



Original article

Pollen functional ability in two indigenous grapevine cultivars in Bosnia and Herzegovina

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Summary

A large number of grapevine cultivars have functionally female flowers. The causes of male sterility in these cultivars in the spheres of microsporogenesis and pollen morphology are only partially tackled. This study examines microsporogenesis and pollen functional ability in two most economically important indigenous grapevine cultivars in Bosnia and Herzegovina by the evaluation of permanent histological sections and the use of a scanning electron microscope. When observed at the cytogenetic and embryological level, microsporogenesis in cvs. Blatina with sterile pollen and Žilavka with fertile pollen shows regularity, they coincide and there are no differences in the sequence of differentiation events, except in their timing and duration. In 'Žilavka', the development of pollen in the anther locule at all sequential events, including pollen release from the tetrad, formation of the structural elements of the exine, physiological and morphological preparations for pollen to be dispersed and its release from the anthers, progresses normally with typical – vital cytogenetic characteristics maintained. In 'Blatina', during pollen development in the anther locule upon release from the tetrads and during the formation of the structural elements of the exine, uncontrolled coverage of pollen by the exine occurs, ultimately resulting in the formation of inaperturate pollen – morphologically sterile pollen grains.

Keywords

microsporogenesis, karyiokinesis, SEM, histology, morphological sterility

Significance of this study

What is already known on this subject?

- The grapevine cultivar Blatina with functionally female flowers have disfunctional pollen grains without pores.

What are the new findings?

- Cv. Blatina has inaperturate pollen regardless of normal microsporogenesis and normal cytogenetic characteristics of pollen grains.

What is the expected impact on horticulture?

- The fact that pollen of 'Blatina' has a normal cytogenetic constitution and does not germinate due to a complete coverage of germination pores with sporopollenin (exine) indicates the possibility of detecting markers for this phenomenon, which is important for the selection of grape wines.

Introduction

The grapevine flowers have a specific anatomy and they have a genetically and morphologically functional predisposition to self-pollination and self-fertilization. At the same time the large number of grapevine cultivars have functional female flowers, among which are many indigenous cultivars (Kevan et al., 1985; Caporali et al., 2003). These indigenous cultivars are mainly maintained in production together as locally grown genetic material because they are adapted to the conditions in which they are grown locally and can be pollinated and fertilized among themselves (Lombardo et al., 1976; Silva et al., 2001; Slimane-Harbi et al., 2004; Büyükkartal et al., 2005; Abreu et al., 2006). Most of the literature only partially tackled the causes of male sterility in these cultivars in the spheres of microsporogenesis and pollen morphology and no definitive explanation has been found in the sphere

of meiotic division (Büyükkartal et al., 2005). Male sterility in grapevine genotypes with functionally female flowers has been described in the literature and identified as morphological sterility, with statements made about pollen grains being spherical in shape and having no apertures (Lombardo et al., 1976, 1978; Kevan et al., 1985, 1988; Silva et al., 2001; Caporali et al., 2003; Slimane-Harbi et al., 2004; Büyükkartal et al., 2005; Abreu et al., 2006; Gallardo et al., 2009; Najmaddin et al., 2011). In some grapevine cultivars with functionally female flowers, irregularities have been detected in microsporogenesis, which are associated with tapetum degradation. Namely, Slimane-Harbi et al. (2004) observed that not until the stage of tetrad membrane dehiscence does tapetum degradation in cv. Rezzegui start. Büyükkartal et al. (2005) reported that tapetum degradation in cv. Cavaş does not occur until pollen is released from the anther locule, this being identified as the cause of pollen sterility. However, all these studies do not analyse the cytogenetic characteristics of these pollen grains.

This study involved a detailed cytohistological examination of the process of meiosis in microsporogenesis and endomitosis during tapetum formation and resorption in cultivar Blatina with sterile pollen and inaperturate pollen grains and cultivar Žilavka with functional pollen. At the same time, these two cultivars are the two most important grapevine cultivars in Bosnia and Herzegovina. Both cultivars are old autochthonous cultivars grown in the south-eastern part of Bosnia and Herzegovina (Herzegovina region) for centuries, primarily because they are used for the production of high-quality wines with geographical origin (Beljo et al., 2014). The aim of comparison of these processes with Blatina and Žilavka cultivars is to show in which sequence of the



pollen grain formation comes the disorder which ultimately results in the formation of inaperturate pollen grains of Blatina cultivar.

Material and methods

Grapevine inflorescence samples for laboratory analyses were collected from Ortiješ Vineyard in the south-eastern part of Bosnia and Herzegovina, Mostar region. Inflorescences of grapevines for histological slides were collected from April to middle of June in 2009 and 2010. Sampling was carried out at five-day intervals in April and at two-day intervals in May and June, between 9:00 and 10:30 am, from the shoot and inflorescence growth, flowering and pollination stages to the berry setting. 10 inflorescences of each cultivar were taken in each term.

