Ciência Téc. Vitiv. 34(2) 84-90. 2019

DETERMINATION OF FLORAL DEVELOPMENT STAGES IN CABERNET SAUVIGNON (VITIS VINIFERA L. CV.): HIGHLIGHTING THE MANIFESTATION OF STAMENS AND PISTIL PRIMORDIA WITH NEW INTERMEDIATE STAGES LINKING THE PHENOLOGICAL STAGES

DETERMINAÇÃO DOS ESTÁGIOS DE DESENVOLVIMENTO FLORAL EM CABERNET SAUVIGNON (VITIS VINIFERA L. CV.): DESTACANDO A MANIFESTAÇÃO DOS PRIMÓRDIOS DOS ESTAMES E DO PISTILO COM NOVOS ESTÁGIOS INTERMEDIÁRIOS LIGADOS AOS ESTÁGIOS FENOLÓGICOS

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(Received 13.11.2018. Accepted 16.10.2019)

SUMMARY

Despite relatively intensive work on the development of inflorescence primordia during grapevine growth in season one, some material gaps are present in the flower and floral organ development in the season two. In addition, current events of phenology and formation of flowers and floral parts has not been dealt with. With the aid of digital imaging, this research had three objectives; a) describe the developmental events that take place during and after bud break in the buds and in the individual flowers in terms of differentiation, b) match these events with phenological stages, and c) determine related growth of the floral organs. After careful dissecting and examination of the samples under microscopy, taken every 5-10 days between March 20 and May 10 in 2016, the results indicated that highly esteemed works regarding the reproductive anatomy of grapevines needed some additional stages to fully describe events in the stamen and pistil primordia after the appearance of petal primordia. Five intermediate stages were added to the stages of inflorescence primordia. Anthers and pistil in the flowers occurred in the second season as the buds swelled in the spring. Stamens and pistil could be seen about 3 weeks later and completed their initial growth in another 3 weeks. Flower primordia was visible on April 1 and showed a more than 9-fold increase over the course of 5 to 6 weeks. Petals flowers increased their width and their length more than 8- and 15-fold, respectively, between stage 8.1 (April 1) and 10.3 (May 10). At first, they were wider than they were longer, but at later stages they grew longitudinally. Reproductive organ primordia were visualized around the time of 2-4 leaves separated on the shoots. Signs of generative parts become apparent in late April. Anthers were the smallest in the flower. Filaments, on the other hand, elongated almost 7-fold in a period of 20 days. Gynoecium growth was the most impressive and total pistil length increased from 52.8 to 162 µm, ovary width from 40.4 to 99.8 µm, and stigma diameter from 9.96 to 44.9 µm in twenty days. By the time the pistil took its final shape, 6-8 leaves grew on the shoot during which inflorescence could also be seen.

RESUMO

Apesar do trabalho relativamente intensivo no desenvolvimento dos primórdios da inflorescência durante o crescimento da videira no ano 1, algumas lacunas informativas estão presentes no desenvolvimento de órgãos florais e florais no ano n. Além disso, eventos atuais de fenologia e formação de flores e partes florais não foram tratados. Com o auxílio da imagem digital, esta pesquisa teve três objetivos; a) descrever os eventos de desenvolvimento que ocorrem durante e após o abrolhamento nos gomos e nas flores individuais em termos de diferenciação, b) combinar esses eventos com estágios fenológicos; e c) determinar o crescimento relacionado com os tamanhos dos órgãos florais. Após cuidadosa dissecação e exame das amostras microscópicas, realizadas entre 5 e 10 dias entre 20 de março e 10 de maio de 2016, os resultados indicaram que trabalhos altamente reputados sobre a anatomia reprodutiva das videiras precisavam de algumas etapas adicionais para descrever completamente os eventos no estame e primórdios do pistilo após o aparecimento dos primórdios das pétalas. Cinco estágios intermediários foram adicionados aos estágios de primórdios de inflorescência. Anteras e pistilo estavam presentes nas flores nos botões inchavam na primavera. Estames e pistilo puderam ser vistos cerca de 3 semanas depois e completaram seu crescimento inicial em mais 3 semanas. Os primórdios das flores foram visíveis em 1 de abril e mostraram um aumento de mais de 9 vezes ao longo de 5 a 6 semanas. As flores aumentaram sua largura e comprimento em mais de 9 e 15 vezes, respectivamente, entre o estágio 8.1 (1 de abril) e 10,3 (10 de maio). A princípio, eram mais largas do que longas, mas em estágios posteriores cresceram longitudinalmente. Os primórdios dos órgãos reprodutivo visualizados por volta de 2 a 4 folhas separadas nos brotos. Sinais de peças generativas se tornam aparentes no final de abril. As anteras

