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Full Length Research Paper

Pollen morphology and variability of *Tulipa hungarica* Borb

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Pollen morphology of the endemic species *Tulipa hungarica* Borb was investigated with the goal of diversity protection and conservation. Morphology of pollen was evaluated using a scanning electron microscopy. Pollen grain was monosulcate with perforate striate exine surface. Also, pollen of *T. hungarica* showed differences in size, shape, and staining intensity after aceto-orceine treatment. The smallest aceto-orcein treated microspore had a diameter of 13.65 µm, while the largest had a diameter of 60.15 µm. 4,6-Diamidino-2-phenylindole (DAPI) staining showed that fifty percent of investigated microspores had one nucleus, and the other half had two nuclei. A rapid method with fluorescein diacetate – FDA was used to determine pollen viability. Up to (80%) of analyzed microspores showed high viability.

Key words: Microspore size, pollen grain, pollen viability, staining intensity, Tulipa.

INTRODUCTION

This study is the first to investigate the pollen morphology of *Tulipa hungarica. T. hungarica* Borb (Liliaceae) is a well known endemic plant of Flora Europaea (Grey-Wilson and Mathews, 1980). It is a critically endangered species (Diklić, 1999). The only population of *T. hungarica* exists in Đerdap National Park. The Đerdap National Park (Figure 1) is situated in eastern Serbia, on the south side of the Danube canyon. The park is an internationally protected region which continues into the Romanian Iron Gate Nature Reserve (Parcul Natural Portile de Fier), along the north bank of the Danube. The area of the park in Serbia covers 63.608 ha, with a surrounding protective area of 93.968 ha. The specific climatic conditions, relief and soils, and hydrology unique to the Danube canyon have endowed this region with some of the most complex and richest relict vegetation in South Eastern Europe. Only two species of genus Tulipa grows in Đerdap National Park: wide spreaded *T. sylvestrys* and endangered *T. hungarica.*

Pollen size varied extensively among angiosperm species. This variation partially reflects evolutionary adaptation to each pollinator species and the overall fertilization environment. Hence, a species characteristic pollen size may balance the competitive advantages of large pollen against the numerical advantages of small pollen, given its specific reproductive environment (Sarkissian and Harder, 2001). Pollen size varies extensively among angiosperms (Erdtman, 1952). This variation partially reflects evolutionary adaptation to each species pollination and fertilization. Hence, small pollen has reproductive advantages over large pollen of *Brassica rapa* (Sarkissian and Harder, 2001). Pollen dimorphism has been detected in anthers of *Zea mays* (Demerec, 1924), *Nicotiana tabacum* (Horner and Street, 1978), *Ephedra (*Ickert-Bond et al., 2003), *Thymus capitatus* (Karabournioti et al., 2007), *Vitis vinifera* (Gallardo et al., 2009), *Aesculus hippocastanum* (Radojević, 1989, 1991; Ćalić et al., 2003a), *Aesculus carnea* (Marinković and Radojević, 1992), *Aesculus flava* (Ćalić-Dragosavac et al., 2008) and *Aesculus parviflora* (Ćalić-Dragosavac et al., 2009) and many other species. Pozhidaev (1995) confirmed significant differences of *Aesculus* pollen size and shape with scanning and transmission electron microscopy.

Endemics are mostly present among the non-tree

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Figure 1. View of the Đerdap National Park (source: Google Earth). 1. *Tulipa hungarica* site location.

species and one of the well-known endemics of the Đerdap gorge is Đerdap tulip.

One possible method for protection of *T. hungarica* is multiplication and conservation of plants with the help of *in vitro* cultures. The technique of multiplying plants by means of anther and microspore culture is called *in vitro* androgenesis. This method could be the only way of micropropagation of this rare species, as a few plants remain in the wild. With the aim of androgenesis induction we investigate the pollen characters.

The aim of this study was to investigate Đerdap tulip pollen characters and viability and to discover the best stage of microspore development for androgenesis induction.

MATERIALS AND METHODS

Closed flower buds of 5 cm size (Figure 2A) of *T. hungarica* originating from Đerdap National Park (Figure 1) were harvested during April 2009. Samples were transported and stored in the dark at 4°C. Anthers (Figure 2B) were isolated from the buds. Two hundred pollen grains per anther were analysed. The total number of pollen grains was 600.

