize seedlings (Tab. 2), with reductions of 9% in chlorophyll-a (Chl-a), 6% in chlorophyll-b (Chl-b), and 7.79% in total chlorophyll content compared to the control (Tab. 2). Moreover, the inhibitory effect of Hg on Chl-a content was more pronounced than on Chl-b. However, the application of NO treatment (SNP + Hg) to Hg-exposed plants led to significant increases of 12.7% in Chl-a, 28% in Chl-b, and 19.45% in total chlorophyll content compared to control plants (Tab. 2). Remarkably, the SNP treatment seemed to have a greater impact on Chl-b content in Hg-exposed seedlings. Interestingly, SNP alone led to a decrease in chlorophyll content compared to control plants (Tab. 2).

Tab. 1. Effects of SNP (as a donor of NO) and Hg on fresh and dry weight, and height of maize seedlings. Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, SNP + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, SNP 0.1 μM SNP. Values are means of three independent experiments ± standard errors. Different letters in the same column indicate statistically significant differences between means ($P < 0.05$).

Treatments	Fresh weight (g)	Dry weight (g)	Height (cm)
Control	2.56 ± 0.19a	0.15 ± 0.003b	39.09 ± 0.96a
Hg	1.63 ± 0.16c	0.12 ± 0.007c	31.71 ± 0.46c
SNP + Hg	2.02 ± 0.21b	0.15 ± 0.005b	33.61 ± 0.51b
SNP	2.76 ± 0.32a	0.17 ± 0.001a	39.92 ± 0.85a

Tab. 2. Changes in the content of chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), and total chlorophyll (total chl) of maize seedlings under Hg treatment with or without SNP (as a donor of NO). Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, SNP + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, SNP – 0.1 μM SNP. Values are means of three independent experiments ± standard errors. Different letters in the same column indicate statistically significant differences between means ($P < 0.05$).

Treatments	Chl-a (mg g^{-1} $_{FW}$)	Chl-b (mg g^{-1} $_{FW}$)	Total chl (mg g^{-1} $_{FW}$)
Control	2.126 ± 0.03a	1.59 ± 0.01ab	3.722 ± 0.01b
Hg	1.936 ± 0.04b	1.498 ± 0.05b	3.434 ± 0.02c
SNP+Hg	2.182 ± 0.06a	1.916 ± 0.09a	4.102 ± 0.01a
SNP	1.99 ± 0.01b	1.464 ± 0.02b	3.462 ± 0.04c

Reactive oxygen species, lipid peroxidation and electrolyte leakage

Table 2 displays the outcomes of ROS such as H_2O_2 and $O_2^{\cdot-}$, lipid peroxidation (MDA) and electrolyte leakage (EL) measurements for the control groups and the Hg-treated groups. NO treatment under normal conditions did not have any effect ($P > 0.05$) on H_2O_2 and $O_2^{\cdot-}$ contents. Conversely, exposure to Hg increased H_2O_2 content by 44% and the $O_2^{\cdot-}$ content by 8% compared to the control (Tab. 3).

Tab. 3. Effects of SNP (as a donor of NO) and Hg on reactive oxygen species (H_2O_2 and $O_2^{\cdot-}$), lipid peroxidation (MDA) and electrolyte leakage (EL) levels of maize seedlings. Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, SNP + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, SNP – 0.1 μM SNP. Values are means of three independent experiments ± standard errors. Different letters in the same column indicate statistically significant differences between means ($P < 0.05$).

Treatments	H_2O_2 (μg g^{-1} $_{FW}$)	$O_2^{\cdot-}$ (μg g^{-1} $_{FW}$)	MDA (nmol g^{-1} $_{FW}$)	EL (%)
Control	101.4 ± 0.43c	3.62 ± 0.15c	1.13 ± 0.10c	14.3 ± 0.27c
Hg	146.03 ± 0.52a	3.91 ± 0.16b	1.67 ± 0.09a	72.6 ± 0.46a
SNP+Hg	115.07 ± 0.51b	3.48 ± 0.08c	1.41 ± 0.01b	45.3 ± 0,19b
SNP	97 ± 0.18c	4.25 ± 0.27a	1.15 ± 0.07c	16.5 ± 0.31c

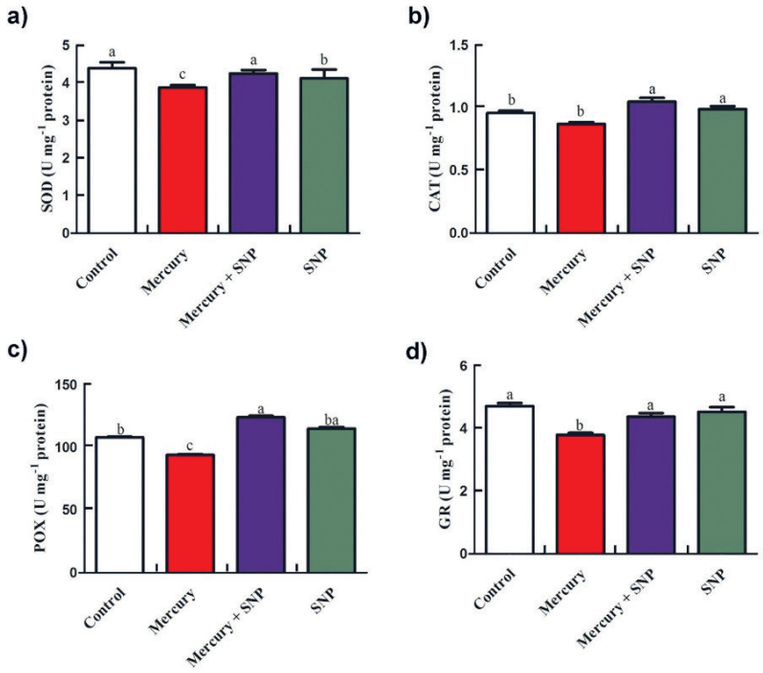

Fig. 1. Effects of SNP (as a donor of NO) and Hg on activities of antioxidant enzymes in maize seedlings. a) Superoxide dismutase (SOD) b) Catalase (CAT) c) Peroxidase (POX) d) Glutathione reductase (GR). Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, SNP + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, SNP – 0.1 μM SNP. Values are means of three independent experiments; bars indicate standard errors. Different letters show significant differences ($P < 0.05$) between maize groups.

However, in the SNP + Hg seedlings, there was a significant decrease in generation of H_2O_2 and $O_2^{\cdot-}$ by 21% and 11%, respectively (Tab. 3). Moreover, toxic Hg increased MDA content by 47% and EL level by 408% indicating oxidative damage to membranes but SNP + Hg significantly reduced MDA content by 15.6% and EL level by 37.6% (Tab. 3).

Activities and isozyme profiles of antioxidant enzymes

Figure 1 and Figure 2 present the activities and isoenzyme profiles of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and glutathione reductase (GR). Exposure to Hg led to reductions in the activities of SOD, CAT, POX, and GR by 11.6%,

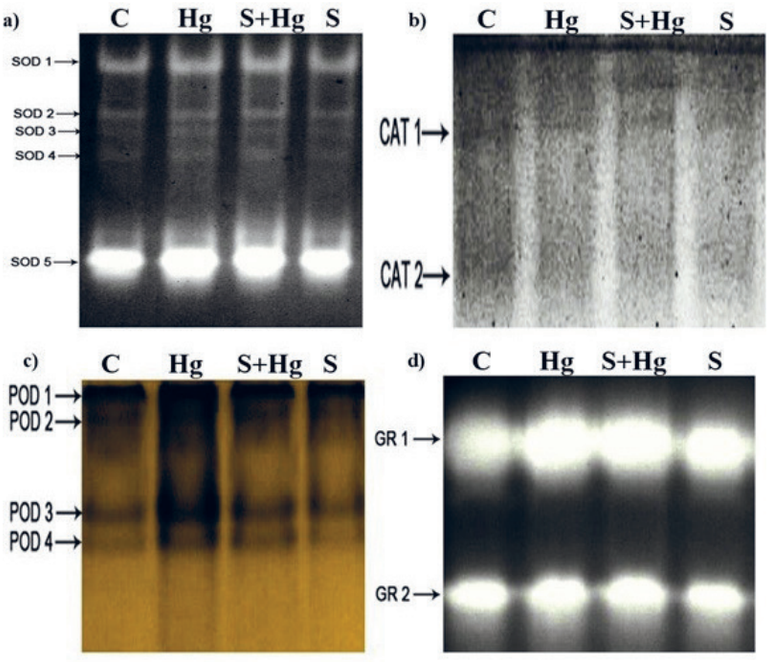

Fig. 2. Effects of SNP (as a donor of NO) and Hg on isoenzyme profiles of maize seedlings a) Superoxide dismutase (SOD), b) Catalase (CAT), c) Peroxidase (POX), d) Glutathione reductase (GR). Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, S + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, S – 0.1 μM SNP.

Tab. 4. Effects of SNP (as a donor of NO) and Hg on glutathione (GSH), ascorbate (AsA) and proline contents of maize seedlings. Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, SNP + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, SNP – 0.1 μM SNP. Values are means of three independent experiments ± standard errors. Different letters in the same column indicate statistically significant differences between means ($P < 0.05$).

Treatments	GSH (nmol g^{-1} $_{FW}$)	AsA (nmol g^{-1} $_{FW}$)	Proline (μg g^{-1} $_{FW}$)
Control	3947.5 ± 9.86b	1098.1 ± 5.78c	0.3196 ± 0.02c
Hg	4796 ± 7.128a	1342.5 ± 4.56b	0.419 ± 0.01b
SNP+Hg	4865 ± 5.82a	1495.6 ± 8.46a	0.53 ± 0.02a
SNP	3990 ± 8.92b	1160.5 ± 6.11c	0.338 ± 0.03c

9.4%, 13.9%, and 19.5%, respectively. Interestingly, the addition of SNP + Hg significantly stimulated the activities of these enzymes by 9.5%, 20.6%, 31.9%, and 15.2%, respectively (Fig. 1).

The isoenzyme profile analysis, depicted in Fig. 2, revealed distinct bands for SOD (SOD 1–5), CAT (CAT 1–2), POX (POX 1–4), and GR (GR 1–2). Notably, exposure to Hg resulted in increased band densities for some isoenzymes of SOD, POD, and GR compared to the control. Furthermore, some isoenzymes appear thicker in maize treated with SNP + Hg, indicating a potential enhancement of the enzyme activities.

