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Note

Reproductive Biology of the Balkan endemic *Sideritis scardica* (Lamiaceae)

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ABSTRACT: *Sideritis scardica* is one of the 270 endemic Balkan species in Bulgarian flora that occurs also in Albania, Greece, Serbia, R Macedonia and Turkey. The excessive exploiting of this valuable medicinal plant, known in Bulgaria as "Pirin mountain tea" or "Mursalitza tea", affects the state of its natural reserves. In this study, three main parameters of reproductive biology of *S. scardica* in the population from the Pirin Mts (Southern) were investigated: pollen viability (with acetocarmine staining), seed viability (with tetrazolium test) and seed germination (with/without addition of growth regulator GA_3). These standard parameters of plant reproductive biology were used for an estimation of reproductive capacity of *S. scardica*, to predict the future state of its natural populations in Bulgaria. The results of the study revealed that a strong relationship between percentage of seed viability, seed germination and duration of seed storage does not exist in this species. Most likely, these parameters depend on the peculiarities of the climatic conditions during times of the year when the seeds are collected.

Key words: *Sideritis scardica*, reproductive capacity, pollen viability, seed viability, germination

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The genus *Sideritis* L. belongs to the family *Lamiaceae* Lindl., one of the most common and diverse angiosperm families in the world. This genus is divided into two subgenera, *Sideritis* and *Marrubiastrum*, present in Europe and the Macaronesian area, respectively. The subgenus *Sideritis*, including four sections, contains approximately 125 species, most of which have a centre of distribution in Mediterranean Europe and Northern Africa. *Sideritis scardica* Griseb., belonging to the section *Empedoclia* (Rafin.) Benth., is a Balkan endemic that occurs in Albania, Bulgaria, Greece, Republic of Macedonia, Serbia and Turkey (Petrova & Vladimirov 2010). It is one of the 270 endemic Balkan species in Bulgarian flora, growing on Mt Slavyanka, Pirin Mts and Rhodopi Mts from 1000 to 2200 m altitude. The species is included in the threat category "endangered" in the Red list of Bulgarian vascular plants (Petrova & Vladimirov 2009) and Red Data Book of the Republic of Bulgaria (Evstatieva 2012).

In Bulgaria, studies on *S. scardica* have been carried out in chorological, karyological and phytochemical aspects (Kozuharov & Kuzmanov 1965; Evstatieva & Bakalova-Protich 1990; Yordanova & Apostolova 2000; Baltisberger 2006; Kostadinova *et al.* 2007). A karyological study of the Rhodopi Mts populations of *S. scardica* revealed that its chromosome number is 2n=32 (Kozuharov & Kuzmanov 1965; Baltisberger 2006). Esra *et al.* (2008) showed that that all species from the section *Empedoclia* of the genus *Sideritis* (including *S. scardica*) in Turkey are diploid (2n=32), with basic chromosome number x=16.

Many chemical compounds have been identified in the genus *Sideritis*: terpenes, flavonoids, essential oils,

