Development and Implementation of an *in vitro* Culture System for Intact Detached Grape Berries

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[Abstract] Grape composition depends on the metabolites accumulated and synthesized during grape development. It is of paramount importance for grape growers because of its major role in shaping wine quality. Therefore, understanding the regulation mechanisms that control the accumulation of quality-related metabolites in grape is of both scientific and agronomical interests. The composition of grape berry at harvest is under complex regulation and can be affected by many factors (Conde *et al.*, 2007). The study of the effects of these factors on berries still attached to intact plants can be highly challenging because of the large size of the plants, interplant, intercluster and interberry variability; and because it is complicated to precisely control the nutrients and hormones imported by the berries, and the environment. Therefore, *in vitro* cultured grape berries are a good model system, which better represents berry anatomy structure (skin and flesh) than grape cell suspensions and nevertheless largely reduces the system complexity compared to whole plant (Bravdo *et al.*, 1990; Pérez *et al.*, 2000; Gambetta *et al.*, 2010). To this end, an *in vitro* culture system of intact detached grape berries has been developed by coupling greenhouse fruiting-cuttings production and *in vitro* organ culture techniques (Dai *et al.*, 2014). The cultured berries are able to actively absorb and utilize carbon and nitrogen from the culture medium, and exhibit fruit ripening features such as color changing and softening. This *in vitro* system may serve to investigate the response of berry composition to environmental and nutrient factors.

Materials and Reagents

1. Grapevine berries from greenhouse-grown fruiting-cuttings of *Vitis vinifera* L. cv. Cabernet Sauvignon at various developmental stages (*e.g.* pea size, green berry, veraison, or later stages). The fruiting-cuttings (*i.e.* only one primary shoot axis with a single cluster per plant) were prepared as described in Mullins and Rajasekaran (1981) and grown in a naturally illuminated and semi-regulated greenhouse (mean seasonal temperature amplitude 20-35 °C) with fungicide treatments every two weeks (Figure 1).
   Note: Efficient fungicide treatments are essential to maximally exclude pathogen infection to grape berries and minimize the sterilization step for the in vitro culture. For example, we continuously provide sulfur contained in sulfur diffusion hotbox (Nivola

Sulphur fl 220 v 3 m, Figure 1B) in the greenhouse and treated the vines with armicarb® at 5 g/L every two weeks.

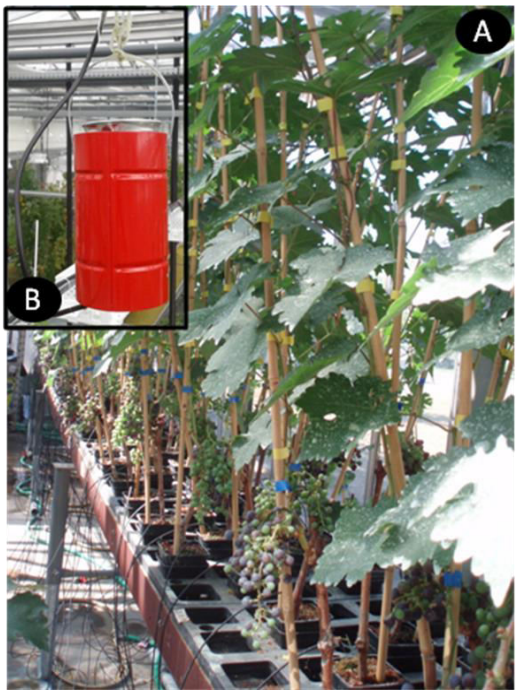

Figure 1. Fruit-bearing cuttings grown in a semi-controlled greenhouse (A) and the sulfur diffusion hotbox (B)

2. 70% ethanol
3. NaClO with available chlorine 2% (Sigma-Aldrich, catalog number: 425044-1L)
4. MS (Murashige & Skoog) medium (Duchefa Biochemie, catalog number: M 0221)
5. Sucrose (Duchefa Biochemie, catalog number: S0809)
6. N-Z-Amine A (Sigma-Aldrich, catalog number: C7290)
7. Vitamines
   Myo-inositol 100 mg/L (Sigma-Aldrich, catalog number: I5125)
   Nicotinic acid 1 mg/L (Sigma-Aldrich, catalog number: N0765)
   Pantothenic acid 1 mg/L (Duchefa Biochemie, catalog number: C0604)
   Biotin 0.01 mg/L (Sigma-Aldrich, catalog number: B4639)
   Pyridoxine HCl 1 mg/L (Sigma-Aldrich, catalog number: P9755)
   Thiamine HCl 1 mg/L (Sigma-Aldrich, catalog number: T4625)
8. 0.5 M NaOH
9. Agar (Kalys, catalog number: HP 696)
10. EDTA (Sigma-Aldrich, catalog number: E5134)
11. Sterile water
12. MS medium with sucrose and vitamins (see Recipes)
13. 200x vitamin (see Recipes)