AsA, GSH, and proline contents

Exposure to Hg significantly elevated the contents of glutathione (GSH) by 21.5%, ascorbate (AsA) by 22.5%, and proline by 31.1% compared to control plants (Tab. 4).

Notably, SNP + Hg treatment also increased the contents of AsA (11.4%) and proline (26.4%), although GSH levels remained unchanged (Tab. 4). These findings indicate the participation of NO in augmenting non-enzymatic antioxidant defenses.

Some nutrient element contents

Exposure to toxic Hg increased sodium (Na) content by 29% compared to the control plants but reduced the content of manganese (Mn) and calcium (Ca) by 39.4% and 13.3%, respectively. The potassium (K) content, however, remained unchanged (Tab. 5). Intriguingly, treatment with NO increased Mn and K content by 7.3% and 8.7%, respectively, while reducing Na content by 26% in maize seedlings exposed to Hg compared to control plants (Tab. 5). SNP alone increased the amount of K, Ca, Na and Mn in maize seedlings compared to control plants. These findings highlight NO's potential in modulating essential element balances.

Discussion

Mercury (Hg), a heavy metal known for its high toxicity despite being non-essential, poses a global threat as it is readily absorbed by plants and accumulates across various plant tissues, jeopardizing crop yield and food safety (Wani et al. 2021). The imperative to counteract heavy metal-induced phytotoxicity has driven the search for effective, safe, and economically feasible solutions. Many studies have been carried out on plant signaling molecules that regulate antioxidant defense mechanisms in plants to increase their tolerance to stressful environments including heavy metals such as As, Cd, Al, Hg (Chen et al. 2015, Ahmad et al. 2021, Wani et al. 2021). Especially, previous studies have demonstrated the potential of salicylic acid and carbon monoxide to alleviate Hg toxicity (Zhou et al. 2007), while other research has unveiled the detoxification effects of nitric oxide (NO) on heavy metals like As, Cd, Al, and Ni (Singh et al. 2009, Saxena and Shekhawat 2013). The present study investigated the effects of NO treatment on maize seedlings exposed to Hg toxicity. The results revealed significant alterations in various physiological parameters, ROS levels, antioxidant enzyme activities, antioxidant molecules contents, chlorophyll content, and elemental composition.

The negative impact of toxic Hg on the growth of maize seedlings was evident from the reduction in fresh weight, dry weight, and shoot length. These findings are in agreement with earlier studies that reported the inhibitory effects of heavy metal stress on plant growth (Zhou et al. 2007, Hasanuzzaman et al. 2011). Furthermore, we observed that Hg toxicity in maize induced the generation of excess ROS such as H_2O_2 and $O_2^{\cdot-}$ along with an increase in lipid peroxidation and electrolyte leakage. These outcomes reflect the oxidative stress imposed by Hg on the seedlings, thus leading to cellular damage and inhibition of growth (Gao et

Tab. 5. Effects of SNP (as a donor of NO) and Hg on contents of elements of some nutrients of maize seedlings. Treatments: Control – half-strength Hoagland's solution, Hg – 100 μM $HgCl_2$, SNP + Hg – 0.1 μM SNP + 100 μM $HgCl_2$, SNP – 0.1 μM SNP. Values are means of three independent experiments ± standard errors. Different letters in the same column indicate statistically significant differences between means ($P < 0.05$). LOD – limit of detection, LOQ – limit of quantitation.

Treatments	mg kg^{-1} $_{dry\ weight}$			
	Mn	Na	K	Ca
Control	24.35 ± 0.15b	3.519 ± 0.05c	33351.53 ± 12.05c	872.85 ± 5.9b
Hg	14.75 ± 0.33d	4.543 ± 0.08b	33222 ± 12.54c	756.23 ± 8.5c
SNP+Hg	15.83 ± 0.12c	3.364 ± 0.03d	36127 ± 23.57b	751.21 ± 6.3c
SNP	26.04 ± 0.36a	4.710 ± 0.09a	38361.38 ± 31.12a	1026.3 ± 9.7a
LOD	0.00051	0.48	2.40	2.98
LOQ	0.01	5.00	5.00	5.00

al. 2010, Sahu et al. 2012, Chen et al. 2015). Similar findings have been reported in studies focusing on heavy metal-induced oxidative stress (Zhou et al. 2007, D'Souza Myrene and Devaraj 2013). The possible reason for increased ROS despite the observed increase in content of antioxidants (ascorbic acid, glutathione, and proline), could be the reduced activities of key antioxidant enzymes, including SOD, CAT, POX and GR, which we noticed after exposure to Hg. Prior studies have documented heavy metal-induced suppression of antioxidant enzymes (Chen et al. 2018).

The application of NO donor SNP to Hg-exposed maize seedlings led to a significant improvement in FW, DW, and SL. This finding aligns with previous research where exogenous NO application was shown to enhance plant growth and development under stress conditions (Kopyra and Gwóźdź 2003). Moreover, Chen et al. (2015) showed that excessive Hg-induced root growth inhibition and oxidative stress in rice plants can be effectively mitigated through application of NO released from sodium nitroprusside (SNP). NO at low concentrations is a second messenger in plants. It prompts the plant response system into an unfavorable situation. Previous research has demonstrated the antioxidant properties of NO and its ability to attenuate oxidative stress (Kopyra and Gwóźdź 2003, Singh et al. 2009, Kazemi et al. 2010, Cui et al. 2010). In the present study, the application of NO significantly reduced the levels of H_2O_2 and $O_2^{\cdot-}$ in SNP + Hg-treated seedlings, confirming its role in ROS scavenging. Additionally, NO treatment attenuated MDA content and EL level, signifying its protective effect against Hg-induced lipid peroxidation and membrane damage and resulting in improved growth. Remarkably, SNP treatment provided enhanced antioxidant enzyme activities. Isozyme analysis revealed alterations in the density and thickness of bands under Hg stress and SNP treatment. This might signify changes in enzyme isoforms or post-translational modifications in response to NO treatment, indicating a potential regulatory role of NO in the antioxidant defense system (Ahmad et al. 2021). Moreover, SNP treatment further enhanced the contents of AsA and proline indicating its involvement in strengthening the adaptive mechanisms that can counteract Hg-induced oxidative stress as has been already reported in plants under As and Cd toxicity (Hsu and Kao 2004). These observations are in line with the results of Ahmad et al. (2021) showing that the correlation between SNP application and the mitigation of Hg toxicity in soybean cultivars is reinforced by an enhanced antioxidant response and improved AsA – GSH cycle (Ahmad et al. 2021). It is also possible that NO may have acted as an antioxidant molecule as NO produces peroxynitrite, less harmful oxidants, by reacting with $O_2^{\cdot-}$ (Saxena and Shekhawat 2013, Chen et al. 2015).

One of the most used methods to understand the effects of abiotic stress factors on plants is to determine chlorophyll (Chl) content (Kupper et al. 1996). Hg exposure led to a reduction in chlorophyll content in maize seedlings, which is consistent with previous reports of heavy metal-induced chlorophyll degradation (Cho and Park 2000, Amooaghaie and Enteshari 2017). Specifically, Chl-a was more adversely affected than Chl-b. This reduction in chlorophyll content indicates impaired photosynthetic capacity, which is crucial for plant growth and development. Strikingly, the addition of NO through SNP treatment reversed this trend, resulting in a significant increase in Chl-a, Chl-b, and total chlorophyll content in SNP + Hg-treated plants. These findings suggest that NO treatment can mitigate Hg-induced chlorophyll degradation, potentially by maintaining chlorophyll synthesis or preventing its breakdown.

The disbalance in plant nutrients may disrupt ion homeostasis and interfere with various metabolic processes. The altered elemental composition under Hg stress, including increased sodium (Na) and reduced manganese (Mn) and calcium (Ca) contents, corroborates with established findings on heavy metal-induced disruptions in mineral uptake (Fang et al. 2019). NO treatment led to a rebalancing effect by decreasing Na and enhancing Mn and potassium (K) contents. Nutrient elements such as Mn, K and Ca have an important role as cofactors in cells and it is essential that under stress conditions the cytosol maintain a high K^+/Na^+ ratio for optimal metabolic functions. NO may protect the maize seedlings against toxic Hg by increasing the K^+/Na^+ ratio as increase of Na^+ in cells disrupts the function of many proteins. This suggests a potential role of NO in modulating ion uptake, transport mechanisms and ion homeostasis, a critical factor in stress tolerance (García-Mata and Lamattina 2013).

In conclusion, the findings of this study highlight the protective role of exogenous NO (SNP) against mercury (Hg) toxicity in maize seedlings. The observed improvements in physiological parameters, attenuation of oxidative stress, and restoration of antioxidant enzyme activities, modulation of non-enzymatic antioxidants, chlorophyll preservation, and essential element balance suggest NO as a valuable candidate for enhancing plant resilience against heavy metal toxicity.

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Effects of artificial sweeteners on antioxidant enzymes and physiological parameters in *Triticum aestivum* (Poaceae)

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Abstract – Due to increased consumption, artificial sweeteners are often present in the environment but their effects on plants are largly unknown. In this research, the effects of four artificial sweeteners on plant stress markers in *Triticum aestivum* L. were investigated. Wheat seedlings were grown from seeds in soil containing artificial sweeteners (saccharin, sodium cyclamate, sucralose, aspartame) in different concentrations (0, 25, 50, 100 mg kg^{-1}). Plants were irrigated at regular intervals to maintain field capacity moisture and harvested after 15 days of growth. Electrolyte leakage, chlorophyll and carotenoid content, and antioxidant enzyme (superoxide dismutase, peroxidase, catalase) activities were determined in harvested leaves. Comparisons between control samples and test samples were statistically evaluated at a 95% confidence interval to determine significant differences. Overall, significant increases in chlorophyll and carotenoid content, and some antioxidant enzyme activities were observed in wheat plants exposed to artificial sweeteners in the soil. A significant increase in electrolyte leakage was observed with saccharin and aspartame treatment, indicating that these sweeteners can cause membrane damage in wheat. Chlorophyll *a* and POX activity were the most sensitive stress parameters in wheat. This study showed the importance of evaluating the potential impact of anthropogenic pollutants that may be present in treated wastewater and consequently affect plants.