A histological analysis of male gametophyte of grapevine cultivars was performed by a modified paraffin technique (Mičić, 1993), with fixing of inflorescences in fixative according to Navašin (1936) (1% chromic acid : formaldehyde : glacial acetic acid; in a ratio of 6 : 3 : 1) and preparing of permanent histological slides. The inclusion in paraffin (Merck, Germany, melting point 56°C) was done through a series of ethylalcohol and xylol solutions (Merck, Germany). Cutting of paraffin blocks to sections of 8–10 µm thickness was done on Leica RM 2245 Microtome (Leica Biosystems, Germany). The preparations were glued to the subject glasses by laboratory-prepared Majer Glue (Mičić, 1993). The preparations were subsequently dried at 32°C in a drier (Sutjeska, Serbia). The colouring of the preparations was preceded by

deparaffining with purification in ammonia solution of 60% ethyl alcohol and in saturated aqueous solution of picric acid (Merck, Germany). The colouring was performed with the laboratory-prepared Delafild's hematoxylin (Mičić, 1993). Colour differentiation was performed under a light microscope (Olympus SZH, Olympus, Japan) in acid solution of 70% ethyl alcohol (Merck, Germany). The preparations were incorporated into the Canadian Balm (Merck, Germany). The observation of histological sections and photodocumentation of the process were done using light microscope Leica DM 6000 B (Leica Biosystems, Germany) with magnification 1000–1.500×. A total of 1,850 permanent histological preparations were made and a total of 1,656 microphotographs of histological sections were recorded.

Anatomical and morphological pollen characteristics were analysed by a scanning electron microscope (SEM) JSM-6390 LV (Jeol, USA) at 500–5.000× magnification. Samples of pollen for analysis on SEM were taken during anther pollination (full blossoming) in both years and preserved in petri vessels prior to analysis. The preparations were readied for observation by placing a two-layer tape on the sample carrier, onto which pollen was applied with a brush. After that, vapour deposition of pollen with a layer of 20 nm thick gold was applied to the BAL-TEC SCD 005 (Bal-tec™, USA). Pollen of both cultivars was tested in dry and hydrated condition. Pollen hydration was carried out with 96% ethyl alcohol solution. 30 pollen grains were analysed for each cultivar. A total of 280 microphotographs of pollen were recorded.

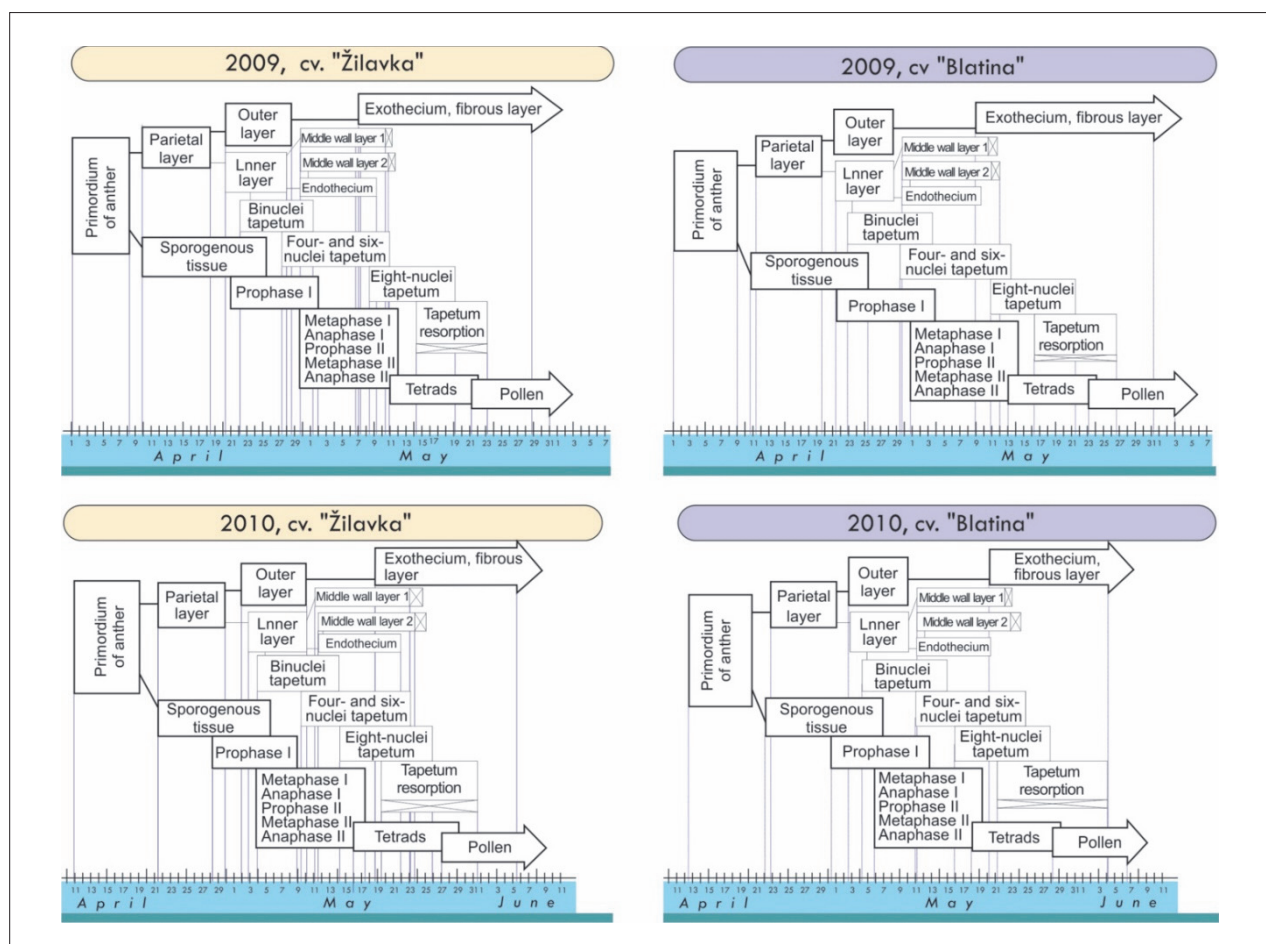


FIGURE 1. Algorithm of dynamics and flow of the process of microsporogenesis in grapevine cultivars Žilavka and Blatina in 2009 and 2010.