14. 20 mM EDTA (see Recipes)

Equipment

1. Laminar flow cabinet (Steril-Helios, Figure 2A)
2. Shaker (Dragon lab, sk-330-pro, Figure 2A)
3. Autoclave machine
4. Growth room with constant temperature of 26 ± 0.5 °C, light period 16 h/8 h day/night, and light ~50 µmol m^{-2} s^{-1} (Figure 4H)
5. Sterilized 6-well plates (Dutscher, catalog number: 353046, Figure 5B)
6. Sterilized plastic boxes with filter (Dutscher, catalog number: E 1650, Figures 3F, 4G, and 5A)
7. Tip plate (Figure 3A)
8. Polystyrene (Figure 3C)
9. Culture dish (145/20 mm, Greiner Bio-One GmbH, Figure 4E)
10. Sterilization solution container (Figure 2C-D)
11. Colander, forceps, scissors, and blade (Figure 2B)

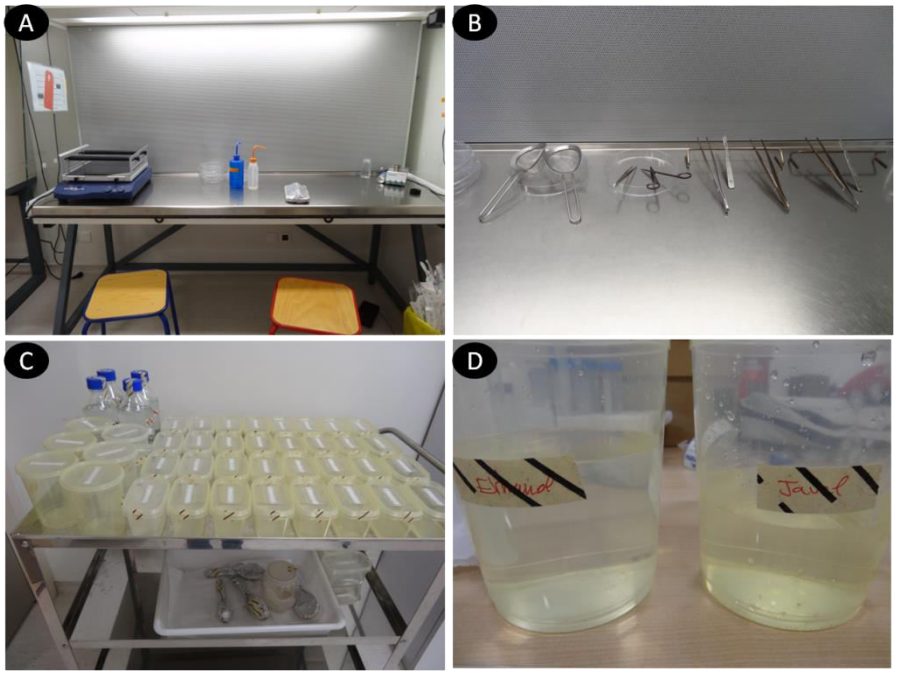

Figure 2. Equipment used in grape berry culture. A. Laminar flow cabinet with a shaker. B. colander, forceps, scissors, and blade. C. solution container, culture boxes, and small materials just after autoclave. D. Sterilized containers for ethanol and NaClO.

Procedure

A. Preparation of the floater for liquid medium
   1. Collect tip plates and cut them into suitable size for the culture box (Figure 3A and B).
   2. Prepare polystyrene cuboids corresponding to the size of tip plates (Figure 3C).
   3. Put the polystyrene cuboids on the two sides of the tip plates to form a floater (Figure 3D-F).
   4. Sterilize floaters with 10% NaClO and 90% ethanol during 20 min each following three rinses with sterile water and then install them in the tissue culture box containing liquid medium.