Keywords: artificial sweeteners, wheat, electrolyte leakage, catalase, peroxidase, superoxide dismutase

Introduction

Today, artificial sweeteners are produced and consumed for a wide variety of purposes (Li et al. 2021a) in the food, cosmetic, and pharmaceutical industries (Stolte et al. 2013). These sweeteners are substitutes for sugar in terms of flavor and serve as alternative food additives since they provide the desired taste in very small amounts and without calories (Chattopadhyay et al. 2014). Artificial sweeteners have been frequently observed in the environment in recent years because of their high consumption, persistence in the environment, and solubility in water (Lange et al. 2012, Li et al. 2020a). Despite their widespread use, the potential risks of artificial sweeteners to the environment and human health are still unclear (Li et al. 2021b). They are classified as anthropogenic trace pollutants with high concentrations in groundwater, surface waters, and drinking water (Lange et al. 2012). The main source of artificial sweeteners released into surface waters is wastewater treatment plants. Artificial sweeteners like many other chemicals cannot be effectively removed by conventional wastewater treatment and are often part of wastewater discharges to surface water (Li et al. 2021b).

Plants, stable organisms in ever-changing environmental conditions, are inevitably exposed to various biotic and abiotic stress factors. These stress factors negatively affect both quality and crop yield. The underlying reason is oxidative damage caused by increased accumulation of reactive oxygen species (ROS) (Miller et al. 2010). Under optimal physiological conditions, reactive oxygen species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide (O_2^-), and hydroxyl radical (OH^-) are frequently produced as by-products of aerobic metabolism. The increased accumulation of ROS in stressed plants is due to the disturbed balance between ROS production and their removal (Mittler et al. 2004). Increased levels of ROS produced under stress

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can cause the oxidation of lipids, proteins, and nucleic acids; these life-threatening reactions activate plant stress responses and defence pathways. The environmental impacts depend on the delicate balance between ROS generation and ROS scavenging, which can be influenced by the intensity of stress and stress duration (Sharma and Dietz 2009). In plants, prevention or clearance of stress-induced ROS accumulation occurs with the active roles of molecules such as enzymatic and non-enzymatic soluble sugars (Miller et al. 2010). Abiotic stress is estimated to be the main cause of excessive crop loss, which is 50% worldwide (Mittler 2002, Gill and Tuteja 2010). Therefore, it is important to understand the responses to stress of ROS signaling pathways and their interaction(s). Previous studies on the impacts of pharmaceutical and personal care products on plants (Osma et al. 2018, Turkoglu et al. 2019, Elveren and Osma 2022) led to the hypothesis that artificial sweeteners might also negatively impact plants and induce changes in plant stress markers such as antioxidant enzymes.

The artificial sweeteners evaluated in this study (saccharin, sodium cyclamate, sucralose, aspartame) are some of the most widely consumed commercial products. Sucralose is a calorie-free high-intensity artificial sweetener that is widely used in a variety of foods and beverages worldwild (Li et al. 2020b). Saccharin is approximately 300 times sweeter than sucralose and is especially found in carbonated beverages in Türkiye (Bayındır Gümüş et al. 2022). Aspartame has low calorie content; hence it is a non-nutritive sweetener (More et al. 2021). Today with an annual production of 3000–5000 metric tons, aspartame is one the world's most widely used artificial sweeteners. It is an ingredient in more than 5000 food and beverage products including cereals, chewing gum, pharmaceuticals, and instant coffee (Landrigan and Straif 2021). Sodium cyclamate is utilised as non-nutritive sweetener. Its equivalent calcium salt is used particularly in low sodium diets. Cyclamate is 30 times sweeter than sucrose. Owing to its heat-stable properties, it is suitable for cooking and baking (More et al. 2021).

These sweeteners can reach the soil (including agricultural areas) in a variety of ways: spreading fertilizer and sewage sludge and using treated wastewater for irrigation (water recycling). Subsequently, they enter the food chain through vegetation consumption or impact plant physiology in a negative manner. In this study, the effects of artificial sweeteners applied to soil at different concentrations were evaluated by monitoring electrolyte leakage, antioxidant enzyme activities, and chlorophyll and carotenoid content in bread wheat plants. Bread wheat (*Triticum aestivum* L.) is one of the most important plants representing more than half of the calories consumed in human nutrition and approximately 50% of the protein (Aydogan and Yagdı 2021). It is grown throughout the world making it an ideal plant for evaluating the potential impact of anthropogenic pollutants that may be present in treated wastewater.

Materials and methods

Plant material and treatments

Seeds of the licensed wheat variety "Esperia" were supplied from the Agricultural Credit Cooperatives of Türkiye. The artificial sweeteners employed, sucralose ($C_{12}H_{22}O_{11}$), saccharin ($C_7H_5NO_3S$), aspartame ($C_{14}H_{18}N_2O_5$) and sodium cyclamate ($C_6H_{11}NHSO_3Na$) were purchased from Sigma Company.

Different concentrations (0, 25, 50, 100 mg kg^{-1}) of artificial sweeteners were mixed as powder into the soil mixed with 650 g of animal manure and then 7 g of wheat seeds were sown on it and covered with 100 g of soil. There were 3 pots with seeds for each of sweetener/concentration combination and 250 mL of water was added. Wheat grown without the addition of artificial sweetener served as the control group. After the seeds germinated, the wheat seedlings were irrigated at regular intervals to maintain field capacity moisture. Plants were grown in a laboratory at 5–10 °C temperature and under 60% humidity. Following germination, the ambient temperature was set at 10–15 °C. At the end of the 15th day, the wheat leaves were harvested and some of them were used fresh to determine the amount of electrolyte leakage, while others were frozen for subsequent physiological and biochemical studies that served as markers of plant stress. For each parameter 10 replicates were prepared.

Determination of electrolyte leakage in leaf samples

The determination of electrolyte leakage was conducted on 0.1 g of fresh leaves in test tubes. 4 mL of distilled water was added to each test tube and incubated at 4 °C for 24 h. The amount of ions present in water was determined using an electrical conductivity meter (Elveren and Osma 2022).

Determination of antioxidant enzyme activity

Leaves (0.5 g) were weighed and placed in a porcelain mortar. 5 mL of cold homogenate buffer (0.1 M KH_2PO_4, pH 7.0 containing 1% polyvinylpyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA)) were added, and the mixture was transferred to a centrifuge tube; the mixture was centrifuged (15000 x *g*, 4 °C) for 15 minutes. The supernatant ("enzyme extract") was used as a source for the activity measurements of antioxidant enzymes as described below. The chemicals and procedures used to measure the activities of each antioxidant enzyme followed those of Turkoglu et al. (2019).

Peroxidase (POX) activity determination was based on monitoring the absorbance increase at 470 nm caused by a colored product of the reaction in which guaiacol and H_2O_2 are substrates. 10 µL of enzyme extract was added to a spectrophotometer cuvette containing 3 mL of the substrate solution (100 mL of 0.1 M NaH_2PO_4, pH 5.5 with the addition of 5 mM guaiacol). The increase in absorbance was recorded at 1-minute intervals for 5 minutes. The amount of enzyme that increased the absorbance by 0.01 units in 1 minute at 25 °C was accepted as 1 enzyme unit, and the results were

presented as enzyme unit per g of fresh tissue (EU g^{-1}_{FW}) (Osma et al. 2018).

The method developed by Havir and McHale (1987) was used for the determination of catalase (CAT) activity. This method is based on monitoring the absorbance decrease at 240 nm following the addition of the plant extract to phosphate buffer containing H_2O_2. Absorbance against the blank was read at 1-minute intervals for 3 minutes. The decrease in absorbance per minute was calculated from the interval in which the absorbance decreased linearly. The amount of enzyme that reduced the absorbance of 1 mole H_2O_2 in 1 minute at 25 °C was accepted as 1 enzyme unit, and the results were presented as enzyme unit per g of fresh tissue (EU g^{-1}_{FW}) (Osma et al. 2018).

Superoxide dismutase (SOD) activity was based on the spectrophotometric determination of the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture (3 mL) contained 50 mM KH_2PO_4, pH 7.8 with the addition of 13 mM methionine, 75 M NBT, 2 M riboflavin, and 0.1 mM EDTA. For the activity measurement, 2.84 mL of the reaction mixture without riboflavin was added to a 3-mL spectrophotometer cuvette, then 100 µL of enzyme extract was added. The reaction was initiated by pipetting 60 µL of a 100 M riboflavin solution into the tube and placing it in front of a white light source immediately after mixing. The tube is kept in front of the light source for 15 minutes; the reaction was stopped by turning off the light source. The absorbance of NBT was read against the blank at 560 nm. The blank consisted of an enzyme-free sample from the same process as above. The amount of enzyme causing 50% inhibition of NBT reduction was accepted as 1 enzyme unit (EU), and the values were presented as EU g^{-1}_{FW} (Turkoglu et al. 2019).

Determination of photosynthetic pigments

Leaves (0.5 g) were homogenized in 5 mL of 80% acetone. The homogenate was filtered using filter paper and then brought to 10 mL volume with acetone. The homogenate was centrifuged for 10 minutes, the supernatant was collected, and absorbance at 663, 646, and 440 nm was recorded. The following formulas were used to calculate chlorophyll and carotenoid (Porra et al. 1989):

$$\text{Chlorophyll } a \text{ (mg mL}^{-1}) = 12.25 \times A_{663} - 2.55 \times A_{646}$$

$$\text{Chlorophyll } b \text{ (mg mL}^{-1}) = 20.31 \times A_{646} - 4.91 \times A_{663}$$

$$\text{Chlorophyll } a + \text{chlorophyll } b \text{ (mg mL}^{-1}) = 17.76 \times A_{646} + 7.34 \times A_{663}$$

$$\text{Carotenoids (mg mL}^{-1}) = 4.69 \times A_{440} - 0.267 \times (\text{chlorophyll } a + \text{chlorophyll } b)$$

Statistical analysis

Analysis of variance (ANOVA) in SPSS was used to determine the significance of treatment effects relative to control (untreated) plants for each individual artificial sweetener. Post hoc tests (Tukey-Kramer multiple comparisons) were performed when significant treatment effects were observed ($P \leq 0.05$).

Results

In this study, four artificial sweeteners (at different concentrations) were applied to soil in which wheat seeds were germinated and grown. The presence of these artificial sweeteners was found to adversely impact the plant stress markers we evaluated in comparison to wheat plants grown in the absence of these artificial sweeteners. In general, the plant biomarkers did not follow a typical monotonic dose-response pattern i.e. incremental increases in concentration did not always produce only incremental increases or only incremental decreases in plant stress response.