Results and discussion

The analyses of the microsporogenesis processes in cvs. Blatina and Žilavka on the cytogenetic and embryonic level in 2009 and 2010 show there is no difference between these two cultivars in the sequence of differentiation phases, except in their timing and duration, which suggests regularity and coinciding of the processes in both cultivars (Figure 1).

Endomitotic cycles in tapetal cells in both cultivars are coincide and have no abnormalities detected, i.e., endomitotic cycles in tapetal cells are synchronous with the process of karyokinesis in pollen mother cells. The final microsporogenesis phase includes dehiscence of the tapetal cell walls, release of their vacuole contents into the anther locule and subsequent release of young microspores from the tetrads (Figures 2 and 3).

Dehiscence of the callose membranes of tapetal cells takes place almost simultaneously with dehiscence of the callose membranes of the tetrads. This indicates that these events are synchronised to allow the development of microspores – young pollen grains in the anther locule after their release from the tetrads. Such events in microspore development have also been identified in other grapevine cultivars (Cardoso et al., 2010), as well as in representatives of wild grapevine populations (Caporali et al., 2003). As determined in *V. vinifera* ssp. *silvestris*, at the stage of microspores, the tapetal wall is completely resorbed, regardless of pollen fertility, i.e., fertile pollen (the pollen of male wild vine plants) or sterile pollen (the pollen of female wild vine plants).

The observations suggest that the microspores – young pollen grains, during their release from the tetrads, are of normal structure and constitution in both cultivars, i.e., in the course of microsporogenesis, the causes of pollen sterility in cv. Blatina cannot be detected and confirmed at this stage of observation.

After tapetal cell resorption, the pollen grains in 'Žilavka' undergo physiological and morphological preparations to be released from the anthers, basically involving the withdrawal of the exine from the colpus area into the pollen grain, i.e., colpus closure and, hence, aperture closure, with only the exine on the pollen grains protruding and upon dehiscence of the anther exothecia, closed pollen grains are released and pollen is at the dispersal stage (Figures 4 and 5).

The final stages of the physiological behaviour of pollen to be dispersed in 'Žilavka' observed in this study are normal and result in the formation of fertile pollen grains with closed colpi (Figure 6), as in many other fertile cultivars (Lombardo et al., 1976, 1978; Caporali et al., 2003; Abreu et al., 2006).

In 'Blatina', pollen grains undergo physiological and morphological behaviour to be released from the anthers, become completely covered by the structural elements of the exine, thus remaining without colpi and apertures and after tapetal cell resorption, when the pollen grains physiologically prepare themselves to be released from anthers, specifically, this process in 'Blatina' ends with the collapse of pollen grains (Figure 7).

The results on process of microsporogenesis in 'Blatina' in this research, i.e., the process of pollen grain development in the anther locules, pollen grain morphology and cytogenetic characteristics at the dispersal stage show that the pollen grains completely covered by the structural elements of the exine, which causes morphologically sterile pollen grains. These sterile pollen grains maintain normal cytogenetic characteristics until they are released from the anthers and the stigma becomes receptive. This research shows that the processes of microsporogenesis in Žilavka and Blatina cultivars, on a cytogenetic and embryologic level, are regular and mutually consistent. The difference in the processes of pollen formation in Žilavka and Blatina cultivars occurs at the moment

