horticulturae

MDPI

Article

Optimization of an In Vitro Embryo Rescue Protocol for Breeding Seedless Table Grapes (*Vitis vinifera* L.) in Italy

Angelica Giancaspro [1,*], Andrea Mazzeo [2], Antonio Carlomagno [3], Agata Gadaleta [1], Stefano Somma [4] and Giuseppe Ferrara [2,*]

[1] Department of Agricultural and Territorial Sciences (DiSAAT), University of Bari Aldo Moro, Via G. Amendola 165/A, 70126 Bari, BA, Italy; agata.gadaleta@uniba.it
[2] Department of Soil, Plant and Food Sciences (DiSSPA), University of Bari Aldo Moro, Via G. Amendola 165/A, 70126 Bari, BA, Italy; andrea.mazzeo@uniba.it
[3] Agriproject Group, Via delle Orchidee 20, 70018 Rutigliano, BA, Italy; antonio.carlomagno85@gmail.com
[4] Agrisoil, Via Vittorio Veneto 89, 76011 Bisceglie, BT, Italy; somma@agrisoil.it
* Correspondence: angelica.giancaspro@uniba.it (A.G.); giuseppe.ferrara@uniba.it (G.F.)

Abstract: Conventional crossing of stenospermocarpic grapes for the obtainment of seedless cultivars presents some technical constraints causing embryo abortion in the early berry developmental stages. Embryo rescue technique partially overcomes these limitations, but the obtainment of viable plantlets relies on the optimization of several genetic and methodological issues. This work aimed to regenerate viable plants from immature ovules of stenospermocarpic table grape hybrids by applying a three-step in vitro culture protocol consisting of embryo development, embryo germination-rooting, and plantlet formation. The influence of parental genotypes (six "seedless × seedless" crosses), ovule sampling time (30, 40, 50 days after pollination (DAP)), and extent of embryo germination induction (4, 6, 8 weeks) was assessed on ovule fertilization, embryo development and germination, rooting, and plantlet formation to establish the best rescue time for each combination hybrid. Our optimized protocol included immature ovule isolation for 40 DAP and embryo germination induction for 8 weeks. As for genotypes, the most efficient embryo germination was recovered from hybrids of Thompson, Superior, and Regal cultivars, whereas the highest percentage of viable plants was derived from 50-DAP ovules of Luisa × Thompson progeny. Such an optimized protocol could be useful to maximize the efficiency of future breeding programs for grape seedlessness.

Keywords: table grape; seedless; embryo rescue; in vitro culture; stenospermocarpy; hybrids; *Vitis vinifera*

Citation: Giancaspro, A.; Mazzeo, A.; Carlomagno, A.; Gadaleta, A.; Somma, S.; Ferrara, G. Optimization of an In Vitro Embryo Rescue Protocol for Breeding Seedless Table Grapes (*Vitis vinifera* L.) in Italy. *Horticulturae* **2022**, *8*, 121. https://doi.org/10.3390/horticulturae8020121

Academic Editors: Federica Blando and Claudia Ruta

Received: 31 December 2021
Accepted: 26 January 2022
Published: 29 January 2022

1. Introduction

Grapevine (*Vitis vinifera* L.) is among the most important fruit crops in the world, native to Asia (Middle East) and today largely grown in Europe, America, and Asia for both table grape and wine consumption. *Vitis* improvement has always drawn much attention from breeders interested in improving quality and yield, postharvest storage, resistance to biotic and abiotic stresses, seedlessness, etc. [1–4]. More recently, grapevine has been greatly valorized for its renewed nutraceutical value due to the high content of bioactive molecules with antioxidant properties conferring anti-inflammatory and anti-cancer activity (e.g., resveratrol) [5–8].

Seedlessness is one of the most appreciated agronomic traits among consumers of fresh table grapes [9,10]. Several seedless cultivars are grown in different climates throughout Europe, the USA, and the Middle East. Seedless grapes traditionally preferred for dried raisins include varieties such as cv. Thompson Seedless (also known as Sultanina), but their commercial importance is reasonably growing, as they are appreciated also for fresh table consumption and easy post-harvest processing into derived products [11,12]. For these reasons, recent decades have seen enhanced efforts aimed at breeding new seedless cultivars

endowed with other valuable agronomic traits such as increased yield and resistance to pests and abiotic stresses [3,13–15].

Seedless grapes are botanically classified as parthenocarpic or stenospermocarpic [16], depending on the occurrence of fecundation after pollination, which leads to the development of berries with none (ovule development without fertilization—Corinto types) to varying degrees of rudimental weedy seed traces (abortion of seeds soon after fertilization—Sultanina types). Parthenocarpic cultivars develop into small-sized berries most suitable for raisin production [17], whereas stenospermocarpic genotypes carry larger berries mostly preferred for fresh table consumption. Stenospermocarpic grapes are heritable, as they can pass the seedless trait to their progenies and are widely used as the parents in classical breeding of elite cultivars [4]. Most commercially important seedless grapevine cultivars are intraspecific hybrids of *V. vinifera* L. Many new cultivars were selected by embryo rescue in the United States [11,18], Argentina [19,20], China [3,21], Japan [12], Italy [22], India [2,23], and Australia [1]. Breeding for seedless grapes is traditionally based on "seedless × seeded" hybridization consisting of crossing stenospermocarpic females (Sultanina variety is the most usual source of stenospermocarpic phenotype in crosses) with male parents with interesting traits, or hybridizing genotypes with different ploidy levels (seedlessness deriving from unbalanced chromosome sets) [17,24]. Compared to "seedless × seeded" crosses, hybridization of both stenospermocarpic parents is more efficient, as the number of seedless progenies is higher [25]. However, the higher percentage of embryos recovered from "seedless × seeded" crosses was correlated to a higher heterozygosity and the wider genetic base with respect to the narrow relatedness of both stenospermocarpic parents, leading to a better survival and development rate during ovule culture [26].

Conventional breeding to obtain viable progeny from intraspecific crosses between seedless cultivars is cost-expensive and time/space-consuming. Furthermore, it involves the generation and selection of thousands of hybrids each year. Moreover, traditional approaches cannot be successfully applied to stenospermocarpic grapes, as hybrid viability is severely reduced by embryo abortion in the early stages of berry development, leading to underdeveloped seeds or seed traces and very low percentages of seedless F_1 progenies ranging from 0 to 16% [27]. For both parthenocarpic and stenospermocarpic cultivars, the recovery rate of viable embryos can be significantly increased, and the time necessary to regenerate drastically reduced by applying an in vitro embryo rescue protocol. The embryo rescue technique was firstly developed by Emershad and Ramming [28] for the development of seedless grape cultivars via ovule culture of stenospermocarpic hybrids. Culturing stenospermocarpic hybrids is still the most efficient approach used to breed seedless grape cultivars. [2,21,29,30]. In vitro culture prevents immature embryo death in the stenospermocarpic berries firstly by medium plating fertilized ovules to allow embryo growth beyond the stage of abortion, and then by opportunely culturing the newly developed embryos until germination and plantlet formation. Weak embryos may be aseptically excised from fertilized ovules following the period of medium culture, or immature seed traces may be directly medium plated upon isolation from berries and induced to embryo germination [31,32]. In both cases, standard procedure involves a three-step protocol including ovule culture, embryo germination, and plantlet formation [9,33].