Electrolyte leakage

Electrolyte leakage in plants significantly increased with increasing soil concentrations of saccharin and aspartame; the effect was especially true for plants exposed to saccharin (Fig. 1). This indicates that the presence of saccharin or as-

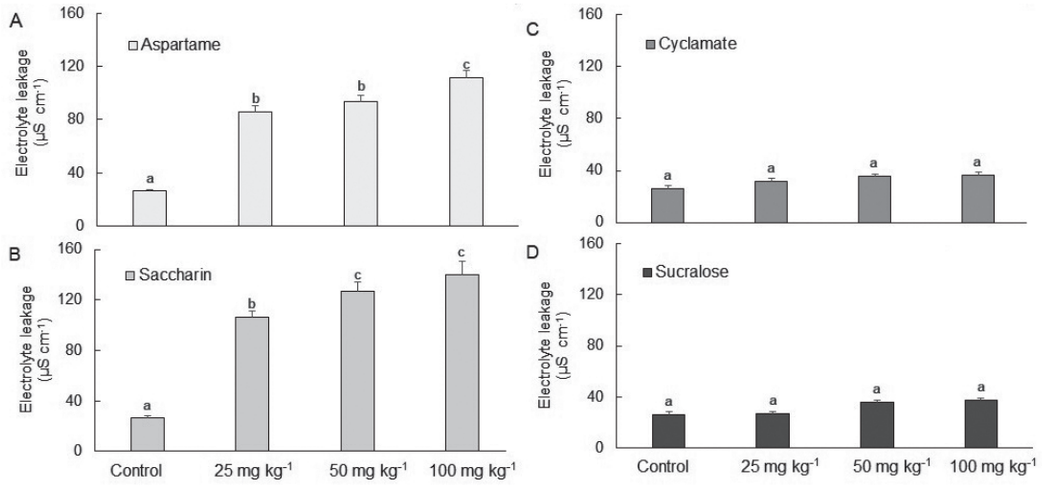

Fig. 1. Electrolyte leakage in wheat *(Ttriticum aestivum* L.) exposed to different concentrations of artificial sweeteners in soil for 15 days. A – sucralose, B – saccharin, C – cyclamate, D – aspartame. Results represent mean ± standard error, n = 10. Bars with different letters are statistically different within a sweetener treatment.

partame in soil produces a general stress response by compromising cell membranes which could negatively impact plant growth.

Sucralose and sodium cyclamate at soil concentrations as high as 100 mg kg^{-1} had no impact on electrolyte leakage compared to control (untreated) plants, suggesting that these sweeteners did not compromise plant cell membranes during the 15 days study.

Photosynthetic pigments

Significant changes in plant pigments in wheat following exposure to all artificial sweeteners were observed (Tab. 1).

The most dramatic changes in plant pigments occurred in the aspartame treatment (Tab. 1). Depending on the sweetener type and the pigment, changes mostly followed a monotonic pattern. In other words, as artificial sweetener concentration in soil increased, plant pigment concentrations increased compared to control plants. Chlorophyll *a* concentrations mostly increased after treatments with all artificial sweeteners.

For aspartame in soil, concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids increased already at the low soil concentration, but did not further increased at higher soil concentrations (Tab. 1).

For saccharin, the increase in concentrations of chlorophyll *a* and chlorophyll *b* was sighnificantly higher at lower than at higher concentrations (non-monotonic increase) (Tab. 1).

For sucralose, a non-monotonic increase of chlorophyll *a* content and a monotonic increase of carotenoids was noticed, while chlorophyll *b* content showed a monotonic decrease (Tab. 1).

For cyclamate, chlorophyll *a* content showed a monotonic increase, chlorophyll *b* a non-monotonic increase while carotenoids initially increased at the lowest concentration followed by no change at the higher concentrations (Tab. 1).

Antioxidant enzymes

Three of the four artificial sweeteners significantly increased CAT activity in wheat plants compared to control (untreated) plant. It was mostly a monotonic increase, although for aspartame the increase was significant already at the lowest concentration while for sucrose only at the highest concentration applied.

All four artificial sweeteners tested significantly increased POX activity in wheat plants compared to control (untreated) plants (Tab. 2). For aspartame a monotonic increase but for saccharine and cyclamate a non-monotonic increase in POX activity was observed. For sucralose, the increase in POX activity was observed only at the lowest (25 mg kg^{-1}) and the highest (100 mg kg^{-1}) concentrations.

Three of the four artificial sweeteners significantly changed SOD activity in wheat plants relative to control plants (Tab. 2). For cyclamate, a monotonic increase in SOD activity with increasing concentration of sweeteners was noticed. For aspartame, a significant increase was observed only at 50 mg kg^{-1} concentration while for sucralose only the highest concentration caused an increase. For saccharin, a decrease in SOD activity was observed at the lowest (25 mg kg^{-1}) concentration; while 50 mg kg^{-1} and 100 mg kg^{-1} concentrations did not significantly affect SOD activity compared to control plants.

Tab. 1. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, and carotenoids (mean ± standard error, n = 10) in wheat grown in soil containing artificial sweeteners for 15 days. For values with a different superscript letter within a treatment, the difference between the treatment means and control was statistically significant (ANOVA, $P \leq 0.05$).

Treatment	Chlorophyll *a* (mg mL^{-1})	Chlorophyll *b* (mg mL^{-1})	Chlorophyll *a*+*b* (mg mL^{-1})	Carotenoids (mg mL^{-1})
Control	5.55 ± 0.05^{a}	2.94 ± 0.12^{a}	8.54 ± 0.18^{a}	0.82 ± 0.08^{a}
Aspartame, 25 mg kg^{-1}	13.55 ± 0.08^{b}	5.28 ± 0.16^{b}	19.59 ± 0.43^{b}	2.26 ± 0.02^{b}
Aspartame, 50 mg kg^{-1}	13.05 ± 0.60^{b}	5.17 ± 0.25^{b}	17.62 ± 0.39^{c}	2.25 ± 0.07^{b}
Aspartame, 100 mg kg^{-1}	13.63 ± 010^{b}	5.28 ± 0.16^{b}	18.96 ± 0.25^{b}	2.21 ± 0.16^{b}
Saccharin, 25 mg kg^{-1}	12.66 ± 0.10^{b}	5.69 ± 0.18^{b}	18.25 ± 0.25^{b}	2.28 ± 0.05^{b}
Saccharin, 50 mg kg^{-1}	10.81 ± 0.17^{c}	4.30 ± 0.15^{c}	15.03 ± 0.31^{c}	2.07 ± 0.04^{c}
Saccharin, 100 mg kg^{-1}	9.17 ± 0.10^{d}	3.55 ± 0.07^{d}	12.64 ± 0.13^{d}	1.94 ± 0.43^{b,c}
Sucralose, 25 mg kg^{-1}	6.49 ± 0.09^{b}	3.0 ± 0.14^{a}	9.57 ± 0.27^{b}	1.25 ± 0.05^{b}
Sucralose, 50 mg kg^{-1}	6.14 ± 0.09^{c}	2.97 ± 0.07^{a}	8.86 ± 0.15^{c}	1.20 ± 0.03^{b}
Sucralose, 100 mg kg^{-1}	6.28 ± 0.02^{c}	2.52 ± 0.14^{b}	8.62 ± 0.05^{a}	1.41 ± 0.30^{b}
Cyclamate, 25 mg kg^{-1}	5.17 ± 0.06^{a}	2.53 ± 0.12^{b}	7.94 ± 0.31^{b}	1.16 ± 0.03^{b}
Cyclamate, 50 mg kg^{-1}	5.22 ± 0.30^{a}	2.34 ± 0.13^{b}	7.09 ± 0.23^{b}	0.91 ± 0.02^{a}
Cyclamate, 100 mg kg^{-1}	6.54 ± 0.07^{b}	3.39 ± 0.13^{c}	9.84 ± 0.17^{c}	0.86 ± 0.02^{a}

Tab. 2. Antioxidant enzyme (catalase – CAT, peroxidase – POX, superoxide dismutase – SOD) activity (mean ± standard error, n = 10) in wheat grown in soil containing artificial sweeteners for 15 days. For values with a different superscript letter within a treatment, the difference between the treatment means and control was statistically significant (ANOVA, $P \leq 0.05$).

Treatment	Catalase activity (EU g^{-1} FW)	POX activity (EU g^{-1} FW)	SOD activity (EU g^{-1} FW)
Control	3650 ± 65^{a}	99742 ± 1982^{a}	624 ± 17^{a}
Aspartame, 25 mg kg^{-1}	4221 ± 247^{b}	131830 ± 1921^{b}	645 ± 10^{a}
Aspartame, 50 mg kg^{-1}	4383 ± 96^{b}	134496 ± 1893^{b}	685 ± 4^{b}
Aspartame, 100 mg kg^{-1}	4878 ± 257^{c}	136736 ± 5524^{b}	649 ± 10^{a}
Saccharin, 25 mg kg^{-1}	3903 ± 127^{a}	129680 ± 3750^{b}	571 ± 9^{b}
Saccharin, 50 mg kg^{-1}	5054 ± 153^{b}	129920 ± 1698^{b}	626 ± 6^{a}
Saccharin, 100 mg kg^{-1}	5025 ± 121^{b}	124418 ± 1189^{b}	628 ± 8^{a}
Sucralose, 25 mg kg^{-1}	3864 ± 145^{a}	115920 ± 2553^{b}	644 ± 8^{a}
Sucralose, 50 mg kg^{-1}	4292 ± 209^{a}	103547 ± 1681^{a}	645 ± 6^{a}
Sucralose, 100 mg kg^{-1}	4691 ± 95^{b}	118920 ± 1697^{b}	653 ± 7^{b}
Cyclamate, 25 mg kg^{-1}	3688 ± 126^{a}	139573 ± 3408^{b}	650 ± 7^{b}
Cyclamate, 50 mg kg^{-1}	3690 ± 48^{a}	119804 ± 2498^{c}	657 ± 6^{b}
Cyclamate, 100 mg kg^{-1}	3715 ± 65^{a}	120667 ± 2770^{c}	676 ± 5^{c}

Discussion

Only a few studies on the effects of artificial sweeteners on plants have been reported. Kobetičová et al. (2016) evaluated the effect of two artificial sweeteners (aspartame and saccharin) on aquatic (duckweed, *Lemna minor*; algae, *Desmodesmus subspicatus*) and terrestrial (white mustard, *Sinapis alba*; lettuce, *Lactuca sativa)* plants. Aspartame had a negative impact on duckweed growth. Unlike aspartame, saccharin had no negative effects on duckweed and the production of chlorophyll increased. It was suggested that it could be a stress response as enhanced photosynthesis could be related to the maintenance of the metabolism under stress (Kobetičová et al. 2016). In this study, both sweeteners (aspartame and saccharin) increased chlorophyll content in plants relative to controls, although saccharin treatment increased chlorophyll *a* and chlorophyll *b* content more dramatically at the lowest soil concentrations which could be consistent with plant response to stress.

