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Overcoming germination barriers through *in vitro* culture of mature zygotic embryos of grapevine cultivars

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ABSTRACT

Grape seeds have physical and physiological dormancy. Conventional germination methods are not very efficient. The *in vitro* culture of seeds and of mature zygotic embryos can be an alternative for overcoming germination barriers. The objective was to develop methodologies that increase seed germination and reduce the time to obtain seedlings. The following assays were developed: (1) conventional *ex vitro* germination, in a 2 × 3 factorial arrangement, of seeds with or without stratification for 90 days, from cultivars 'Niágara Rosada', 'Itália' and 'Red Globe'; (2) *in vitro* seed germination, in a 3 × 3 factorial arrangement, of the three cultivars and three explants (intact seeds, seeds sectioned in the micropyle region and seeds with a cross-section); and (3) *in vitro* germination of mature zygotic embryos, in a 4 × 2 factorial arrangement represented by four concentrations of mineral salts (MS, ½MS, WPM and ½WPM) and two cultivars ('Niágara Rosada' and 'Itália'). The conventional *ex vitro* germination assay resulted in a low seed germination for the three cultivars. The two types of section associated with *in vitro* culture provided an increase in seed germination. However, the highest germination rate (over 90%) in the shortest time (17 days) was achieved by the embryos grown *in vitro*. This study defines an efficient methodology to obtain high germination in grape seeds— over 85%—with uniformity and in a short time, using mature zygotic embryos grown in ½WPM culture medium. This methodology can accelerate grapevine breeding programs.

Index terms: *Vitis vinifera; Vitis labrusca*; dormancy; seed scarification.

INTRODUCTION

Grapevines are commercially propagated by grafting and cutting (Smith et al., 2012). The seed propagation is commonly adopted in breeding programs to obtain new hybrids (Ritschel et al., 2014; Santos et al., 2019). However, grape seeds have dormancy resulting in low germination rates which hinders and delays grapevines breeding programs (Conner, 2008; Ellis; Hong; Roberts, 1983; Generoso et al., 2019; Maeda; Pereira; Terra, 1985; Pommer; Maeda; Ribeiro, 1988; Val; Alvarenga; Cecon, 2010).

Physical dormancy in grape seeds is due a waterproof integument (Conner, 2008). Physiological dormancy in grape seeds is associated with high endogenous levels of abscisic acid (Rajasekaran; Vine; Mullins, 1982). Cold stratification can lead to a reduction in the levels of this hormone. To break dormancy in grape seeds, the Rules for Seed Testing (RST) (Brasil, 2009) recommend stratification in moist substrate at temperatures between 3 and 5 °C for 90 days, before germination tests. However, this strategy requires a significant period of time and results in germination that hardly exceed 50% (Conner, 2008; Ellis; Hong; Roberts, 1983; Pommer; Maeda; Ribeiro, 1988).

In vitro germination of seeds and zygotic embryos is aimed at accelerating germination, breaking the different types of dormancy and obtaining uniform seedlings. This technique has been used in different crops such as grapevine (Generoso et al., 2019; Val; Alvarenga; Cecon, 2010), pepper

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(Walter et al., 2018), oil palm (*Elaeis guineensis* Jacq.) (Kingsley et al., 2016; Sparjanbabu et al., 2020), murici (*Byrsonima cydoniifolia* A. Juss.) (Martendal et al., 2013) and castor bean (*Ricinus communis* L.) (Louis; Okafor; Okezie, 2018).

In vitro rescue of immature embryos is a quite common practice that is used to produce seedlings from seedless grapes (Li et al., 2018; Li et al., 2020; Tang et al., 2009; Tian et al., 2008). However, no studies have been found that investigate the *in vitro* germination of mature zygotic embryos to overcome germination barriers in grapes. This technique can be especially useful in breeding programs of seed-bearing grapes, as it provides increased germination rates and uniform seedlings produced in a shorter time. This technique can accelerate breeding programs for seed-producing grapevines.

The present study proposes the breaking of dormancy in grape seeds through *in vitro* germination of seeds and mature zygotic embryos. The objective was to develop methodologies that increase seed germination and reduce seedling production time.

MATERIAL AND METHODS

Experimental unit

The experiments were carried out in the Horticulture Sector of the Phytotechnic Laboratory of the Agricultural Science and Technology Center of the State University of North Fluminense Darcy Ribeiro in Campos dos Goytacazes, RJ. The municipality is located at latitude 21° 45' S and longitude 41° 20' W and has an average altitude of 11 meters.

Germplasm

Seeds extracted from ripe fruits of cultivars 'Niágara Rosada' (*V. labrusca* L.), 'Itália' (*V. vinifera* L.) and 'Red Globe' (*V. vinifera* L.) were used (Figure 1A and 1B).

Ex vitro seed germination

The standards recommended by the RST (Brasil, 2009) were followed. The experiment was laid out in a completely randomized design (CRD) with a 3 × 2 factorial arrangement (cultivars and stratification), in eight replicates. Each replicate consisted of a plastic germination box (11 × 11 × 3.5 cm) containing 25 seeds. The seeds of the three cultivars were stratified at a temperature of 5 °C for a period of 90 days (stratified seeds) and another part of the seeds was set to germinate immediately after removal from the fruits (unstratified seeds). Seeds were placed in a plastic germination box that was kept in a germination chamber regulated to an alternating temperature of 20 °C (dark) and 30 °C (light) and photoperiod adjusted to 16:8 h (dark:light, respectively). The final determination of the percentages of normal seedlings, abnormal seedlings, primary-root protrusion, ungerminated seeds and cumulative germination was conducted at 28 days. Normal seedlings were considered only those that exhibited expanded root and shoots. Seeds that did not germinate were removed and tested for viability by the tetrazolium vigor test, following the methodology proposed by Conner (2008) with modifications. For this, the seeds were cross-sectioned with tweezers and a scalpel and left to soak in water for one day. Subsequently, the seeds were sectioned lengthwise and half of them were placed in a solution of 0.5% 2,3,5-tetrazolium triphenyl chloride for 2 h at 40 °C in a germination chamber. Vigor was assessed only in normal seedlings, by measuring the following variables: seedling length (cm) and main-root length (cm), with a digital caliper; and number of leaves. Germination speed index (GSI) (Maguire, 1962) was calculated based on the number of seeds that produced primary root.