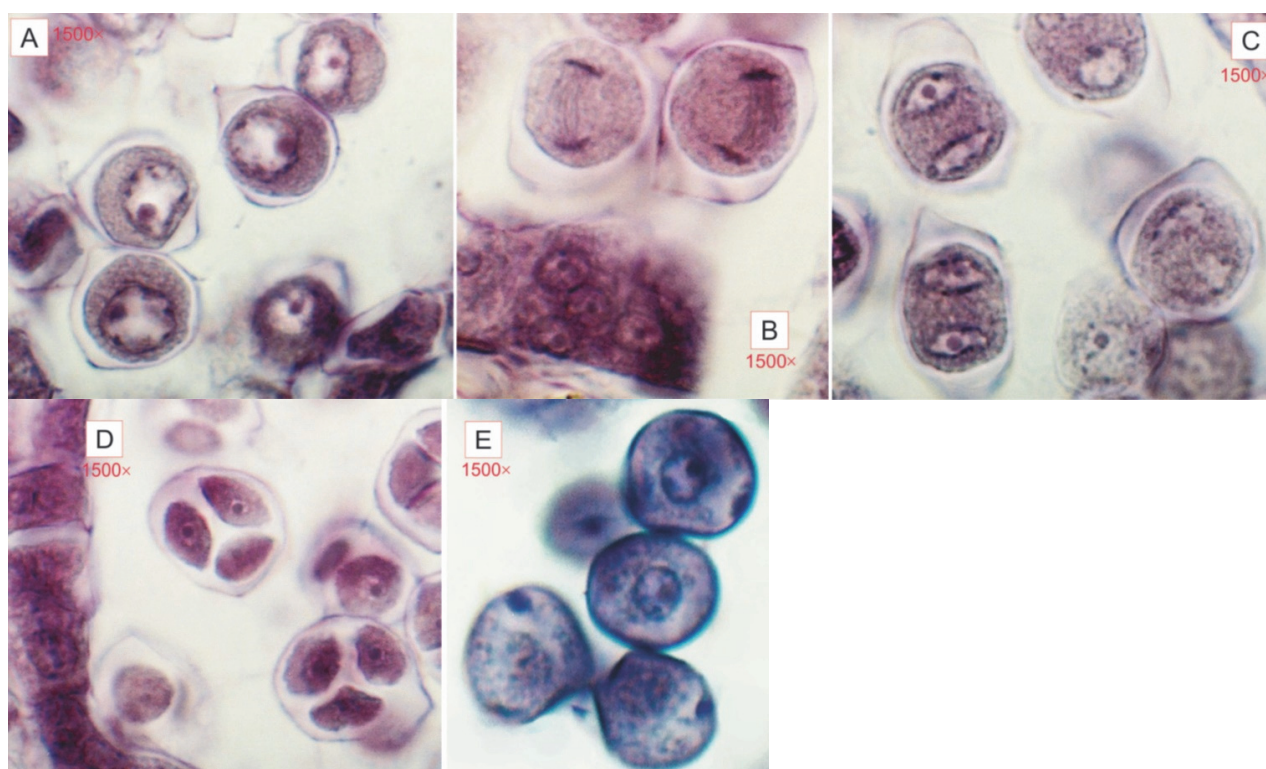


FIGURE 2. Basic stages of microsporogenesis in grapevine cultivar Blatina. A) prophase I – diakinesis; B) telophase I, with 4–8 nuclei in tapetal cells; C) interphase II – simultaneous-type meiotic division; D) tetrads; E) microspores immediately after release from the tetrads.

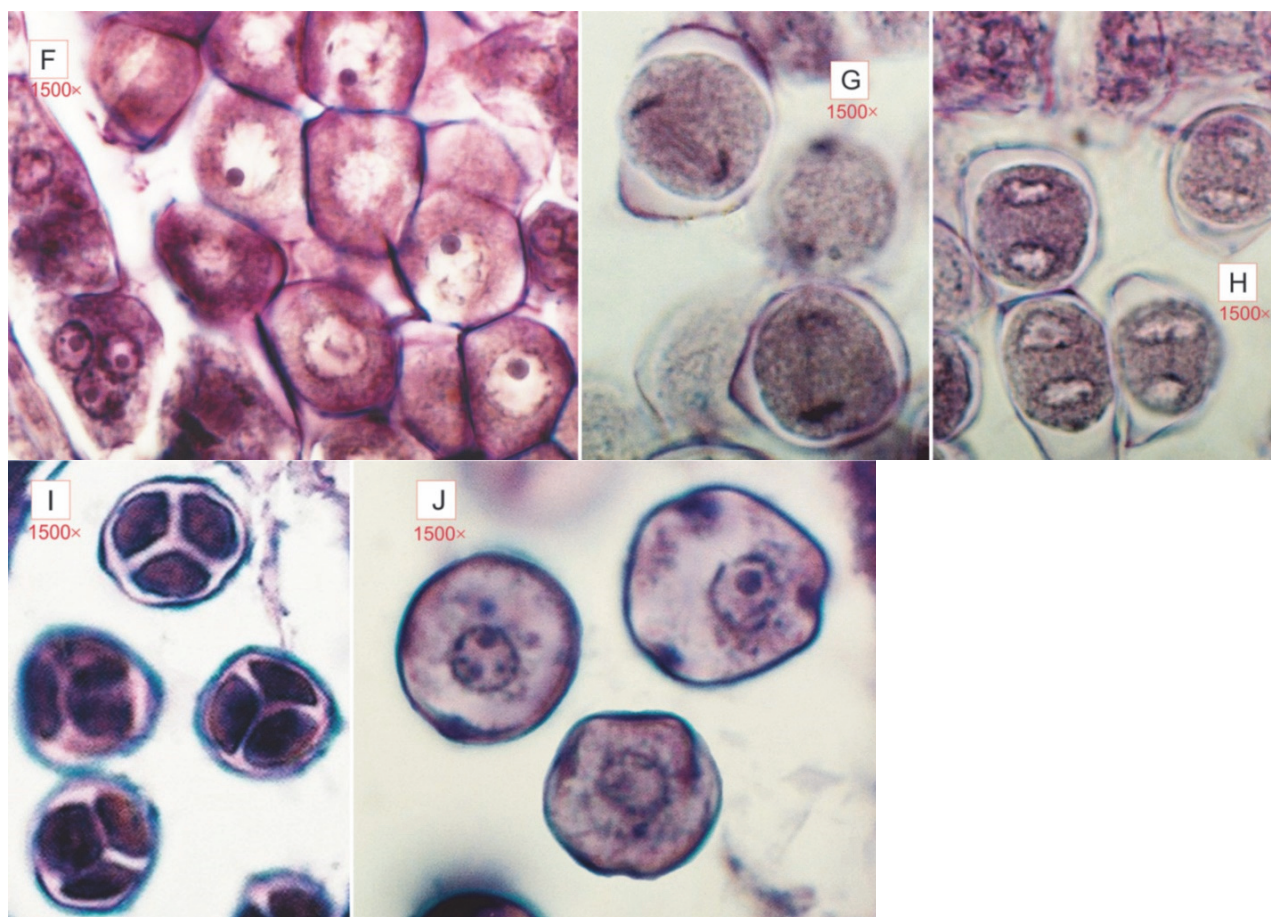


FIGURE 3. Basic stages of microsporogenesis in grapevine cultivar Žilavka. F) prophase I – diakinesis; G) telophase I, with 4–8 nuclei in tapetal cells; H) interphase II – simultaneous-type meiotic division; I) tetrads; J) microspores immediately after release from the tetrads.

