

# Reproductive potential of the functionally female native Croatian grapevine 'Grk bijeli'

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## Summary

**A native Croatian grapevine 'Grk bijeli', sharing a parent-offspring relationships with 'Tribidrag' (aka 'Zinfandel'), is grown exclusively on the Adriatic island of Korčula. It is one of the grape cultivars with female-only functional flowers, causing reduced fertilisation and leads to problems in grape production and wine quality. A typical cluster of 'Grk bijeli' at maturity contains fully developed seeded berries, in addition to a highly variable proportion of undersized seedless berries. The aim of this study was to identify the cause of the reduced reproduction potential of 'Grk bijeli' in order to establish a better growing environment for improved yield and grape quality. 'Grk bijeli' female gametophyte develops normally and at maturity contains both egg and central cell, together with two synergid cells and three antipodal cells. On the other hand, 'Grk bijeli' pollen grains show developmental deviations. Specifically, most of the pollen grains undergo the first pollen mitosis and contain one vegetative cell and one generative cell, while 20 % of ungerminating grains also accomplish the second pollen mitosis, giving rise to two sperm cells and one vegetative cell. Moreover, 'Grk bijeli' pollen has acolporate morphology, which prevents germination and contributes to reduced reproduction. Furthermore, fertilisation after pollination with other varieties results in various degrees of ovule abortion depending on the pollinator, revealing Croatian cultivars 'Plavac mali crni' and 'Pošip bijeli' as favourable varieties. Although this study describes a highly valuable cultivar of local importance, it also contributes to fundamental knowledge of grapevine reproductive biology and offers a strategy for improvement of wine production and oenological performance of semi-fertile varieties in general.**

**Key words:** *Vitis vinifera*; gametophyte; ovule; fertilisation; stenopericarp; parthenocarpy.

## Introduction

In flowering plants, including grapevine, haploid spores produced by meiosis (sporogenesis) undergo three succe-

sive mitotic divisions (gametogenesis) giving rise to the multicellular gametophytes, which are the basis for double fertilisation. Female megagametophyte, also called the embryo sac, is produced in an ovule located within a pistil ovary. As in *Arabidopsis*, grapevine has a polygonum type female gametophyte (PRATT 1971). It originates from a single haploid spore, which undergoes three incomplete mitotic division cycles to develop into an eight-nucleate syncytium (YADEGARI and DREWS 2004, MA and SUNDARESAN 2010). Following cellularisation, the distinct cell types are formed: two synergid cells and one egg cell, which are located at the pollen tube entrance point (micropyle), three antipodals, which are formed at the opposite chalazal end, and a large diploid cell, which occupies the central region (YANG *et al.* 2010). Male gametophyte or pollen grain develops within an anther from a single haploid microspore, which undergoes two mitotic divisions. Tricellular pollen grain contains two sperm cells within the cytoplasm of a larger vegetative cell. In grapevine, under optimal climatic conditions, pollen formation and development starts from sporogenous cells in the anthers, as soon as the inflorescences emerge from the growing shoot (TELLO *et al.* 2018). Meiosis starts after a few days, giving rise to tetrads, which rapidly release microspores in the loculus. Microspores vacuolate thereafter, and pollen mitosis begins just before anthesis, when pollen grains are mature enough to be released from the anthers (LEBON *et al.* 2004, 2005). Before germination, in hermaphrodite grapevine varieties pollen grain is normally tricolporate and bicellular, consisting of a large vegetative cell and a small generative cell, which divides into two haploid sperm cells during pollen germination (ABREU *et al.* 2006).

Pollen germination involves guided polar growth, which enables the pollen tube to reach and penetrate the embryo sac, where its bursts and sperm cells are delivered to the vicinity of the two female gametes. During the process of double fertilisation, one sperm cell fuses with the haploid egg cell, triggering embryogenesis, and the second sperm cell fuses with the diploid central cell, producing the endosperm.

*Vitis vinifera* (Vitaceae family) is one of the most important cultivated fruit species with a long history of cultivation (first evidence dates from 6,000-5,000 B.C.; MC GOVERN and MONDAVI 2007). Cultivation in different climate conditions, genome instability, natural and human selection and vegetative propagation over a long time peri-

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od created a striking number of *Vitis vinifera* cultivars that exist today. According to the "The International Organisation of Vine and Wine" (OIV 2017), about 6,000 varieties exist, while at least 5,000 unique genotypes are proposed based on DNA profiling data (THIS *et al.* 2006). Wild *Vitis vinifera* are dioecious with either male or female flowers on separate plants (CARMONA *et al.* 2008, RAMOS *et al.* 2014), while the majority of cultivated grapevine cultivars possess hermaphrodite flowers with well-developed male and female organs and gametophytes (HEAZLEWOOD and WILSON 2004, MENEGHETTI *et al.* 2006, VASCONCELOS *et al.* 2009). Those cultivars are fit for self-pollination and successful fertilisation which ensures a "normal" proportion (> 50 %) of ovaries in the inflorescence that become seeded fruits (BESSIS 1993, VASCONCELOS *et al.* 2009, DRY *et al.* 2010).

However, in rare *Vitis vinifera* varieties one set of reproductive organs undergoes developmental arrest, leading to the formation of nonfunctional organs (CAPORALI *et al.* 2003). It is known that *Vitis vinifera* is a predominantly self-pollinating (self-compatible) species (PRATT 1971, MAY 2000, MUNOZ-RODRIGUEZ *et al.* 2011) with only 1-2 % of cross-pollination (HEAZLEWOOD and WILSON 2004, CHKHARTISHVILI *et al.* 2006, MUNOZ-RODRIGUEZ *et al.* 2011). Therefore, defects in development of flower organs or gametophytes lead to reduced fertilisation (VAROQUAUX *et al.* 2000) leading to a significant proportion of undeveloped and seedless berries in some *Vitis vinifera* cultivars (MAY 2000, TELLO *et al.* 2018).

The appearance of seedless berries could be a consequence of parthenocarpy (BOUQUET and DANGLLOT 1996, VAROQUAUX *et al.* 2000, VARGAS *et al.* 2007), a process that represents fruit development without fertilisation and gives rise to small berries with no seeds at all. Two types of parthenocarpy have been described in grapevine: a vegetative (autonomic) parthenocarpy in which fruit develops without any pollination ultimately producing a purely vegetative structure; and a stimulative parthenocarpy in which pollination does occur, but pollen grains fail to germinate (STOUT 1936).

However, seedless berries can also occur in some fertile cultivars or cross-pollinated female cultivars as an outcome of stenospermocarpy (PRATT 1971, MAY 2000, VASCONCELOS *et al.* 2009, MUNOZ-RODRIGUEZ *et al.* 2011) where fertilisation occurs, but embryo development is blocked, resulting in varying degrees of perceptible seed traces or no seeds whatsoever (PRATT 1971, TIAN *et al.* 2008).