In vitro seed germination

Seeds were disinfected in an aseptic environment in a laminar flow hood by immersion in 70% alcohol for 30 s and in 1% sodium hypochlorite (NaClO) solution with two drops of detergent (Tween® 20) for 75 mL of solution for 20 min and then rinsed three times in sterile, deionized water for 5, 10 and 10 min. Subsequently, the seeds were left to soak for a period of 24 h in sterile deionized water. The seeds were then sectioned and inoculated in test tubes (25 × 150 mm) containing 10 mL of ½MS culture medium containing half the concentrations of mineral salts and White vitamins (Murashige; Skoog, 1962), 30 g L^{-1} sucrose (Vetec®), 200 mg L^{-1} polyvinylpyrrolidone (PVP) (Sigma®) and 100 mg L^{-1} myo-inositol (Sigma®). The pH was adjusted to 5.7 ± 0.1 and then the medium was solidified with 6 g L^{-1} bacteriological agar (Sigma®), before autoclaving at 121 ºC and 1.1 atm of pressure for 15 min.

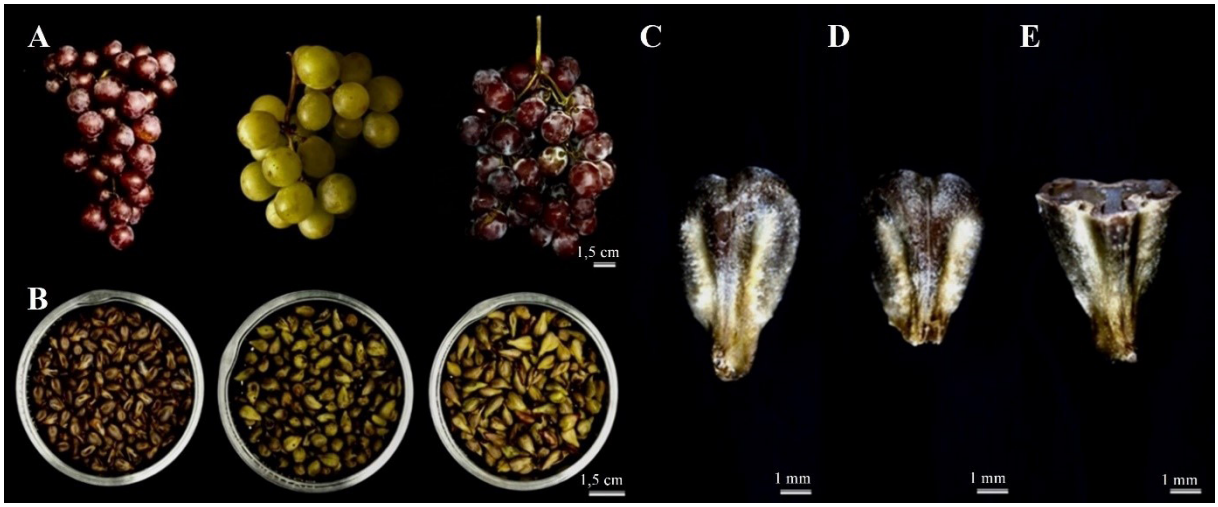

Figure 1: Fruits (A) and seeds (B) of cultivars 'Niágara Rosada', 'Itália' and 'Red Globe', respectively, and seeds used as explants: intact seeds (C), seeds sectioned in the micropyle region (D) and seeds with a cross-section (E).

The experiment was laid out in a CRD with a 3 × 3 factorial arrangement (three cultivars and three types of explants), in eight replicates. Each replicate consisted of 10 test tubes (25 × 150 mm) containing one explant each. The explants were intact seeds, seeds sectioned in the micropyle region or seeds with a cross-section (Figure 1C, 1D and 1E). This assay took place in a growing room with temperature set at 27 ± 2 ºC, under a photoperiod of 16:8 h (light:dark), irradiance of 50 µmol m^{-2} s^{-1} provided by daylight fluorescent lamps (Osram®). At the end of 54 days, the percentage of normal seedlings, abnormal seedlings, primary-root protrusion, ungerminated seeds and cumulative germination were determined. Vigor was assessed only in normal seedlings, by measuring the following variables: seedling length (mm) and main-root length (mm), with a digital caliper; and number of leaves. Germination speed index (Maguire, 1962) was calculated based on the number of seeds that produced primary root.

In vitro germination of mature zygotic embryos

To reduce the thickness of the seed coat, the methodology proposed by Zarek (2007) was applied, with modifications. Seeds were scarified in 50% sulfuric acid (H_2SO_4) (Vetec®) for 15 min and then washed under running water, disinfected, and left to soak for 24 h, following the same methodology described in item 2.3. Using a stereoscopic microscope (Tecnival®), forceps and a scalpel, mature zygotic embryos were isolated (Figure 2) and inoculated in Petri dishes (82 × 15 mm) containing 10 mL of culture medium. The culture media were MS (Murashige; Skoog, 1962) and woody plant medium (WPM) (Lloyd; McCown, 1981) with the total or half the concentrations of mineral salts and White vitamins (½MS and ½WPM). Then, 20 g L^{-1} sucrose (Vetec®), 200 mg L^{-1} PVP (Sigma®) and 100 mg L^{-1} myo-inositol (Sigma®) were added. The pH was adjusted to 5.7 ± 0.1 and the media were solidified with 2 g L^{-1} Phytagel (Sigma®), before autoclaving at 121 ºC and 1.1 atm of pressure for 20 min. Subsequently, the culture media were poured into sterile Petri dishes (82 × 15 mm) in a laminar flow hood.

