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Immature Embryo Rescue and *in Vitro* Development Evaluation of Intraspecific Hybrids from Brazilian Seedless Grapevine "Superior × Thompson" Clones

Eiryanne Fonseca de Menezes[1], Eliene Matos e Silva[2], Adriana Mayumi Yano-Melo[3], Patrícia Coelho de Souza Leão[4], Natoniel Franklin de Melo[4*]

[1]State University of Feira de Santana—PPG on Plant Genetic Resources, Feira de Santana, Brazil
[2]Federal University of Pernambuco—PPG in Biology of Fungi, Recife, Brazil
[3]Federal Universityof Vale do São Francisco-CCA/CZOO, Petrolina, Brazil
[4]Embrapa Semi-Arid Tropical, Biotechnology Laboratory, Petrolina, Brazil
Email: [*]natoniel.melo@embrapa.br

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Abstract

The fruit production for export is an economically significant activity in the Valley of São Francisco River, especially in the irrigated lands of Petrolina-PE/Juazeiro-BA, Brazil. The development of new genetic material most suitable to the tropical climate and the demands of the consumer market have led to the selection of new seedless grapes cultivars. In this case, the use of the embryo rescue technique has produced satisfactory results for obtaining such materials, especially in the semiarid region. This study aimed to evaluate the *in vitro* development of intraspecific hybrids of grapevine (*Vitis vinifera* L.), derived from the rescue of immature embryos resultant from the crossing of "Superior Seedless" and "Thompson Seedless" Brazilian clones. To establish and develop the cultivation, the culture media was supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol, 0.002 g/L glycine, 0.1 mg/L indoleacetic acid (IAA), 6.5 g/L of agar, adjusted pH to 5.7. The experiment was evaluated after 90 days. The variables measured were: number of nodes, number of leaves, plant height (cm), number of roots and length (cm) of the root system and internodes. The period of 60 days of *in vitro* culture of ovules resulted in the highest values of embryos (about 50%), as well as better characterized developmental stages with higher germination (47.3%). The three types of hybrid grapes evaluated in micropropagation showed very similar values of the measured parameters, even having originated from embryos of different developmental stages.

[*]Corresponding author.

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Keywords

***Vitis vinifera*, Seedless Grapes, *in Vitro* Embryo Cultivation**

1. Introduction

The grapevine culture presents special economic and social importance in the Lower Basin of the São Francisco Valley, involving a large annual turnover meant for the domestic and foreign markets, especially standing out among the irrigated crops of the region, such as that presents the highest coefficient of direct and indirect jobs generation [1].

The viticulture in the region has some specificity because of its adaptation and behavior in those different climatic conditions. The plant physiological processes are accelerated. The spread is very fast and the first harvest begins in about eight months after the planting period. Considering that the cycle fluctuates around 120 days, it's possible to obtain even two and a half harvests a year, through the watering management and by conducting programmed pruning. This region is the only one in Brazil that exports seedless grape.

The seedless varieties represent 75% of the total number of fine table grapes exported by the region. As its preference grows among consumers, there is an expansion of cultivation areas in the Irrigation Pole of the region of Petrolina and Juazeiro, reaching about 2500 irrigated hectares with expansion trend [2].

Because of these trends, the priority is the development of new varieties of fine grapes, mostly seedless ones, adapted to different regions, and presenting high natural fertility, compatible quality with the market demands and that is less demanding in specialized labor practices as the berry thinning [3].

The biotechnology contribution to the vine culture in Vale do São Francisco lies mainly in eliminating viruses, in selection, plant maintenance and multiplication with desirable traits, and in the obtaining of new seedless varieties [4]. In this case, the improvement and the routine use of the technique of embryo rescue are crucial for increasing the efficiency of the vine genetic breeding program, making it possible to obtain large numbers of seedless plants per crossing cycle.

The technique of recue of immature embryos provides important genetic gains for the seedless feature, with the seed traces size reduction resulting from crosses between seedless parents [5]. The advantages of using this technique were evaluated by [6] under environmental conditions of the Brazilian semiarid region, quantifying the efficiency of plant regeneration by controlled intersection when applying the technique of embryo rescue.

The present study aimed to evaluate the *in vitro* development of intraspecific grapevine hybrids (*Vitis vinifera* L.) originated from the "Superior Seedless" and "Thompson Seedless" varieties crosses, in order to generate new genotypes for the vine genetic breeding program.

2. Material and Methods

The grape hybrids used in this study were derived from the "Thompson Seedless" and "Superior Seedless" Brazilian clones crossing, obtained from cultivated plants in an experimental field of Embrapa Tropical Semiarid, according to the procedures proposed by [7]. Eight weeks after the crossing, 152 ovules were collected from the obtained fruits, and inoculated into the culture media proposed by [8] under aseptic conditions. After 30 days of culture, it was performed the isolation of the immature embryos in 76 ovules and, in the other 76 ovules, embryos were isolated after 60 days of cultivation. In this case, the embryos were classified according to their development stage (**Figure 1**) and inoculated into a new culture media, using [8] formulation (**Table 1**). A third group of ovules was grown for 180 days in the initial culture media as an additional treatment.