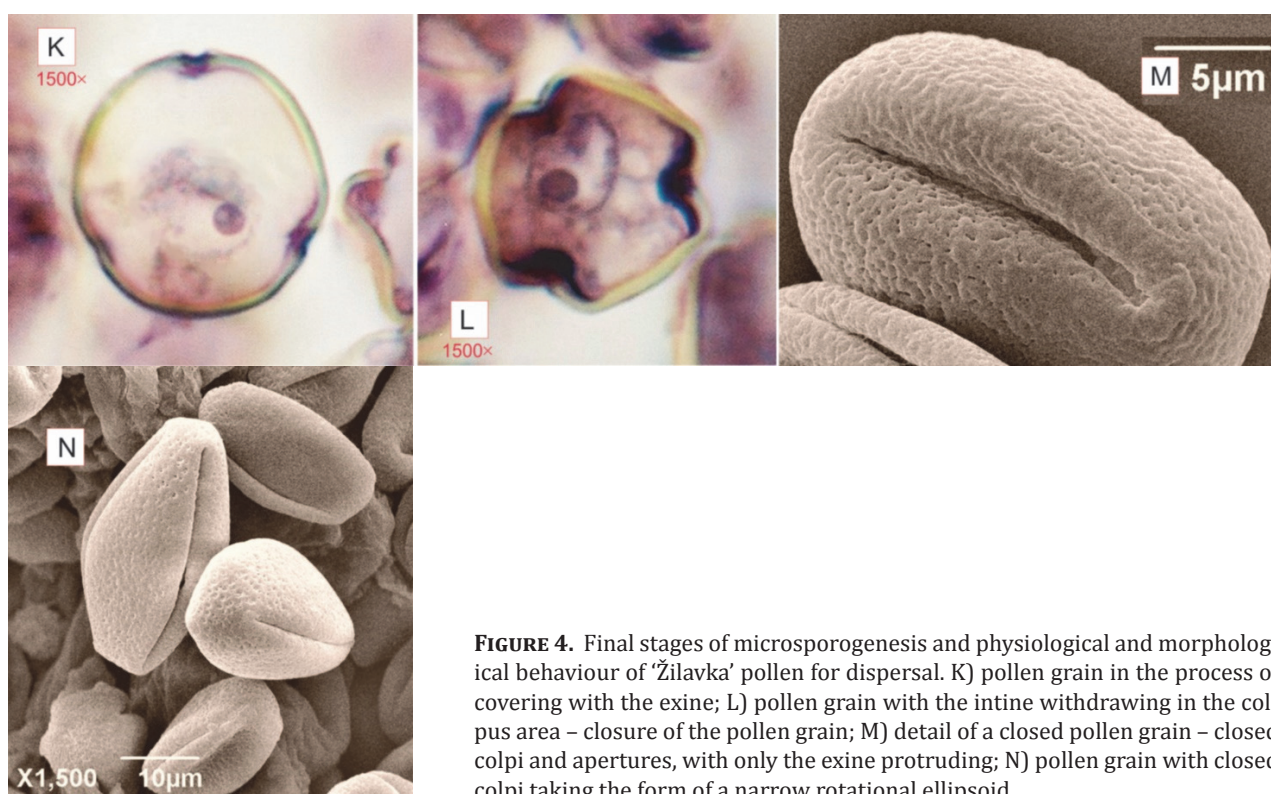


FIGURE 4. Final stages of microsporogenesis and physiological and morphological behaviour of 'Žilavka' pollen for dispersal. K) pollen grain in the process of covering with the exine; L) pollen grain with the intine withdrawing in the colpus area – closure of the pollen grain; M) detail of a closed pollen grain – closed colpi and apertures, with only the exine protruding; N) pollen grain with closed colpi taking the form of a narrow rotational ellipsoid.

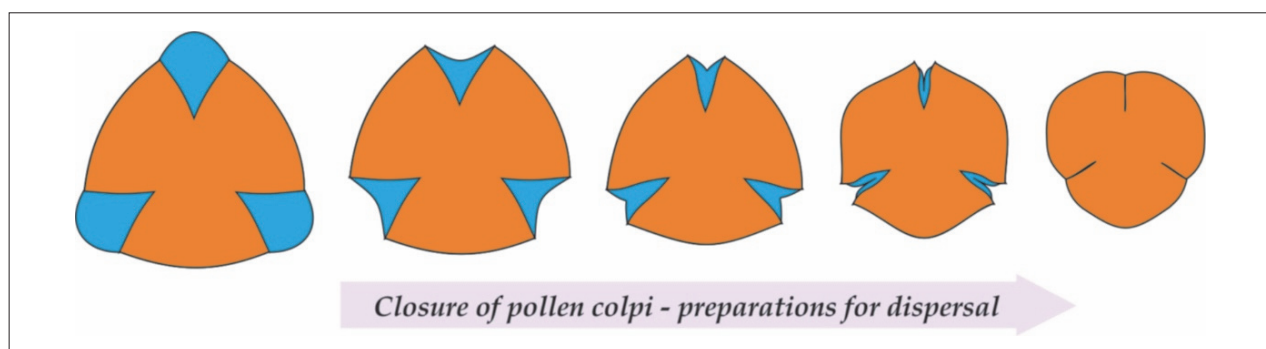


FIGURE 5. Graphical presentation of the sequence of the pollen grain closure in the process of physiological and morphological behaviour for the release from anthera and dispersal in cultivar Žilavka. In the process of physiological behaviour for dispersal, pollen is released from excessive water (facilitated transport), whilst the closing of colpi and the openings for germination protects the interior of the pollen and puts structural elements of the exine in the first place as an element of susceptibility.