The experiment was laid out in a CRD with a 4 × 2 factorial arrangement (four culture media and two cultivars), in six replicates. Each replicate consisted of a Petri dish (82 × 15 mm) with four explants. The culture media were MS, ½MS, WPM and ½WPM, and the cultivars used were 'Niágara Rosada' and 'Itália'. Petri dishes were placed in a growing room under the same environmental conditions as in item 2.3. At the end of 28 days, the percentage of normal seedlings, abnormal seedlings, ungerminated embryos and cumulative germination were determined. Vigor was assessed only in normal seedlings, by measuring the following variables: seedling length (mm) and main-root length (mm), with a digital caliper; and number of leaves. Germination speed index (Maguire, 1962) was calculated based on the number of seeds that produced primary root.

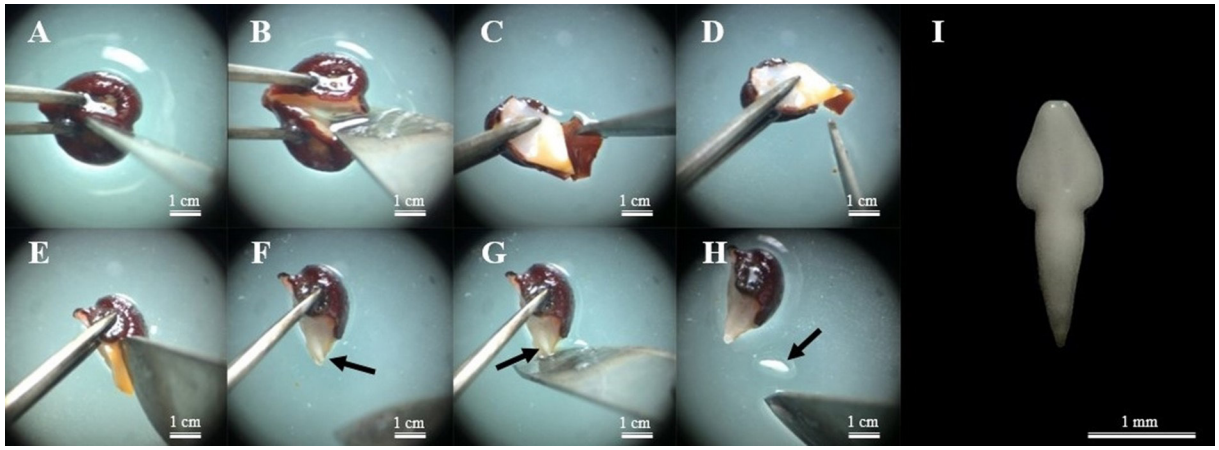

Figure 2: Process of excision of mature zygotic embryos of grapevines. Intact seed scarified in sulfuric acid (A); longitudinal section on the seed (B); removal of the integument that constitutes and covers the micropyle region (C and D); removal of part of the endosperm that covers the embryo (E and F); embryo removal (G) and embryo isolated (H and I). Arrow = embryo.

Statistical analysis

Due to insufficient data obtained in the *ex vitro* seed germination assay, descriptive analysis was performed, in which the mean of the treatments was calculated and the standard deviation of the mean was applied.

Data from *in vitro* germination assays of seeds and mature zygotic embryos were subjected to normality assumption testing by the Shapiro Wilk test. Subsequently, the data were transformed and subjected to analysis of variance. When significant by the F test, means were compared by Tukey's test at 5% probability, using SISVAR statistical software version 5.6 (Ferreira, 2011).

RESULTS

Ex vitro seed germination

The unstratified seeds of any of the three cultivars germinated (Table 1).

The seeds of cultivar 'Niágara Rosada' that were stratified for 90 days did not germinate. The stratified seeds of cultivars 'Red Globe' (Figure 3A, 3E) and 'Itália' (Figure 3B, 3D) showed low germination and abnormal seedlings. Only the seeds of cultivar 'Red Globe' developed normal seedlings (Table 1; Figure 3C).

The tetrazolium test revealed that all seeds—stratified and unstratified—that did not germinate were viable (Figure 3F).

Seed germination started on the 15th day after sowing, with germination being observed until the 27th day. The highest cumulative germination, 12.5%, was obtained by cultivar 'Red Globe', on the 27th day (Figure 4).

In vitro seed germination

For *in vitro* seed germination, the variables of GSI, normal seedlings, abnormal seedlings and ungerminated seeds were significantly affected by the interaction between the studied cultivars and explant types (p <0.05). There was no effect of cultivar and explant type on primary-root protrusion (p >0.05). For the vigor variables of plant length, main-root length and number of leaves, a significant difference was observed for cultivar and explant type (p <0.05).

The highest GSI values were observed when the seeds were cross-sectioned, for the three cultivars. Cultivar 'Red Globe' showed the highest GSI for this type of section (Table 2).

The cross-section of the seeds also provided the highest percentage of normal seedlings for the three cultivars, although in 'Itália' there was no difference between this section and intact seeds (Table 2).

For cultivar 'Red Globe', the cross-section provided the highest germination percentage, resulting in the highest values of normal and abnormal seedlings and the lowest percentage of ungerminated seeds (Table 2).

Table 1: Means and standard deviation of the variables of germination speed index, primary-root protrusion, normal seedlings, abnormal seedlings, ungerminated seeds, seedling length, main-root length and number of leaves, after *ex vitro* germination of cultivars 'Niágara Rosada', 'Itália' and 'Red Globe' for 28 days in germination chamber. Seeds stratified for 90 days (SS) and unstratified seeds (US).