Kobetičová et al. (2018) also evaluated potential growth effects of four artificial sweeteners (aspartame, saccharin, sucralose, and acesulfame K) and a natural sweetener (stevioside) on duckweed (*Lemna minor*). They determined that aspartame and sucralose inhibited growth parameters (frond number and frond area), but did not affect chlorophyll concentrations. In this study, aspartame as well as saccharin increased the photosynthetic pigments in all concentrations investigated while sucralose increased chlorophyll *a* and carotenoids but decreased chlorophyll *b* at the highest soil concentration. Amy-Sagers et al. (2017) examined the effect of sucralose on *Lemna minor* and determined that the plant used sucralose instead of sugar to increase photosynthetic capacity and green leaf area. They determined that duckweed utilizes sucralose in metabolism as a carbon source.

Electrolyte leakage in plants significantly increased with increasing concentrations of saccharin and aspartame in the soil, indicating that these two sweeteners are more toxic to wheat than sucralose and cyclamate, causing oxidative damage of membranes. This could be also related to the observed increase in photosynthetic pigments especially carotenoids which could serve as antioxidants in an effort to protect the plant from stress (Mittler 2002, Gill and Tuteja 2010). It could be hypothesized that aspartame and saccharin may cause ion escape by increasing the permeability of the cell membrane more than other sweeteners. However, observed electrolyte leakage was not consistently related to antioxidant enzyme activities. Saccharin and aspartame caused increases in CAT activity and POX activity, probably as a result of increased H_2O_2 as the role of these enzymes is to scavenge H_2O_2. However, the activity of SOD, enzyme that catalyzes the dismutation of superoxide anion to H_2O_2 was not affected in a consistent manner. Sucralose and cyclamate at soil concentrations as high as 100 mg kg^{-1} had no impact on electrolyte leakage. However, sucralose increased CAT activity, while cyclamate caused an increase in SOD and POX activity indicating that they can induce some change in plant metabolism which is reflected in the activity of antioxidative enzymes.

Chlorophyll *a* content and POX activity proved to be sensitive parameters that could be used as stress markers in wheat. It is consistent with studies investigating the effect of active ingredients of drugs and personal care products on wheat (Turkoglu et al. 2019, Elveren and Osma 2022). SOD activity was the least sensitive plant stress marker in this study. It is possible that some sweeteners can react with superoxide radicals, causing a decrease in the amount of superoxide in the environment and a subsequent decrease in SOD activity.

Generally, the plant biomarkers did not follow a typical monotonic dose-response pattern. This pattern is consistent with previous observations on the impacts of pharmaceuticals and perosonal care products to wheat plants (Osma et al. 2018, Turkoglu et al. 2019, Elveren and Osma 2022).

Conclusions

The artificial sweeteners present in the soil could exert significant changes in plant metabolism. All four artificial sweeteners (saccharin, sodium cyclamate, sucralose, aspartame) inreased the concentration of chlorophyll *a* and POX activity, altough not always in a consistent monotonic manner. Saccharin and aspartame in the soil were more toxic to wheat plants inducing oxidative damage to membrane and activating antioxidative enzymes CAT and POX, thus suggesting that oxidative stress might be a mechanism which explains their toxicity.

Individual photosynthetic pigments or oxidative stress markers can be useful for evaluating the impact of different chemicals in the environment on plants provided they have adequate sensitivity to the stress. However, it is always more appropriate to use several markers, as different chemicals can impact plants differently.

The ecotoxicological effects of artificial sweeteners remain to be determined; the possible physiological, biochemical, and genetic changes that they may produce in plants should be further investigated.

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Short communication

Diversity of fungal endophytes isolated from the invasive plant *Solanum rostratum*

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Abstract – A culture-dependent method was used to isolate fungal endophytes from the leaves, stems, and roots of the invasive plant *Solanum rostratum* Dunal. growing in Xinjiang Province, China. All isolates were identified according to ITS (internal transcribed spacer) region of ribosomal DNA sequences and analyzed by Nucleotide BLAST according to NCBI GenBank and Mycobank database. Altogether 176 endophytic fungal isolates corresponding to 44 OTUs were identified, which were classified into 12 genera, with *Penicillium* (59.66%) and *Aspergillus* (23.29%) being the highly dominant genera. Ten endophytic isolates (OTU1, OTU15, OTU16, OTU21, OTU23, OTU25, OTU26, OTU30, OTU37 and OTU44) were identified as potential new species.

Keywords: Culture-dependent endophyte isolation, endophytes, *Penicillium*, *Mucor circinelloides*

Introduction

Solanum rostratum Dunal. is an annual weed with a strong capacity for propagation and adaptation. A notorious invader, it also serves as the primary host of the potato leaf-roll virus and *Leptinotarsa decemlineata* (potato beetles), which pose substantial threats to biodiversity and the environment in China (Zhao et al. 2013, Liu et al. 2020). Additionally, *S. rostratum* contains abundant amounts of secondary metabolites, primarily flavonoids, alkaloids, steroids, and other compounds (Liu et al. 2020).

Endophytic fungi live in plants for all or part of their lives without harming the host (Ripa et al. 2019). They may affect a plant's ability to reproduce, grow, or resist abiotic stress or natural enemies (Rho et al. 2018). To the best of our knowledge, previous papers largely concentrated on the biological traits and phytochemical profile of *S. rostratum* and there is no report about the diversity of endophytic fungi of this invasive plant. The main goal of this study is to explore the community of the fungal endophytes of *S. rostratum*. Identification of the endophytes may help explain the invasive success of *S. rostratum* from the perspective of plant-microbe interaction; these endophytes are also potentially valuable resources of bioactive substances that have various biological activities.

Materials and methods

Forty-five mature *S. rostratum* plants at flowering stage were collected on June 28, 2018 from 3 different locations (15 plants from each location) in Urumqi and Changji city of Xinjiang province: location 1: 43° 55'60'' N, 87° 20'41'' E (Loc-1); location 2: 43° 56'0'' N, 87° 20'41'' E (Loc-2); location 3: 43° 46'14'' N, 87° 46'31'' E (Loc-3). Endophytes were isolated using a culture-dependent method within two days of collection of plants. Surface sterilization of plant parts (roots, stems, and leaves) and isolation of the endophytes were conducted following the protocol of Schulz et al. (1993). Colonization rate (CR) was counted by following Petrini et al. (1982).

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DNA of the endophytic isolates was extracted by using the DNA Extraction Kit for fungi (Solarbio Life Sciences, Beijing, China), according to the manufacturer's instructions (Abd-Elsalam et al. 2003). PCR amplification of the rDNA ITS (internal transcribe spacer) region was conducted with the use of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') fungal primer pairs (White et al. 1990). The sequences of the fragments were identified using the basic local alignment search tool BLAST (http://www.ncbi.nlm.nih.gov/) of the NCBI and Mycobank (https://www.mycobank.org) databases. Fungal identities were generated by sequence alignment analysis with those previously submitted to GenBank.

Results and discussion

In total, 176 endophytic fungal isolates corresponding to 44 OTUs (operational taxonomic units) were isolated and were classified into 12 genera (Tab. 1). Among the isolates, 55 (31.25%) were obtained from leaves, 55 from stems (31.25%), and 66 from roots (37.50%); 34 out of 44 OTUs had between 97.14% and 100.00% sequence similarity with relevant entries in Mycobank and GenBank databases, whereas OTU1, OTU15, OTU16, OTU21, OTU23, OTU25, OTU26, OTU30, OTU37 and OTU44 had between 70.13% and 95.87% sequence similarity with species belonging to the genera *Aspergillus*, *Penicillium*, *Microascus*, *Purpureocillium* and *Mucor* (Tab. 1), indicating they might be potential new species. *Penicillium* and *Aspergillus* were the dominant genera of the endophytic fungal community (Cheng et al. 2018). OTU23 (closest hit *Purpureocillium lilacinum* CBS 284.36) was chosen for further study on its secondary metabolites due to its high plant growth regulatory activity, which resulted in the isolation and identification of 3 bioactive compounds, i.e., adenosine, cerevisterol, and thymine, which were found to possess significant plant growth regulatory activity (Kuchkarova et al. 2020).

The percentage of endophytic isolates belonging to *Penicillium* (59.66%; 105/176) was much higher than those identified as *Aspergillus* (23.29%; 41/176), *Purpureocillium* (6.25%; 11/176), *Emericella* (3.41%; 6/176), *Fusarium* (2.27%; 4/176), *Paecilomyces* (1.14%; 2/176), *Geotrichum* (1.14%; 2/176) as well as *Altenaria*, *Microascus*, *Mucor, Pichia* and *Talaromyces,* which were detected only sporadically (< 1%). The CR of the roots of the plant was higher (43.33%) than that of the stems (30.00%) and leaves (26.67%) of the identical plants. Furthermore, the CR of fungal endophytes of plants acquired from Loc-1 was much higher than that from Loc-2 and Loc-3.

To the best of our knowledge, this is the first report on the diversity of the endophytic fungi isolated from the invasive plant *S. rostratum*. This study demonstrated the comparatively high multiplicity of the endophytic fungi of *S. rostratum* from three locations in Xinjiang. Our work revealed that the invasive plant *S. rostratum* harbours a variety of fungal endophytes in its leaves, stems, and roots. Given the fact that endophytes are able to produce biologically

Tab. 1. List of identified endophytic fungi isolated from *Solanum rostratum* plant parts. [a]BLASTN max score; [b]level of identification for pairwise alignments by calculating using the Martinez-Needleman-Wunsch algorithm; [c]level of similarity for pairwise alignments with the closest match, using the NCBI and Mycobank database; Accession number of the closest database match; [d]accession number of the closest database match. OUT - operational taxonomic unit.