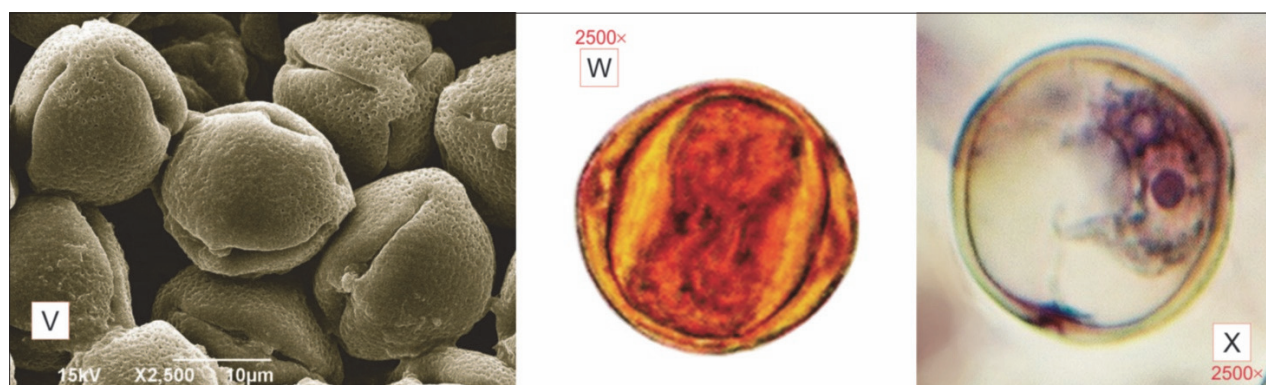


FIGURE 6. Pollen grains of 'Žilavka' hydrating on the stigma: V) partially hydrated pollen grains at the event of colpus opening; W) hydrated pollen grains are spherical to slightly elliptic, and at this stage of development, functionally able pollen grains are binucleate (X).

of physiological preparation of pollen for dispersal (Figure 8).

Pollen grain sterility in cv. Blatina can be identified as morphological sterility, since pollen grains confirm normal and maintainable cytogenetic characteristics in the following sequence of developmental events: 1) immediately after release from the tetrads; 2) undergoing coverage by the structural elements of the exine; 3) physiological behaviour for pollen to be released from the anthers – collapsed pollen grains; and 4) upon hydration of collapsed pollen grains. Thus, in our research, pollen grain sterility of cv. Blatina can be identified as morphological sterility, since the complete coverage of the grain by the structural elements of the exine acts as a mechanical obstacle to germination, while pollen grains in all developmental events have typical cytogenetic characteristics.

Conclusion

The study of the process of microsporogenesis, tapetum resorption, the development of pollen grains in the anther after release from the tetrads, the physiological and morphological preparation of anther release and spreading, the morphology of pollen to be dispersed and hydration on the stem beam, as well as cytogenetic constitution in all sequences of development, in Blatina cultivar with sterile pollen and Žilavka cultivar with fertile pollen shows as follows:

- Processes of microsporogenesis observed at the cytogenetic and embryologic level do not differ in the sequences of differentiation, except in the time of their occurrence and duration, therefore it can be concluded that the process of microsporogenesis in both cultivars is regular and

mutually consistent.

- Endomytotic cycles in the tapetum cells in both cultivars are consistent and without the observed anomalies, i.e., endomytotic cycles of the tapetum cells follow the process of karyokinesis in pollen mother cells.
- In Žilavka cultivar, the development of pollen in the anther locule from the release from the tetrad, the formation of structural elements of exine, physiological and morphological preparation for dispersal (closure of colpi and germination aperture), until release from the anther, flows normally with a typical – vital cytogenetic constitution.
- In Blatina cultivar in the process of pollen development in the anther locule upon release from the tetrads and during the process of formation of structural elements of the exine, uncontrolled coverage of pollen by the exine occurs, ultimately resulting in the formation of pollen without colpi and germination apertures – pollen grains are morphologically sterile.
- Morphologically sterile pollen grains in Blatina cultivar, in all development sequences, from the physiological preparation for dispersal, the release from the anther and hydration on the gynoecium of the stigma, maintain a typical – vital cytogenetic constitution, but germination is prevented because they are completely covered with structural elements of exine.