Cultivar	Germination speed index	Primary-root protrusion (%)	Normal seedlings (%)	Abnormal seedlings (%)
Niágara Rosada (US)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Niágara Rosada (SS)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Itália (US)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Itália (SS)	0.3±0.1	4.0±1.4	0.0±0.0	2.0±1.4
Red Globe (US)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Red Globe (SS)	0.7±0.2	3.0±1.1	0.5±0.4	9.0±2.7
Cultivar	Ungerminated seeds (%)	Seedling length	Main-root length	Number of leaves
Niágara Rosada (US)	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Niágara Rosada (SS)	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Itália (US)	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Itália (SS)	94.0±2.5	0.0±0.0	0.0±0.0	0.0±0.0
Red Globe (US)	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Red Globe (SS)	87.5±3.1	0.3±0.3	0.4±0.3	0.2±0.2

Figure 3: Results of *ex vitro* germination of grape seeds after 28 days in germination chamber. Primary-root protrusion in cultivars 'Red Globe' (A) and 'Itália' (B); normal seedling of cultivar 'Red Globe' (C); abnormal seedlings of cultivars Itália' (D) and 'Red Globe' (E) and ungerminated seeds after viability test in 2-3-5-triphenyl tetrazolium chloride (F).

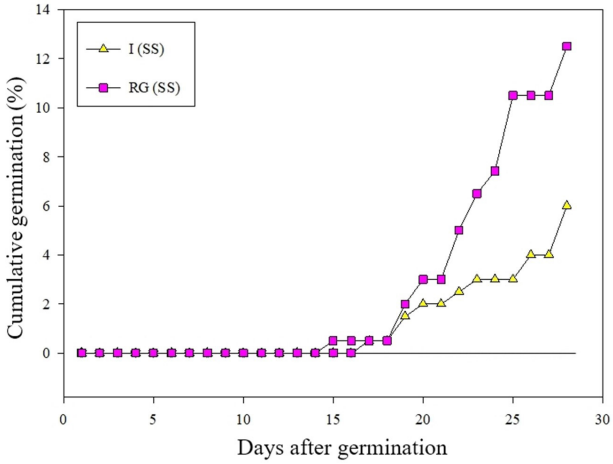

Figure 4: Cumulative *ex vitro* germination after 28 days of germination chamber culture of cultivars 'Itália' (I) and 'Red Globe' (RG). Seeds stratified for 90 days (SS) and unstratified seeds (US).

As regards the vigor variables, cultivars 'Itália' and 'Red Globe' and the cross-sectioned seeds showed seedlings with higher values for shoot length, main-root length and number of leaves (Table 3).

For all evaluated cultivars, germination started on the fifth day after *in vitro* sowing in the treatments in which the seeds were sectioned. The highest cumulative germination, 56.3%, was observed for cultivar 'Red Globe', using seeds with a cross-section as an explant, on the 46th day. The lowest cumulative germination, 1.25%, was found in intact seeds of cultivar 'Niágara Rosada', on the 44th day (Figure 5).

Seeds germinated and normal seedlings were obtained in all treatments (Figure 6).

In vitro germination of mature zygotic embryos

There was no effect of cultivars 'Niágara Rosada' and 'Itália' or culture media on GSI or percentage of ungerminated embryos (p >0.05). GSI was high regardless of the cultivar and culture medium used, with rates of 6.7 for 'Niágara Rosada' and 6.4 for 'Itália'. The percentage of ungerminated embryos was 8.3% for 'Niágara Rosada' and 11.4% for 'Itália'. Culture medium influenced the variables of normal seedlings, plant length and number of leaves (Table 4) (p <0.05). There was a significant interaction effect between cultivars and culture media only for abnormal seedlings and main-root length (Table 5) (p <0.05).

Table 2: Decomposition analysis of the variables of GSI, normal seedlings, abnormal seedlings and ungerminated seeds, as a function of the interaction between grapevine cultivars ('Niágara Rosada', 'Itália' and 'Red Globe') and explant type (intact seed, seed sectioned in the micropyle region and seed with a cross-section), after 54 days of *in vitro* germination.

Cultivar	GSI		
	Intact seed	Section at micropyle	Cross-section
Niágara Rosada	0.0 bC	0.8 aB	3.1 bA
Itália	1.2 aB	1.6 aB	3.1 bA
Red Globe	0.4 bB	1.1 aB	7.3 aA
Cultivar	Normal seedlings (%)		
	Intact seed	Section at micropyle	Cross-section
Niágara Rosada	1.2 bB	6.2 aB	31.2 aA
Itália	10.0 aAB	8.7 aB	21.2 aA
Red Globe	3.7 bB	6.2 aB	32.5 aA
Cultivar	Abnormal seedlings (%)		
	Intact seed	Section at micropyle	Cross-section
Niágara Rosada	0.0 bA	0.0 aA	2.5 bA
Itália	10.0 aA	1.2 aB	5.0 bAB
Red Globe	1.2 bB	0.0 aB	23.7 aA
Cultivar	Ungerminated seeds (%)		
	Intact seed	Section at micropyle	Cross-section
Niágara Rosada	98.7 aA	92.5 aA	63.7 aB
Itália	78.7 bA	87.5 aA	73.7 aA
Red Globe	93.7 abA	92.5 aA	43.7 bB

Means followed by the same lowercase letter in the column and uppercase letter in the row do not differ from each other by Tukey's test ($p \leq 0.05$).

The culture media with half the concentrations of mineral salts and vitamins (½MS and ½WPM) provided a higher percentage of normal seedlings. These media also provided the highest values for plant length and number of leaves, although they did not differ from the WPM medium at full concentration (Table 4).

For both cultivars, the lowest number of abnormal seedlings was obtained with the ½WPM culture medium. For cultivar 'Niágara Rosada', this treatment did not differ from the WPM medium, and for cultivar 'Itália', this treatment did not differ from the ½MS medium (Table 5).