OTU	Accession no.	Best Blast hit					Number of isolates						
		Closest taxa match	Score[a]	Query coverage (%)[b]	Ident (%)[c]	Accession no[d]	By tissue type			By location			Total observed
							Leaf	Stem	Root	1	2	3	
1	ON149677	*Aspergillus lentulus*	556	94	85.21	PWQ2395	0	0	1	1	0	0	1
2	ON149678	*Penicillium oxalicum*	843	95	100.00	FMR 14261	6	4	18	18	4	6	28
3	ON149679	*Pichia kudriavzevii*	711	96	99.36	CNRMA6.98	1	0	0	1	0	0	1
4	ON149680	*Aspergillus quadrilineatus*	786	92	99.40	IHEM 22705	1	1	0	1	0	1	2
5	ON149681	*Aspergillus rugulosus*	762	92	99.20	UOA/HCPF 10020	2	0	3	5	0	0	5
6	ON149682	*Emericella nidulans*	775	94	98.22	WM 06.100	4	2	0	6	0	0	6
7	ON149683	*Aspergillus creber*	637	94	97.42	FMR 14364	1	0	0	1	0	0	1
8	ON149684	*Fusarium verticillioides*	775	94	100.00	IHEM 9835	0	1	0	1	0	0	1
9	ON149708	*Penicillium citrinum*	795	96	99.60	NRRL 1841	0	0	2	1	1	0	2
10	ON149685	*Aspergillus niger*	857	94	100.00	WM 10.76	7	0	1	7	0	1	8
11	ON149686	*Aspergillus nidulans*	805	95	99.42	WM 11.60	0	0	2	1	1	0	2
12	ON149687	*Penicillium brasilianum*	843	94	100.00	FMR 14296	0	1	0	1	0	0	1

Tab. 1. Continued.

OTU	Accession no.	Best Blast hit					Number of isolates						
		Closest taxa match	Score[a]	Query coverage (%)[b]	Ident (%)[c]	Accession no[d]	By tissue type			By location			Total observed
							Leaf	Stem	Root	1	2	3	
13	ON149688	*Aspergillus oryzae*	852	95	99.81	WM 10.120	0	0	1	1	0	0	1
14	ON149689	*Aspergillus tubingensis*	848	94	100.00	IHEM 17440	3	3	2	4	0	4	8
15	ON149690	*Penicillium rolfsii*	695	95	95.10	FMR 14307	0	0	1	1	0	0	1
16	OM698374	*Microascus cirrosus*	166	62	70.13	FMR 12256	0	1	0	1	0	0	1
17	ON149691	*Penicillium chrysogenum*	835	94	100.00	FMR 14008	14	19	15	5	15	28	48
18	ON149692	*Fusarium pseudonygami*	735	89	100.00	U34563	0	0	1	1	0	0	1
19	ON149693	*Fusarium oxysporum*	732	93	98.77	UOA/HCPF AB82	0	0	1	1	0	0	1
20	ON149694	*Aspergillus terreus*	848	92	100.00	WM 03.218	0	0	2	2	0	0	2
21	ON149695	*Aspergillus calidoustus*	667	95	95.87	UOA/HCPF 9236	1	0	0	1	0	0	1
22	ON149696	*Aspergillus fumigatus*	863	95	100.00	ATCC 1022	1	0	0	1	0	0	1
23	ON149697	*Purpureocillium lilacinum*	624	76	95.59	CBS 284.36	4	5	2	4	3	4	11
24	ON149698	*Penicillium coprophilum*	817	99	99.24	FMR 13998	1	1	0	1	1	0	2
25	ON149699	*Penicillium glabrum*	516	75	94.12	FMR 14292	0	2	0	2	0	0	2
26	ON149700	*Penicillium frequentans*	513	75	94.09	FMR 14318	0	1	0	1	0	0	1
27	ON149701	*Talaromyces pinophilus*	816	94	99.42	FMR 14017	0	0	1	1	0	0	1
28	ON149702	*Aspergillus aculeatus*	795	92	100.00	CBS 172.66	0	0	4	1	0	3	4
29	ON149703	*Paecilomyces lilacinus*	791	83	98.85	WM 04.457	0	1	1	0	2	0	2
30	ON149704	*Aspergillus brasiliensis*	732	95	95.48	ATCC MY-A4553	1	0	0	0	1	0	1
31	ON149705	*Aspergillus flavus*	836	93	100.00	PWQ 2335	4	0	0	0	4	0	4
32	ON149706	*Penicillium echinulatum*	770	94	98.48	FMR 13945	0	0	1	0	1	0	1
33	ON149707	*Penicillium rubens*	827	93	99.81	FMR 13874	0	0	1	0	1	0	1
34	ON149709	*Penicillium crustosum*	726	85	99.16	FMR 1430	0	6	1	0	1	6	7
35	ON149710	*Penicillium allii*	808	93	99.04	FMR 14251	0	0	1	0	1	0	1
36	ON149711	*Penicillium commune*	764	93	97.14	CBS 311.48	0	0	1	0	0	1	1
37	ON149712	*Mucor circinelloides f. circinelloides*	754	91	92.19	IHEM 24129	1	0	0	0	0	1	1
38	ON149713	*Geotrichum candidum*	482	90	99.03	WM 07.304	1	0	0	0	0	1	1
39	ON149714	*Geotrichum bryndzae*	436	82	98.93	PMM09-440L	1	0	0	0	0	1	1
40	ON149715	*Fusarium keratoplasticum*	798	93	100.00	FRC S-2465	0	1	0	0	0	1	1
41	ON149716	*Penicillium brevicompactum*	738	93	97.67	WM 06.340	0	5	2	0	0	7	7
42	ON149717	*Alternaria alternata*	817	95	100.00	WM 04.486	0	1	0	0	0	1	1
43	ON149718	*Penicillium griseofulvum*	813	94	99.44	CBS 185.27	0	0	1	0	0	1	1
44	OM698376	*Penicillium palitans*	507	72	91.09	FMR 14268	1	0	0	0	0	1	1
Total							55	55	66	72	36	68	176

active secondary metabolites that affect the growth of their hosts, we speculate that the endophytic fungi might contribute to the invasive success of *S. rostratum*.

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Short communication

First record of *Prangos trifida* (Apiaceae) in Croatia

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Abstract – Three individuals of *Prangos trifida* (Mill.) Herrnst. & Heyn (Apiaceae) were found in Croatia for the first time in 2013. The population has increased in the last ten years and in 2023, 30 individuals were found. *Prangos trifida* grows on the small islet of Zmorašnji Opuh (Puh) in the Kornati National Park (Dalmatia) within the vegetation of salt-sprayed rocky cliffs. The taxonomic status, ecology and origin of the species are briefly discussed.

Keywords: eastern Adriatic, ecology, islets, NE Mediterranean, *Prangos*

Introduction

The genus *Prangos* Lindl. (Apiaceae, Apioideae) comprises 50 accepted species, distributed from Europe to Mongolia and the western Himalayas (POWO 2023). Most species occur in Asia, and the centre of diversity of the genus is the Iranian-Turanian region (Pimenov and Leonov 1993). *Prangos* is polymorphic and varies considerably in habit and flower and fruit morphology, making it difficult to determine the genus boundaries (Lyskov et al. 2017a). A monographic treatment of *Prangos* was published by Herrnstadt and Heyn (1977), while subsequent taxonomic revisions have involved significant changes in the system of the genus and related genera (for details see Pimenov and Tikhomirov 1983, Lyskov et al. 2017 a,b, and references therein).

In Europe, *Prangos* species occur from Portugal to South European Russia (POWO 2023). For the Euro-Mediterranean region, 23 *Prangos* species and four subspecies have been listed (Euro+Med 2006-2023).

Of the *Prangos* species in Croatia, only *P. ferulacea* (L.) Lindl. has been reported from the southern part of the country (Visiani 1852). Lovrić (1995) also reported the association "*Opopanaci-Prangetum ferulacei* (Adamović 1911) Lovrić 1987" from the Dubrovnik region. However, the presence of *P. ferulacea* has never been confirmed in the field.

Here we report the first record of *Prangos trifida* (Mill.) Herrnst. & Heyn found in Croatia during fieldwork on the islands of the Middle Adriatic (Dalmatia).

The taxonomic status of *P. trifida* and of the closely related species *Cachrys alpina* M.Bieb. must be briefly highlighted. Tutin et al. (1981) recognised these species as separate taxa with different geographical ranges – *C. trifida* (later replaced to *Prangos*) is the western Mediterranean plant found in Albania, France, Italy, Portugal, Spain, "Yugoslavia", while *C. alpina* occurs in SE Europe, from North Macedonia to SE Russia. This taxonomic concept is widely accepted in different sources (WCVP 2020, WFO 2023, POWO 2023, Musolino et al. 2023). In these databases, "Yugoslavia" is given as the range for both species. This is still confusing and does not help to clarify the boundaries of the species. Indeed, an improvement of these databases, after more than 30 years of the collapse of the former state, might be obligatory.

In this report we followed the concept that *P. trifida* and *C. alpina* are two species, according to the descriptions in keys and books (Tutin et al. 1981, Josifović 1973, etc.). Anyway, taxonomic inconsistencies appear both in the literature (e.g. Duran et al. 2005, Teofilovski 2015, Barina et al. 2015, etc.) and in databases (GBIF Secretariat 2022, Euro+Med 2006-2023).