*Vitis vinifera* 'Grk bijeli' is an autochthonous Croatian grapevine cultivar grown almost exclusively on the island of Korčula. Although its name ('Grk bijeli' in Croatian means 'Greek') and long cultivation history suggest that the cultivar was probably introduced by the Greek during the colonisation of Korčula, because of its close genetic relationship with *Vitis vinifera* 'Tribidrag' (aka 'Zinfandel'; MALENICA *et al.* 2011), and its relationship with a whole group of Dalmatian cultivars (MALETIĆ *et al.* 2004), it is almost certain that *Vitis vinifera* 'Grk bijeli' is an indigenous Croatian variety. Despite the relatively small population of 'Grk bijeli' (the most productive vineyards are located exclusively on sandy soils near the Korčula town of Lumbarda), its wine is considered to be of the highest quality standard (PREINER *et al.* 2012).

Cultivar 'Grk bijeli' possesses morphologically hermaphrodite flowers with only female parts appropriately developed, while stamens are characterised with bent filaments that prevent anthers to outgrow and successfully pollinate the stigma (PREINER *et al.* 2012, MIROŠEVIĆ *et al.* 2012). Clusters of *Vitis vinifera* 'Grk bijeli' at full maturity contain a combination of fully developed seeded berries and a variable proportion of undersized seedless berries (locally called 'pasoline'). These seedless berries, when present in optimal proportion in the cluster, positively influence the chemical composition of the grape and have a very positive impact on wine quality (PREINER *et al.* 2012, MALETIĆ *et al.* 2015).

The aims of this study were to analyse and describe the reproductive potential of Croatian autochthonous grapevine 'Grk bijeli' by evaluating male and female fertility, and to study the course of its postpollination/postfertilisation development. We showed that due to pollen sterility, 'Grk bijeli' is preferentially a cross-pollinated variety in which seedless berries are a consequence of either fertilisation absence (parthenocarpy), or of ovule abortion after cross-pollination (stenospermocarpy), where the frequency of ovule abortion depends on the cultivar used as a pollen donor.

This work represents the first study of *Vitis vinifera* 'Grk bijeli' reproductive biology and will be used for optimising growing conditions and pollination environment in order to obtain an appropriate ratio of normal and undersized berries that will ensure the best oenological characteristics. In addition, detailed morphological and developmental observations made in this study will route future molecular studies of 'Grk bijeli' reproductive potential, but could be also applicable to similar grapevine varieties of local importance such as Blatina (JOVANOVIĆ-CVETKOVIĆ *et al.* 2016), Dišeća ranina and Cetinka (MALETIĆ *et al.* 2015) or numerous other varieties with functionally female flowers (TISCHELMAYER 1998).

## Material and Methods

All experiments were performed during 2012 and 2013 on samples collected from the National collection of indigenous grape varieties of the Republic of Croatia "Jazbina", Faculty of Agronomy, University of Zagreb, located on the southern foothill of mountain Medvednica. In this environment, adjacent to vines of 'Grk bijeli' (IIVC variety number 5066) on both sides are 'Plavac mali crni', 'Ljutun crni', 'Primitivo', 'Malvazija istarska' and 'Ranfol'. The evaluation of fertilisation success in hand-pollinated 'Grk bijeli' clusters was performed in the experimental vineyard, located in Baštica (near Zadar, Dalmatia), established in 2008. Rootstock is Kober 5BB. In this environment, 'Grk bijeli' grows isolated from other cultivars.

**Morphometric analysis:** Ovules, ovaries and berries were characterized in three *Vitis vinifera* cultivars: 'Grk bijeli', 'Plavac mali crni' and 'Chardonnay'. Ovules and ovaries were isolated from unopened flowers, at flower opening and multiple times after flower opening, up to 20 days after pollination (DAP). The size of ovules and young ovaries was measured as a diameter, using a binocular microscope upgraded with a Zeiss camera (AxioCam ERc 5c)

and AxioVision software. The mean values are based on measurement of 50 randomly selected ovules or ovaries.

**Female gametophyte analysis:** Cytological observations of ovules and female gametophytes were carried out on *Vitis vinifera* 'Grk bijeli', 'Plavac mali crni' and 'Chardonnay'. The aim was to reveal the structure and development of ovules and embryo sacs 5 DAP. The most developed ovule was isolated from an individual ovary and analysed.

For each cultivar, a total of 70 ovules was analysed. In addition, for cultivar 'Grk bijeli', 70 ovules from undersized ovaries were analysed at the same time point. Collected samples were fixed in modified FAA (formaldehyde – acetic acid – ethanol) solution (90 mL ethanol, 2 mL formaldehyde and 5 mL acetic acid) for 24 h. After fixation, the samples were gradually dehydrated by immersing in a series of solutions containing increasing concentrations of ethanol, for 20 min each (50 %, 75 % and 96 % v/v). Thereafter, samples were immersed in chloroform overnight. Paraffin embedding and sectioning of plant tissue was performed according to protocol: [www.molecularinfo.com/MTM/J/J2/J2-1/J2-1-2.html](http://www.molecularinfo.com/MTM/J/J2/J2-1/J2-1-2.html) (Copy Right © 2001/ Institute of Molecular Development LLC), in serial longitudinal and cross-sections of 6-8  $\mu\text{m}$  thickness. Sections were transferred to a water bath (56 °C) for 1 min, mounted on a microscope slide pretreated with adhesive substance and left to dry overnight. Paraffin was removed from the tissue sections by immersing in xylol and was gradually rehydrated by washing in solutions with decreasing concentrations of ethanol (from 96 %, 80 %, 70 %, 60 % to 50 % v/v). Before microscopy, tissue sections were treated with 10  $\mu\text{g}\cdot\text{mL}^{-1}$  DAPI (4',6-diamidino-2-phenylindole).

**Male gametophyte analysis:** Male gametophyte, pollen viability and germination were analysed in *Vitis vinifera* 'Grk bijeli', 'Plavac mali crni' and 'Chardonnay'. Pollen was collected in the field from inflorescences with fully opened flowers by strong shedding in paper bags.

The germination ability of pollen grains was tested in a germination medium containing a 40 % sucrose solution with 200  $\text{mg}\cdot\text{L}^{-1}$  of boric acid and 600  $\text{mg}\cdot\text{L}^{-1}$  of calcium nitrate ( $\text{CaNO}_3$ ). The pollen grains were applied on the surface of the media. To protect pollen integrity, the pollen grains were covered with a cover glass after 2 min of hydration (except for propidium iodide staining) and left in a humid chamber at 25 °C until the analysis. The germination rate was determined after 1 h and 16 h.