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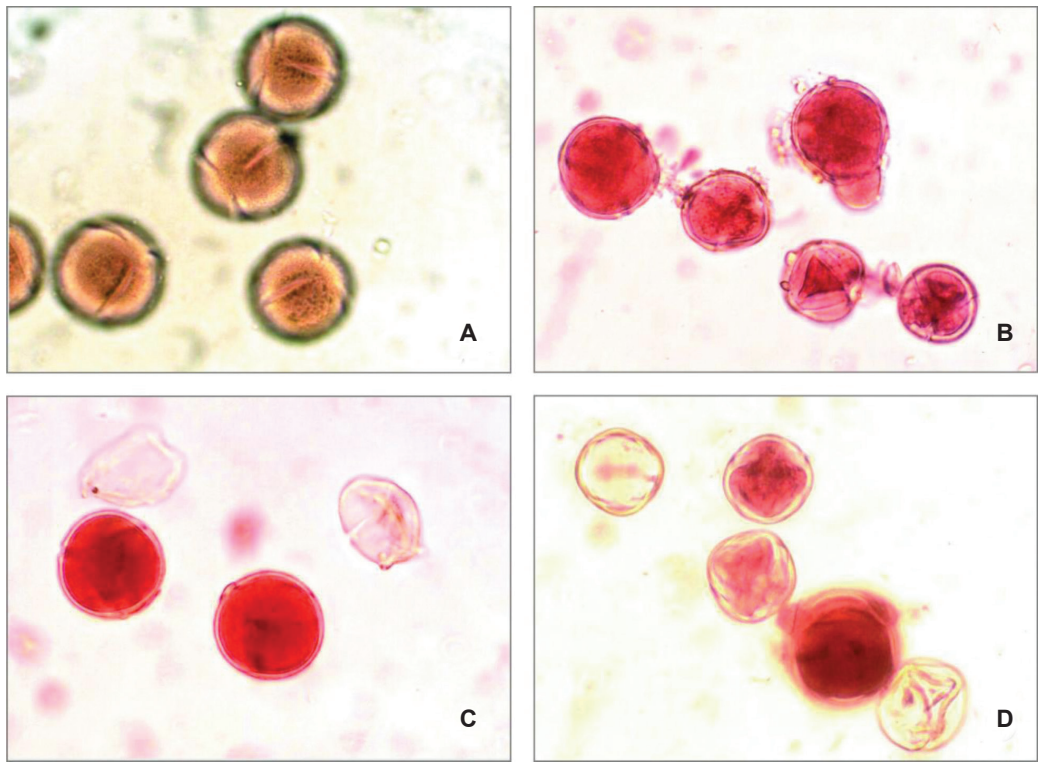

Fig. 1. Pollen viability tested by acetocarmine staining: A – mature pollen grains not treated with acetocarmine; B – tricolpate viable pollen stained in red; C – tricolpate viable pollen stained in red and nonviable colorless pollen; D – tetracolpate viable pollen stained in red and nonviable colorless pollen

iridoids, coumarins, lignans and sterols. The activity of *Sideritis* species, in particular *S. scardica,* is mainly due to their flavonoid and terpenoid contents. In Bulgaria, the infusion of the aerial parts of *S. scardica*, known as "Pirin mountain tea" or "Mursalitza tea", is employed largely as an expectorant for the treatment of pulmonary emphysema and angina pectoris (Ivancheva & Stancheva 2000). The excessive exploiting of this valuable medicinal plant affects the state of its natural reserves and reproductive capacity.

The aim of this study is to evaluate three main parameters of the reproductive biology in *S. scardica*, connected with its reproductive capacity: pollen viability, seed viability and seed germination, to predict the future state of its natural populations in Bulgaria as a valuable medicinal plant.

The study was carried out on a population of *S. scardica* from the Pirin Mts (Southern), the peak "Orelek" at 2099m a.s.l., during three successive years (2009, 2010 and 2011), and allowed a comparative analysis of the main parameters of its reproductive capacity to examine: pollen viability, seed (embryo) viability and seed germination.

A common method to assess pollen viability is by staining and direct count (Heslop-Harrison 1992). For this purpose, anthers from open flowers were collected and placed in 1% acetocarmine solution (Singh 2003). After that, the pollen was dispersed on the slide and the number of stained (viable) and unstained (nonviable) grains were counted. Pollen viability was examined of the three years. The mature pollen grains were counted in up to 30 anthers (visual field – at a magnification 100x), using a light microscope. The total number of pollen grains examined for viability was 137952. When the cytoplasm and nuclei of the pollen grains were stained in red they were considered viable, whereas the colorless pollen grains were considered non-viable and sterile. Observations on pollen viability were made using a light microscope "Olympus" CX21 and micrographs – with Digital camera (1.4 Mpx).

