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A highly efficient embryo rescue protocol to recover a progeny from the microvine

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Summary

The grapevine is a difficult species for genetic studies due to the plant size and long life cycle. With the microvine, these limitations can be overcome thanks to its dwarf stature, continuous flowering, short juvenile phase and generation cycle. The advantages of the microvine allow scientists to undertake genetic studies 2-5 times more rapidly than the current situation with normal grapevines. However, the seeds obtained from microvine parents have a low germination rate, and therefore some approaches to improve seed germination are required. Four microvine lines (ML1, V19, Pico x FLB 225, and AB x ML1) and a classical grapevine variety ('Syrah' cl. 174) were experimented in embryo rescue experiments. To evaluate embryo germination rate during berry development, seeds were collected from four different berry developmental stages including 2 weeks before veraison (WBV), veraison, 3 and 6 weeks after veraison (WAV). For all microvine varieties, the age of seed or berry stage influenced the development of the embryos. The highest percentage of germinating embryos (100 %) and normal developed plantlets (100 %) were recorded at veraison stage followed by 2 WBV, 3 WAV and 6 WAV, respectively. In addition, growth and development of embryos derived from veraison berries were also faster than the other phases. This study concluded that veraison is the most suitable berry developmental stage for microvine embryo extraction and culture, whereas, the best stage for embryo rescue in 'Syrah' grapevine is 2 WBV. The information obtained from this experiment will be useful for microvine breeding programs and expand their germplasm base in the future.

K e y w o r d s : dwarf grapevine, *Vitis vinifera*, berry stage, embryo rescue, *in vitro* culture.

Introduction

The microvine originated through somatic embryogenesis from L1 cell layer of 'Pinot Meunier', a traditional cultivar from the Champagne region of France (Boss and Thomas 2002, Franks *et al*. 2002). The microvine presents the *Vvgai1* (*Vitis vinifera* GA insensitive) mutant allele that confers a semi-dwarf stature (*VvGAI1/ Vvgai1* called microvine) or dwarf stature (*Vvgai1/ Vvgai1* called picovine), continuous flowering, a short juvenile phase and generation cycle (Boss and Thomas 2002, Chaïb *et al*. 2010, Cousins 2012). These innovative biological features allow the scientists to speed up genotypic and phenotypic studies (*i.e.* for reproductive traits) approximately 2-5 times more rapidly than in classical, non dwarf grapevine (Fig. 1) (Thomas *et al*. 2010). Moreover, the microvine model provides opportunities to study grape morphology, physiology and development at any time of the year under greenhouse or growth chamber conditions (Cousins and Tricoli 2007,

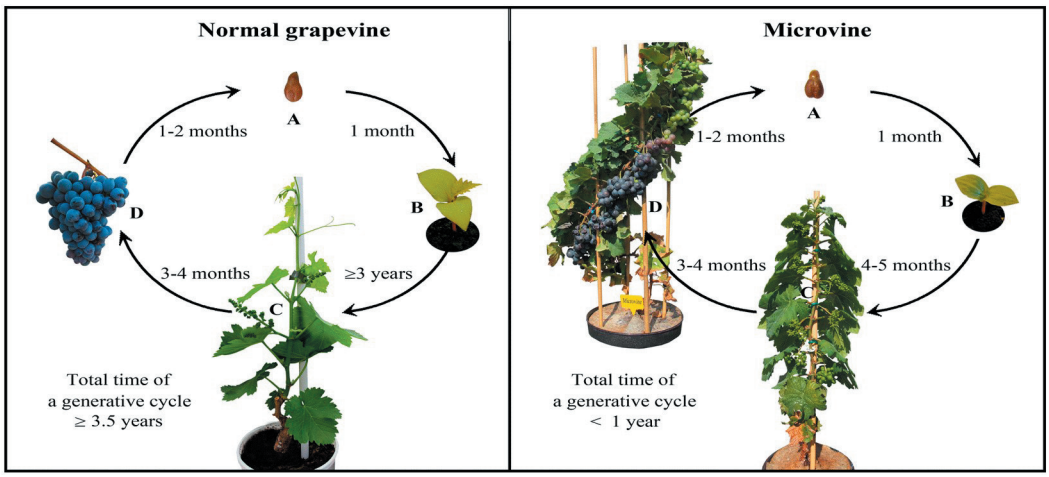

Fig. 1: A comparison of the duration of each stage within a reproductive cycle in the normal grapevine (non dwarf) and the microvine: **A-B** (the stage of seed to seedling), **B-C** (the stage of seedling to flowering), **C-D** (the stage of flowering to berry ripening), **D-A** (treatment for breaking seed dormancy).