Pollen staining

Scanning electron microscopy was employed for observation of anthers, polen grains and microspores. Samples were coated with a thin layer of gold (ion sputtering coating) in a BALETECSCD 005 Sputtering Device and examined using a JSM-6460 LV (JEOL, Tokyo, Japan) scanning electron microscope operated at 20 kV. Pollen terminology follows mainly that of Wodehouse (1935), Erdtman (1952) and Punt et al. (1994).

Anthers were longitudinally dissected and free microspores and pollen grains were stained with 1% orcein solution, prepared in 45% acetic acid for measurement of pollen grain diameter. Aceto-orcein treated microspores orginating from one anther were viewed with Leica, DMRB microscope (Wetzlar, Germany), and analysed by UTHSCSA Image Tool version 3.0 (San Antonio, USA) software program. The results were analysed using completely randomized design and tested according to least significant difference (LSD) test.

The number of nuclei was determined by 4', 6-diamidino-2-phenylindole (DAPI) (Coleman and Goff, 1985). The content of anthers was squeezed out and stained with 1-2 drops of DAPI (1 µg ml^{-1}) solution, prepared in distilled water.

A rapid method with fluorescein diacetate – FDA (Heslop-Harrison and Heslop-Harrison, 1970) was used to determine microspores and pollen viability. These reagents were also used to observe cell divisions in the microspores. FDA (2 mg l^{-1}) dissolved in acetone was diluted by (1:1) 0.5 M sucrose solution. DAPI and FDA- treated microspores and pollen grains were viewed with a BX 51 Olympus microscope fitted with an ultraviolet exciter filter B 12 or BP 340-380 for DAPI in combination with barrier filter Y 50.