Pollen grains were scored as germinated when the length of the pollen tube was more than twice the diameter of the pollen grain. For each cultivar, 200 pollen grains were analysed. To determine the number of nuclei, existence of furrows and viability rate, pollen was stained with propidium iodide and fluorescein diacetate. Propidium iodide (100  $\mu\text{g}\cdot\text{mL}^{-1}$  aqueous solution) was added directly to the medium to a final concentration of 10  $\mu\text{g}\cdot\text{mL}^{-1}$  and pollen grains were covered with a cover glass immediately, omitting the hydration step, to enable penetration of the dye into the pollen grains. Fluorescein diacetate (2  $\text{mg}\cdot\text{mL}^{-1}$  dissolved in acetone) was added to the pollen solution in a final concentration of 2  $\mu\text{g}\cdot\text{mL}^{-1}$ . Prepared samples were examined under the microscope.

**Grapevine seed germination:** The seeds were separated from the flesh of ripe grapevine berries and left to dry overnight at room temperature (RT). The following day, the dry remaining berry flesh was removed from the seeds and the seeds were then left to fully dry at RT for 2 weeks on a paper towel in an open Petry dish. After drying, the seeds were immersed in distilled water for 5 d at 4-8 °C in a conical 50 mL tube (Falcon). Seeds were rinsed daily with distilled water. Finally, the water was drained and wet seeds were tightly closed in a conical tube and stratified at 4-8 °C for 2-3 months. During the incubation period, seeds were washed in distilled water if fungal contamination was observed. After the stratification period, seeds were placed on 2-3 layers of wet filter paper in a Petry dish, sealed with Parafilm and incubated at 28 °C in the dark for 5-6 d. After germination, the seeds were transferred to soil-filled pots.

**Chromosome number determination:** Chromosome number was determined from the root meristems of seedlings generated after hand pollination of 'Grk bijeli' with pollen of 'Plavac mali crni' and 'Chardonnay', respectively. Root tips were pre-treated with a 2 mM aqueous solution of 8 hydroxyquinoline for 2 h at 4 °C and 2 h at 20 °C. After the pretreatment, the root tips were fixed in Carnoy's solution containing ethanol and acetic acid (3 : 1 v/v) for at least 24 h at room temperature and stored in 70 % ethanol at -20 °C. Chromosome preparations were done according to MLINAREC *et al.* (2006). Chromosomes were stained with DAPI (2  $\mu\text{g}\cdot\text{mL}^{-1}$ ) for 15 min at RT. The preparations were mounted in Dako Fluorescence Mounting Medium (Dako North America Inc., USA) and stored at 4 °C. At least 10 metaphase cells were observed and the best metaphase plates were photographed.

**Microscopy:** Images were acquired by Axiovert 200 M fluorescence microscope operated by the AxioVision software 4.5 (Zeiss, Göttingen, Germany). The filter set 02 for DAPI (excitation 365 nm, emission LP420), filter set 38 for FDA (excitation 470/40 nm, emission BP 525/50) and set 14 for propidium iodide (excitation BP 510-560, emission LP 590) were used. All images were recorded using an AxioCam camera (MrC, Zeiss, Göttingen, Germany).

**Fertilisation success in hand-pollinated 'Grk bijeli' clusters:** In order to determine the phenological compatibility, *Vitis vinifera* 'Grk bijeli' flowers were pollinated with pollen of the 'Plavac mali crni', 'Pošip bijeli' and 'Chardonnay' (in collection of indigenous grape varieties "Baštica") during the flowering period. Four basal clusters on each of the 40 randomly selected 'Grk bijeli' vines were emasculated 10 d before anthesis. The isolation was carried out by fixing paper bags around the clusters. For hand pollination, anthers from donor plants ('Chardonnay', 'Plavac mali crni' and 'Pošip bijeli') were isolated from flowers at anthesis and pollen grains were applied with a tiny brush on the pistil stigma of isolated clusters. On every vine, each isolated cluster was pollinated with pollen of a different cultivar, while the fourth cluster was left in conditions of isolation, without pollination and used as a control. The paper bags were removed from the clusters two weeks after full bloom to ensure normal development. The clusters from the experiments were harvested at full maturity and the percentage of fertilised berries with seeds was calculated.

**Statistical analyses:** Data are presented as mean  $\pm$  SE of at least two independent experiments with two biological replicates, and have been analysed using ANOVA and the Duncan's Multiple Range (DMRT) Test (Statistica 7.1, StaSoft, USA). Significance level was determined at the  $P \leq 0.05$  level.

## Results and Discussion

**'Grk bijeli' flowers – ovary and anther development:** The reproductive organs in *Vitis vinifera* flowers, five stamens and a bicarpellate pistil, develop completely hidden inside the calyptra until the anthesis when the calyptra is pushed away and flower organs become visible, indicating the maturity and morphology of the flower organs (MULLINS *et al.* 1998).

In functionally female 'Grk bijeli' at anthesis, the pistil was well developed consisting of a large ovary and a short style with a papillate stigma. The stigma was yellowish in color, with the appearance of secretion. Three days after flower opening, the stigma darkened, indicating the optimal fertilisation time. The stamen appearance was typical for a morphological hermaphrodite and functional female flower, with bent filaments, which did not outgrow the pistil stigma (PREINER *et al.* 2012). At flower opening, anthers were yellowish to light brown in color, then underwent intensive darkening, and in most cases would fall off after three days.

**Post-pollination ovary growth and development in 'Grk bijeli':** Reaching maturity, clusters of 'Grk bijeli' contain fully developed and seeded berries, approximately 16 mm in diameter and undersized seedless berries, 7 mm in diameter. The proportion of seedless berries is variable between different clones selected from Korčula vineyards, differing from 28 % to 85 % of total berry number (PREINER *et al.* 2012).