Table 3: Mean values of the vigor variables of shoot length, main-root length and number of leaves according to grapevine cultivar ('Niágara Rosada', 'Itália' and 'Red Globe') and explant type (intact seed, seed sectioned in the micropyle region and seed with cross-section), after 54 days of *in vitro* culture.

Cultivar	Shoot length (mm)	Main-root length (mm)	Number of leaves
Niágara Rosada	14.3 b	33.6 a	2.8 b
Itália	27.7 a	61.8 a	6.3 a
Red Globe	38.3 a	64.7 a	5.9 a
Explant	**Shoot length (mm)**	**Main-root length (mm)**	**Number of leaves**
Intact seed	15.2 b	24.2 b	2.5 c
Seed sectioned at the micropyle	26.1 b	47.2 b	5.0 b
Seed with a cross-section	38.9 a	88.7 a	7.6 a

Means followed by the same lowercase letter in the column do not differ from each other by Tukey's test ($p \leq 0.05$).

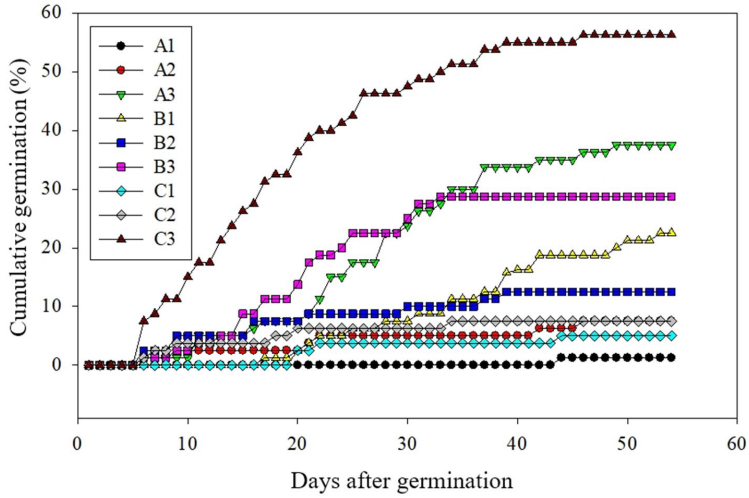

Figure 5: Cumulative germination after 54 days of *in vitro* culture. Cultivar 'Niágara Rosada' in A1 (intact seeds), A2 (seeds sectioned in the micropyle region) and A3 (seeds with a cross-section); cultivar 'Itália' in B1 (intact seeds), B2 (seeds sectioned in the micropyle region) and B3 (seeds with a cross-section); and cultivar 'Red Globe' in C1 (intact seeds), C2 (seeds sectioned in the micropyle region) and C3 (seeds with a cross-section).

The culture media did not influence the length of the main root in cultivar 'Niágara Rosada'. For 'Itália', the highest main-root length values were obtained in the treatments with half the concentrations of mineral salts (½MS and ½WPM). There was no difference in main-root length between the cultivars, except in the ½MS medium, where cultivar 'Itália' showed a longer main root than cultivar 'Niágara Rosada' (Table 5).

Germination started on the fourth day after *in vitro* inoculation of the mature zygotic embryos of the cultivars used. Root elongation was observed from the second week onwards. Additionally, expansion of cotyledon leaves above the culture medium was also noted. From that day on, a trend towards rapid and uniform germination was seen (Figure 7 and 8). After the sixth day of culture, more than 50% germination was observed in all treatments. The greatest cumulative germination occurred on the 17th day after embryo inoculation, with 87.5% to 95.8% of embryos germinating in all treatments (Figure 9).

The *in vitro* culture of mature zygotic embryos resulted in a higher percentage of cumulative germination, with uniformity and in less time, in comparison with the other assays.