Statistics and repetition

Microspore size of *T. hungarica* was investigated. The results were

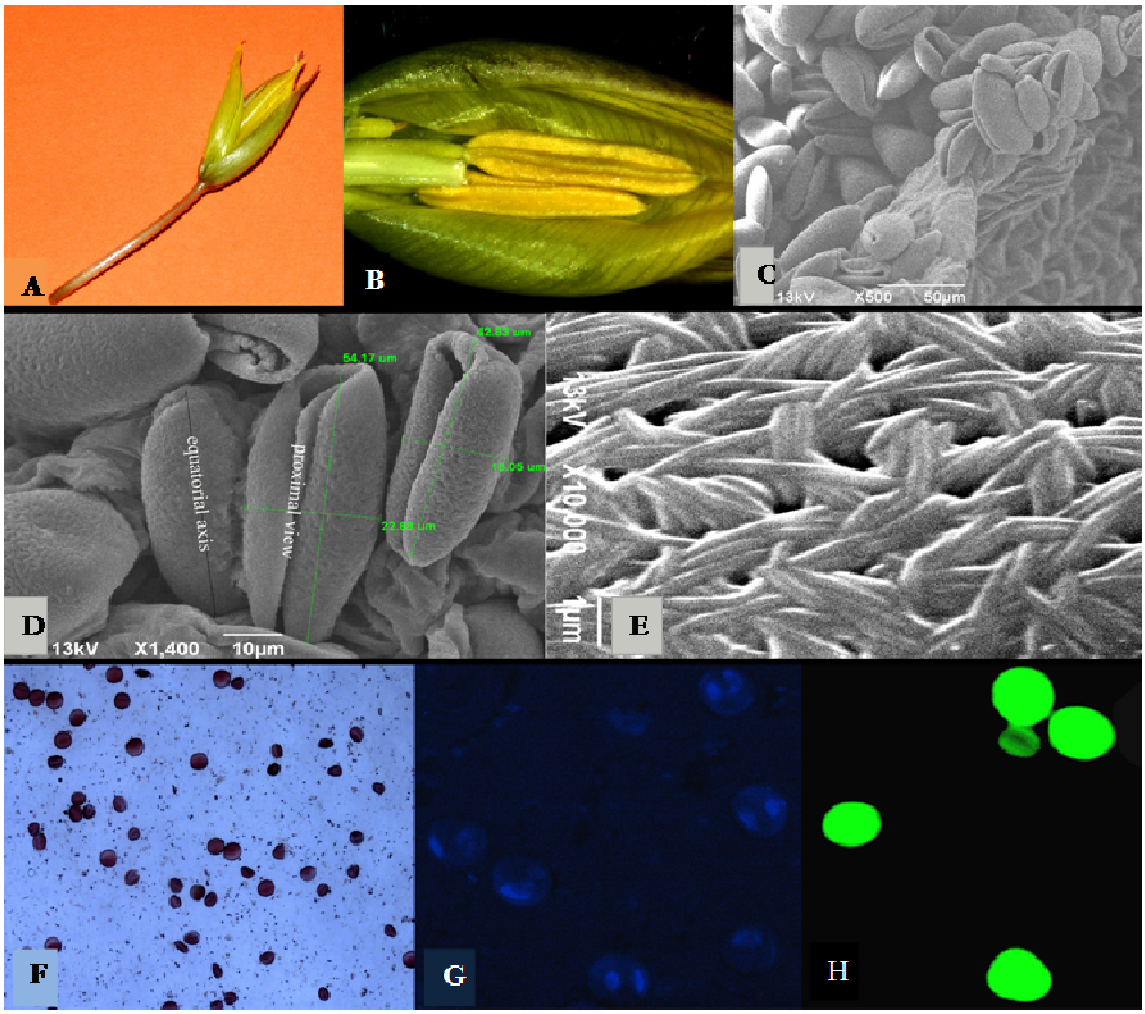

Figure 2. Flower, anthers and pollen grains of *Tulipa hungarica*. Flower (A) and anthers (B) of *Tulipa hungarica*; C to D: scanning micrographs of pollen grains; E: exine ornamentation (SEM); F: Aceto-orcein stained microspores (smaller microspore – arrow; 100 x); G: microspores and pollen stained with DAPI (ultraviolet exciter BP340-380 and Y50 barrier filter; 200x); H: FDA treated microspores (x 200).

assessed using the variation analysis. The means were compared by the least significant difference (LSD) test (significance level α = 0.05).

RESULTS

Uninuclear and binuclear microspores (pollen grains) were isolated from anthers (Figure 2B) of *T. hungarica*. Scanning electron microscopy showed that microspores had an elongated elliptical shape (Figure 2C and D). Pollen grains are monosulcate, heteropolar, operculate (Figure 2D). The long axis of elipsoidal grains ranges from 39.25 to 56.07 µm, while the short axis ranges from 16.82 to 23.40 µm. The sulcus extends full length of the grain and is from 1.8 to 6.7 µm wide. Surface of sulcus membrana has a one-banded, operculate. The exine sculpture was perforate-striate (Figure 2E) with striae of different length and orientation. Exine thickness was from 1.9 to 2.1 µm.

Microspores treated with aceto-orceine, DAPI and FDA had oval like shapes due to glass cover. The smallest aceto-orcein treated microspore had diameter of 13.65 µm, while the largest pollen had diameter of 60.15 µm (Figure 2F). It has been suggested that microspores can be grouped in two classes: small, lightly staining (with aceto-orceine), and large densely staining (Figure 2F). Fifty percent of investigated *T. hungarica* microspores had one nucleus, and the other half had two nuclei after DAPI-staining (Figure 2G). Up to (80%) of FDA-treated microspores had a high level of viability (Figure 2H).

The variability of microspore size was confirmed by the presence of a bimodal distribution, with lower and higher peaks (Figure 3). The two characteristic peaks had different values (21.5 and 31.5 µm) for microspores derived from closed flower buds of the same length of 5 cm