The appearance of underdeveloped seedless berries is a known characteristic in many vine varieties, but it rarely occurs at levels that exist in 'Grk bijeli'. Although underdeveloped berries are rare and insignificant for production in 'Chardonnay', MAY (2004) classifies *Vitis vinifera* berries in seven categories according to the period when berries and seeds have been blocked in development. The largest berries belong to category 1 and together with categories 2, 3 and 4 are defined as normal. Category 5 consists of those berries that are far smaller than the category 4, but they mature and thus contribute to the cluster mass. Category 6 includes green berries, whereas category 7 comprises dried, black berries that do not fall off. Differences in berry categories are correlated with differences in seed content. Categories 5 and 6 contain no seeds or only seed traces, depending on the post-pollination time point at which the seed development was arrested. Another, simpler categorisation is given for *Vitis vinifera* 'Merlot'. It includes three groups: normal seeded berries with diameter between 15-17 mm at harvest time ("hens"); mid-sized seedless and ripe berries between 4-7 mm at harvest time ("chickens"), and small berries of diameter 2-3 mm ("shot") (CHOLET *et al.* 1998, 2002). The same author states that the minimum size for a normally fertilised and fully developed berry is 9 mm at

maturity. According to classification given by MAY (2004), undersized 'Grk bijeli' berries belong to group 5, or to "chickens" according to CHOLET (1998, 2002). The improper seed development inside an underdeveloped berry, or its complete decay, depends mostly on the success of pollination and fertilisation, the properties influenced by physiological processes such as male or female sterility, parthenocarpy, stenospemocarpy, insufficient plant nutrition, etc. (KIMURA *et al.* 1997, MAY 2004, DA SILVA 2001, ABREU *et al.* 2006). At the time of fertilisation, which was determined by the color change of the stigma, the diameter of berries in 'Grk bijeli' and 'Plavac mali crni' were between 1.5 and 2.0 mm. This size was approximately 0.5 mm bigger than the size of corresponding stage in 'Chardonnay'.

Three days after pollination, most of 'Grk bijeli' ovaries were between 2.0 and 2.5 mm in diameter. In contrast to roundish berries with a regular number of four ovules (Fig. 1a; PRATT 1971), 25 % of 'Grk bijeli' berries and 47 % of 'Plavac mali crni' berries contained five or six normally developed ovules (Fig. 1b). Those berries had a shape of double cones fused at the prominent central part. At 5 DAP, obvious diversification in berry diameter started to appear (Fig. 1e). In addition to berries that were 3-4 mm in diameter, we observed the "undersized" berries of less than 3 mm in diameter. More significant divergence was obvious 10 DAP when at least one ovule was developed (approx. dimensions 2 mm x 3 mm) in normal berries (Fig. 1c), in contrast to none developed ovules (0.2 mm) in undersized berries (Fig. 1d). Intensive or decreased growth and almost double the size difference between normal (7.5 mm) and undersized berries (4.0 mm) occurred extensively between 8 and 15 DAP (Fig. 1e). This period coincides with the rapid endosperm development as described for seedless 'Concord'. Later, the endosperm undergoes subsequent degeneration due to failed fertilisation (BARRITT 1970).

Diameter and growth dynamics of undersized berries in field-grown conditions (exposed to pollination) was positively correlated with berry diameter of clusters which were mechanically isolated (bagged) to prevent cross-pollination. Accordingly, the "normal"-sized berry diameters were positively correlated with the diameter of hand-pollinated berries in controlled pollination experiments using 'Chardonnay', 'Plavac mali crni' or 'Pošip bijeli' pollen. Berries from bagged clusters reached a diameter of approximately 3 mm at 10 DAP. Similar to undersized berries in exposed conditions, they did not contain any developing ovules. Instead, the ovules underwent subsequent growth cessation and dehydration resulting in ovules no wider than 0.2 mm 10 DAP, as shown in Fig. 1d. These results indicate that the absence of fertilization leads to immediate and synchronized degeneration of all ovules in an individual ovary, followed by their complete disappearance up to 15 DAP. This kind of early and synchronised ovule degeneration was observed in 57.1 % of 'Grk bijeli' ovaries that were exposed to cross-pollination (Fig. 1f), while in 42.9 % of the ovaries at least one ovule, considered as fertilised, continued to develop (Fig. 1c, f). For comparison, immediate and synchronised ovule degeneration in 'Chardonnay' was only 7.1 %, while in 'Plavac mali crni' 47.4 % of ovules underwent synchronised degeneration (Fig. 1f).

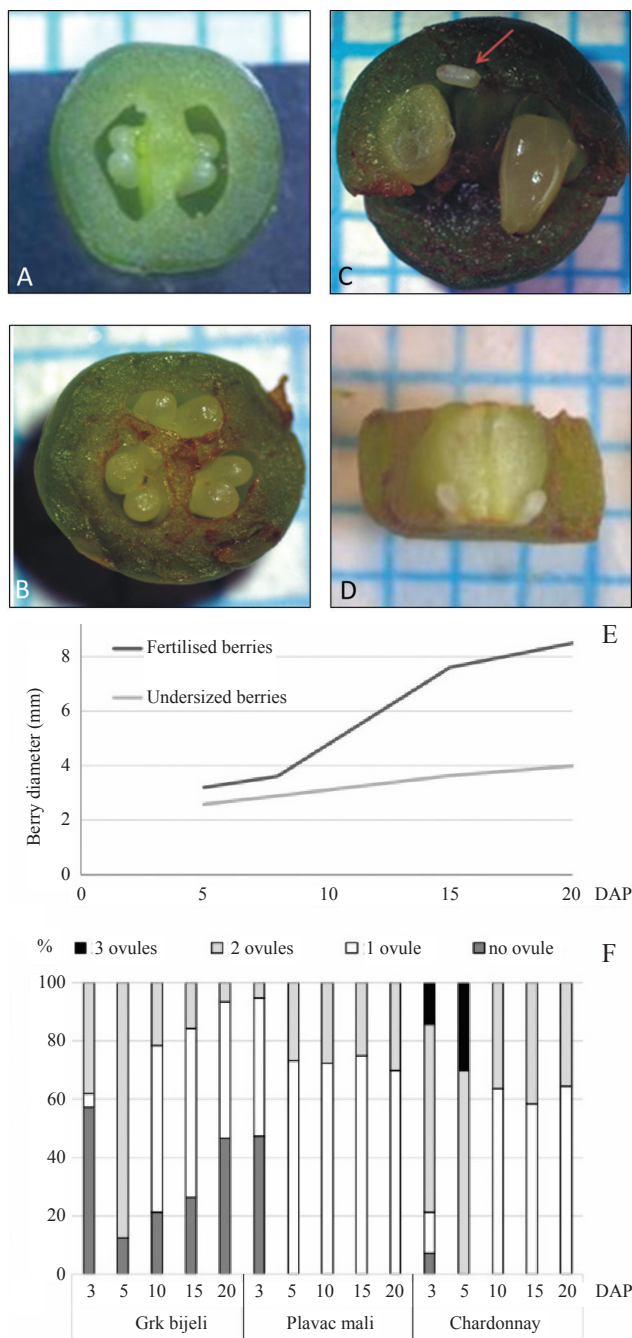


Fig. 1: Growth dynamics for normal and undersized berries of 'Grk bijeli'. Ovaries with 4 (A) and 6 (B) ovules isolated 3 DAP. Fertilised ovary containing normal and aborted ovules (C) and undersized ovary with 4 degenerated ovules (D) 10 DAP. Ovary growth represented as berry diameter (E) and dynamics of ovule abortion represented as a percentage of ovaries with a particular ovule number (F) between 3 and 20 DAP.