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Rienth *et al*. 2012, 2013 and 2014, Luchaire *et al*. 2013). One of most important steps of future genetic studies is to produce progenies, and this process requires the seeds to generate new individuals. The microvine shows some limitations as regards propagation through seeds in terms of strong seed dormancy. Thus, these seeds can remain dormant for more than a year under normal germination conditions and even the cold stratification method failed to stimulate seed germination. However, GA_3 application and scarification can enhance seed germination processes (Boss and Thomas 2002, Cousins and Tricoli 2007). Chaïb *et al*. (2010) reported that seed germination rates from the picovine x Ugni Blanc cross were 24-59 %, which is similar with wild-type seeds treated with GA_3 and scarified.

In the grapevine, zygotic embryo rescue is widely used in breeding programs to assist in the development of weak or immature embryos that might abort before seed maturation, for instance, stenospermocarpic seedless (Cain *et al*. 1983, Emershad and Ramming 1984, Burger and Goussard 1996, Singh *et al*. 2011, Ji *et al*. 2013) crosses and interspecific hybrid (Yang *et al*. 2007, Tian and Wang 2008, Guo *et al*. 2011, Sun *et al*. 2011). In addition, the low germination rate of seeds was successfully improved by immature embryo culture (Ramming *et al*. 1990).

The above-cited publications revealed that the low germination rate of the microvine may be caused by the balance of hormones, especially gibberellins (GAs) and abscisic acid (ABA) within the seed at harvest. The equilibrium of synthesis and catabolism between GAs and ABA plays an important role in seed germination and the regulation of dormancy (Rodríguez-Gacio *et al*. 2009). In the berry, the concentration and ratio of both hormones considerably change from the lag phase to berry ripening (Keller 2010). Thus, the present study was carried out to investigate the evolution of embryo germination rate throughout the development of microvine berries *via* the embryo rescue technique.

Material and Methods

Plant materials: Seeds were obtained from self-pollination of three microvine varieties (ML1, V19, and Pico × FLB 225), a cross between 'Alicante Bouschet' and microvine (AB × ML1), and a self-pollination of 'Syrah' grapevine (Tab. 1). Berries of each variety were harvested at different stages including; i) 2 weeks before veraison (lag phase), ii) veraison (80 to 90 % of berry softening in a cluster), iii) 3 weeks after veraison (mid-maturation) and iv) 6 weeks after veraison (full-maturation). Eight to ten berries of each stage were randomized from the clusters to measure the total soluble solids (TSS) by a hand refractometer.

Surface sterilization and embryo extraction: Berries were separated from the clusters and the seeds were extracted from these berries and placed into a cylindrical 125 mL PP container. They were rinsed three times with tap water and surface-sterilized with 2 % NaOCl with a drop of tween-20 for 30 min. The sterilized seeds were transferred to a laminar flow hood (ADS Laminaire, France) and rinsed twice with sterile water. Embryos were aseptically excised under a stereo-microscope.

Media and embryo culture: Half-strength Murashige and Skoog (½MS) medium supplemented with activated charcoal (2.5 $g \cdot L^{-1}$) and antibiotics (200 $mg \cdot L^{-1}$ of Augmentin and Cefotaxime) was utilized as a media culture. Isolated embryos were cultured in petri dishes (Ø 55 mm) and placed in an incubator in the dark at 28 ± 2 °C. The percentage of embryo germination and normal plantlet development were evaluated at 8 and 14 days after commencing the culture.