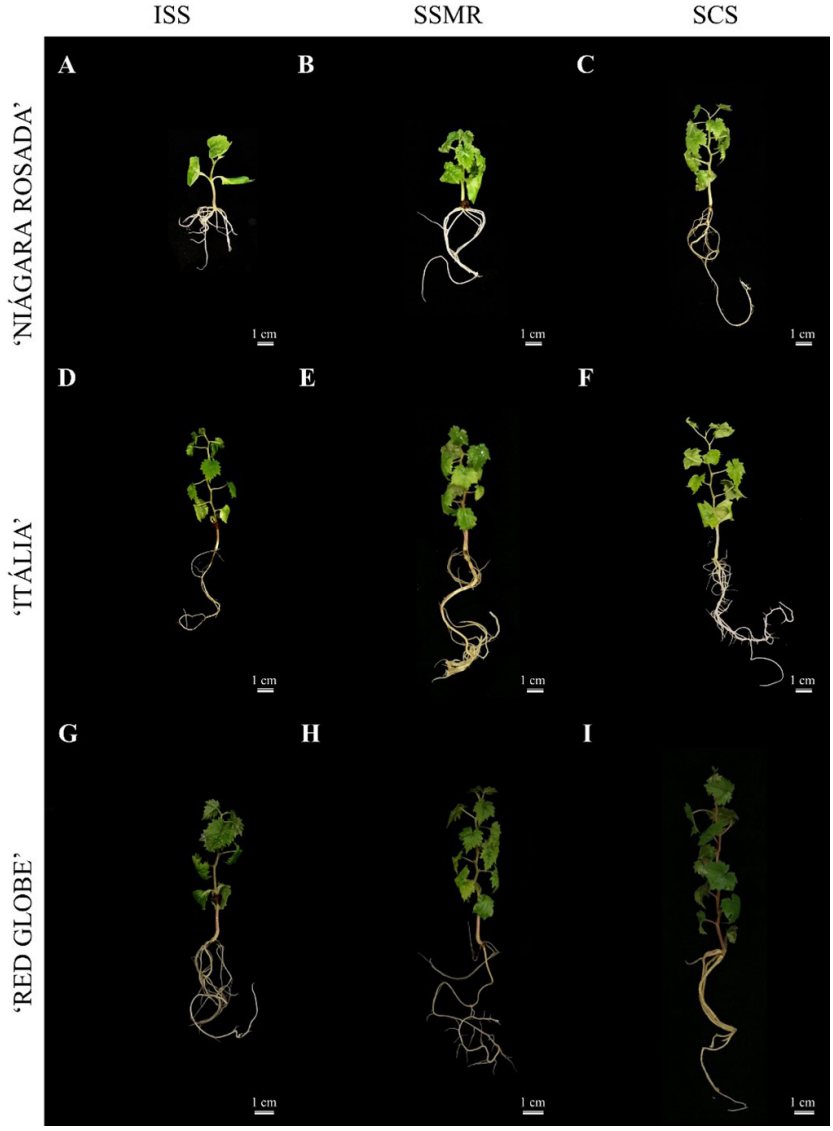

Figure 6: Results of *in vitro* culture at 54 days. 'Niágara rosada' in A, B and C; 'Itália' in D, E and F; and 'Red Globe' in G, H and I, with intact seeds (IS), seeds sectioned in the micropyle region (SSMR) and seeds with a cross-section (SCS).

DISCUSSION

Ex vitro seed germination

As expected, the grape seeds that were not stratified at a low temperature did not germinate (Figure 4). We used this treatment as a control. According to RST, stratification is recommended to break dormancy in these seeds (Brasil, 2009).

The seeds of cultivar 'Niágara Rosada' that were stratified for 90 days also did not germinate. Because the evaluated species was *V. labrusca*, stratification tends not to be efficient in breaking dormancy (Ellis; Hong; Roberts, 1983; Pommer; Maeda; Ribeiro, 1988). This germination difficulty in *V. labrusca* can be attributed to the hardiness of this species.

The seeds of cultivars 'Itália' and 'Red Globe' (*V. vinifera*) that were stratified for 90 days showed low germination percentages, which did not exceed 12.5%. According to Ellis, Hong and Roberts (1983), Pommer, Maeda and Ribeiro (1988) and Conner (2008), germination rates in grape seeds hardly reach 50%, even after stratification.

Table 4: Mean values of the variables of normal seedlings, plant length and number of leaves for the different culture media used (MS, ½MS, WPM and ½WPM), after 28 days of *in vitro* culture of mature zygotic embryos of grapevine cultivars 'Niágara Rosada' and 'Itália'.

Culture medium	Normal seedlings (%)	Plant length (mm)	Number of leaves
MS	12.5 c	4.9 b	1.5 b
½MS	54.1 ab	9.1 a	3.0 a
WPM	45.8 b	9.9 a	2.3 ab
½WPM	87.5 a	11.8 a	3.4 a

Means followed by the same lowercase letter in the column do not differ from each other by Tukey's test ($p \leq 0.05$).

Table 5: Decomposition analysis of the variables of abnormal seedlings and main-root length, as a function of the interaction between grapevine cultivars ('Niágara Rosada' and 'Itália') and the different culture media used (MS, ½MS, WPM and ½WP), after 28 days of *in vitro* culture of mature zygotic embryos.

Abnormal seedlings (%)				
Cultivar	Culture medium			
	MS	½MS	WPM	½WPM
Niágara Rosada	79.1 aA	50.0 aAB	29.1 bBC	8.3 aC
Itália	66.6 aA	25.0 bBC	54.1 aAB	8.3 aC
Main-root length (mm)				
Cultivar	Culture medium			
	MS	½MS	WPM	½WPM
Niágara Rosada	7.2 aA	12.4 bA	14.5 aA	18.7 aA
Itália	6.7 aB	34.5 aA	10.9 aB	33.0 aA

Means followed by the same lowercase letter in the column and uppercase letters in the row do not differ from each other by Tukey's test ($p \leq 0.05$).

All ungerminated seeds were viable, demonstrating that this methodology proposed by RST (Brasil, 2009) is not suitable for breaking dormancy and for germinating grape seeds.

In vitro seed germination

Intact seeds of cultivar 'Niágara Rosada' in culture medium exhibited low germination *in vitro*. Germination increased when the seed was sectioned. According to Val, Alvarenga and Cecon (2010), exposing the embryo to the culture medium is fundamental for the germination of 'Niágara Rosada' seeds. In our study, the cross-sectioned seeds showed greater germination than the seeds sectioned in the micropyle region. Because the micropyle is closer to the embryo, sectioning in this region may have caused damage to the embryo. However, in the study of Val, Alvarenga and Cecon (2010) the section in the micropyle region provided better germination outcomes than the section in the transverse region. Apparently, success in this technique is dependent on the ability of the handler to perform the section.

The *V. vinifera* cultivars 'Red Globe' and 'Itália' also exhibited greater germination when the seeds were sectioned and the embryos were exposed to the culture medium. Therefore, sectioning is fundamental for breaking physical dormancy in grape seeds. *In vitro* germination provides a more controlled and aseptic environment for the germination of these seeds whose embryos have been exposed.

Overall, the cross-section of seeds associated with *in vitro* culture surpassed the conventional germination method, reducing the time to obtain seedlings from 120 to 54 days. This methodology removed the physical dormancy, directly exposing the embryo to the culture medium and increasing germination percentage. However, the results are dependent on the genotype.