In addition to seedlessness in grape, embryo rescue has recently been applied in breeding programs for early ripening [25], triploidy [34–36], or interspecific crosses [2,21,36–38] in many other fruit crops such as apple [39], citrus [40], banana [41], mango [42], persimmon [43], and peach [44]. However, despite continuous efforts to optimize the in vitro rescue technology, the number of obtained F_1 hybrids is often inadequate for breeding programs of seedlessness [24], as protocol efficiency is deeply affected by several endogenous and exogenous factors. Parental crossing genotypes, ripening season, berry ripening stage upon ovule removal, seed trace size, culture medium composition, addition of growth regulators, and plantlet acclimation conditions are among the most influential factors affecting rescue efficiency [45]. Moreover, embryo viability and germination seem to be influenced also by

more intrinsic factors such as shape (globular, heart, and torpedo—from the earliest to the latest stage), thus the more immature the embryo is at excision time, the less efficient the germination. [3,46–48]. However, the embryo developmental stage at excision time does not affect the morphological features of regenerated plants [21,49,50]. Considering all the above-mentioned limitations, to date only a few seedless grape cultivars have been successfully developed [51], as the methodology is still challenging and requires the simultaneous optimization of several parameters to maximize viable plant recovery.

Very helpful tools in grape breeding programs are represented by the identification and mapping of genetic loci controlling the seedlessness trait and the development of suitable trait-associated markers to fasten the selection pipeline and enable early identification of seedless progeny. Quantitative trait loci linked to seedlessness were firstly identified on chromosome 18 [52–54], and later, Mejía et al. [55] developed an intragenic simple sequence repeats (SSR) marker mapped on the regulatory region of the *VviAGL11* candidate gene (p3_VvAGL11). More recently, discovery and exploitation of new DNA-based markers associated with seedlessness were carried out by Karaagac et al. [56], Akkurt et al. [10], Li et al. [15], Zhu et al. [3], and Muñoz-Espinoza et al. [57].

The aim of the present work was to investigate the influence of genetic and methodological factors on the efficiency of embryo rescue technique for the regeneration of seedless hybrids from crosses between some table grape cultivars (*V. vinifera* L.). Time after pollination for ovule excision (sampling time), extent of ovule culture, and ovule stage for setting up embryo induction were evaluated for several couples of crossing genotypes, and an optimized protocol for in vitro culture of stenospermocarpic grapes was thus finally established.

2. Materials and Methods

2.1. Plant Materials: Parent Vines and Hybridization

All the table grape cultivars used in this study belonged to the species *V. vinifera* and were grown in vineyards located in Bari province, Puglia region, southeastern Italy. Six different hybridizations were carried out: the stenospermocarpic soft-seeded cultivar Luisa (Stella®)was used as the female parent in all the crosses, whereas the following cultivars were used as pollen donors: Thompson Seedless® (Sultanina), White Seedless, Superior Seedless® (Sugraone), Princess Seedless® (Melissa), Crimson Seedless, and Regal Seedless. All the male grapes were stenospermocarpic, and cultivars differed for some berry characteristics briefly reported in Table 1.

Table 1. Seedless table grape cultivars used for hybridizations. Berry morphological features and identification codes are reported for each cross.

Cultivar	Species	Berry Characteristics	Cross	Id.
Luisa (♀)	*Vitis vinifera* L.	Seedless (herbaceous seed traces), white skin, elliptic shape, muscat taste		
Thompson Seedless® (♂)	*V. vinifera* L.	Seedless, white skin, oval shape, medium size, neutral taste	Luisa × Thompson S.	SG-1
White Seedless (♂)	*V. vinifera* L.	Seedless, white skin, medium size, neutral taste	Luisa × White S.	SG-2
Superior Seedless® (♂)	*V. vinifera* L.	Seedless, white skin, medium-large size, crunchy, neutral taste	Luisa × Superior S.	SG-3
Princess Seedless® (♂)	*V. vinifera* L.	Seedless, white skin, cylindrical shape, medium-large size, muscat taste	Luisa × Princess S.	SG-4
Crimson Seedless (♂)	*V. vinifera* L.	Seedless, red skin, medium-large size, elliptic shape, crunchy, neutral taste	Luisa × Crimson S.	SG-5
Regal Seedless (♂)	*V. vinifera* L.	Seedless, white skin, ovoidal shape, medium-large size, crunchy, neutral taste	Luisa × Regal S.	SG-6

2.2. Emasculation and Pollination

The pollen was collected by taking the clusters off the vines from the different 'male' cultivars at the beginning of flowering when calyptras (caps) started to fall; clusters were stored in paper bags at room temperature to allow a better dehiscence of the anthers and the collection of pollen grains at the bottom of the bag. With regards to the female flowers, the key step was to prevent accidental pollination before performing hand pollination with the selected pollen sources. The emasculation to prepare 'female' cultivar took place 4–6 days before the beginning of flowering, and both the caps and the anthers were removed all together by using forceps with very fine tips, avoiding damage to the ovary. Clusters were successively bagged to avoid contamination with other pollen sources, and both forceps and hands were sterilized with 70% ethanol between pollinations.

Once the emasculation was accomplished, the collected pollen was brushed onto the female flowers and poured into the bag to spread and adhere the grains onto the stigma of female flowers. Each bagged cluster was successively shaken to better spread the pollen; the shaking of clusters (for pollination) was repeated each day for 1 week. Paper bags were only removed when the set berries were collected for embryo rescue. Each cluster was opportunely tagged with all the necessary information on the cross.

2.3. Sampling Time

Sampling time represents the number of DAP for immature fruit collection and fertilized ovule excision. Grape clusters from each parental cross were collected after pollination at three different ripening stages: 30, 40 and 50 DAP in the vegetative season running from mid-May to mid-October 2017. Thirty berries were collected from a single cluster at each sampling time. Berries were picked from each cluster, washed for 5 min in sterile water (previously autoclaved at 121 °C for 20 min and cooled at room temperature) and then surface-sterilized twice using 70% (*w*/*v*) ethanol for 1 min. Alcohol was poured off and replaced with 10% (*w*/*v*) sodium hypochlorite for 10 min, then berries were finally rinsed three times in sterile water for 5 min each, and finally air-dried for 15 min under a laminar flow hood.

2.4. Ovule Culture and Embryo Growth

Immature fertilized ovules with the consistency of herbaceous, soft seed traces were gently excised from stenospermocarpic berries using sterile forceps and plated on Petri dishes (100 × 80 mm) containing a solid ovule culture medium-or embryo formation medium—(see paragraph below for composition). Fertilization rate was calculated as the number of immature ovules isolated from 30 berries collected from a single cluster of each cross. Ovules were maintained for 8 weeks at 25 °C in the dark at a density of 10 samples per dish. Twice-weekly transfers were performed onto fresh medium to allow increases in seed size and optimal development of the inner immature embryos.

Following the growth period, the percentage of ovule survival was recorded for each cross at each sampling time by reporting the number of survived ovules in the total number of plated explants. In this stage, the number of survived ovules was expected to correspond to the number of viable *in-ovulo* embryos, so the percentage of ovule survival was also referred to as the percentage of embryo formation (number of developed *in-ovulo* embryos in the total number of plated ovules, ×100).

2.5. Embryo Germination, Rooting, Plantlet Formation, and Acclimation

In the second phase of the rescue protocol, growing embryos were not excided from ovules, but the biggest healthy seeds that developed from the 8-week culture of immature ovules—approximately 5 mm long and 3 mm wide—were directly plated onto a solid woody plant medium (WPM, [58]), at a density of five samples per plate, to undergo induction of embryo germination (see paragraph below for germination medium composition). Immature seeds were carefully half-cut and gently embedded with the cut surface in contact with the solid jelly medium to allow a more efficient adsorption of culture nutrients

by small *in-ovulo* embryos. Embryo germination was conducted for 8 weeks in a growth chamber under a 16-h photoperiod with a light intensity of 40 μE s^{-1} m^{-1} provided by a cool-white fluorescent lamp, at a temperature of 20 ± 2 °C. Embryos showing newly formed cotyledonal leaves were observed and recorded regularly after 4, 6, and 8 weeks. Embryos that did not germinate by the 8th week were further cultured for an additional month. The percentage of embryo germination was calculated as the number of germinated embryos in the total number of formed embryos (culture-survived ovules).