In optimal environmental conditions, the absence of fertilisation is caused either by the absence of pollination (vegetative parthenocarpy) or by functionally inadequate pollen germination (stimulative parthenocarpy). Vegetative parthenocarpy is a phenomenon that generally enhances fruit abscission (DRY *et al.* 2010, DAUELSBERG *et al.* 2011). In 'Grk bijeli', fruit abscission of seedless berries did not occur in natural field-grown conditions. However, fruit abscission was absent even in controlled conditions where pollination was mechanically prevented and the only possible mecha-

nism driving the production of seedless berries was vegetative parthenocarpy. Because their expected dispersal from the cluster was inhibited, these berries eventually contributed to the total cluster mass of 'Grk bijeli'. In the case of stenospERMOCARPIC varieties, a normal process of fertilisation and initial seed development occurs primarily due to the early onset of endosperm development (BARRITT 1970), but at a certain point this development ceases and seed growth halts. Beside abnormal endosperm development, degeneration of integuments, nucellus and embryo sac occur (BARRITT 1970, CARRARO *et al.* 1979, LEDBEITER *et al.* 1994, KORKUTAL 2005).

As mentioned above, 5 DAP 42.9 % of 'Grk bijeli' ovaries (3-4 mm in diameter) were considered as fertilised and normally growing. Among them 87.5 % contained 2 developing ovules while 12.5 % did not contain developing ovules (Fig. 1f). Ten DAP, 21.4 % ovaries (4-5 mm in diameter) did not contain developing ovules, 21.4 % contained two developing ovules, while 57.1 % contained only one developing ovule. After this developmental stage, an obvious decrease in number of developing ovules occurred, leading to the subsequent increase of berries with no developing ovules at later stages. Thus, at 20 DAP in 'Grk bijeli', 46.7 % of berries were either 1-ovuled or containing only ovule traces, while only 6.6 % contained 2 developing ovules. At the same developmental stage in 'Plavac mali crni' and 'Chardonnay', only 1-ovuled and 2-ovuled berries were present (Fig. 1f). Seed decay in 'Grk bijeli' after regular fertilisation was most intensive between 15 and 20 DAP (double increase in number of ovaries with no developing ovules, Fig. 1f), which is in agreement with stenospERMOCARPIC development described by BARRITT (1970). The author's observation that endosperm degeneration starts after a week of its rapid development (between 8 and 15 DAP) and precludes its cellularisation, supports our findings in 'Grk bijeli' where an intensive growth period between 8 and 15 DAP was followed by synchronised degeneration of fertilised ovules in a high proportion of ovaries. Since embryogenesis in *V. vinifera* begins no earlier than 14 DAP and in some varieties even one month after pollination, the cause of stenospERMOCARPIC ovule abortion in 'Grk bijeli' is most likely aborted endosperm development, which occurs before the onset of embryogenesis. As stated above, 57.1 % of 'Grk bijeli' ovaries were aborted up to 5 DAP. Of the remaining 42.9 % (classified as fertilised), an additional 46.7 % ceased to develop in the time between 5 and 20 DAP. In summary, these potentially seedless berries comprised 77.1 %, while successfully fertilised seeded berries comprised only 22.9 % of the cluster. The statistical data presented above refers to results obtained in environmental conditions of vineyard "Jazbina", as described.

'Grk bijeli' pollen accomplishes microgametogenesis but is unable to germinate: Grapevine pollen grain shape is subspherical to triangular due to the presence of three furrows with the circular pores with narrow but distinct costae (tricolporate morphology; ABREU *et al.* 2006, GALLARDO *et al.* 2009). Inside the anthers, the pollen grain is bicellular, consisting of a large vegetative cell and a small generative cell. In some *Vitis vinifera* cultivars, acolporate round shaped pollen or pollen polymorphism are observed (LOMBARDO *et al.*

1978, CAPORALI *et al.* 2003, ABREU *et al.* 2006, ALVA *et al.* 2015). Often, the sterile pollen in female varieties shows additional post-meiotic irregularities such as degeneration of the generative, vegetative or both nuclei, and the absence of the secondary mitotic division of the generative cell (DA SILVA 2001), which normally gives two haploid sperm cells during pollen germination (CRESTI and CIAMPOLINI 1999). Here we analysed the pollen cellular structure, morphology, viability and germination capability in cultivars 'Grk bijeli', 'Plavac mali crni' and 'Chardonnay'. Two nuclei were detected before germination in mature pollen grains of all tested cultivars, and generative and vegetative nuclei could be distinguished according to their shapes. A vegetative nucleus is regularly oval-shaped while the generative nucleus is lens-shaped (CHKHARTISHVILI *et al.* 2006). In some pollen grains of tested cultivars, the difference in nuclear shape was obvious (Fig. 2), clearly distinguishing the flattened generative cell (arrow on Fig. 2a-c). However, in 'Grk bijeli', more than 20 % of pollen grains were in the trinucleate stage (arrowhead on Fig. 2c), which indicates that pollen mitosis II occurred in ungerminated pollen grains. This data shows that 'Grk bijeli' grains have functional meiotic and mitotic development unlike a pollen sterile 'Corinto bianco' (a parthenocarpic seedless somatic variant of seeded 'Pedro Ximenes'), in which a set of genes responsible for pollen development and maturation is downregulated along with genes that control pollen tube growth (ROYO *et al.* 2016). Before the analysis of pollen germination capacity, we analysed the metabolic activity in pollen grain by FDA reagent staining in germination-induced pollen grains. The intensive metabolic activity (indicated by the fluorescent signal) was confirmed in 97 %, 78 % and 68 % of pollen grains in 'Chardonnay', 'Plavac mali crni' and 'Grk bijeli', respectively (Fig. 3a, c, e). However, in germinating pollen grains of 'Chardonnay', the fluorescence was localized in the cytoplasm at the pollen-germinating zone (Fig. 3a). Moreover, 'Chardonnay' had a very

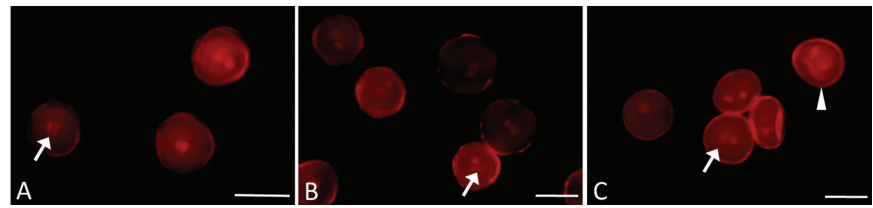


Fig. 2: Propidium iodide staining of 'Chardonnay' (A), 'Plavac mali crni' (B) and 'Grk bijeli' (C) pollen grains. The generative lens-shaped nuclei are indicated by arrows. A three nuclear pollen grain in 'Grk bijeli' is indicated by an arrowhead (C). Scale bar 20  $\mu$ m.