Article available at https://www.ctv-jve-journal.org or https://doi.org/10.1051/ctv/20193402084

menores da flor. Os filamentos, por outro lado, alongaram quase 7 vezes em um período de 20 dias. O crescimento do gineceu foi o mais impressionante e o comprimento total do pistilo aumentou de 52,8 para 162 µm, a largura do ovário de 40,4 para 99,8 µm e o diâmetro do estigma de 9,96 para 44,9 µm em vinte dias. No momento em que o pistilo assumiu a sua forma final, 6-8 folhas cresceram na parte aérea, durante a qual a inflorescência também era vista.

Key words: grapevine, flower primordia, pistil, phenology, flower formation and development.
Palavras-chave: primórdios florais, pistilo, fenologia, formação e desenvolvimento de flores.

INTRODUCTION

Investigating the sequence and timing of floral development in horticultural species is of importance in terms of determining productivity and deciding on application time of cultural practices. Different from most woody species in horticulture, grape flowers do not attract any special attention at maturity due to their lack of variation in appearance. Therefore, they are not a crucial material of ampelographic identification (Jackes, 1984). On the other hand, the flowering process is unusual and covers two growing seasons in temperate regions. In the first season, an uncommitted primordium (anlage) becomes visible in the apices of latent buds on shoots and, at optimum conditions, inflorescence primordia (IP) might develop from these specialized meristematic structures. In the second season, individual flowers are formed on previously developed inflorescence primordia (Li-Mallet *et al.,* 2016).

The first studies on floral organogenesis in grapevine flowers included micro techniques that involves sectioning of grapevine buds in a serial order (Barnard and Thomas, 1933; Snyder, 1933). With the advent of microscopy, especially epi-illumination microscopy, the development and structures of floral inflorescences and/or individual flowers were examined in different *Vitis* species (Gerrath and Posluszny, 1988; Timmons *et al.,* 2007). Scanning electron microscopy has enabled researchers to 3-D visualize the sequential developmental events in the flowers both in *Vitis* species, including the wild grape (Caporali *et al.,* 2003; Spada *et al.,* 2003; Ramos *et al.,* 2014) and *Vitis vinifera* L. cultivars, such as 'Sultana' (Scholefield and Ward, 1975), 'Shiraz' (Srinivasan and Mullins, 1976), 'Chardonnay' (Watt *et al.,* 2008) and 'Pinot noir' (Jones *et al.,* 2009). However, these studies were only descriptive without providing quantification or size information.

Literature on the dimensions of grape flower in the *Vitaceae* family at full development stage before anthesis state that its size depends on species, being 2 mm in *Vitis berlandieri* Planch. and 6-7 mm in *Vitis labrusca* L. (Keller, 2015). In one study, Caporali *et al.* (2003), presented through SEM observations some dimension information on pistil and anther length of wild grape, *V. vinifera* subsp. *sylvestris* (C.C. Gmel.) Hegi. Noyce *et al.* (2015, 2016) conducted a series of studies in order to provide more detail into development of IP in cv. 'Chardonnay' (*V. vinifera*) using dissecting microscope and quantified the number and the size of IP in the buds. To the best of our knowledge, no specific size information regarding the sizes of primordia of flowers and/or floral organs in *V. vinifera* are presented in previous studies.