The third phase of the rescue protocol was set up by individually transferring young grape shoots (approximately 1–2 weeks old and 2–3 cm long) to glass Perkin Elmer flasks (250 mL) containing a solid rooting medium (see paragraph below for composition) to allow root formation and elongation. Shoots underwent a period of in vitro growth—up to 10 weeks depending on parental genotypes and individual samples—to allow root development, elongation, and thickening in the same growth chamber and under the same conditions as germination induction. In this phase, plantlets were maintained in the same medium without sub-culturing. For acclimation, well-rooted shoots were finally counted and transplanted, without any root pruning, from glass flasks to sterile pots containing a synthetic soil mixture of perlite:peat:soil (3:1:1, *v*/*v*/*v*), covered with thin transparent plastic film to maintain high humidity. After an additional 8- to 12-week growth period, young grape plantlets were gradually uncovered to allow hardening and moved to the greenhouse with natural daylight and temperature. Plantlet numbers were recorded, and the percentage of plant development was thus estimated as the number of plantlets regenerated from the recovered embryos.

2.6. Culture Media Composition

Ovule culture medium or embryo formation medium contained the inorganic ingredients of Emershad and Ramming medium (ER, [28]) supplemented with 6% (*w*/*v*) sucrose, 4 mM asparagine, 0.3% (*w*/*v*) activated charcoal, and 0.7% (*w*/*v*) agar, adjusted to pH 6.0. Composition of ER is reported in Supplementary Table S1.

Embryo germination and shoot development medium contained 2.3% of solid WP [58] supplemented with 2% (*w*/*v*) sucrose, 4 mM asparagine, 0.3% (*w*/*v*) activated charcoal, 0.7% (*w*/*v*) agar, 5.7 μM Indolacetic acid (IAA), 4.4 μM 6-Benzylaminopurine (6-BAP), 1.4 μM Gibberellic acid (GA3), adjusted to pH 6.0.

Rooting medium was composed of half-strength Murashige and Skoog salt mixture (MS, [59]) without activated charcoal, supplemented with 0.3% agar and 1.7 μM Indolacetic acid (IAA), adjusted to pH 6.

For all the media, pH was adjusted to 6.0 using appropriate NaOH solutions prior to autoclaving at 121 °C for 20 min. The use of activated charcoal in the culture media was aimed to adsorb any inhibitory substances derived from oxidation and browning of phenolic compounds concentrated in grape ovules [21,32,36,60].

2.7. Experimental Design and Statistical Analyses

The efficiency of the embryo rescue technique for the recovery of viable hybrids from seedless grapes was evaluated under variation of the following genetic, phenological, and methodological parameters: crossing genotypes, time after pollination for initiating ovule culture (sampling time), ovule stage upon embryo growth induction, extent of germination period. Embryo rescue was applied to the progenies of six different "seedless × seedless" crosses (Table 1). Berries were collected at three different ripening stages to isolate immature fertilized ovules: 30, 40, and 50 days after pollination (DAP). After collection, seed traces with a consistency varying between soft herbaceous and hard woody were allowed to undergo a three-phase in vitro culture consisting of the following steps: immature ovule culture and embryo growth (8 weeks), embryo germination induction (4–8 weeks), and rooting and plantlet formation (2–10 weeks). Percentage of fertilization, percentage of ovule survival (embryo formation), percentage of embryo germination, percentage of shoot

rooting, and percentage of plantlet formation were recorded at each sampling stage to determine the best embryo rescue time for each seedless hybrid.

Analysis of variance (ANOVA) was performed with XLSTAT-Pro software (Addinsoft, Paris, France) at the 0.05 P level. The assumptions of variance were verified with the Levene test (homogeneity of variance) and Shapiro–Wilk test (normal distribution). The mean values obtained for the different factors were statistically separated by using the REGWQ test.

3. Results and Discussion

Since its first application by Emershad and Ramming in 1982 [28], in vitro embryo rescue has been the most promising technique to recover immature embryos and obtain viable progenies from crosses between stenospermocarpic grapes. However, the efficiency of the method is highly variable because different genetic and methodological issues must be carefully taken into account: the genotypes of crossing parents, the suitable fruit development stage for berry collection and immature ovule isolation, the right extent of ovule culture upon induction of embryo germination, the optimal composition of culture media to boost embryo development and rooting, and finally, the best environmental conditions for plantlet hardening and healthy growth (reviewed in [32]). In this study, all these parameters were considered for the application of an embryo rescue protocol for the recovery of viable hybrids from some seedless table grape cultivars.

3.1. Effect of Genotype and Sampling Time on Ovule Fertilization Rate

Many studies reported the great importance of choosing the right time for initiating in vitro ovule culture, as this seems to be the key step to ensure a successful embryo rescue [9,32,61–63]. The main reason for embryo abortion in stenospermocarpic berries is the under-development of seed endosperm, which prematurely perishes, reducing nutrients to the newly formed germ [32]. Embryo formation induces growth hormone production (i.e., cytokinins and gibberellins) that allow cell division and expansion, but berry development prematurely stops, leading to embryo death and very small fruits [64,65]. In this context, berry size has been reported to be a reliable indicator of ovule fertilization and seed development in the sense that larger berries are likely to carry bigger ovules, which are presumed to hold more-viable embryos [66]. For these reasons, to maximize the chances of progeny recovery and minimize the experimental errors due to culturing embryo-less ovules, only berries with adequate size (at least 5–6 mm in diameter) were used for our experiments.

In addition to berry size, embryo viability has also been related to sampling time in the sense that abortion seem to be more likely if the ovule is excised too early or too late, depending on the genotypes used [61]. For this reason, we collected berries at three different stages to identify that giving the highest ovule fertilization.

In the present study, ovule fertilization was found to be influenced by both berry collection stage and the genotypes of crossing parents. In fact, for each hybridization event, a difference was observed among the various sampling times in terms of fertilization rate (total number of excised ovules from 30 berries) and average ovule number per berry (Table 2). At each collection time, a significantly different number of ovules were isolated from berries of the six crosses; in detail, the fertilization rate ranged from 0 to 93 for 30-DAP berries, 30 to 83 for 40-DAP, and 15 to 99 for 50-DAP. However, two or three hybrids with closely similar values could be identified in each case: Superior S./Princess S. at 30 DAP, White S./Superior S./Regal S. at 40 DAP, and Crimson R./Regal S. at 50 DAP. "Luisa × Thompson S." was the only hybrid to show dissimilar fertilization rates compared to any of the other hybrids (Table 2). Moreover, the total number of isolated ovules was significantly higher at 50 DAP (431), followed by 40 DAP (334) and 30 DAP (274) (Table 2).

The six crosses also differed for the mean number of ovules per berry, which ranged between 0 ("Luisa × Thompson S.") and 3.1 ("Luisa × Crimson S.") at 30 DAP, 1 ("Luisa × White S.") and

2.8 ("Luisa × Princess S.") at 40 DAP, and 0.5 ("Luisa × White S.") and 4.1 ("Luisa × Princess S.") at 50 DAP (Table 2).