Statistics and data analysis: This experiment was arranged in a completely randomized design (CRD) with 5 replications per treatment (*i.e.* stage x genotype). Each replicate consisted of 2 petri dishes (10 embryos per petri dish). Data analysis carried out with SPSS 17.0 software for windows.

Results and Discussion

Total soluble solids (TSS): The sugar content of berries derived from 5 grape varieties showed similar concentrations at each stage (2 WBV = 5.0-5.9 °Brix, Veraison = 11.0-12.2 °Brix, 3 WAV = 17.2-17.5 °Brix, and 6 WAV = 20.6-21.5 °Brix), excepting the berries of Pico × Flb 225 plants at 3 WAV and 6 WAV (Tab. 2). The sugar concentration of Pico × Flb 225 berries was lower than the other varieties because this microvine variety is very fruitful and gave large bunches, resulting in high yield per plant and delaying maturation. A negative relationship between crop load and sugar accumulation in berries was previously

Table 1

The list and origin of plant material

Plant material	Origin and description
ML1	This microvine was regenerated in Montpellier from L1 layer cells of Pinot Meunier mutant
V19	An offspring derived from the cross between the picovine and Grenache (referenced as 04C023V0019 in Chaïb *et al*. (2010)
Pico × Flb 225	An offspring derived from the cross between picovine (00C001V0008 in Chaïb *et al*. 2010) and Ugni Blanc fleshless berry mutant (Fernandez *et al*. 2006).
AB × ML1	The seeds obtained from the cross between Alicante Bouschet and ML1 pollen.
Syrah	A classical grapevine cultivar with a (non dwarf stature)

Table 2

The total soluble solids (TSS) at harvest and germination rate of embryo rescue (14 days after culture) from different stages of five tested genotypes

Grapevine varieties	Stages of berry development	TSS (°Brix)	Germination rate (%)		
			Total plantlets	Normal plantlets	Abnormal plantlets
ML1 (microvine)	2 weeks before veraison	5.0 ± 0.1^{d}	100 ± 0^{a}	70±3^{b}	30 ± 3^{a}
	Veraison	11.8 ± 0.1^{c}	100 ± 0^{a}	100±0^{a}	0 ± 0^{c}
	3 weeks after veraison	17.3 ± 0.3^{b}	80 ± 3^{b}	70±3^{b}	10 ± 3^{b}
	6 weeks after veraison	20.6 ± 0.2^{a}	14 ± 6^{c}	10±2^{c}	10 ± 4^{b}
V19 (microvine)	2 weeks before veraison	5.9 ± 0.1^{d}	88 ± 1^{b}	74±4^{b}	14 ± 3^{ab}
	Veraison	11.0 ± 0.2^{c}	100 ± 0^{a}	99±1^{a}	1 ± 1^{b}
	3 weeks after veraison	17.2 ± 0.3^{b}	76 ± 5^{c}	56±10^{b}	20 ± 7^{a}
	6 weeks after veraison	20.9 ± 0.1^{a}	58 ± 4^{d}	30±5^{c}	28 ± 7^{a}
Pico × Flb 225 (microvine)	2 weeks before veraison	5.0 ± 0.1^{d}	96 ± 2^{a}	84 ± 5^{a}	12 ± 5^{ab}
	Veraison	11.4 ± 0.2^{c}	100 ± 0^{a}	100 ± 0^{a}	0 ± 0^{b}
	3 weeks after veraison	14.6 ± 0.1^{b}	72 ± 6^{b}	59 ± 8^{b}	13 ± 3^{a}
	6 weeks after veraison	18.4 ± 0.4^{a}	64 ± 10^{b}	43 ± 6^{b}	21 ± 6^{a}
AB × ML1 (pollinated with microvine)	2 weeks before veraison	-	-	-	-
	Veraison	12.2 ± 0.2^{b}	100 ± 0^{a}	99 ± 0^{a}	1 ± 0^{a}
	3 weeks after veraison	-	-	-	-
	6 weeks after veraison	21.5 ± 0.2^{a}	54 ± 3^{b}	43 ± 4^{b}	11 ± 2^{b}
Syrah	2 weeks before veraison	5.2 ± 0.1^{d}	100 ± 0^{a}	100 ± 0^{a}	0 ± 0^{c}
	Veraison	12.0 ± 0.1^{c}	76 ± 7^{b}	60 ± 7^{b}	16 ± 4^{ab}
	3 weeks after veraison	17.5 ± 0.2^{b}	62 ± 2^{c}	44 ± 2^{c}	18 ± 2^{a}
	6 weeks after veraison	21.2 ± 0.2^{a}	38 ± 4^{d}	28 ± 4^{d}	10 ± 0^{b}