One of the most significant advances in seed testing technology in recent years is the application of a tetrazolium method – a quick chemical test conducted in a short time with minimal equipment (Peters 2000). The quality of mature seeds can be determined by studying their germination and viability potential after the application of this test. Initially, the tetrazolium solution is colorless, but changes to red when it contacts the hydrogen (reduction process) deriving from enzymes of the respiration process of the seed. Embryos showing active respiration turn red and are considered as viable (the darker the color, the greater the respiratory activity in the seed). Light pink indicates an embryo with lower

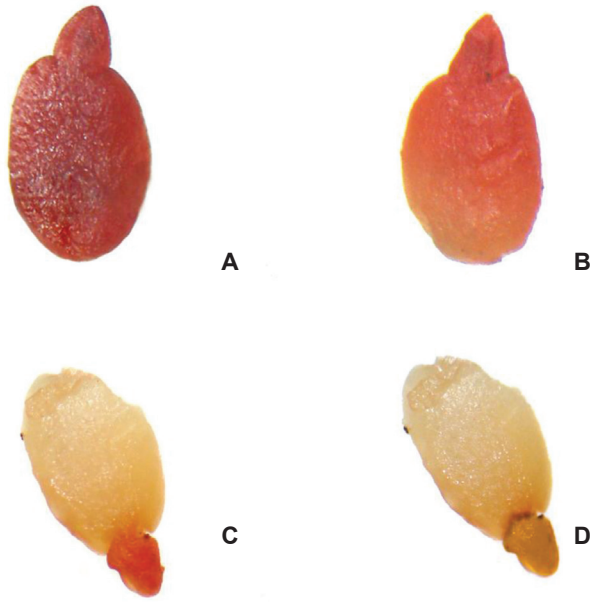

Fig. 2. Seed viability according to tetrazolium test: A – dark red colored viable embryo; B – light red colored viable embryo; C – nonviable embryo; D – nonviable colorless embryo

viability than those staining dark red. Thus, the staining pattern after using the tetrazolium test reveals the live and dead areas of the embryo and enables the capacity of seeds to produce normal seedlings to be determined (Copeland & McDonald 2001).

In our study, an evaluation of the seed (embryo) viability of *S. scardica* was made on approximately 400 mature seeds tested with the tetrazolium method in each year. Seed viability was evaluated by the color pattern of the isolated embryos.

Seed germination potential was estimated by a germination test carried out in laboratory conditions (room temperature, 25°C, long-day conditions), and treatment with or without 0.01% gibberellic acid (GA_3). Five replicates of 20 seeds (100 seeds for each year and treatment) were sown on moistened filter paper disks in Petri dishes. The criterion of germination was visible radicle protrusion. Every day, after the count, the germinated seeds were discarded and unfilled or mouldy seeds were not counted. Non-germinated seeds were analyzed to identify empty or dead seeds. The germination potential was expressed as percentage of germinated seeds.

Pollen viability has been defined as "having the capacity to live, grow, or develop" (Lincoln *et al.* 1982). Pollen quality is usually associated with pollen viability, i.e. the proportion of pollen grains that are viable (Kearns & Inouye 1993). Besides the important role of pollen for different processes connected with reproductive biology, its viability has particular importance in ensuring effective seed set following pollination (Smith-Huerta & Vasek 1984). The highest pollen viability after acetocarmine stain technique was determined for samples collected in 2011 (88.4%) and lowest 84.7% for those in 2009 (Table 1, Fig. 1). This high pollen viability will contribute to effective pollination and fertilization, as evidenced by the strong sexual reproduction and successful seed formation established in *S. scardica* in a preliminary study on its reproductive biology, in particular embryological structures and processes of this species (Yurukova-Grancharova & Yankova-Tsvetkova 2012).

Seed viability evaluated after the application of the tetrazolium test aimed to determine which seed tissues had the potential to germinate under optimum conditions, as many seeds were neither completely dead nor completely alive. The staining pattern of this test reveals the live and dead areas of the embryo and determines the capacity of seeds to produce normal seedlings (Copeland & McDonald 2001). On the grounds of intensity of staining with tetrazolium solution, the embryos were classified in four groups (Fig. 2): I group – viable embryos (stained in red); II group – nonviable embryos (only root stained in red); III group – nonviable embryos (colorless), IV group – nonviable embryos (seeds without embryo, empty seeds). The highest seed viability (70.0%) was found in 2010 and the lowest (41.0%) in 2011 (Table 2).