Material and methods

The study is based on field research in the Kornati National Park (KNP) in 2013 and 2023. The KNP (223.75 km^2) is an area of 91 islands, cliffs and sea reefs in the eastern part

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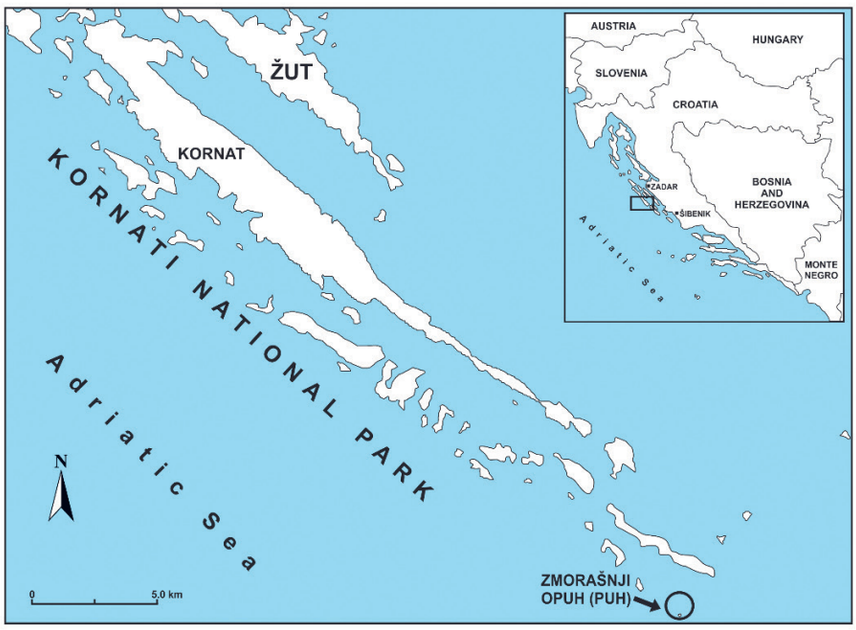

Fig. 1. Map of the study area with location (circled) of the islet of Zmorašnji Opuh (Puh) in the southeastern part of the Kornati National Park. The square on the map in the upper right corner shows the study area in the SE European context.

of the Middle Adriatic (Fig. 1). The bedrock consists of carbonate rocks, mainly Cretaceous limestones and dolomites (Pandža 2010). Brown soil (calcareous cambisol) of shallow to medium depth is the most widespread type. The climate is Mediterranean, with an average annual air temperature of 16.3 °C and an average annual precipitation of 571.8 mm. North winds prevail. The KNP is classified among Croatia's Important Plant Areas (IPAs) and is part of the Natura 2000 ecological network (Official Gazette 2019). Halophilic and halotolerant vegetation overlap largely due to the constant influence of the sea (waves, salt spray and strong winds) over a small altitudinal gradient on most of the islets. The increase in tourism, the abandonment of traditional agriculture, physical changes in ecosystems, the invasion of exotic species and global climate change are the factors that contribute most to environmental risks (Pandža 2010).

A phytosociological relevé was collected using the Braun-Blanquet approach (Braun-Blanquet 1964, Westhoff and van der Maarel 1980). The nomenclature of the taxa follows the International Plant Names Index (IPNI 2023). The collected plant was deposited in the herbarium CNHM (Thiers 2023).

Results and discussion

On 2 July 2013, *P. trifida* was recorded for the first time in this country. It was found as follows: Croatia, North Dalmatia, Šibenik-Knin County, Murter-Kornati Municipality, Kornati National Park, Donji Kornati, islet of Zmorašnji Opuh (Puh; an area of 0.014 km^2), altitude 10 m a.s.l., geographical coordinates 43.676164 N, 15.495563 E, date 10 July 2023, leg. M. Pandža, det. N. Jasprica and M. Pandža, herbarium code: CNHM, 600:ZAG; 9206:BOB.

Prangos trifida was found within the rupicolous herb-rich vegetation of salt-sprayed rocky cliffs of the alliance *Limonion anfracti-cancellati* (Horvatić 1934) Mucina in Mucina et al. 2016 (Fig. 2). The phytosociological relevé [plot size: 50 m^2; coordinates (WGS84): latitude 43°40'34.2", longitude 15°29'44.0"; altitude 10 m a.s.l.; aspect: -; stoniness 10%; rockiness 20%; vegetation cover 70%; vegetation height 1 m; date: 10 July 2023] includes list of taxa as follows: *Smilax aspera*, 3; *Prangos trifida,* 2; *Prasium majus*, 2; *Allium commutatum*, 2; *Daucus carota* subsp. *hispanicus*, 1; *Crithmum maritimum*, 1; *Asparagus acutifolius*, 1; *Silene vulgaris*, 1; *Limonium cancellatum*, +; *Lotus cytisoides*, +; *Reichardia picroides*, +; *Elymus athericus*, +; *Mercurialis annua*, +.

On the first visit to the islet, three individuals were found. A decade later (10 July 2023), 30 individuals were counted on the islet. The species was not found during an exploration of the surrounding islets of the archipelago. It is unclear whether this population is native or introduced. The population of *P. trifida* found in this study is located between those reported from the province of Liguria (NE Italy) in the north and Montenegro (GBIF Secretariat 2022) and Albania (Barina et al. 2015) in the south. Interestingly, Conti et al. (2005) described the Italian population as "no longer recorded".

Although some recent studies (e.g., Wojewódzka et al. 2019) reject, at least partly, any role for anemochory in the dispersal of Apioideae fruits, we think that both zoochorous dispersal modes (endozoochory, epizoochory) are eligible because (i) the islet is a nesting site for gulls, (ii) the area is on one of the major migration routes in the region and includes habitats important for the conservation of migratory bird species (Kralj et al. 2013, Purger 2015). Furthermore, the influence of the environment (habitat, wind frequency, etc.) on the survival of the plants cannot be ignored (Wen et al. 2020).

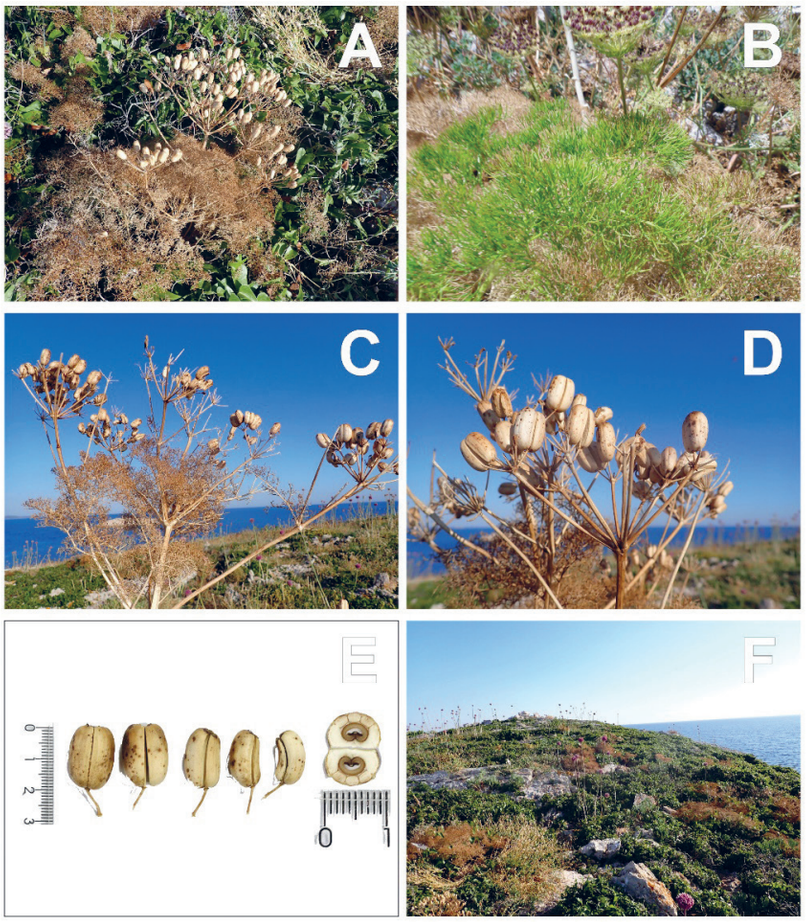

Fig. 2. *Prangos trifida* (Mill.) Herrnst. & Heyn in Croatia, Middle Adriatic, Kornati National Park, islet of Zmorašnji Opuh (Puh). A – habit, B – lower leaves, C – upper leaves with fruits, D – fruits, E – fruits (left) and cross sections through fruit (right), scale: cm, F – habitat (photo: M. Pandža, N. Jasprica).

In the present case, *P. trifida* occurs within the low rocky coastal vegetation belt. In contrast, Pignatti et al. (2017-2019) reported an altitudinal range of the Ligurian population from 800 to 1600 m a.s.l. In Albania, it was found on limestone within the *Buxus sempervirens* L. dominated community at 981 m a.s.l. (Barina et al. 2015). According to Jury (2003), *P. trifida* occurs in the Iberian Peninsula on uncultivated places in the altitudinal range 0-1700 m.a.s.l. In France, it occupies garrigues and rocks (MNHN, OFB (2003-2023). Although the GBIF network contains a large data set on the distribution of *P. trifida*, especially from the southern coast of Portugal (Faro area, Algarve) and the Mediterranean coasts of Spain and France, the data are based on the field observations which do not provide details on the type of the communities or habitats (GBIF Secretariat 2022).

In our opinion, further field surveys are essential to determine the actual distribution and ecology of *P. trifida* in Croatia. Furthermore, it would be desirable to monitor the species in order to assess the conservation status of the population described here. Last but not least, the biological potential of some substances and the phytochemical content of the essential oil isolated from *P. trifida* are of importance (Abad et al. 2001, Palá-Paúl et al. 2004).

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Short communication

Differences in soil chemistry between early and late succession of oak-hornbeam forest after grassland abandonment

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Abstract – Changes in C and nutrient cycling during succession are well studied, however, results can be contrasting for different nutrients and successional sequences. We analyzed soil chemical differences between early and late succession of oak-hornbeam forest. Late forest succession efficiently retained plant-available P, and total Mn, Zn, Fe, Cu, and Ni pools in the soil, as their concentrations were similar to those of early-successional grasslands. Available K, soil organic C, and organic matter content, as well as C:N and C:S ratios were higher in late than in early succession. Soil organic N and S concentrations did not differ between the stages.

Keywords: ecosystem development, *Epimedio-Carpinetum betuli*, forest restoration, oak forest development, soil-vegetation relationships

Introduction

Generally, due to increment of litter rich in lignocellulosic components during late forest succession, nutrient mineralization rate is expected to decelerate, and large amounts of nutrients become captured within tree biomass. Also, due to the different functional compositions of most grassland and forest communities, it can be expected that these two vegetation types will exert different influences on nutrient retention and cycling. Plants in most early successional stages such as grasslands (except those growing on very nutrient-poor soils) are often characterized by lower C:N and C:P ratios, higher N and P contents, and lower content of lignocellulosic components in their tissues than those of species from late successional stages, such as forests (Vitousek et al. 1988, Poorter et al. 2004, Cortez et al. 2007). Therefore, it can generally be expected that litter mineralization will be much faster in early successional grasslands than in late successional stages, especially because the high content of lignocellulosic components (primarily that of lignin) in the tissues of late successional tree species delays the release of nutrient forms available to plants.

Here, we report local-scale differences in soil chemical properties between early and late succession of oak-hornbeam forest (association *Epimedio-Carpinetum betuli* (Horvat 1938) Borhidi 1963) after cessation of agricultural land use (i.e. grassland abandonment) in NW Croatia.