Note: Represented data are mean values ± standard error (5 replications). Values with different letters in the same column (within variety) are significantly different at $p < 0.05$.

reported (Čuš 2004) and showed to be usually related to a poorly balanced sink/source ratio (Davies *et al*. 2012).

Embryo size: In microvine progenies (ML1, V19, Pico × Flb 225 and AB × ML1), the embryos obtained from earliest stage of berry development (2 WBV) exhibited smaller sizes than those at other developmental stages (veraison, 3 WAV, and 6 WAV). Moreover, the embryos at this phase were found heterogeneous in shapes and sizes while the others appeared more uniform (Fig. 2). We observed that embryo growth of the microvine ceases before veraison, at the end of green growth phase of the berry as described in other seeded grapevine cultivars (Pratt 1971, Keller 2010). Conversely, the size of embryos from 'Syrah' did not show any obvious morphological differences whatever the stage of berry development (Fig. 2).

Embryo development & Embryo germination rate: For all tested varieties, embryo growth and development could be observed from the third

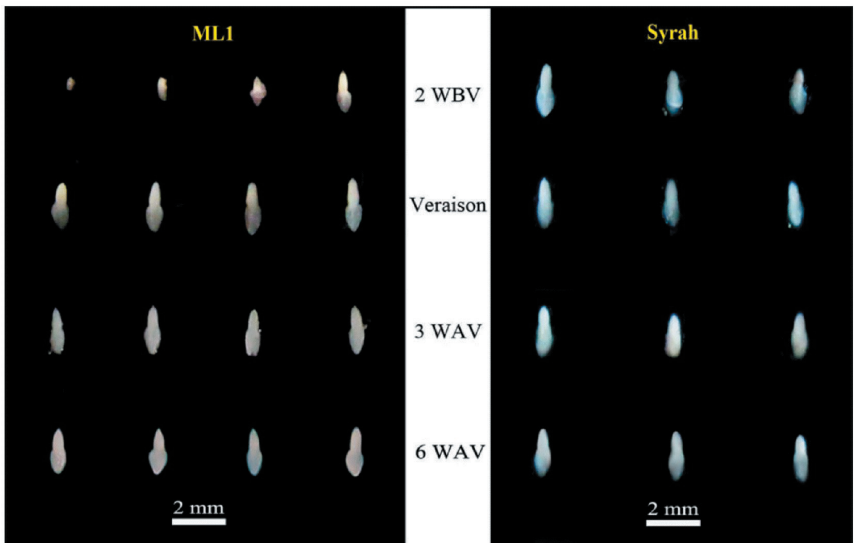

Fig. 2: The embryo sizes of ML1 microvine (left) and 'Syrah' grapevine (right) were isolated from four different stages of berry development: 2 weeks before veraison (2 WBV), veraison, 3 and 6 weeks after veraison (3, 6 WAV).