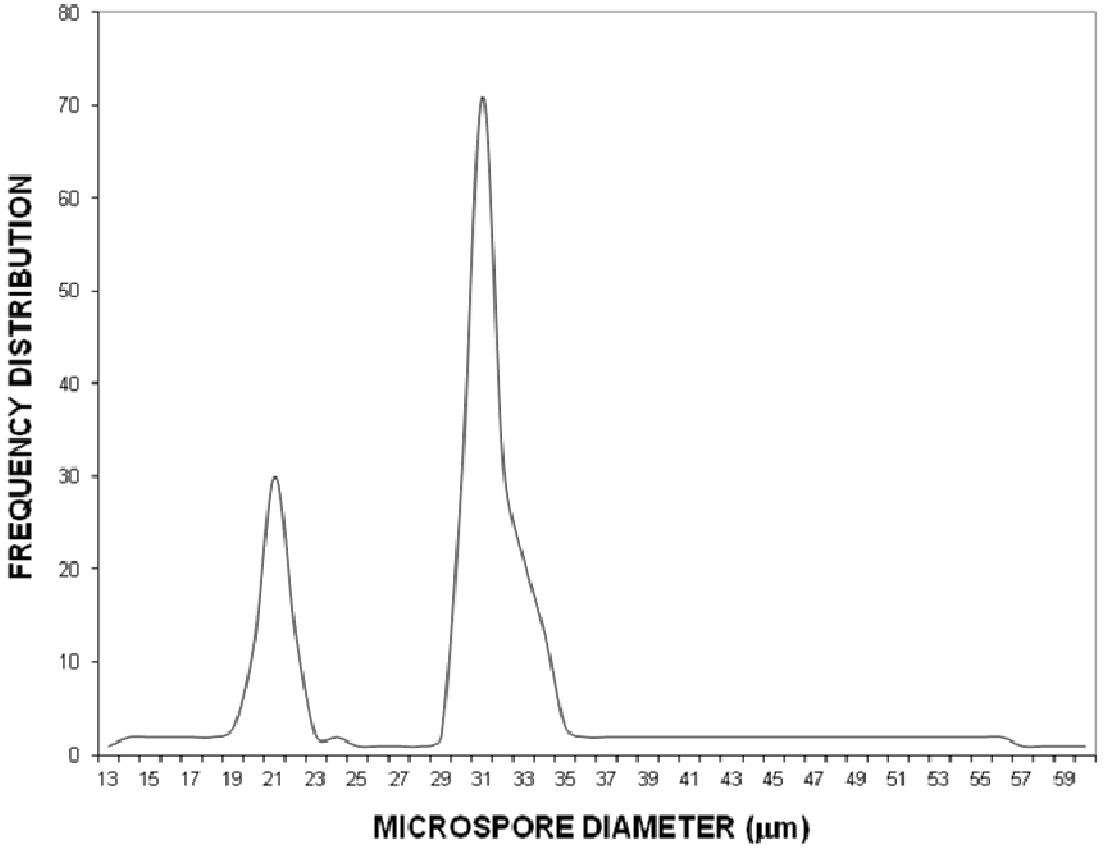

Figure 3. Bimodal distribution of *T. hungarica* microspore and pollen size.

(Figure 3).

DISCUSSION

Species *T. hungarica* posses a monosulcate pollen grains with single longitudinal aperture and perforate striate exine surface. The genus *Tulipa* is heterogeneous in both aperture type and exine ornamentation (Koshenko, 1999). The pattern of exine ornamentation and the structure of the aperture and its membrane are peculiar features for species and genera.

The presence of pollen dimorphism has been detected in anthers of many other species as *Zea mays* (Demerec, 1924), *Nicotiana tabacum* (Horner and Street, 1978), *Ephedra (*Ickert-Bond et al., 2003), *Thymus capitatus* (Karabournioti et al., 2007), *Vitis vinifera* (Gallardo et al., 2009).

Pollen dimorphism in *T. hungarica* may be in relation with the induction of androgenesis. Our results that microspores of *T. hungarica* isolated from flower buds showed differences in size, shape, staining intensity, fluorescence and viability are in agreement with results on *N. tabacum* (Horner and Street, 1978), *A. hippocastanum* (Radojević 1989, 1991; Radojević et al., 1989, 2000; Ćalić et al., 2003a), *A. carnea* (Marinković and Radojević, 1992), *A. flava* (Ćalić-Dragosavac et al., 2008), *Prunus armeniaca* (Asma, 2008) and *A. parviflora* (Ćalić-Dragosavac et al., 2009). Anthers of many species were cultured at the uninuclear microspore stage, and varying embryogenic potential was recorded (Höfer et al., 1999; Nägeli et al., 1999; Bueno et al., 2003; Ćalić et al., 2003b; Germana and Chiancone, 2003; Höfer, 2004). Growth of the microspores was accelerated after their release from the anthers, suggesting that anther walls contain some growth inhibitors. This means that further information at the level of endogenous hormones and other metabolites, not only in sporogenic but also in somatic anther tissue, is necessary for the full understanding of the androgenesis process and for to explain of which different factors affect this process (Radojević, 1991).

Microspore culturing proved to be an effective technology for the production of haploids, due to the successful separation of small embryogenic microspores which was critical for androgenesis induction.

Microspore separation based on size (small embryogenic and large nonembryogenic) and further research on the capacity of *T. hungarica* androgenesis and an *in vitro* and *in vivo* increase in the number of plants will be the

focus and goal of our future research.

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