Time-course fertilization in the different seedless crosses is depicted in Figure 1. When Thompson S. was used as the pollen donor, no seed traces were recovered at 30 DAP because the berries were too small and immature. Thompson S. could be classified as a "late" cultivar, as ovules from its hybrid started to be collected no earlier than 40 days following fertilization in this cross. On the contrary, the earliest hybrid was from Crimson S., which displayed the highest number of fertilized ovules (93) from the very first dissection stage (30 DAP). At 40 DAP, the hybrid showing the lowest number of fertilized ovules was "Luisa × White S." (30), whereas the progeny with the most abundant seed traces were derived from Princess S. (83). Hybrid from Princess S. exhibited the highest ovule fertilization also at the last sampling time (50 DAP), with 124 isolated ovules versus only 15 excised from "Luisa × White S.". Based on these observations, Princess S. and White S. were respectively the best and the worst performing pollen donors in terms of fertilization rate among the analyzed table grape genotypes.

In the present study, fertilization trends over the whole sampling period (from 30 to 50 DAP) differed among the various genotypes (Figure 1). Cultivars Princess S. and Crimson S. gave the most fertile hybrids, as they exhibited the highest number of total ovules isolated over the three sampling times (252 and 251, respectively); on the contrary, the less productive seedless hybrid was derived from White S. (75 total ovules isolated from 30 to 50 DAP). The hybrid "Luisa × Crimson S." displayed a nearly constant fertilization rate during the whole sampling time, as suggested by the similar numbers of seed traces collected at the three sampling times (93, 75, 83). This stenospermocarpic hybrid showed only a slight reduction in the ovule fertilization rate during the overall maturation process, with a final 10.8% reduction with respect to the early sampling time. Conversely, the hybrid from White S. displayed a sharp decrease in fertilization rate from the early to the last sampling time, yielding 50% fewer fertilized ovules at 50 DAP compared with the number at 30 DAP. Finally, the widest variation in fertilization rate was exhibited by the hybrid from Princess S. with a nearly 3-fold increase in rescued ovules from 30 to 50 DAP (Figure 1).

Table 2. Effect of genotype and sampling time (days after pollination, DAP) on seedless hybrid (*V. vinifera* L.) ovule fertilization rate.

Cross (♀× ♂)	Id. Code	30 DAP		40 DAP		50 DAP		Total (n.)
		Total (n.)	Mean (n.)	Total (n.)	Mean (n.)	Total (n.)	Mean (n.)	
Luisa × Thompson S.	SG-1	0 e	0	76 b	2.5	37 d	1.2	113 c
Luisa × White S.	SG-2	30 d	1	30 c	1	15 e	0.5	75 d
Luisa × Superior S.	SG-3	48 c	1.6	36 c	1.2	99 b	3.3	183 b
Luisa × Princess S.	SG-4	45 c	1.5	83 a	2.8	124 a	4.1	252 a
Luisa × Crimson S.	SG-5	93 a	3.1	75 b	2.5	83 c	2.8	251 a
Luisa × Regal S.	SG-6	60 b	2	34 c	1.1	73 c	2.4	167 b
Total n.		276 C	1.8	334 B	1.9	431 A	2.4	1041

The total number of isolated ovules at each sampling time refers to 30 berries collected from each cluster. The mean was calculated by dividing the total number of ovules by 30. Letters within columns indicate significant differences ($p < 0.05$) in the number of isolated ovules for each cross at the different sampling times and for the total number, according to REGWQ test. Capital letters in the last row indicate significant differences ($p < 0.05$) in the number of isolated ovules at each sampling time, according to REGWQ test.

Ovule fertilization in seedless table grape hybrids is strongly affected by sampling time, but interestingly, is also quite different in each cross combination, thus confirming the strong influence of genotype, in this case of the male pollen donor. Only the hybrids from Thompson S. and White S. exhibited a decrease in fertilized ovule numbers in the last sampling time, whereas in all the other crosses, the latest sampling stage yielded the highest

numbers of ovules. Thus, sampling time is a key factor for obtaining the highest rate of ovules, and this knowledge can hardly be known *a priori*. As the significant differences observed between crosses are due to the simultaneous influence of berry ripening stage and genotype of crossing parents, experimental assessments such as those described in the present study are crucial to detect the right and most efficient rescue time to maximize the number of fertilized ovules collected.

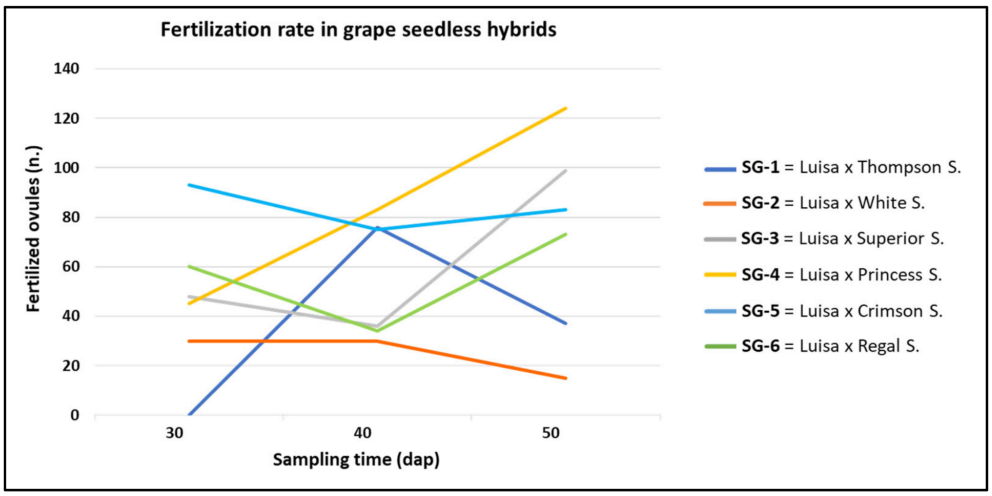

Figure 1. Patterns of ovule fertilization in six hybrids derived from seedless table grape cultivars (*V. vinifera* L.). Data refer to three sampling times: 30, 40, and 50 days after pollination (DAP).

3.2. Effect of Genotype and Sampling Time on Embryo Formation and Germination

According to recent studies, a crucial methodological issue for the recovery of viable hybrids from seedless crosses seems to be the extent of the ovule culture period. Liu et al. [26] reported that a greater embryo rescue efficiency could be achieved by extending embryo growth during the ovule culture phase, in the sense that survival and development into plantlets was greater for more advanced embryos at the time of excision. For this reason, we maintained ovule culture from all the hybridization events onto a solid medium (ovule culture medium) until formation of big, healthy seeds approximately 5 mm long and 3 mm wide. Ovule culture medium is fundamental to increase the recovery rate of potentially abortive embryos [1,22,30,32,33]. The ovule culture phase was maintained for 8 weeks at 25 °C in the dark, with twice-weekly transfers onto fresh medium to allow swelling of seeds and optimal development of inner immature embryos. To avoid lowering the hybrid recovery rate, we did not carry ovule culture too long (over 8 weeks) [26].

Following the in vitro culture period, the percentage of viable ovules (percentage ovule survival) was calculated for each cross and for each sampling time as the number of survived ovules in the total number of plated explants (Table 3). As each survived ovule was presumed to carry a developed *in-ovulo* embryo, the percentage of ovule survival corresponded to the percentage of embryo formation. We found that the highest survival rates were gained by culturing fertilized ovules isolated at 40 DAP (86.1%, "Luisa × Superior S.") and 50 DAP (83.8%, "Luisa × Thompson S."); conversely, in vitro culture was less efficient in rescuing immature ovules when applied to seed traces excised from berries at 30 DAP (0% for "Luisa × Thompson S." and 26.7% from "Luisa × Princess S."). The only exception was represented by the hybrid from "Luisa × White S.", which reached the highest percent embryo formation from ovules sampled at 30 DAP (40.0%), followed by a slight decrease in the successive ripening stages (Table 3). However, the best values for this hybrid were significantly lower than in all the other crosses. The most efficient embryo formation was obtained by applying in vitro culture to immature ovules isolated from hybrids of Thompson S., Superior S., and Regal S.