Grape seeds do not have physical dormancy only. Physiological dormancy has been reported in several studies (Rajasekaran; Vine; Mullins, 1982; Ellis; Hong; Roberts, 1983; Maeda; Pereira; Terra, 1985; Pommer; Maeda; Ribeiro, 1988; Conner, 2008; Val; Alvarenga; Cecon, 2010; Generoso et al., 2019). In the work of our group, Generoso et al. (2019) proved the existence of physiological dormancy using GA_3 to break it and increase *in vitro* seed germination of cultivars 'Niágara Rosada', 'Itália' and 'Red Globe'. Val, Alvarenga and Cecon (2010) also demonstrated the breaking of this dormancy using GA_3 to increase germination in seeds of 'Niágara Rosada'. In both studies, the seed had to be sectioned to break physical dormancy, and GA_3 was necessary to break physiological dormancy.

However, the reviewed papers do not specify where in the seed are the germination inhibitors, responsible for physiological dormancy. One way to determine whether these inhibitors are present in the embryo is by removing and culturing the embryos in an *in vitro* growth medium.

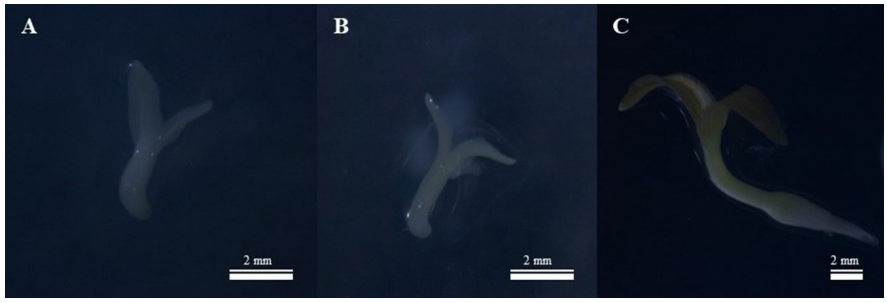

Figure 7: Embryos germinated after the fourth day of *in vitro* culture (A and B) and embryo with elongated root system and expanded cotyledon leaves in the second week of *in vitro* culture (C).

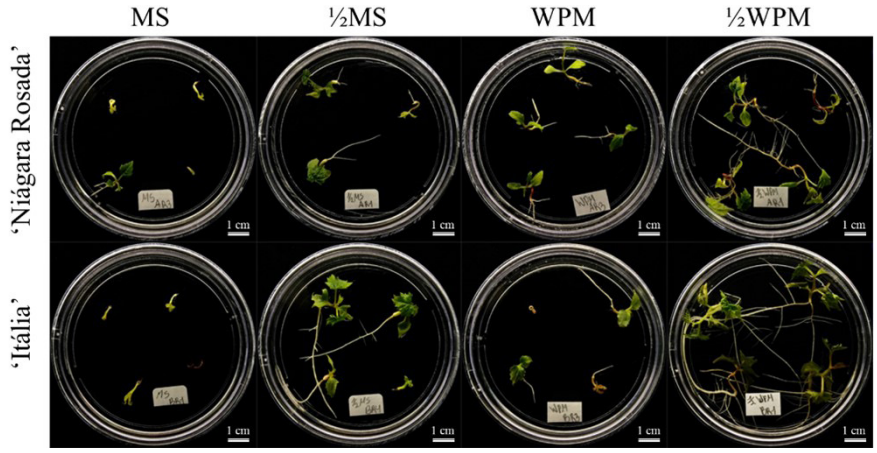

Figure 8: Germination of mature zygotic embryos after 28 days of *in vitro* culture. 'Niágara Rosada' in A, B, C and D in the MS, ½MS, WPM and ½WPM culture media, respectively, and 'Itália' in E, F, G and H in the MS, ½MS, WPM and ½WPM culture media, respectively.

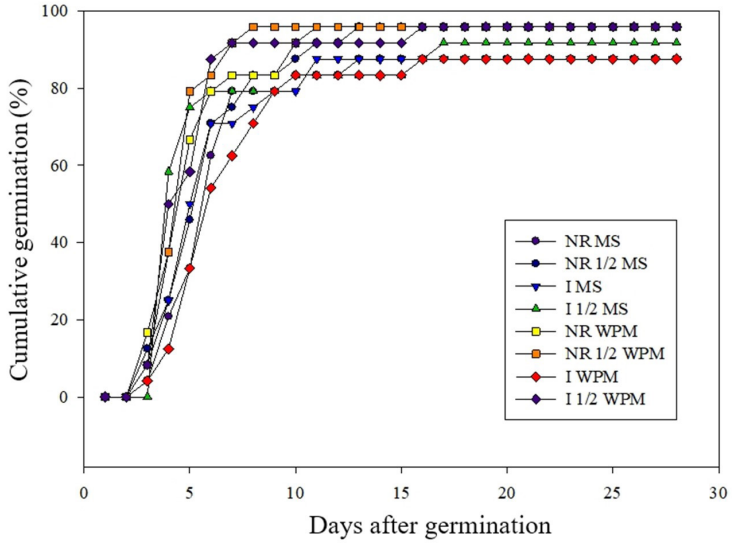

Figure 9: Cumulative germination after 28 days of *in vitro* culture of mature zygotic embryos. Cultivar 'Niágara Rosada' (NR) in culture media (MS, ½MS, WPM and ½WPM) and cultivar 'Itália' (I) in culture media (MS, ½MS, WPM and ½WPM).

In vitro germination of mature zygotic embryos

For cultivars 'Niágara Rosada' (*V. labrusca*) and Itália' (*V. vinifera*), the removal of the embryo from the seed and its culture in growth medium provided the highest GSI values (over six) and germination percentages (over 85%). Generoso et al. (2019) worked with the same cultivars and achieved similar germination percentages only when using sectioned seeds and GA_3 in the culture medium, with the result depending on the concentration of GA_3 and the cultivar. Although germination percentage was similar, it resulted in a lower number of normal seedlings when compared with our results. In addition, the maximum GSI obtained was 2.8 at the end of 45 days of germination and was dependent on the cultivar, whereas for embryos, this value was higher than six in as little as 28 days of germination.