Acknowledgments

The work has been supported by the Ministry of Science and Technology of the Republic of Srpska within the projects "Anatomorphological and cytohistological evaluation of

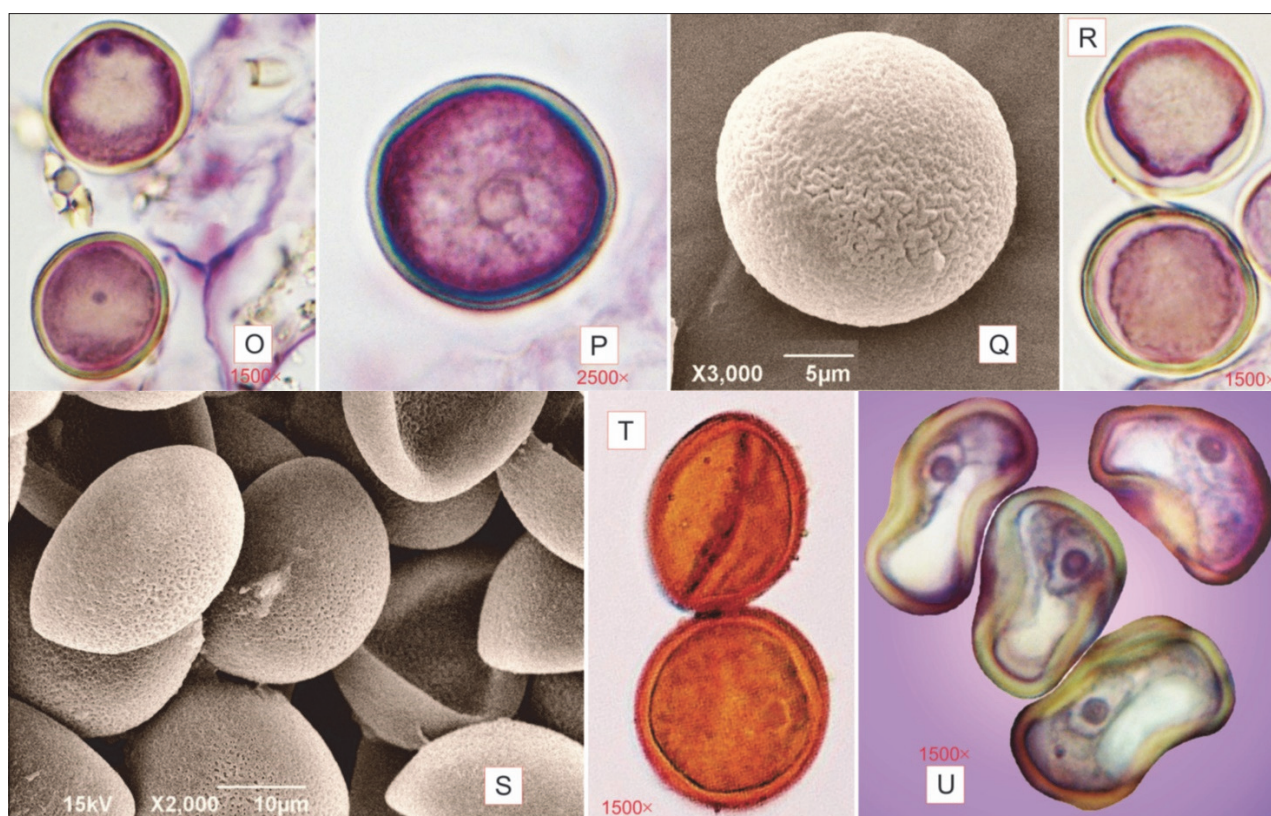


FIGURE 7. Final stages of microsporogenesis and physiological and morphological behaviour of 'Blatina' pollen for dispersal: O) pollen grain being covered by the external layer – the exine; P) pollen grains have normal cytogenetic characteristics prior to physiological and morphological behaviour for their dispersal; Q) pollen grains completely covered by the exine – no differentiated colpi and apertures formed; R) pollen grains have no colpi, i.e., during exine formation, the pollen grain is enclosed by the structural elements of the exine; S) pollen grains of 'Blatina' after physiological behaviour for dispersal take the form of collapsed pollen grains; T) however, histological sections of pollen grains in the collapsed state show that their cytogenetic characteristic is preserved; U) morphologically irregular forms of inaperturate pollen grains after physiological preparations for their release from the anthers, described as collapsed pollen grains, are the result of complete coverage of pollen grains by the structural elements of the exine.

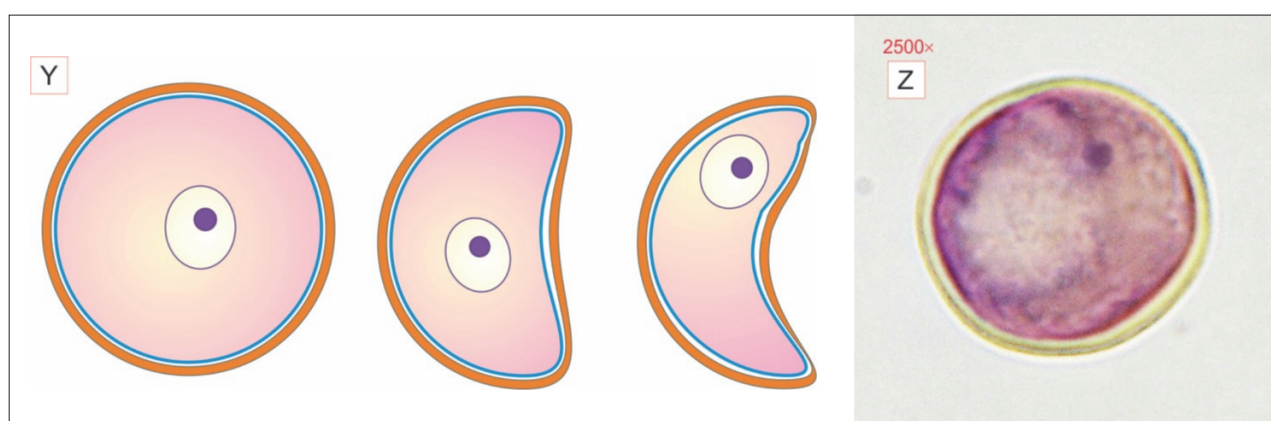


FIGURE 8. Graphic presentation of the establishment of a "collapsed" form of inaperturate pollen grains of 'Blatina' in the process of physiological preparation of pollen for release from anthera (Y). By hydration, inaperturate pollen grains have normal physiological and cytogenetic characteristics, but germination is not possible because pollen is completely covered by structural elements of exine (Z).

reproductive organs of indigenous grape cultivars in Bosnia and Herzegovina” (Contract No. 06/6-020/961-119/08) and “Genotype characterization of cv. Blatina male sterility” (Contract No. 19/6-020/961-177/09).