Even though grapevine floral morphogenesis has been well understood and explained in detail, the morphological development and size increment over time in the floral, specifically generative, parts has not been indicated. There are some vital gaps in the development of grape inflorescence primordia in the season two after inflorescence differentiation. The most regarded study of Srinivasan and Mullins (1981) described flower development in season two, to start with stage 8 and end with stage 11 without any specific reference to stamen and pistil organogenesis between stage 10 and 11.

There are some widely known descriptive systems for identifying growth cycle (phenology) of grapevine, such Baggiolini (1952), Eichhorn and Lorenz (1977), the BBCH system by Lorenz *et al.* (1995) and Coombe (1995). Unfortunately, none of these systems indicate any relationship between progress of flower development and phenology. This study aimed to unveil primordial development of the reproductive parts in a *Vitis vinifera* L. flower (cv. 'Cabernet Sauvignon') from the beginning of bud break to the completion of pistil formation. This would enable determining the fate of the differentiated inflorescence primordia turning into a complete flower cluster and quantifying the size increase in the individual flower parts well before anthesis. Also, an attempt to associate all these events with the phenological stages in the life cycle of grapevine was also done.

MATERIAL AND METHODS

Plant material and study site

Plant material was obtained from *Vitis vinifera* L. cv. 'Cabernet Sauvignon' at the Research Station of Department of Horticulture, located at Dardanos campus of Canakkale Onsekiz Mart University in

Çanakkale, Turkey. The grapevines were ten years old, grafted on 5 BB rootstock, with goblet training and winter spur pruning (2-3 buds per spur). The planting density was 2x3 m. Management was all identically applied to all vines, with no irrigation (rain-fed), throughout the season.

Studies on flower morphology

Sampling time was from the end of dormant season (March) to the mid-May 2016. Samples (compound latent buds or individual flowers) were taken every 5-10 days at each sampling date and the phenological stages, between 2-15, were recorded according to the modified Eichorn-Lorenz (E-L) system by Coombe (1995) (Fig. 1). The central tendency of each stage was measured by taking the median count at each sampling time. Blooming took place 3 weeks later the last sampling time. Sampling size was at least 15 buds or flowers per sampling time.

Figure 1. Phenological stages (modified E-L system by Coombe 1995) according to which the *Vitis vinifera* L. 'Cabernet Sauvignon' samples were taken.

Estados fenológicos (sistema E-L modificado de Coombe 1995) segundo as quais as amostras de Vitis vinifera L. 'Cabernet Sauvignon' foram tiradas.

Microscopy and storage of samples

Samples were immediately placed in FAA solution (formalin 10%, ethyl alcohol 50% and glacial acetic acid 5%) for 24 or longer hours. Samples were dissected using an Olympus SZX7 stereo zoom microscope (Olympus Corp., Japan). The overwintering buds were rid of their scales and wooly hairs under the stereo microscope with the help of dissecting needles and mini double lancets. For the microscopic examination of the stamen and pistil primordia structures, sepals and petals were removed with the help of the arrowhead two-edge sharp needles and protruding conical stylet. These operations were done in distilled water to prevent the samples from drying.

Images were photographed using a digital microscope camera (LC20, Olympus DF PLAPO X_{-4}, Japan) mountable on the microscope. The measurements in µm were enabled by a software program (LC20-Bundle LCmicro, Olympus Corp., Japan). Samples at the same phenological stages were taken for flower and flower organ primordia measurements. Measurements were taken for flower width and length, anther width and length, filament length, pistil length, ovary width and stigma diameter at the sampling times.

Determination of the floral development stages

In order to determine the stages of development of flower and floral primordia, initial guidance was

obtained from the work of Srinivasan and Mullins (1981). Since no clear evidence on formation of stamen and pistil primordia was given in this highly respected study, a need to add intermediary stages relating the formation of these primordia existed. Additionally, a link between the phenological stages of the grapevine and morphological stages of floral development in grapevine flowers was tried to be established.

RESULTS AND DISCUSSION

The morphological development of floral primordia in the inflorescence was detected with the aid of microscopy and it was possible to visualize when the floral development occurred in the grapevine cultivar 'Cabernet Sauvignon'. Development of flower and floral organ primordia in this cultivar was depicted in Figure 2.