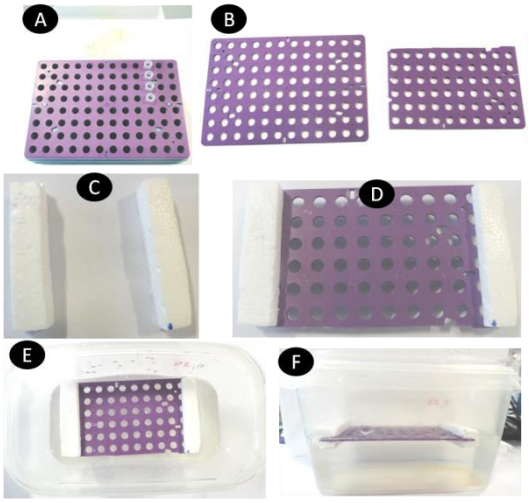

Figure 3. **Preparation of the floater for liquid medium.** A. Boxes of micropipette tips. B. Cut tip plates to a suitable size for the culture box. C. Prepare two polystyrene cuboids. D. Assemble polystyrene cuboids with tip plate to form the floater. E. Vertical view of the floater inside of a culture box. F. Side view of the floater inside of a culture box.

B. Berry *in vitro* culture
   1. Fruit-bearing cuttings were selected and brought to culture room after removing all leaves (Figure 4A).
   2. Grape clusters were excised from the mother plant, and berries were subsequently excised from peduncle with berry pedicel (about 3 mm) and dropped immediately into tap water (Figure 4B).
   3. Keep tap water running for 15 min to clean the berries.
   4. Put berries into 70% ethanol for 2 sec and transfer immediately into NaClO with 2% available chlorine with a sterilized colander (Figure 4D).
      Note: The volume ratio between berry and sterilization solution is important for an efficient sterilization, and a minimum ratio 1:3 (berry/solution) is recommended.

5. Shaking the container of NaClO with a shaker at 350-450 rpm for 2 min (Figure 4C).
   Note: Dependent on the status of the greenhouse-grown berries, this step can be extended to 3-5 min. Longer sterilization will hurt berry skin.
6. Transfer berries into sterilized de-ionized water with colander (Figure 4D) and shake at 350-450 rpm for 2 min.
7. Repeat step B6 three times.
8. Transfer fully rinsed berries into 20 mM EDTA solution, which is contained in a big culture dish (Figure 4E).
9. Cut berry pedicel again to about 2 mm under EDTA solution (Figure 4E), in order to exclude cavitations and to prevent plugging of sieve tubes by callose synthase, a strictly calcium-dependent enzyme.
10. Gently open the culture boxes and quickly put the berries on the solid or liquid culture medium with berry pedicel rooting into culture medium (see Recipes, Figure 4F and Figure 5).
11. Gently put the cover of culture box (Figure 4G) and transport all boxes to culture room with constant temperature of 26 ± 0.5 °C, light period 16 h/8 h day/night, and light ~50 μmol m^{-2} s^{-1} (Figure 4H).