Healthy grape seeds derived from 8-week cultured ovules—presumably carrying viable and developed embryos—were directly plated onto a solid WPM medium to undergo

induction of germination and formation of cotyledonal leaves. WPM was used in the germination stage because of its referenced beneficial effects on embryo germination and plantlet development as reported in literature [21,33,60]. The practice of half-cutting seeds and placing them with the cut surface in contact with the solid medium was well-reported in literature as an efficient method to increase the germination rate of embryos deriving from soft seed coated grape varieties. On the contrary, higher germination rates of embryos belonging to cultivars with hard seed coat were achieved by completely exciding embryos from seeds and plating them on the appropriate medium [20,32,47,49].

For each hybridization event and sampling time, germinated embryos were recorded after 4, 6, and 8 weeks of induction and compared to the total number of plated ovules (percentage of embryo germination). In seedless grape breeding, the identification of the right sampling time for each cultivar is fundamental to obtain the best embryo rescue efficiency through the application of an in vitro culture protocol. Best rescue time is usually referred to as the ideal number of DAP for the optimal ovule excision and embryo germination. In the present study, in vitro culture was successfully applied to all the tested hybridization events, but embryo rescue efficiency was widely influenced by several factors represented by parental cross, collection time, and extent of germination period. Effects of crossing genotypes, excision time, and induction period are reported in Table 3 and Figure 2a–c.

As reported in Table 3, each cross exhibited some variability for the rate of embryo recovery and embryo germination within the three sampling times. Except for "Luisa × Crimson S.", which showed statistically different values of embryo recovery in all the three sampling stages, for all the other crosses similar values were observed for two out of three sampling times. The best rescue time was dependent on each seedless hybrid under investigation and ranged from 0 ("Luisa × Thompson S.") to 86.1 ("Luisa × Superior S."). In all cases, the optimal embryo recovery was obtained from ovules isolated at 40 or 50 DAP, except for "Luisa White S." (30 DAP).

For each hybridization event, variability among the three sampling times was observed also for embryo germination: no cotyledonal leaves were scored after 4 weeks of induction on WPM, maybe because this was too short a time to promote the germination process. At 6 weeks of induction, statistically different values for the germination rate were observed in two out of three sampling stages for each hybrid, except for "Luisa × Princess S." and "Luisa × Regal S.", which showed significant dissimilarities at 30, 40, and 50 DAP. Six-week germination efficiency ranged between 0 ("Luisa × Thompson S.", "Luisa × White S.", "Luisa × Princess S.") and 22.3 ("Luisa × Thompson S."). After 8 weeks of WPM induction, each cross exhibited similar germination efficiency at two out of three sampling times. Additionally, in this case, the best rescue time was dependent on each seedless genotype under investigation and ranged from 0 ("Luisa × Thompson S.", Luisa × White S.") to 52.2 ("Luisa × Crimson S.").

Except for "Luisa × Thompson S." hybrid—for which no fertilized ovules were isolated from berries at 30 DAP—germination was recorded from immature explants excised at every sampling time, but the stage of ovules and the extent of embryo induction on WPM were fundamental in determining the efficiency of embryo rescue. As reported in Table 3, the most efficient embryo rescue was obtained by applying in vitro culture to immature ovules isolated at the intermediate sampling time (40 DAP). In fact, considering the total number of embryos germinated after 8 weeks of induction on WPM, the numbers of grape shoots derived from 30 and 50 DAP were almost identical (36 vs. 40), whereas a significantly higher number of embryos (58) were rescued from immature ovules excised at 40 DAP. The correlation between excision time and percentage of embryo germination observed in this study was in accordance with literature that reported weaker embryo viability in the case of ovules excised in the earliest or in the latest berry ripening stages [63].

Table 3. Effects of parental crossing genotypes, sampling time (DAP), and induction period on ovule survival (embryo recovery) and embryo germination of table seedless grape hybrids (*V. vinifera* L.).

Cross (♀× ♂)-Sampling Time	Isolated Ovules [a]	Embryo Recovery [b]		Embryo Germination [c]				
	(n.)	(n.)	(%)	4 Weeks (n.)	6 Weeks (n.)	6 Weeks (%)	8 Weeks (n.)	8 Weeks (%)
Luisa × Thompson S.-30 DAP	0 c	0 b	0	0	0 b	0	0 b	0
Luisa × Thompson S.-40 DAP	76 a	30 a	39.5	0	1 b	3.3	9 a	30.0
Luisa × Thompson S.-50 DAP	37 b	31 a	83.8	0	7 a	22.6	7 a	22.6
Luisa × White S.-30 DAP	30 a	12 a	40.0	0	0	0	3 a	25.0
Luisa × White S.-40 DAP	30 a	9a b	30.0	0	0	0	3 a	33.3
Luisa × White S.-50 DAP	15 b	5 b	33.3	0	0	0	0 b	0
Luisa × Superior S.-30 DAP	48 b	28 b	58.3	0	3 b	10.7	11 ab	39.3
Luisa × Superior S.-40 DAP	36 c	31 b	86.1	0	6 ab	19.4	13 b	41.9
Luisa × Superior S.-50 DAP	99 a	74 a	74.8	0	9 a	12.2	9 a	12.2
Luisa × Princess S.-30 DAP	45 c	12 b	26.7	0	0 c	0	6 b	50.0
Luisa × Princess S.-40 DAP	83 b	55 a	66.3	0	6 a	10.9	17 a	30.9
Luisa × Princess S.-50 DAP	124 a	50 a	40.3	0	3 b	6.0	3 b	6.0
Luisa × Crimson S.-30 DAP	93 a	42 b	45.2	0	3 b	7.1	9 b	21.4
Luisa × Crimson S.-40 DAP	75 b	23 c	30.7	0	5 b	21.7	12 a	52.2
Luisa × Crimson S.-50 DAP	83 ab	60 a	72.3	0	8 a	13.3	8 b	13.3
Luisa × Regal S.-30 DAP	60 b	18 b	30.0	0	0 c	0	7 b	38.9
Luisa × Regal S.-40 DAP	34 c	29 b	85.3	0	4 b	13.8	4 b	13.8
Luisa × Rega S.l-50 DAP	73 a	60 a	82.2	0	13 a	21.7	13 a	21.7
Total n. of shoots from 30 DAP-ovules					36 b			
Total n. of shoots from 40 DAP-ovules					40 b			
Total n. of shoots from 50 DAP-ovules					58 a			

Letters within columns indicate significant differences ($p < 0.05$) for the parameters examined for each specific cross at the three different sampling times, according to REGWQ test. [a] isolated ovules = number of fertilized ovules collected from 30 berries of each hybrid at each sampling time. [b] embryo recovery (percentage embryo formation) = number of developed embryos (viable ovules) in the total number of plated ovules, ×100. [c] percentage embryo germination = number of germinated embryos in the total number of developed embryos (viable ovules), ×100.