The cultivars did not influence the evaluated variables. This can be a good indicator that the culture of mature zygotic embryos of grapevine can be used for seed germination regardless of the cultivar or the species of the genus *Vitis*. However, further work on other species, hybrids and cultivars should be carried out for confirmation.

The culture media with half the concentration of MS and WPM salts provided the highest values for all evaluated variables. The high concentration of mineral salts in the culture medium may have caused necrosis in the apical region of some explants (Figure 8). The WPM medium is less concentrated than the MS medium and has shown superior results to the latter. In a study led by Ebadi et al. (2016) with *in vitro* culture of immature zygotic embryos of *V. vinifera*, the WPM medium provided a higher percentage of normal seedlings than the MS medium. The balance of mineral salts in the culture medium is essential for the proper nutrition of seedlings when these are grown *in vitro* (Monfort et al., 2018).

The successful germination and high percentage of normal seedlings obtained with the *in vitro* culture of mature zygotic embryos can be attributed to the total exposure of the embryos to the culture medium and to their advanced embryonic stage. Another possible contributing factor is the absence of tissues containing germination inhibitors. We should expect lower percentage of inhibitors on mature seeds and higher percentage on the removed tissues like endosperm (Figure 8).

The use of mature zygotic embryos from the studied grapevine cultivars provided rapid and uniform germination (Figure 9). Rapidity and uniformity in germination are the main characteristics sought in the germination not only of mature zygotic embryos of grapevines, but of several other species such as oil palm (*Elaeis guineensis* Jacq.) (Kingsley et al., 2016; Sparjanbabu et al., 2020), murici (*Byrsonima cydoniifolia* A. Juss.) (Martendal et al., 2013) and castor bean (*Ricinus communis* L.) (Louis; Okafor; Okezie, 2018). Our research group has studied in recent years several crosses between grapes species for resistance to nematode, downy mildew and powdery mildew. A total of 1.597 seeds from the main crosses were placed to germinate (V. romanetti C 166-043 x Nocera, V. romanetti C 166-043 x (Cabernet Sauvignon x Carignane), 06354-047 x Cereza, 06354-047 x Nocera). All seeds were placed to germinate following a protocol of pre-treatment of grapes seeds as a way to increase germination (Brasil, 2009). Even with this strategy, only 558 seeds showed germination and vigor (35%), from the different crosses studied. This demonstrates the ability of *in vitro* cultivation strategies to support plant breeding programs in the conduction and conservation of germplasm. (Unpublished data).

Grape seeds have physical and physiological dormancy (Cadot; Miñana-Castelló; Chevalier, 2006; Val; Alvarenga; Cecon, 2010; Çelik, 2014; Generoso et al., 2019 Kara; Doğan; Vergili, 2020; Wang et al., 2022). By working with *in vitro* culture of mature zygotic embryos in grapevine, we were able to eliminate physical and physiological dormancy and obtain a high percentage of germination of normal seedlings, quickly and uniformly. Both of these factors are known to naturally hinder germination, which was not observed when mature zygotic embryos were cultured *in vitro* in an appropriate medium that met the nutritional requirements for the studied cultivars.

Based on a study carried out by our group, Generoso et al. (2019) managed to break the physical and physiological dormancy present in seeds of the same cultivars with the use of seed cuttings and GA_3. Nevertheless, the authors were unable to prove where the germination inhibitors are located in the seeds. Thus, from the isolation of the embryo and its culture in medium without phytoregulators, we could demonstrate that

physiological dormancy in grape seeds is not located in the embryo, but in the tissues that surround it.

The absence of external structures that cover the embryo, coupled with the availability of nutrients in essential quantities without the use of phytoregulators and the presence of a carbon source in the culture medium were sufficient to provide a high percentage of germination of normal seedlings in a rapid and uniform manner, resulting in 87.5 to 95.8% germination after only 17 days of culture.

Overall, the first assay following the recommendations described by RST required 90 days of seed stratification and another 28 days of germination, totaling approximately 120 days to obtain the seedlings. For the second assay, the time required was 54 days, and for the third, only 28 days. It is worth mentioning that for the *in vitro* assays, the seeds were not stratified for 90 days.

This is the first study in the literature that employs the technique of *in vitro* culture of mature zygotic embryos for breaking physical and physiological dormancy in mature seeds of grapevine cultivars.

Considering that grapevine is an ancient crop and that it has increasingly consumption of its fruits and processed products, there is a major need for new cultivars to be produced in a short time. These cultivars originate from breeding programs that must release cultivars increasingly productive and resistant to biotic and abiotic factors; hence, the great need for methods to accelerate this process.

The methodology developed in this study for the excision of mature zygotic embryos will enable the advance of new research aimed at breaking dormancy in seeds with some germination barrier.

CONCLUSIONS

This study defines an efficient methodology to obtain high germination percentages in grape seeds (over 85%) in a rapid and uniform manner, using mature zygotic embryos grown in ½WPM culture medium. This methodology allows an increase in the number of germinated seeds from different crosses; a reduction in the time for germination and plant production; and, consequently, a decrease in the time necessary to obtain new grapevine cultivars, accelerating breeding programs for this crop.

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AUTHORS' CONTRIBUTIONS

Conceptualization: Costa Júnior, O.D.; Carvalho, V.S.; Generoso, A.L.; Viana, A.P. Data acquisition: Costa Júnior, O.D.; Silva, L.M; Sales, R.A. Data analysis: Costa Júnior, O.D.; Carvalho, V.S; Generoso, A.L. Design of Methodology: Costa Júnior, O.D.; Carvalho, V.S.; Generoso, A.L. Writing and editing: Costa Júnior, O.D.; Carvalho, V.S.; Generoso, A.L.; Silva, L.M.; Sales, R.A.; Viana, AP.

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