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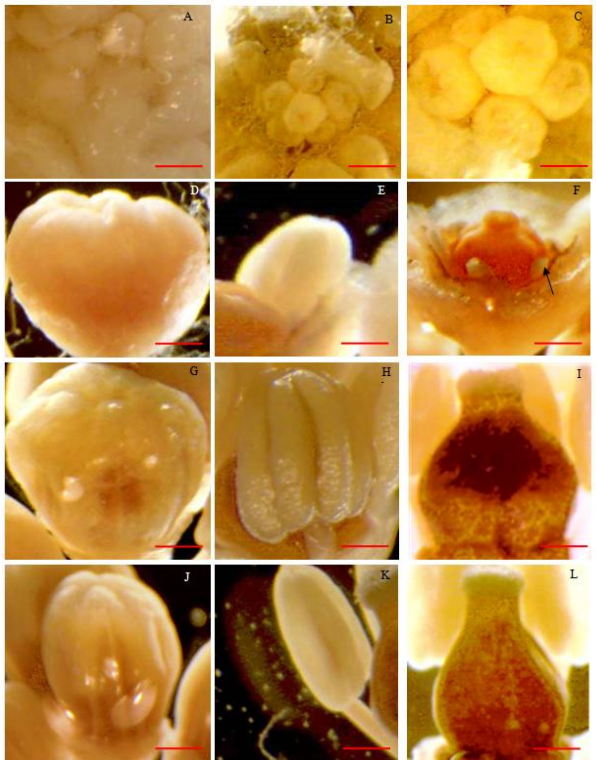

Figure 2. Stereo zoom microscope images of development of flower and flower organ primordia in (*Vitis vinifera* L. cv. 'Cabernet Sauvignon' A- *Stage 8.1*, floral primordium initials with sepal primordia starting, B- *Stage 8.2*, floral primordium with calyx and petal primordia, C- *Stage 9*, floral primordium with sepal and petal which forms an incomplete cover over the flower, D- *Stage 10.1*, flower in which the petals have completely overgrown over the top forming the calyptra, and have continued to push up through the calyx ring, E- *Stage 10.1*, anthers appear bithecal (sepal and petal removed), F- *Stage 10.1*, pistil appears (removed organs are sepal, petal, anthers and filaments (arrow shows filament cut)), G- *Stage 10.2*, flower in which the calyptra lobes are visible through the top of the calyx, H- *Stage 10.2*, anthers taking shapes with short filaments (the calyptra was removed), I- *Stage 10.2*, pistil takes its unique shape and style and stigma become visible (removed organs are sepal, petal, anthers and filaments), J- *Stage 10.3*, flower primordia with calyptra, stamens and pistil (calyptra is so transparent that anthers can be seen, K- *Stage 10.3*, extended anther and filament (removed organs are sepal and petal), L- *Stage 10.3*, fully differentiated pistil with clear short style and disc-like stigma. Bar = 30µm (A to C, G to I), 40µm (D to F) and 50µm (J to L).

Imagens stereo zoom microscópicas do desenvolvimento de primórdios de órgãos florais e florais em Vitis vinifera L. cv. 'Cabernet Sauvignon'. Estádio 8.1, primórdios florais iniciais do primórdio da sépala, B- Estádio 8.2, primórdio floral com cálice e primórdios de pétala, C- Estádio 9, primórdio floral com sépala e pétala que forma uma cobertura incompleta sobre a flor, D- Estádio 10.1, flor na qual as pétalas se cobriram completamente sobre o topo, formando a caliptra, e continuaram a subir através do anel do cálice, E- Estádio 10.1, as anteras aparecem como as duas tecas (sépala e pétala removidas), F- Estádio 10.1, o pistilo aparece (os órgãos removidos foram as sépalas, pétalas, anteras e filetes (seta mostra filamento cortado)), G- Estádio 10.2, flor na qual os lóbulos do caliptra são visíveis através do topo do cálice, H- Estádio 10.2, anteras formadas com filetes curtos (a caliptra foi removida), o I- Estádio 10.2, o pistilo assume a sua forma e estilo únicos e o estigma torna-se visível (os órgãos removidos foram as sépalas, pétalas, anteras e filetes),J- Estádio 10.3, primórdios florais com caliptra, estames e pistilo (a caliptra é tão transparente que as anteras podem ser vistas, o Estádio K 10.3, a antera e o filete desenvolvidos (os órgãos removidos foram as sépalas e pétalas), Estádio L 10.3, pistilo totalmente diferenciado com estilete curto e claro e estigma discoide. Bar = 30µm (A para C, G para I), 40µm (D para F) and 50µm (J para L).