or fourth day after initiating the culture. The percentage of embryo germination at 8 and 14 days after culture (DAC) was identical but the plantlets at 8 DAC were only half the size of 14 DAC (Fig. 3). For the progenies obtained from microvine genotypes, *i.e.* from ML1, V19, Pico × Flb 225 and AB × ML1 used as maternal parent, the most suitable stage of berry development for embryo rescue was veraison. At this stage, cultures displayed not only 100 % embryo germination but also 100 % normal plantlet development (Tab. 2). In terms of success rate, this stage was followed by the berry before veraison (2 WBV), which gave 88-100 % of germination. The embryo recoveries from mid-ripening (3 WAV) and full-ripening (6 WAV) of microvine progenies were only 72-80 % and 14-64 %, respectively. In 'Syrah' grapevines, the seeds from 2 WBV showed the highest percentage of embryo rescue. On the other hand, the germination rate trended downwards as seed maturity progressed (Tab. 2). These changes in the capacity of embryo for further development could be related to the equilibrium in plant growth regulators. Before veraison, it was shown that gibberellins (germination-promoting hormones) produced by embryos reach a high concentration at the end of green growth phase while the level of abscisic acid (a germination-inhibiting hormone) accumulation is low (SCIENZA *et al*. 1978). When berries start to ripen, the abscisic acid concentration increased rapidly and peaked at full fruit ripening (INABA *et al*. 1976) while gibberellin production is gradually decreasing. These changes inhibit embryo growth capacity while the seed is entering into a dormant phase (NAMBARA and MARION-POOL 2005, GUTIERREZ *et al*. 2007).

Percentage of abnormal plantlets: The seeds from all microvine progenitors at veraison stage did not produce (less than 1 %) abnormal plantlets (Fig. 4), whereas the other stages presented 10-30 % of deformed plantlets (Tab. 2). For the 'Syrah', a maximum of normal plantlets was observed for 2 WBV berries while other stages of sampling produced from 10 % to 18 % of abnormal embryogenic structures (Tab. 2). The characteristic of malformed plantlets derived from seeds explanted from of 2WBV berries was the lack of cotyledon as shown in Fig. 4C. This abnormal appearance resulted from the culture of incompletely developed embryos. The typical malformation of plantlets obtained from mid-maturation and full-maturation berry stage was the irregular development of hypocotyls (tap root and root hairs) or epicotyls (cotyledon and/or shoot apex) as presented in Fig. 4D, E. The physical disorder of these plantlets probably resulted from the tissue dormancy which hampers some aspects of organ development.

Plantlet size: This characteristic was only monitored with normal plantlets. In plantlets deriving from ML1, Pico × Flb 225 and AB × ML1 microvine genotypes, growth rates were higher for embryos explanted at veraison as compared with other stages of berry development (Fig. 3). For V19 microvine descendants, the fastest embryo growth and development was observed at veraison and 3 WAV stages followed by 6 WAV and the slowest growth and development was recorded at 2 WBV (Fig. 5). The plantlets of 'Syrah' derived from 2 WBV and veraison stages were the largest size followed by 3 WAV and 6 WAV, respectively (Fig. 6). These observations are in accordance with WINKLER and WILLIAMS (1935) and PRATT (1971) who reported that seeds at veraison or soon after veraison reached their full size (NITSCH *et al*. 1960) with a maximum of gibberellin and auxin accumulations (IWAHORI *et al*. 1968, KELLER 2010) and low ABA concentration (SCIENZA *et al*. 1978).

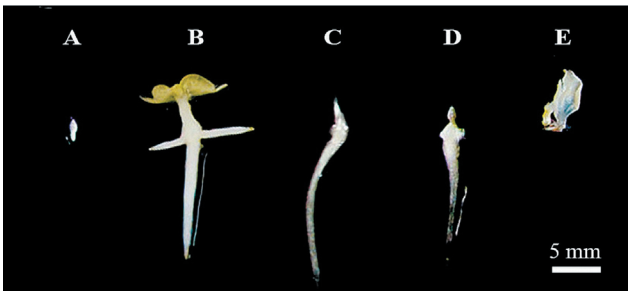

Fig. 4: The characteristics of microvine plantlets at 10 days after culture: **A**) undeveloped embryo, **B**) normal plantlet, **C**) abnormal plantlet; developed both epicotyl and hypocotyl but lose cotyledon, **D**) abnormal plantlet; developed only hypocotyl, and **E**) abnormal plantlet; developed only epicotyl.