The extent of the embryo induction period also influenced the efficiency of embryo rescue from the different sampling times. As depicted in Figure 2a–c, no germination was scored after 4 weeks of WPM culture on immature seeds derived from 30-, 40-, or 50-DAP ovules, but cotyledonal leaves were observed in the 4th–6th and 6th–8th weeks of induction. After 6 weeks, germination was detected on immature embryos isolated at 30 DAP only for two out of six hybrids ("Luisa × Thompson S." and "Luisa × Superior S."), whereas the remaining 60% of progenies exhibited shoot formation from ovules excised at 40 DAP or 50 DAP. In detail, embryo germination ranged as follows: from 0% ("Luisa × White S.", "Luisa × Princess S." and "Luisa × Regal S.") to 10.7% ("Luisa × Superior S.") for immature ovules collected at 30 DAP; from 3.3 ("Luisa × Thompson S.") to 21.7% ("Luisa × Crimson S.") for ovules isolated at 40 DAP, and from 6% ("Luisa × Princess S.") to 22.6% ("Luisa × Thompson S.") for explants excised at 50 DAP. Overall, the best embryo rescue percentage was obtained for 50-DAP ovules of the hybrid "Luisa × Thompson S.".

After 8 weeks of germination induction, only two out of six seedless hybrids (33.3%) showed the highest percentage of embryo germination from ovules isolated at 30 DAP (50.0% and 38.9%, respectively, for "Luisa × Princess S." and "Luisa × Regal S."), while the most efficient embryo rescue for the remaining four progenies was obtained from 40-DAP ovules, with a range from 30.0% ("Luisa × Thompson S.") to 52.2% ("Luisa × Crimson S."). Finally, embryo germination for explants excised at 50 DAP ranged from 0% ("Luisa × White S.") to 22.6% ("Luisa × Thompson S."). All grape embryos that were not germinated at 8 weeks were further cultured for an additional 2 months with weekly transfers onto fresh medium, and they were finally scored as "not viable".

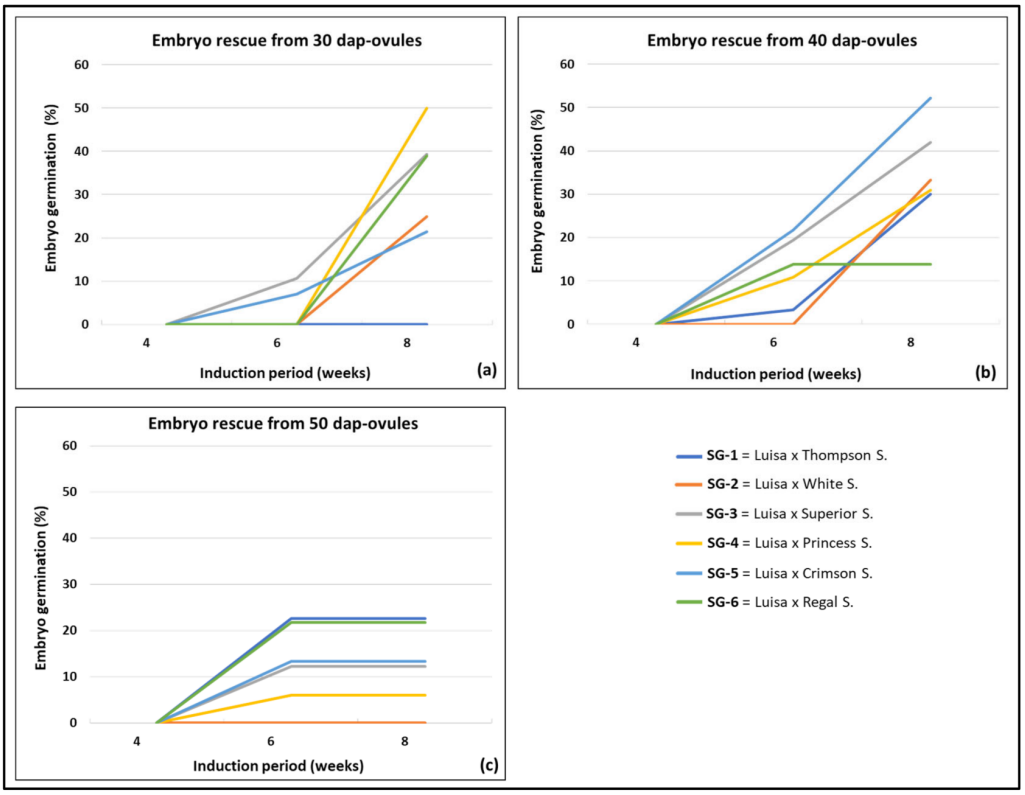

Figure 2. Influence of crossing genotypes, ovule sampling time (DAP), and germination induction period (4, 6, 8 weeks) on embryo rescue of hybrids from seedless table grape cultivars (*V. vinifera* L.). Percentage embryo germination = number of germinated embryos to the total number of formed embryos (viable ovules), expressed as percentage. (**a**) Embryo rescue after induction of 30-DAP ovules. (**b**) Embryo rescue after induction of 40-DAP ovules. (**c**) Embryo rescue after induction of 50-DAP ovules.

Identification of the best sampling times for the different seedless hybrids is also reported in Figure 3, which depicts the time-course trend for embryo germination rates during the tested induction period. Histograms report the increase in germinated embryo numbers during the 0–4th, 4th–6th and 6th–8th week of WPM culture. As reported in Figure 3a, for immature ovules excised in the early ripening stage (30 DAP), the germination rate was very low between the 4th and 6th weeks, but it rose when induction was extended up to 8 weeks; this could be explained by the fact that embryos collected too early need a very long culture period to germinate. On the contrary, Figure 3c clearly shows that the germination rate of 50-DAP ovules underwent a fast increase during the first 6 weeks of WPM culture but slowed down and reached a plateau when induction was prolonged beyond the 6th week. Based on these observations, we speculated that since 50-DAP ovules were collected in an advanced state of maturation, they completely expressed all their germination potential in the very first stage of induction, without responding to further stimulation. Finally, Figure 3b shows that the number of shoots formed from immature ovules isolated at 40 DAP experienced a first increase between the 4th and 6th weeks, and continued growing also when WPM culture was prolonged beyond (6th–8th week). The final number of rescued 40-DAP embryos was higher than those derived from 30-DAP ovules but still lower compared to the number formed by 50-DAP ovules. In the present study, the best sampling time for initiating immature ovule culture and obtaining the highest germination efficiency was 40 DAP for all of the tested seedless progenies, except for "Luisa × White S." and "Luisa × Regal S.", which achieved the best embryo rescue by applying the in vitro culture protocol to ovules excised at 30 and 50 DAP, respectively.

In conclusion, to optimize time, space, and effort to rescue immature embryos from these seedless hybrids, the best choice would seem to be to isolate immature ovules at 40 DAP and induce embryo germination by culturing on WPM for 8 weeks.