Concerning seed germination, in the present study, the highest germination activity (82%) was determined for seeds collected in 2010 that were not treated with GA_3 and the lowest germination (50.50%) forseeds collected in the same year but treated with GA_3 (Table 3). No correlation was found between the time of storage and the germination ability of seeds of *S. scardica*. The seeds conserved their germination capacity with time. Although the growth regulator GA_3 is generally known to increase seed germination capacity, in our study, in contrast, seed germination decreased in *S. scardica* following GA_3 treatment in 2009 and 2010. In 2011 GA_3 treatment had no significant effect on percentage of seed germination (Table 3). Kozuharova (2009), however, did find that GA_3 had a stimulatory effect on the seed germination of *S. scardica*. We found no stimulatory effect of GA_3 on seed germination of this species.

In the present study, the relatively high percentage of germinated seeds not treated with GA_3 and preserved at room temperature (without stratification) as well as the initiation of successful seed germination soon after sowing the seeds, show that seeds of *S. scardica* most likely have no dormancy. Estrelles *et al.* (2010) examined seed germination behavior in two Iberian endemic species of the genus *Sideritis*, namely *S. pungens* Benth. and *S. chamaedryfolia* Cav. from different habitats. They established that seed germination was closely related

Table 1. Pollen viability estimated by acetocarmine staining.

Year	Number of anthers analyzed	Number of pollen grains analyzed	% stainable pollen grains (viable) + SD (standard deviations)
2009	30	43132	84.7 ± 4.8
2010	30	47936	87.2 ± 4.1
2011	30	46884	88.4 ± 4.1
Total	90	137952	87.0 ± 4.5

Table 2. Embryo viability assessed by the tetrazolium test.

Year	Number of embryos analyzed	Number of viable embryos	Viable embryos (%)	Nonviable embryos (%)
2009	406	177	43.6 %	56.4 %
2010	403	282	70.0 %	30.0 %
2011	454	186	41.0 %	59.0 %
Total	1263	645	51.1%	49.9%

Table 3. Estimation of seed germination with or without gibberellic acid (0.01%)

Year	Seed germination (%)	
	with GA_3	without GA_3
2009	52.5	71.0
2010	50.5	82.0
2011	65.7	58.0

to the characteristics of habitats, mainly environmental conditions (temperature and sunlight intensity) and soil structure. Our results concerning the seed germination ability of *S. scardica* support this conclusion and show that the process of seed germination is complex and can be affected at different stages by many factors and interactions of factors such as temperature, water availability, light, maturity of seeds, etc.

The present study on the main reproductive biology parameters in *S. scardica* showed no strong relationship between the percentage of seed viability, seed germination and duration of seed storage. Thus, neither seed germination nor viability depended on the duration of storage of seeds and neither did seed treatment with plant growth regulators. Most likely, the three parameters examined would be dependent on the climatic conditions each year at the time when seeds are collected and examined. This assumption may also be true for other species of the genus *Sideritis*, growing in the Mediterranean area and Balkan Peninsula. The high pollen viability established in *S. scardica* is a precondition for its effective pollination, fertilization and seed set – the main processes that provide a successful reproduction of this species and conservation of its native populations.

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REZIME

Reproduktivna biologija Balkanskog endemita *Sideritis scardica* (Lamiaceae)

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Sideritis scardica Griseb. je endemična vrsta Balkana u Bugarskoj flori, koja takodje nastanjuje i Albaniju, Grčku, Srbiju, Republiku Makedoniju i Tursku. Ekstenzivnom eksploatacijom ove lekovite vrste, koja je u Bugarskoj poznata kao „Pirinski planinski čaj" ili „Mursalitza čaj", ugroženo je stanje njenih populacija u prirodi. U ovom radu su predstavljena tri glavna parametra reproduktivne biologije populacija *S. scardica* na planini Pirin: vijabilnost polena, vijabilnost semena i klijanje semena. Ovi parametri su korišćeni u svrhu procene reproduktivnog kapaciteta, kako bi se predvidela stanje prirodnih populacija ove vrste u Bugarskoj. Na osnovu rezultata može se zaključiti da ne postoji direktna korelacija izmedju vijabilnosti semena, njihovog klijanja i sužine skladištenja istih semena. Pretpostavlja se da su ove reproduktivne karakteristike semena u direktnoj vezi sa klimatskim uslovima u kojima su biljke rasle i semena prikupljana.

Ključne reči: *Sideritis scardica*, reproduktivni kapacitet, vijabilitet polena,vijabilitet semena, klijanje