For the analysis of the micropropagation, three hybrids were evaluated, all of them were derived of 60 days inoculation seedlings, and obtained from embryos of each developmental stage (globular, heart and torpedo), using nodal segments of approximately 1 cm length, containing an axillary bud. The seedlings were micropropagated and inoculated into 15 ml of media culture, using test tubes as containers. The test tubes were sealed with foil lids and PVC plastic film. It was used the inorganic salts and vitamins formula proposed by [8] supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol 0.002 g/L glycine, 0.1 g/L indoleacetic acid (IAA), and 6.5 g/L of agar. The pH was adjusted to 5.7 before autoclaving under a pressure of 1 atm and a 121˚C temperature for 20 minutes. The material was cultured for 90 days in a grow room with a photoperiod of 16 h, at 23˚C ± 27˚C temperature and a photosynthetic active radiation of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Table 1. Development and germination of immature embryos of "Superior × Thompson" grapevine hybrids in relation to number of days of *in vitro* ovule culture.

Number of days after inoculation	Number of ovules inoculated	Number, percentage and development stage of embryos rescued (%)				Number of germinated embryos (%)			
30	76	25				15			
		(32.9%)				(19.7%)			
		G	H	T	U	G	H	T	U
		14	00	03	08	12	00	03	00
		(18.42%)	(0.00%)	(3.95%)	(10.53%)	(15.78%)	(0.00%)	(4.11%)	(0.00%)
60	76	38				36			
		(50.0%)				(47.3%)			
		G	H	T	U	G	H	T	U
		15	12	04	07	15	12	04	05
		(19.73%)	(15.78%)	(5.26%)	(9.21%)	(19.73%)	(15.78%)	(5.26%)	(6.57%)

G (globular); H (heart); T (torpedo); U (undefined).

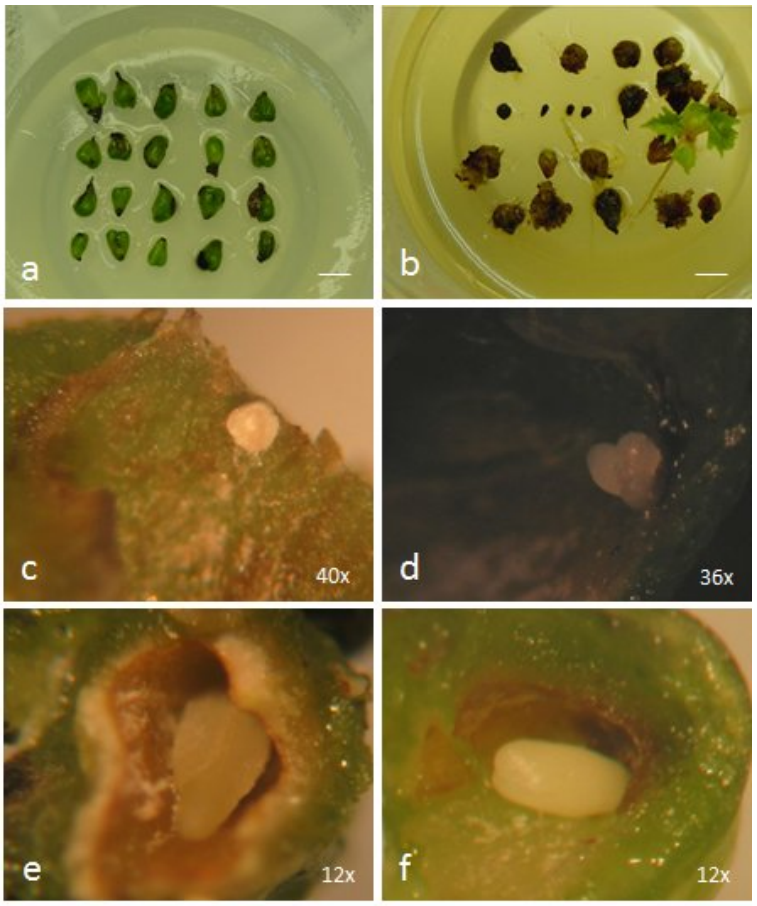

Figure 1. *In vitro* ovuleculture of grapevine "Superior" × "Thompson" seedless hybrids. (a) Ovules after 30 days of *in vitro* culture; (b) *In vitro* germination of ovules after 120 days of inoculation; (c) Ovule containing embryo at the globular stage; (d) Embryo at the heart stage; (e) Embryo detail in undefined developmental stage; (f) Ovule containing embryo at the torpedo development stage. Scales in (a) and (b) correspond to 2 cm with corresponding magnification in (c), (d), (e), and (f).