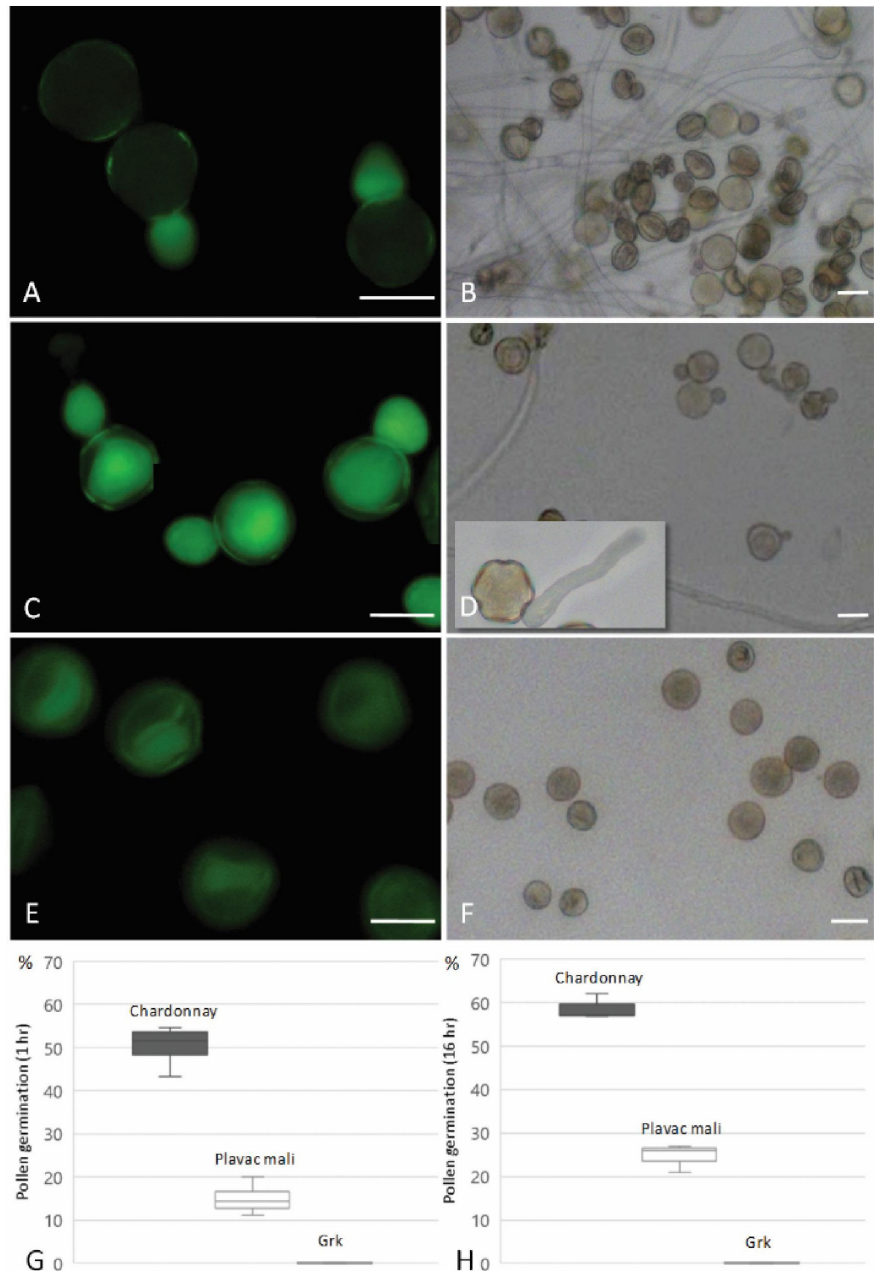


Fig. 3: Pollen viability and germination. Fluorescein diacetate staining of *Vitis vinifera* 'Chardonnay' (A), 'Plavac mali crni' (C) and 'Grk bijeli' (E) pollen. Green colour represents viable pollen grains and high metabolic activity. *In vitro* pollen germination of 'Chardonnay' (B), 'Plavac mali crni' (D) and 'Grk bijeli' (F) pollen after 16 h of incubation on the germination medium at 25 °C. Percentage of germination after 1 h (G) and 16 h (H) of incubation at 25 °C. The represented data from three independent replicates are visualised using boxplots. The mainbox indicates the upper and lower quartile, and the whiskers the upper and lower extreme. Scale bar 20  $\mu$ m.

fast onset of germination and more than 50 % of 'Chardonnay' pollen grains germinated within 60 min cultivation on the induction medium (Fig. 3b, g). This germination rate did not substantially increase during longer (16 h) cultivation on the germinating medium (Fig. 3h). In 'Plavac mali crni' the germination rate was on average 15 % in the first hour, but increased after 16 h, when 23 % of pollen grains have further elongated their pollen tubes (Fig 3d, g, h). However, pollen of 'Grk bijeli' did not germinate even after 16 h of stimulation on the pollen germinating medium (Fig. 3f, g, h). This was probably due to an acolporated pollen morphology (Fig. 3e, f), in which the exine formed a continuous layer, as opposed to a tricolporate pollen morphology of 'Plavac mali crni' (Fig. 3d, inset) and 'Chardonnay'. The lack of germination as a consequence of germinating pore absence in pollen grains and the impossibility to develop a pollen tube was first described by LOMBARDO *et al.* (1978) in functionally female cultivars 'Picolit', 'Bicane', 'Moscato rosa' and in 'Loureiro' (ABREU *et al.* 2006).

Our data show that microgametogenesis occurs properly and pollen grains of 'Grk bijeli' undergo regular pollen mitosis I and even pollen mitosis II. However, the absence of pollen grain furrow most likely blocks the ability of pollen germination, which makes this cultivar male sterile, disabling delivery of sperm cells to the embryo sac.

'Grk bijeli' female gametophyte is properly developed in both undersized and normal berries: Low grape productivity has also been attributed to anomalous development of the female gametophyte and ovule (MAY 2004, ABREU *et al.* 2006). Thus, we analysed ovule development and morphology of the embryo to inspect possible female defects which could prevent seed formation in 'Grk bijeli'.