References

- Abreu, I., Costa, I., Oliveira, M., Cunha, M., and de Castro, R. (2006). Ultrastructure and germination of *Vitis vinifera* L. cv. Loureiro pollen. *Protoplasma* 228, 131–135. <https://doi.org/10.1007/s00709-006-0167-1>.
- Beljo, J., Mandić, A., Jovanović-Cvetković, T., Dodig, R., Gašpar, M., Ivanković, M., Jakirović, A., Lasić, V., Leko, M., Nikić, A., Prusina, T., and Sivrić, M. (2014). Atlas of Viticulture and Wine of Bosnia and Herzegovina (University of Mostar, FRAM ZIRAL), ISBN 978-9958-16-030-1, 216 str.
- Büyükkartal, H.N., Cölgeçen, H., and Marasali, B. (2005). Development of anther wall throughout microsporogenesis in *Vitis vinifera* L. cv. Çavuş. *Int. J. Agric. and Biol.* 4, 616–620. https://www.fspublishers.org/published_papers/9453_.pdf.
- Caporali, E., Spada, A., Marziani, G., Failla, O., and Scienza, A. (2003). The arrest of development of abortive reproductive organs in the unisexual flower of *Vitis vinifera* ssp. *silvestris*. *Sexual Plant Reprod.* 15, 291–300. <https://doi.org/10.1007/s00497-003-0169-5>.
- Cardoso, H.G., Campos, M.C., Pais, M.S., and Peixe, A. (2010). Use of morphometric parameters for tracking ovule and microspore evolution in grapevine (*Vitis vinifera* L. cv. Aragonez) and evaluation of their potential to improve in vitro somatic embryogenesis efficiency from gametophyte tissues. *In Vitro Cell. Dev. Biol.-Plant.* 46, 499–508. <https://doi.org/10.1007/s11627-010-9295-6>.
- Gallardo, A., Ocete, R., Ángeles Lopez, M., Lara, M., and Rivera, D. (2009). Assessment of pollen dimorphism in populations of *Vitis vinifera* L. subsp. *silvestris* (Gmelin) Hegi in Spain. *Vitis* 48, 59–62. <http://pub.jki.bund.de/index.php/VITIS/article/view/4146>.
- Kevan, P.G., Longair, R.W., and Gadawsky, M.R. (1985). Dioecy and pollen dimorphism in *Vitis riparia* (Vitaceae). *Can. J. Bot.* 63, 2263–2267. <https://doi.org/10.1139/b85-321>.
- Kevan, P., Blades, D., Posluszny, A., and Ambrose, J. (1988). Pollen dimorphism and dioecy in *Vitis aestivalis*. *Vitis* 27, 143–146. <http://pub.jki.bund.de/index.php/VITIS/article/view/5891>.
- Lombardo, G., Cararro, L., Carnagello, G., and Bassi, M. (1976). Ultrastructure of pollen of *Vitis vinifera* L. cv. ‘Picolit giallo’ and its behaviour in experiments of self-and cross-pollination. *Vitis* 15, 73–81. <http://www.vitis-vea.de/admin/volltext/e010992.pdf>.
- Lombardo, G., Cargnello, G., Bassi, M., Gerola, F.M., and Carraro, L. (1978). Pollen ultrastructure in different vine cultivars with low productivity. *Vitis* 17, 221–228. <http://pub.jki.bund.de/index.php/VITIS/article/view/6605/6192>.
- Mičić, N. (1993). The plum organogenesis. Doctoral dissertation (Novi Sad: University of Novi Sad Faculty of Agriculture).
- Najmaddin, C., Khatijah, H., and Maideen, H. (2011). Comparative study on the anatomy and palynology of the three varieties of *Vitis vinifera* variety (family Vitaceae). *African J. Biotechnol.* 10, 16866–16874. <https://doi.org/10.5897/AJB11.2315>.
- Navashin, M.S. (1936). [Методика цитологического исследования для селекционных целей]. М., “ОГИЗ-Сельхозгиз”.
- Silva, P.R., Bione, N., Silva, N., and Paglaini, M.S. (2001). Meiotic behaviour of the Brazilian table grape cultivar Rubi (*Vitis vinifera* L.) with a high proportion of seedless berries. *Vitis* 40, 1–4. <http://pub.jki.bund.de/index.php/VITIS/article/view/4484/4417>.
- Slimane-Harbi, M.B., Chabbouh, N., Snoussi, H., Bessis, R., and Gazzah, M.E. (2004). Etude du germoplasme de vignes autochtones de Tunisie. Précisions sur l’origine du millerandage du «Razzegui».
- Bulletin O.I.V. 77, 487–501. <http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.472.668&rep=rep1&type=pdf>.

Received: Feb. 14, 2017

Accepted: Nov. 14, 2017

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