Samples taken at first phenological stages (E-L stages 2-5) in March 2016, did not yield any observable flower primordia, in agreement with May and Antcliff (1973), Scholefield and Ward (1975), Srinivasan and Mullins (1981), Watt *et al.* (2008) and Jones *et al.* (2009), who also expressed that in the earlier phases of bud development (before or during winter dormancy), flower primordia did not develop. In the samples at E-L stage 7 (April 1), floral organogenesis started and sepal primordia developing on each flower primordia were revealed (Fig. 2a) and this stage was denoted as *8.1*, the first addition to the stage 8 described by Srinivasan and Mullins (1981) as "differentiation of branch primordia at bud burst and initiation of the flower initials". This additional stage involved a bit further development in which sepal primordia started to become visible, hence the new denotation, *8.1*. Keller (2015) stated that individual floral organ development occurs 5 weeks during and after bud break. This was supported with the current observations, although the duration was a bit longer.

On April 5 (E-L stages between 7 and 9), the calyx and the petal primordia became visible on inflorescence (denoted as *8.2*) (Fig. 2b). At the E-L stage 9, the state that corolla development started but was not yet interdigitated at the top of the flower was caught in our samples on April 10 (Fig. 2c). The progress from the flower initial formation to corolla formation took place in a 10-day period. The next event in the flowers were borders of petal lobes being visible and formation of calyptra, named as the stage 10 according to Srinivasan and Mullins (1981) description.

From this point because there are no explanation or representation as to what happens regarding the stamens and/or pistil, we numbered the following stages as *10.1*, *10.2*, and so on. Fig. 2d-f shows that on April 20 (E-L stages between 9-12), the calyptra fully formed (stage *10.1*) and inside, anthers with two lobes were exposed. They did not have any filament formation yet. The pistil, on the other hand, had already started to develop from gynoecial ring and looked like a pressed balloon filled with water sitting on a flat surface. Style was barely noticeable (Stage *10.1*). As the flower continued to grow, on May 5 (E-L stages between 12-15), filaments were formed, and the pistil started to take its shape with a little bit style visibility. A slight asymmetry could be observed on the ovary (stage *10.2*, Fig. 2g-i). Within another five days (May 10, E-L stage 15), the flower lost its puffiness and showed a longitudinal growth. At this stage, the stamens and the pistil were fully shaped and elongated (Fig. 2j-l) and the style and the stigma were clearly visible (stage *10.3*).

In the earlier studies involving the timing of these developments, flower initiation has been reported to include formation of some part of calyx primordia before bud break (Alleweldt and Ilter 1969; Ağaoğlu 1971), but our data are not able to confirm or deny such results. Due to the possible reasons of difference sampling interval, technological incapacities or simply missed because of their extremely miniature size, we inclined to assume that no floral part develops before bud break. Swanepoel and Archer (1988) stated in their study with *V. vinifera* cv. 'Chenin blanc' that the duration between the appearance of inflorescence and completion of floral development was 20 days. In the current study, this was a bit longer, approximately 30 days, which might have been the result of both the cultivar and the environmental conditions, especially temperature.