The experimental design used in this study was completely randomized with three treatments (three grapevine hybrids) in twenty five replicates. The variables measured were: number of nodes, number of leaves, number of roots, plant height (cm), root system length (cm) and internodes' length (cm). The measurements were taken with a digital caliper. The variance analysis was performed using the SisVar program [9], being the average data compared by the Tukey Test at 5% probability.

3. Results and Discussion

Table 1 presents the results obtained in the rescue of immature embryos. Among the 76 ovules opened after 60 days of culture, 38 embryos were rescued, which corresponds to 50% of the total of inoculated ovules. Among these 38 embryos, 36 had germinated, corresponding to 47.3% of inoculated ovules. On the other hand, among the 76 ovules which had been cultured for 30 days, 25 embryos were obtained (35.9% of the total) in which 15 embryos had germinated, resulting in a 19.7% percentage of hybrid seedling development. In additional control, germination occurred only in 3% of the ovules, 120 days after *in vitro* inoculation.

By studying the rescue of immature embryos in some vine varieties, [10] reported no number reduction of recovered embryos, even when seeds were grown for a long period of time. Also [11], by using the technique of rescue of immature embryos as a tool for the gene introgression in varieties of vine, obtained satisfactory results, had achieved 26.7% of rescued embryos in the "Emerald Seedless" variety, with a percentage of developed seedlings in about 19.6% after 60 days of cultivation. On the other hand, the results obtained by [12] when realizing the study about embryo in crosses of diploid and tetraploid vine varieties, had determined to be between 35 - 45 days the best period to remove the embryos of the seed-traits. In the present study, it was observed that the 60 days period after seeds inoculation resulted in an increased number of embryos produced, as well as in better characterized developmental stages with higher germination values.

In both evaluation periods, the analysis of the type of rescued embryos in comparison to the success of the germination shows a high correlation with the globular, heart and torpedo developmental stage. The embryos rescued at the 30th day in undefined stage did not germinate, probably because of the short time for development. Several studies have demonstrated the importance of determining the best period for the isolation of immature embryos from grapevine crosses. In a study by [13], using embryo rescue technique for the "Flame Seedless" vine variety, it was obtained (100%) of embryos, but with a smaller capacity (<20%) of generating seedlings. Reference [14] had obtained 20 seedlings from 44 rescued embryos from the interspecific cross between *V. vinifera* and *V. rotundifolia*. Reference [15] obtained the rate of germination of 12.67% after 28 days of culture. In an experiment with "Centennial Seedless" and "Thompson Seedless" grape varieties by [16], using culture media supplemented with benzylaminopurine, 11.9% of developed embryos to the variety 'Thompson Seedless' were observed. In our case, the values obtained were around 50% of germinated seedlings, demonstrating the good efficiency of this methodology, using 0.1 $mg \cdot L^{-1}$ indoleacetic acid.

The process of crossing between two distinct genomes probably resulted in decreased synchronization of the development of hybrid embryos. In this case, we seek to assess whether this asynchrony could also have been observed on *in vitro* multiplication phase, an effect not seen in the morphological parameters analyzed and presented in **Table 2**. The three types of hybrids in study showed very similar values of the measured parameters, even having originated from embryos of different developmental stages (**Table 2**). In this case, the plants from hybrids 1 and 3 showed slightly higher values in comparison of hybrid 2 in height and internodes length aspects. In general, we can assume that in closer genetic materials which are under the same cultivation conditions, there is a greater uniformity in the *in vitro* growth, showing similar values for all analyzed variables.

Table 2. Mean values for the number of nodes, leaves, height, and number of roots, root system length and internodes of three types of grapevine hybrids, obtained from immature embryos of grapevine at different developmental stages, after 90 days of *in vitro* culture.

Hybrids	Number of nodes	Number of leaves	Plant height (cm)	Number of roots	Root length (cm)	Internodes = length (cm)
Hybrid 1 (globular)	7.72 a	8.72 a	7.37 a	1.36 a	3.65 b	3.79 a
Hybrid 2 (heart)	7.68 a	8.64 a	6.29 b	1.32 a	4.58 a	3.43 b
Hybrid 3 (torpedo)	7.40 b	8.20 b	7.35 a	1.40 a	3.47 b	3.75 a

Average numbers followed by the same letter in each column do not differ in the Tukey test at 5% of probability.

4. Conclusions

The period of 60 days after inoculation of the ovules from the hybrid grapevine "Superior Seedless" vs. "Thompson Seedless" results in an increased number of embryos produced, as well as better characterized developmental stages with higher germination.

The three grapevine hybrids evaluated in micropropagation present very similar average values of the variables measured, even having originated from embryos of different developmental stages.

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