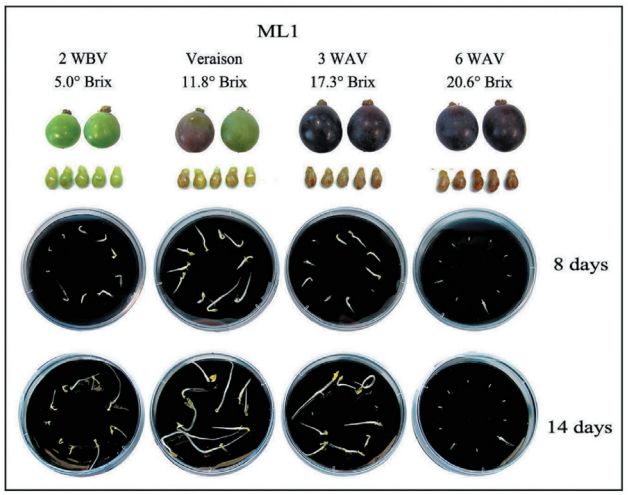

Fig. 3: The plantlets at 8 and 14 days after embryo culture of ML1 microvines from four different stages of berry development.

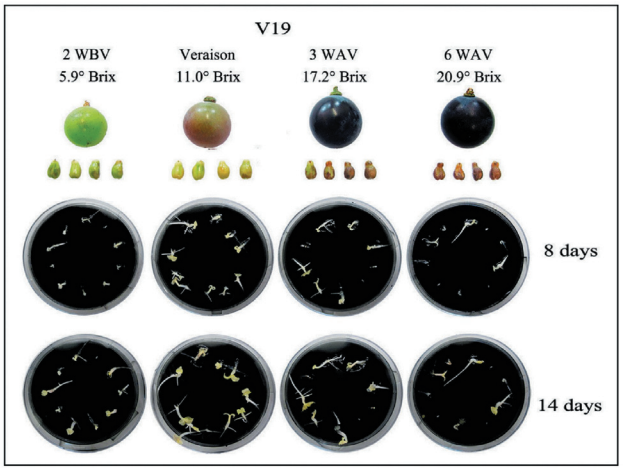

Fig. 5: The plantlets at 8 and 14 d after embryo culture of V19 microvines from four different stages of berry development.

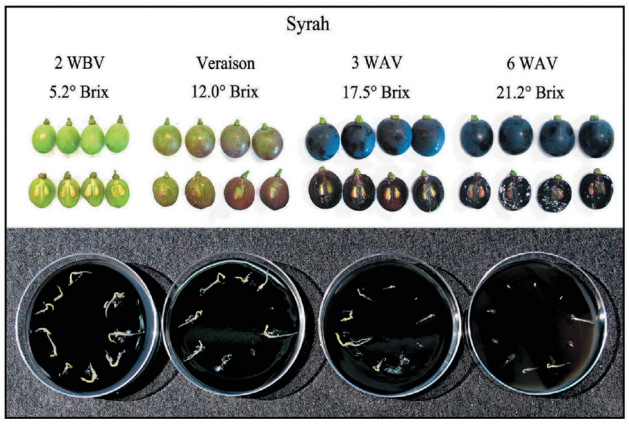

Fig. 6: The plantlets at 8 d after embryo culture of 'Syrah' grapevine from four different stages of berry development.

According to these experiments, the seeds from veraison were found the most suitable for embryo rescue of microvine progenies. The protocol proposed here presents several advantages: i) save time: the seeds can be harvested 1-1.5 months before full ripening. In that way, a new progeny can be obtained around 2 months after flower fertilization occured; ii) simple embryo extraction from seeds, due to the low lignification and dehydration levels of integuments; iii) high degree of embryo rescue with rapid and normal plantlet development, due to the optimal stage of embryo development; iv) inexpensive and simple process due to the absence of treatment to break dormancy for example, cold stratification, scarification, H_2O_2, gibberellins, cytokinins etc. (Katchru *et al*. 1972, Ellis *et al*. 1983, Bouquet and Davis 1989); v) increase the plant copies and reduce the risk of seedling loss: under the *in vitro* condition, a plantlet can be micropagated and conserved in secure conditions. A genotype can be studied for both vegetative and reproductive traits within 1 year.

The optimum size for transferring the plantlets to acclimatation step is 20 ± 3 mm long (or 8-10 d after embryo extraction). However, the suitable transplanting period might be modified due to the grape variety and stage of berry development because both factors directly impact the initial embryo size and the speed of embryo development.

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