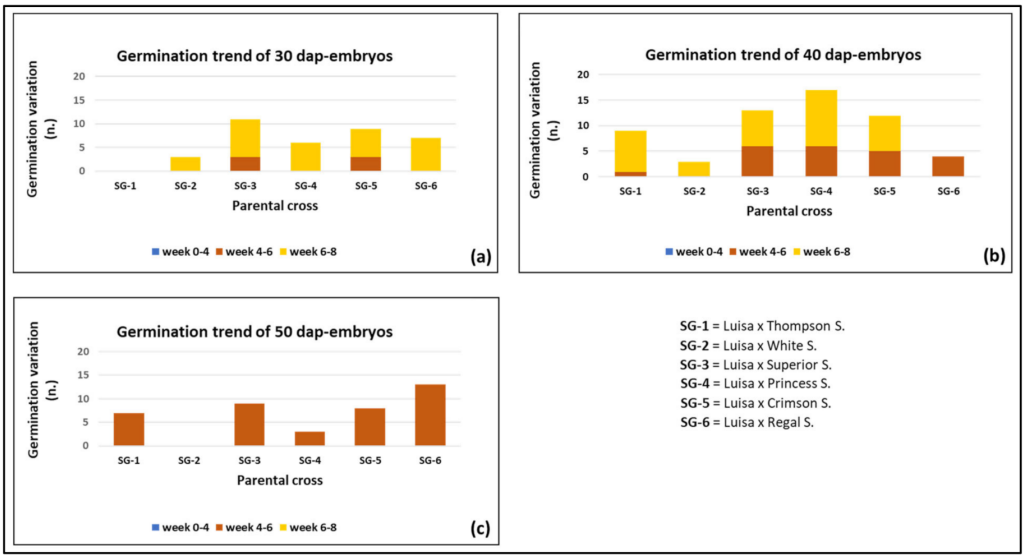

Figure 3. Variation of germination rate during the embryo induction phase of six hybrids from seedless table grape crosses (*V. vinifera* L.). Blue, light grey, and dark grey colors represent germination variation between 0–4th, 4th–6th, and 6th–8th week, respectively. (**a**) Embryo rescue from immature ovules isolated at 30 DAP. (**b**) Embryo rescue from immature ovules isolated at 40 DAP. (**c**) Embryo rescue from immature ovules isolated at 50 DAP.

3.3. Effect of Genotype and Sampling Time on Shoot Rooting and Plantlet Development

The last phase of the rescue protocol was not conducted in vitro; instead, well-rooted shoots were gently transplanted to sterile pots containing a synthetic soil mixture and maintained in a growth chamber. The percentage of plantlet formation was estimated as the number of plantlets developed from the total number of developed embryos (Table 4). After 8 to 12 weeks of the growth period, grape plantlets were moved to a greenhouse with natural daylight and temperature for hardening and acclimation. Table 4 and Figure 4 report shoot rooting and plant recovery efficiency for the six hybridization events under investigation. As for the previous phases of the rescue protocol, they varied based on both parental genotypes and sampling times.

Table 4 shows that, except for "Luisa × Thompson S.", which obtained the best rooting efficiency for shoots derived from 50-DAP embryos, and "Luisa × Superior S.", which yielded the highest number of rooted shoots from 30-DAP explants, most rooted shoots were derived from 40-DAP ovules. For each hybridization event, the maximum value was significantly higher than those of the other sampling times (Table 4). Among the 40-DAP shoots, the best rooting efficiency was observed for shoots of "Luisa × Crimson S." (21.7%), whereas the worst was observed for shoots of "Luisa × Regal S." (6.9%). The overall best rooting efficiency among the six crosses was shown by "Luisa Thompson S." (22.6%).

For three out of six seedless crosses ("Luisa × Princess S.", "Luisa × Crimson S.", "Luisa × Regal S."), the highest plant recovery percentage was achieved from ovules isolated at 40 DAP. Only one hybrid ("Luisa × Superior S.") showed the best plant formation from embryos isolated in the early maturation stage (30 DAP), and the progeny of one seedless cross ("Luisa × White S.") completely failed in forming plants, as it yielded only one single regenerated young shoot that perished in the rooting phase. The overall best plant formation percentage was obtained by applying the embryo rescue protocol to immature ovules of the hybrid "Luisa × Thompson S." isolated at 50 DAP, whereas the least efficient plant recovery derived from immature embryos excised at 30 DAP from hybrids "Luisa × White S.", "Luisa × Princess S.", and "Luisa × Regal S." (no plantlet

formation) (Table 4). The highest percentage of plant formation from 30-DAP ovules was obtained for "Luisa × Superior S." (10.7%), whereas the maximum percentage of plants from 40-DAP ovules was scored for "Luisa × Crimson S." (13.0%).

In the present study, we observed that the pattern of rooting was maintained during plant formation. Except for "Luisa × White S.", which yielded one single rooted shoot that did not mature, the efficiency of plant development was in line with the efficiency of rooting. This was because shoots that formed long and thick roots were more likely to develop into adult plants. Interestingly, this behavior was different from that observed in the previous stages of in vitro culture when the pattern of embryo recovery and germination was not always maintained in the following developmental stages (see Table 3). Based on these observations, we deduced that the early stages of in vitro culture are the most susceptible and variable, whereas a proper rooting process is a stronger guarantee of shoot stability and survival leading to successful plant development. Moreover, this could explain why "Luisa × Superior S." was the only hybrid to show the best plant formation efficiency from embryos isolated in the earliest maturation stage (30 DAP). In fact, as depicted in Figure 5, this hybrid was the one to exhibit the highest shoot rooting efficiency from ovules isolated at 30 DAP. Instead, for other crosses, 30 DAP ranked first in some stages before rooting (i.e., embryo recovery for "Luisa × White S.", embryo germination for "Luisa × Princess S." and "Luisa × Regal S.", ovule isolation for "Luisa × Crimson S.").

More generally, we observed that—except for "Luisa × Superior S."—the best rescue time for each hybrid was the specific sampling stage that simultaneously ensured the highest efficiency in several in vitro culture steps from immature ovule culture to plantlet formation. In this study, the best sampling time was represented by 40 DAP, as for all six crosses, it ensured the highest efficiency in two or three stages of in vitro culture (Figure 5). On the other hand, 30 and 50 DAP were the worst rescue times because they yielded the best efficiency in only one stage of the rescue protocol (Figure 5). In the cross "Luisa × Thompson S.", isolation of immature ovules at 30 DAP was never the best choice in any of the culture stages (Tables 2–4, Figures 1–5). A schematic representation of in vitro culture stages is depicted in Figure 6.

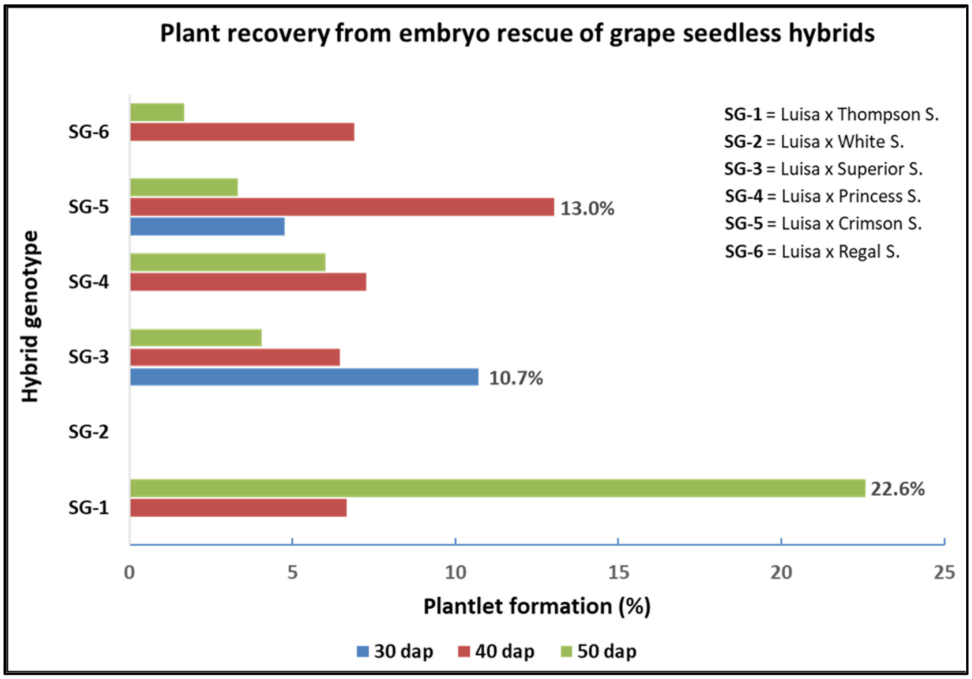

Figure 4. Plant recovery from embryo rescue of seedless table grape hybrids (*V. vinifera* L.). Colors of bars represent the different sampling times (30, 40, 50 DAP). Best plant recovery efficiency is reported for each sampling time. Missing bars indicate no plants obtained from the corresponding hybrid genotype.