Materials and methods

Study area

The study was conducted in the surroundings of Brlog Ozaljski village, near the city of Ozalj in NW Croatia. Existing grasslands at the study site are used as hay-pastures dominated by *Avenula pubescens* (Huds.) Dumort. (i.e. as occasionally grazed meadows), and after abandonment, the succession pathway is the following: successional grasslands (2–5 years), *Cornus sanguinea* L. and *Prunus spinosa* L. shrubs (5–15 years), *Populus tremula* L. stage (15–30 years), and oak-hornbeam forest (> 30 years). This last forest stage represents the *Epimedio-Carpinetum betuli* association. The soil type was slightly leached calcic cambisol on biolithitic and bioclastic limestones.

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Soil sampling and analyses

To analyse the differences in soil chemistry between the early and late successional stages, six pairs were selected, each containing early successional stage of grassland and the late successional stage of forest, as close to each other as possible. Each pair of grassland and forest plots represented a block. The grasslands selected for this purpose were recently used hay-pastures undergoing colonization with successional grasses, whereas forest plots selected for this purpose were late successional mixed stands of *Populus tremula*, *Carpinus betulus* L., and *Quercus petraea* (Matt.) Liebl. and/or *Q. robur* L. Soil was sampled from the top soil at a depth of 0–10 cm. Soil sampling was performed from February 28 to March 2, 2020, before the beginning of the vegetation season.

Soil analyses were carried out using air-dried, homogenized, and sieved soil samples (< 2 mm sieve). The details about soil chemical analyses are given in On-line Suppl. Tab. 1.

Tab. 1. The results of blocked ANOVA for the differences in soil chemical properties between early succession (i.e. grassland stage) and late succession (i.e. forest stage) (n = 6) If the block effect was insignificant (P > 0.25), then the F and P values for the succession effect corresponded to those of simple one-way ANOVA, whereas if the block effect was significant (P < 0.25), then the F and P values for the succession effect corresponded to that of blocked ANOVA (i.e. two-way ANOVA without replication); SOC – soil organic carbon, SOM – soil organic matter, N – soil organic nitrogen, S – soil organic sulfur, C:N – soil carbon to nitrogen ratio, C:S – soil carbon to sulfur ratio, N:S – soil nitrogen to sulfur ratio, P_A and K_A – ammonium lactate-extracted plant available phosphorus and plant available potassium, respectively, Mn – soil total manganese, Zn – soil total zinc, Cu – soil total copper, Ni – soil total nickel, Fe – soil total iron.

Soil feature	F	Block effect	Succession effect
pH 1 M KCl	0.0003	**0.19**	0.99
SOC, g kg^{-1}	8.28	0.81	**0.02**
N, g kg^{-1}	0.29	0.84	0.61
S, g kg^{-1}	1.17	0.55	0.30
C:N	296	**0.03**	**< 0.0001**
C:S	7.32	0.50	**0.02**
N:S	0.20	0.80	0.67
P_A, mg $(100\ g)^{-1}$	2.28	0.57	0.19
K_A, mg $(100\ g)^{-1}$	7.41	**0.01**	**0.04**
Mn, mg kg^{-1}	0.11	0.35	0.75
Zn, mg kg^{-1}	0.55	0.49	0.48
Cu, mg kg^{-1}	0.21	0.75	0.66
Ni, mg kg^{-1}	0.99	**0.16**	0.36
Fe, g kg^{-1}	1.49	0.49	0.25

Data analysis

The differences in soil chemistry between early and late succession were analyzed by two-way analysis of variance (ANOVA) without replication in order to account for inter-site variation (i.e. including the block effect). One-way ANOVA without blocking was used for the variables that were not significantly influenced by inter-site variation (i.e. if the block effect was > 0.25). Threshold value of 0.25 was chosen for assessment of the significance of the block effect because it is more stringent than the usual threshold of 0.05 for the purpose of determining the inter-site variation of the soil chemical properties. The data was analyzed using PAST 4.03 software.

Results and discussion

The results suggested that the SOC content in the top soil was 16.4 g kg^{-1} higher on average (P < 0.05), in the late successional forest stage than in the grassland stage (Tab. 1 and On-line Suppl. Tab. 2). The forest stage also had higher C:N (P < 0.0001) and C:S ratios (P < 0.05) than the grassland stage in the top soil, whereas soil organic N and S concentrations and soil N:S ratio were not different (P > 0.05) (Tab. 1). The K_A concentration in the top soil of the late successional forest stage was higher (P < 0.05) than that of the grassland stage, whereas no significant differences in the concentrations of P_A and total Mn, Zn, Cu, Ni, and Fe were found between the two stages (P > 0.05) (Tab. 1). Detailed results of our soil laboratory analyses are given in On-line Suppl. Tab. 2.

The late successional forest stage had higher SOC contents in the topsoil than the early successional grassland stage, with a mean increase of 39%. This is expected, as the soil organic matter (SOM) in late successional forest has a wider C:N ratio than that of early successional grassland, and originates from the litter rich in lignocellulosic components, thus, being more resistant to degradation than grassland SOM in topsoil. In addition, soil organic N and S were not increased in late-successional forest stage, even though an increase could be expected because of the high SOM content in the forest stages that organic N and S originate from (David et al. 1982). Indeed, litter of early successional grassland should have higher N and S contents, but lower C:N and C:S ratios than that of late successional forest. Therefore, the loss of N and S via mineralization is faster in grassland than in forest stage, in which litter is more stable and decomposes much more slowly, which is expected to result in a longer retention of soil organic N and S in the forest than in grassland stage. On the other hand, soil C:N and C:S ratios in the topsoil were significantly higher in the late successional forest stage than in the early successional grassland stage. Increased soil C:N ratio in the forest stage could indicate that N mineralization in this stage is somewhat slower than in grasslands. Studies have suggested that N mineralization is slower in forest soils than in those of early successional stages, and that this is due to various processes related to the inhibition of nitrification (Rice and

Pancholy 1972). However, the results of studies that compared N mineralization in forest stages to that in early-successional stages vary (Robertson and Vitousek 1981, Vitousek et al. 1989, Yan et al. 2009), thus, a soil C:N ratio alone is not necessarily a reliable measure for evaluating N mineralization rates.

In the present study, soil K_A concentration was significantly higher in the late successional forest than in the grassland stage. As the effects of secondary succession on soil K pool are still understudied, it is hard to make a generalized conclusion about K cycling during succession; however, an increase in soil K pool following secondary succession has been already reported (Liu and Huang 2005). It is hard to conclude which reasons exactly underlie the increased K_A concentration under forest stages. Either trees possess an ability to substitute K^+ from the crystal lattice of clay minerals with H^+, thus efficiently extracting it into the soil labile pool (Boyle and Voigt 1973), or they are also able to efficiently increase its concentration through leaf litter deposition and throughfall. As the soil was sampled from the top soil, all processes are likely to contribute simultaneously to the increase in K supply. Further studies on plant nutritional statuses and litter decomposition rates during succession are required to clarify this.

On the other hand, no differences in concentrations of P_A, and total Mn, Zn, Cu, Ni, and Fe in top soil were found between the grassland and the forest stage. Indeed, overall nutrient pools in the whole food chain of forest ecosystems are higher than in grasslands, however, nutrients are continuously being stored in the living trees and slowly decomposing dead biomass, thus, their turnover to the mineral soil is expected to be slower than that of hay-pastures. Despite this, supplies of the mentioned elements were efficiently retained in the topsoil of the late successional forest stage in the present study.

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INSTRUCTIONS FOR AUTHORS

The interest of the journal is field (terrestrial and aquatic) and experimental botany including plant viruses, bacteria, archaea, algae and fungi, from subcellular level to the ecosystem level with a geographic focus on karstic areas of the southern Europe and the Adriatic Sea (Mediterranean).

The journal welcomes manuscripts for publication in the following categories: original research papers, short communications, book reviews, social news and announcements. Review articles are accepted on editor invitation only.

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In the cover letter addressed to Editor-in-Chief, the authors should explain how the manuscript meets the scope of the journal and indicate why it will be of interest to the general readership of Acta Botanica Croatica. Authors should propose the names and e-mail addresses of at least **five potential reviewers who are experts in the topic of manuscript. Please avoid colleagues with joint publications, or from the same institutions. At least three of them have to be international recognized scientists outside of your home country**. Also, in the cover letter, confirm that the manuscript has not been published or submitted for publication elsewhere and that all authors have read the manuscript and approved it for submission. Include also Founding statement in which any sources of financial support should be specified.

Type of contribution

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Review and mini-review articles are usually accepted for the reviewing process if invited by editor. Authors who wish to contribute a manuscript to this category are encouraged to contact the Editor-in-Chief. The manuscript should be organized according to Acta Botanica Croatica guidelines and there are no limitations on the number of references.

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The metric system should be used throughout the manuscript. If required, equivalent values in other systems may be placed in parentheses immediately after the metric value.

Italicize only the names of genes (e.g. *Arpl* gene), genera, species, subspecies and lower taxonomic units. Genetic information, such as DNA, RNA, or protein sequences, should

be submitted to public data bases (GenBank, EMBL, etc.), and accession numbers should be available in Material and methods. Voucher specimens must be made and deposited in a public herbarium. For endemic and protected taxa, permission has to be obtained from the competent authority. The nomenclature of taxa and syntaxa has to be in strict accordance with international rules (codes).

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ABSTRACT of up to 250 words that highlights the objective, results, and conclusion of the paper.

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Keep the Introduction brief, stating clearly the purpose of the article and its relation to other papers on the same subject. Do not give an extensive review of literature. Provide enough information in the Material and methods section to enable other investigators to repeat the experiments. Report Results clearly. In the Discussion interpret the results, state their meaning and draw conclusions. Do not simply repeat the results. Proceed with Acknowledgments where any sources of financial support as well as any individuals who were of direct help to the authors should be acknowledged. At the end give Author contribution statement in which the contributions of all authors should be described.

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Cite references in the text by name and year in parentheses. Some examples: Wrischer (1998), … Jones and Smith (1987), …. (Jones 1987a, b), ….. Jones et al. (1986), … (Facca et al. 2002, 2003, Socal et al. 2006).

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Books:

Horvat, I., Glavaš, V., Ellenberg, H., 1974: Vegetation Sudosteuropas. Geobotanica selecta 4. Gustav Fischer Verlag, Stuttgart.

Chapter in a book:

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.

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