Measurements taken on the flower and the flower parts of cv. 'Cabernet Sauvignon' were given in Table 1. 'Cabernet Sauvignon' flowers increased their width and their length more than 9- and 15-fold, respectively, between stage 8.1 (April 1) and 10.3 (May 10). At first, they were wider than they were longer, but at later stages they grew longitudinally. Signs of generative parts become apparent at the late part of April. Anther width and length were the ones that kept their growth to minimum compared to the other parts of the flower. Filaments, on the other hand, elongated almost 7-fold in a period of 20 days. Gynoecium growth was the most impressive and total pistil length increased from 52.8 to 162 µm, ovary width from 40.4 to 99.8 µm, and stigma diameter from 9.96 to 44.9 µm in twenty days. Literature paid scant attention to the growth rate of the generative parts of the *Vitis* flower. In an earlier work, Considine and Knox (1979) presented some volume information of the pistil primordia of 'Gordo blanco' (*Vitis vinifera* L. cv.), reaching 0.1 mm^3 around the time of anthesis. Recently Caporali *et al.* (2003) provided some size information of the pistil of *V. vinifera* subsp. *sylvestris* flowers at stages 7 and 8. However, these studies did not present any size increase over time in the floral parts.

TABLE I

Measurements (mean, µm) of floral organ primordia in *Vitis vinifera* L. cv. 'Cabernet Sauvignon' at each date of sampling in 2016 and corresponding phenological stage (range in parenthesis)

Medidas (média, µm) de primórdios de órgãos florais em *Vitis vinifera* L cv. 'Cabernet Sauvignon' em cada data de amostragem em 2016 e estágio fenológico correspondente (intervalo entre parênteses)

Floral stage	8.1	8.2	9	10.1	10.2	10.3	relative growth (%)
Sampling Date	April 1	April 5	April 10	April 20	May 5	May 10	
Mod.E-L syst.	7	7-9	9	9-12	12-15	15	
Flower width	15.9 (8.80 - 19.6)	25.9 (19.3 - 31.6)	36.8 (33.8 - 39.7)	114.5 (99.7 – 125)	196.9 (164 – 223)	170 (164 – 192)	969
length	13.4 (9.42 - 23.3)	25.3 (20.2 - 29.0)	38.0 (34.4 - 44.3)	104.4 (84.3 – 118)	192.1 (181 – 204)	223 (200 – 254)	1564
Anther width				49.2 (41.8 - 61.1)	57.1 (50.5 - 66.7)	81.4 (64.4 - 96.19	65
length				66.4 (60.1 - 73.2)	75.1 (70.8 - 84.5)	104.2 (98.9 – 121)	57
Filament length				15.4 (11.4 - 21.1)	52.4 (45.7 - 65.1)	115.3 (110 – 123)	648
Pistil length				52.8 (43.1 - 63.1)	93.2 (87.1 – 103)	162 (121 – 213)	206
Ovary width				40.4 (39.4 - 33.1)	75.5 (68.6 - 87.1)	99.8 (80.6 – 140)	147
Stigma diameter				9.96 (9.26 - 13.4)	17.2 (12.8 - 24.1)	44.9 (29.4 - 63.1)	350

CONCLUSIONS

Here in this study, establishing a link between the phenological stages and the floral primordia development was accomplished for the cv. 'Cabernet Sauvignon'. No matter how miniscule and hard to spot in the very compressed shoot at the beginning of the bud break in the spring, appearance of the inflorescence marked the formation of reproductive organs of the flower. Ongoing from this point, the progression of the development in the stamen and the pistil primordia was relatively fast. Full formation of the flower with its complete parts was coincided with the time around 7-8 leaves separated from the shoot and inflorescence was visible. Summation of the intermediate floral stages were as follows: 8.1, differentiation of sepal primordia; 8.2, development of calyx and petal primordia; 10.1, calyptra is almost fully formed, stamens with no filament growth, and the pistil starts to show; 10.2, calyptra fully covers the stamens which have a short filament and pistil with distinguishable style and stigma, and 10.3, extended stamens and pistil with clear style and discoid stigma.

The value of recognition of growth stages for the grapevine is indispensable not only for implementing cultural operations in the vineyard but also providing a unanimous understanding among the human partners in grape growing. In addition, establishing a link between these stages with the flowering process in *Vitis vinifera* is also of great importance.

ACKNOWLEDGMENTS

This work was supported by Çanakkale Onsekiz Mart University, The Scientific Research Coordination Unit, Project number: FBA-2015-494.

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