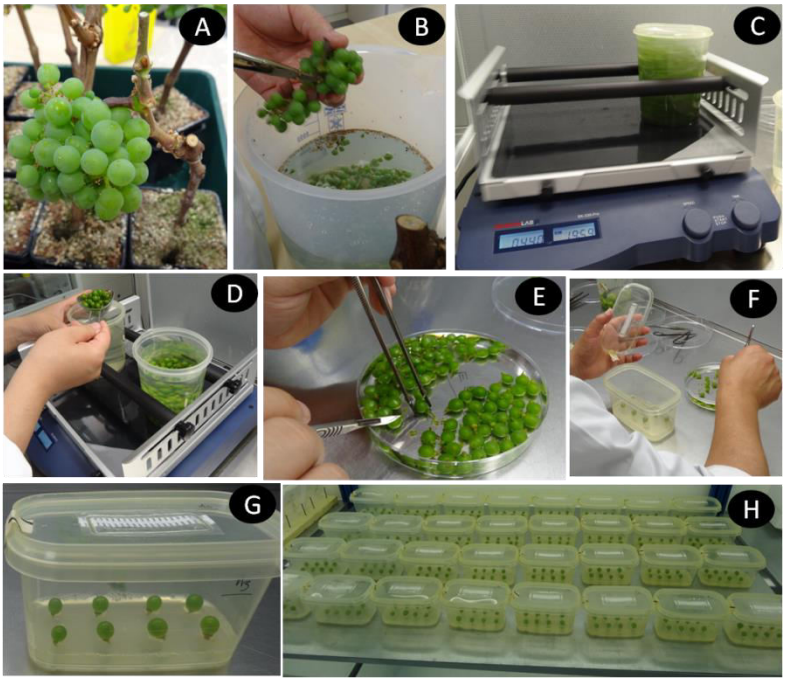

Figure 4. Berry *in vitro* culture procedure. A. Bring fruit-bearing cuttings to the lab after removing all leaves. B. Excise berries from grape cluster and drop them immediately into tap water. C. Sterilize berries with ethanol and bleach solutions with a shaker. D. Transform the sterilized berries to water for rinsing. E. Recut berry pedicel in 20 mM EDTA. F. Install berries into culture medium. G. Put the cover of tissue culture box. H. Install tissue culture boxes into culture room.

12. Alternative culture methods depending on the desired experiment might be chosen (Figure 5).
13. The berry culture can be maintained as long as 3 months. Since the medium volume is much greater than the berries, there is no need for change of medium. However, it will be very easy to change the medium composition of liquid medium with syringe injection.

    Note: Berries are not very tightly fixed on the culture medium, so any movement and transport should be done gently to avoid berry slanting.

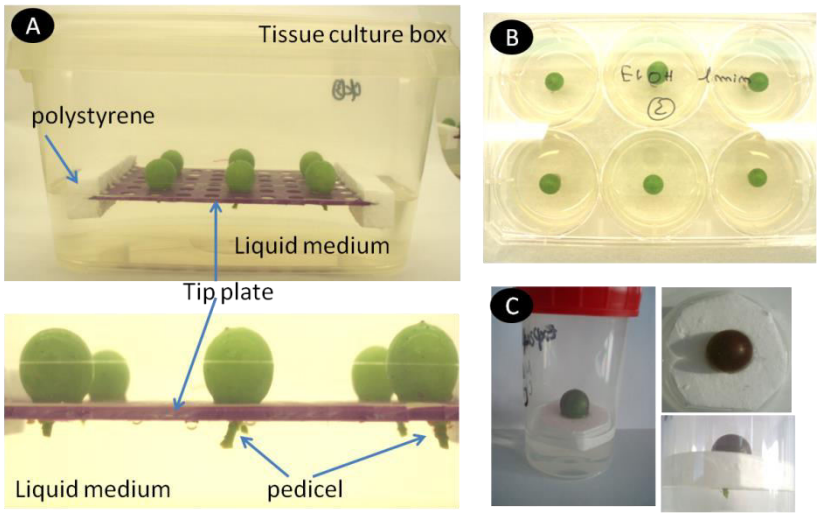

Figure 5. Different types of berry *in vitro* culture. A. Culture with liquid medium and floater. B. Culture with solid medium in a 6-well plate. C. Culture with liquid medium and floater in a small container.

Recipes

1. MS medium with sucrose and vitamins

   4.3 g M0221

   20 g sucrose

   0.25 g N-Z-AmineA

   Dissolve in 800 ml deionized water with vortex

   5 ml vitamin 200x (see below)

   Adjust pH to 5.8 with 0.5 M NaOH

   Complete to 1 L with deionized water

   Add 9 g agar for solid medium

   Autoclave (120 °C, 20min)

   For medium containing agar, pour 4 ml medium to each well of the 6-well plate (Figure 5B); or 50 ml medium to the tissue culture box with special filters allowing gas

exchange (Figure 4G), before medium concretion and inside of the laminar flow cabinet.

For liquid medium, pour 150 ml medium the tissue culture box with special filters (Figure 5A).

Note: A minimum 2% of sucrose is needed to prevent berry crack for green Cabernet Sauvignon berries. Different cultivars may have different sensitivity and therefore a suitable sucrose concentration has to be established for other cultivars.

2. 200x vitamin

   Dissolve 8 mg biotin into 1 ml de-ionized water with one drop of KOH

   25 µl biotin solution

   2 g myo-inositol

   20 mg nicotinic acid

   20 mg pantothenic acid

   20 mg pyridoxine HCl

   20 mg thiamine HCl

   Dissolve and complete to 100 ml with deionized water

   Stored at -20 °C

3. 20 mM EDTA

   Dissolve 7.44 g EDTA in 1 L de-ionized water

   Autoclave (120 °C, 20 min)

   Stored at room temperature

Acknowledgments

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