Because embryo development starts not earlier than 20 DAP (PRATT 1971, BARRITT 1970), our analysis of ovule and embryo sac morphology were conducted 5-20 DAP. Five DAP we separately analysed fertilised ovaries of 3-4 mm in diameter and "undersized" unfertilised ovaries that were smaller than 3 mm. In order to identify regularity in the development of the female gametophyte, we compared the data obtained for 'Grk bijeli', 'Plavac mali crni' and 'Chardonnay'. In all three cultivars, the longitudinal sections of ovules revealed the existence of a normally developed egg

apparatus (egg cell and two synergid cells), the central cell located towards the chalazal pole, and three antipodals at the chalazal pole of the embryo sac (Fig. 4a, b, c). We did not observe any difference in morphology of the embryo sac that could indicate irregular development of undersized or normal berries in 'Grk bijeli'.

The contribution of regularly developed embryo sacs in 'Grk bijeli' was 77.1 % in fertilised and 68.6 % in undersized ovaries, which was higher than the contribution of regularly developed embryo sacs in 'Plavac mali crni' (62.9 %). As expected, 'Chardonnay' showed the best developmental characteristics with 94.3% of regularly developed embryo sacs (Tab. 1).

In approximately 17.1 % of ovules in 'Plavac mali crni' and in undersized ovaries of 'Grk bijeli', degeneration of nucellus and ovule (Fig. 4f) was observed. Additionally, separation of nucellus and integuments (Fig 4a, arrow) or inner and outer integuments (Fig. 4d, arrowhead) was found in normal and undersized 'Grk bijeli' (8.6 %) and 'Plavac mali crni' (5.7 %) ovaries but never in 'Chardonnay' (Tab. 1).

The absence of an embryo sac due to the developmental arrest was detected in *Vitis vinifera* 'Plavac mali crni' (14.3 %) and in undersized 'Grk bijeli' ovaries (5.7 %) (Fig. 4d, e). Developmental arrest during meiotic stages of female gametophyte development that leads to the absence of a mature embryo sac at full bloom is described as an important cause of the high proportion of seedless berries in *Vitis vinifera* 'Corinto bianco'. On the other hand, the sporadic appearance of seeded berries in 'Corinto bianco' produces mostly tetraploid progeny, showing that viable seeds are formed by fertilisation of unreduced gametes produced by meiotic diplospory (ROYO *et al.* 2016).

The chromosome analysis of *Vitis vinifera* 'Grk bijeli' progeny after controlled fertilisation by 'Plavac mali crni' or 'Chardonnay' pollen revealed their diploid nature ( $2n = 38$ , Fig. 5) which additionally confirms normal meiotic division and generation of haploid gametes during development of the female gametophyte. Taken together, the obtained results confirmed normal female gametophyte development in more than 60 % of ovules during both meiotic and mitotic stages in all inspected cultivars. In the undersized berries of 'Grk bijeli', defects were represented at 10 % higher rate than in normal berries.

Table 1  
Degeneration categories of ovules and embryo sacs in berries at 5 DAP.  
Only the most developed ovule in each ovary was analysed

Cultivar	Ovule/embryo sac development				
	Normal	Arrested/no embryo sac <sup>a</sup>	Degenerated nucellus/ovules <sup>b</sup>	Detached nucellus/integuments <sup>c</sup>	Number of ovules examined
Grk bijeli (normal)	54 (77.1%)	-	10 (14.3%)	6 (8.6%)	70
Grk bijeli (undersized)	48 (68.6%)	4 (5.7%)	12 (17.1%)	6 (8.6%)	70
Plavac mali crni	44 (62.9%)	10 (14.3%)	12 (17.1%)	4 (5.7%)	70
Chardonnay	66 (94.3%)	-	4 (5.7%)	-	70

<sup>a</sup>Abnormalities refer to the lack of embryo sac.

<sup>b</sup>Abnormalities refer to compact nucellus and abnormal shape of ovule.

<sup>c</sup>Abnormalities refer to detachment of nucellus from integuments and between integuments.

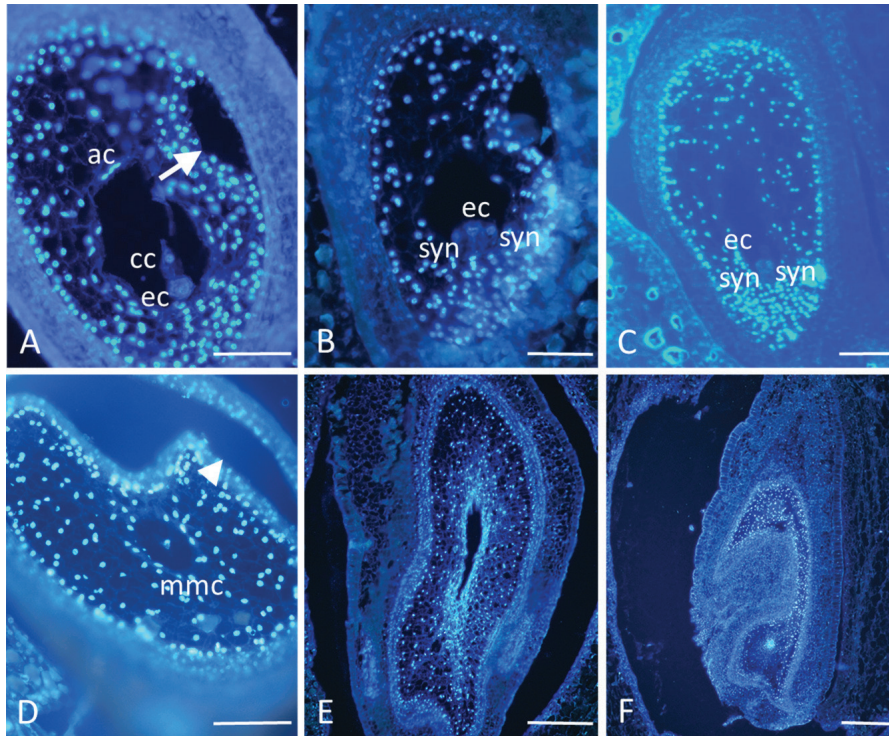


Fig. 4: Megagametophyte morphology in *Vitis vinifera* 'Plavac mali crni' (A, E) and 'Grk bijeli' (B, C, D, F) after DAPI staining. A mature embryo sac of 'Plavac mali crni' (A) and 'Grk bijeli' (B, C) contained the three antipodals (ac), central cell (cc), egg cell (ec) and synergid cells (syn). A non functional ovule of 'Grk bijeli' in developmental arrest, one containing arrested megaspore mother cell (mmc) (D), the other with no embryo sac (E). A non functional degenerated ovule of 'Grk bijeli' with compact nucellus and no embryo sac (F). Arrow represents detachment of nucellus from integuments and arrowhead detachment between integuments. Scale bar 20 µm.

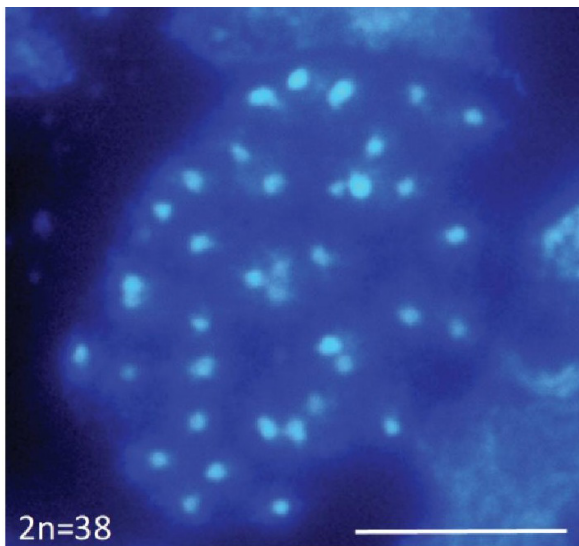


Fig. 5: Chromosome number in root cells of seedling germinated after controlled pollination of *Vitis vinifera* 'Grk bijeli' with *Vitis vinifera* 'Chardonnay' pollen revealed diploid ( $2n = 38$ ) progeny. Scale bar 10 µm.

Determination of fertilisation success in *Vitis vinifera* 'Grk bijeli' after controlled pollination: Cultivars with functionally female flowers are dependent on allogamy with cultivars that produce viable and functional pollen grains. For better fertilisation rates, it is necessary to grow these cultivars in close proximity to suitable hermaphrodite varieties for better pollination (BOEHM 1960). Three cultivars

('Chardonnay', 'Plavac mali crni' and 'Pošip bijeli') were used as pollen donors during two seasons (2012, 2013). The experiment was done in Baštica collection located in Zadar region. In 2012, the flowering time course was correlated for 'Grk bijeli', 'Plavac mali crni' and 'Pošip bijeli', while full bloom in 'Chardonnay' occurred four days earlier. In 2013, 'Chardonnay' was again in full bloom 4 d earlier than 'Grk bijeli'. For 'Plavac mali crni' and 'Pošip bijeli', full bloom occurred four days after 'Grk bijeli'. For hand pollination, pollen was collected from anthers of a particular cultivar and applied to the mature pistil (yellowish in color, with the appearance of secretion) of emasculated flowers of 'Grk bijeli'. Fertilisation success was expressed as the percentage of seeded berries in clusters at full maturity (Tab. 2). The

Table 2

Fertilisation success of 'Grk bijeli' expressed as percentage of seeded berries in clusters at full maturity after exclusive pollination with different pollen donors

Pollen donor	Seeded berries (%)		
	2012	2013	Average
No pollination	0	0	0
Chardonnay	17.27 b*	2.19 b	9.73 b
Plavac mali crni	49.54 a	4.36 b	26.95 a
Pošip bijeli	45.17 a	10.19 a	27.68 a

\* The mean values marked with different letters differ statistically with  $p < 0.05$  using Duncan's multiple range test.



largest number of seeded berries in 2012 was determined in clusters where 'Plavac mali crni' (49.54 %) and 'Pošip bijeli' (45.17 %) were used as pollinators, with no significant difference between the two. However, a significantly lower percentage of seeded berries was obtained after pollination with 'Chardonnay' pollen (17.27 %). In 2013, the fertilisation success of all three pollen donor cultivars were significantly reduced. 'Chardonnay' was again confirmed as the weakest pollinator of 'Grk bijeli' with only 2.19 % of seeded berries. Fertilisation success after pollination with 'Plavac mali crni' pollen grains was significantly reduced (4.36 %) compared to the previous year. In 2013 'Pošip bijeli' induced a significantly higher proportion of seeded berries (10.19 %) than 'Chardonnay' and 'Plavac mali crni'. Taken together, these results point to 'Pošip bijeli' as the most suitable pollinator among the three tested cultivars.

The difference in fertilisation success between the two seasons could be explained by stark differences in climate conditions. In 2012, conditions were favourable for growing vines and ripening grapes, whereas in 2013, this was not the case, primarily due to extremely high rate of rainfall (data obtained from Croatian Meteorological and Hydrological Service). Despite the differences, the results indicate that in cross-pollination the genetic relationship between pollen donor and acceptor might be a crucial factor that positively influences fertility along with sufficient pollen viability and germination rate.

### Conclusion

'Grk bijeli' represents a unique case where undersized berries positively influence wine quality (PREINER *et al.* 2012). Therefore, an analysis of reproduction which would focus on ensuring an optimal ratio between normally developed fertilised berries and undersized berries has the potential to improve both the yield and oenological value of 'Grk bijeli'. This study represents the first detailed analysis of reproductive potential of *Vitis vinifera* 'Grk bijeli'. The data confirm that 'Grk bijeli' is functionally female with morphological defects of the male reproductive organs. In addition to reduced stamen filament elongation and bent shape, 'Grk bijeli' pollen was unable to germinate due to a lack of germination pores. In contrast to the male gametophyte, around 70 % of female gametophytes developed normally, producing the egg and central cell, three antipodal cells and two synergid cells, which is enough to ensure correspondingly high fertilisation rates. However, reduced fertilisation and the high proportion of seedless berries in 'Grk bijeli' (77.1 %) was affected by parthenocarpy (57.1 %) and stenospermocarpy (20.0 %).

Because the optimal proportion of seedless/seeded berries in a cluster has a positive impact on the sensory characteristics of wine (PREINER *et al.* 2012), our research suggests the possibility for controlling this ratio by exposing 'Grk bijeli' vineyards to a combination of specifically chosen pollinators. Here, the ratio of seedless/seeded berries in experiments in which *Vitis vinifera* 'Grk bijeli' was hand-pollinated in isolated conditions by pollen of 'Chardonnay', 'Plavac mali crni' and 'Pošip bijeli', highlighted an

advantage of Dalmatian cultivars as pollinators, despite the lower pollen germination ability of 'Plavac mali crni' compared to 'Chardonnay'. This observation suggests that the genetic relationships have a stronger impact on fertilisation success than the pollen viability. Therefore, to control the proportion of seeded/seedless berries in 'Grk bijeli', Dalmatian cultivars such as 'Pošip bijeli' and 'Plavac mali crni' could be used as pollinators to provide higher fertilisation success and contribute to production of seeded berries, whereas 'Chardonnay' might ensure a higher proportion of seedless berries. A similar approach for improvement of economic importance and oenological properties could be applied to other numerous cultivars with functionally female flowers.

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