Table 4. Effects of crossing genotypes and sampling time (DAP) on plantlet formation from embryo rescue of table seedless grape hybrids (*V. vinifera* L.). Percentages of shoot development, shoot rooting, and plant formation are compared to the number of formed embryos (viable ovules) after immature ovule culture.

Cross (♀× ♂)-Sampling Time	Embryo Recovery [a]	Shoot Development [b]		Shoot Rooting [c]		Plantlet Formation [d]	
	(n.)	(n.)	(%)	(n.)	(%)	(n.)	(%)
Luisa × Thompson S.-30 DAP	0 b	0b	0	0 c	0	0 c	0
Luisa × Thompson S.-40 DAP	30 a	9 a	30	2 b	6.7	2 b	6.7
Luisa × Thompson S.-50 DAP	31 a	7 a	22.6	7 a	22.6	7 a	22.6
Luisa × White S.-30 DAP	12 a	3 a	25.0	0	0	0	0
Luisa × White S.-40 DAP	9 a	3 a	33.3	1	11.1	0	0
Luisa × White S.-50 DAP	5 b	0 b	0	0	0	0	0
Luisa × Superior S.-30 DAP	28 b	11 ab	39.3	3 b	10.7	3	10.7
Luisa × Superior S.-40 DAP	31 b	13 a	41.9	3 b	9.7	2	6.5
Luisa × Superior S.-50 DAP	74 a	9 b	12.2	6 a	8.1	3	4.1
Luisa × Princess S.-30 DAP	12 b	6 b	50.0	0 c	0	0 b	0
Luisa × Princess S.-40 DAP	55 a	17 a	30.9	6 a	10.9	4 a	7.3
Luisa × Princess S.-50 DAP	50 a	3 b	6.0	3 b	6.0	3 a	6.0
Luisa × Crimson S.-30 DAP	42 b	9 b	21.4	2 b	4.8	2	4.8
Luisa × Crimson S.-40 DAP	23 c	12 a	52.2	5 a	21.7	3	13.0
Luisa × Crimson S.-50 DAP	60 a	8 b	13.3	2 b	3.3	2	3.3
Luisa × Regal S.-30 DAP	18 c	7 b	38.9	0 b	0	0 b	0
Luisa × Regal S.-40 DAP	29 b	4 c	13.8	2 a	6.9	2 a	6.9
Luisa × Regal S.-50 DAP	60 a	13 a	21.7	2 a	3.3	1 a	1.7

Letters within columns indicate significant differences ($p < 0.05$) in the parameters examined for each specific cross at the three different sampling times, according to REGWQ test. [a] Embryo recovery = number of developed embryos (viable ovules) to the total number of plated ovules, ×100. [b] Shoot development = number of germinated embryos to the total number of developed embryos (viable ovules), ×100. [c] Shoot rooting = number of rooted shoots to the total number of developed embryos (viable ovules), ×100. [d] Plantlet formation = number of formed plantlets to the total number of developed embryos (viable ovules), ×100.

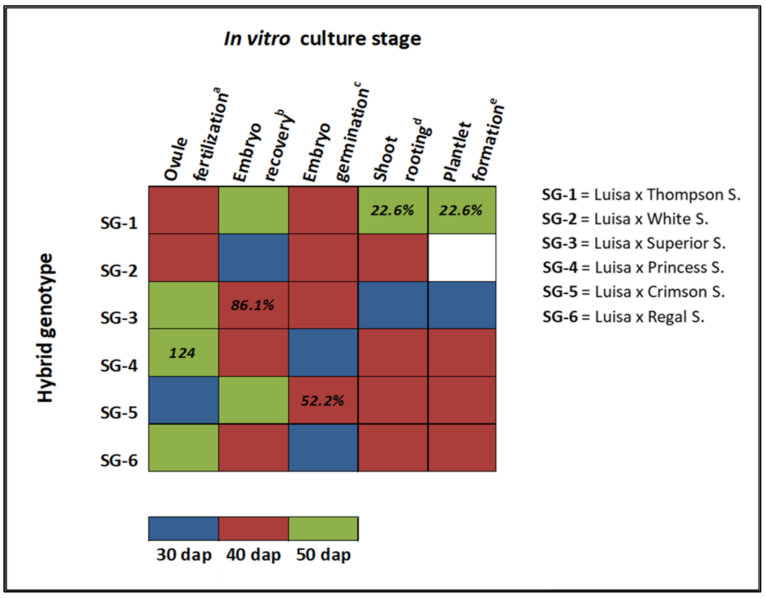

Figure 5. Graphical summary of best sampling times for ovule isolation, embryo recovery, embryo germination, shoot rooting, and plant formation in the embryo rescue of six table seedless grape hybrids (*V. vinifera* L.). [a] = number of fertilized ovules isolated from 30 berries. [b] = percentage of embryos developed from immature ovules. [c] = percentage of shoots developed from the total recovered embryos (germination); [d] = percentage of rooted shoots to total recovered embryos. [e] = percentage of plantlets developed from total rescued embryos. Colors represent different sampling times (30, 40, 50 DAP). Numbers in boxes indicate the best value for each stage of in vitro culture. White box means no plants from SG-2 embryos.

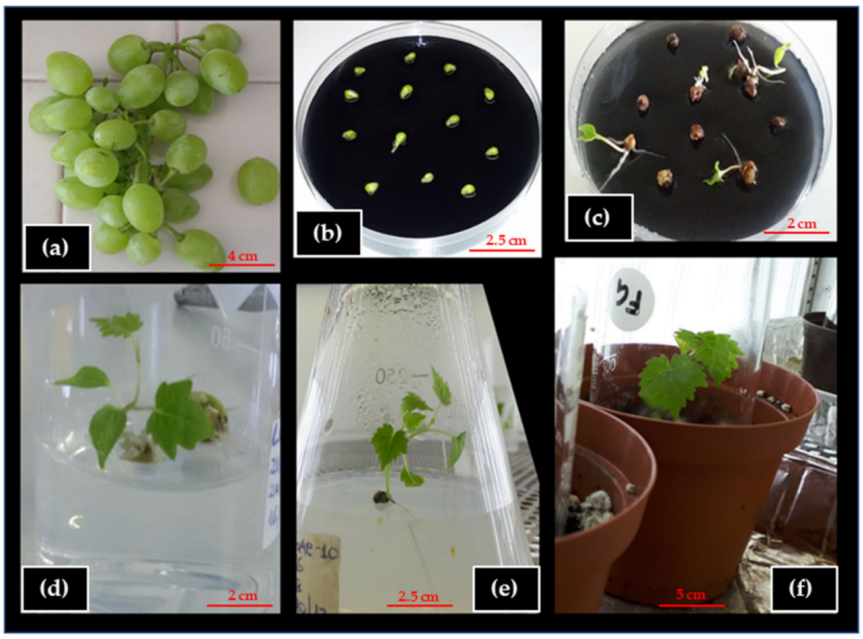

Figure 6. Representative stages of in vitro protocol for embryo rescue of table seedless grape hybrids (*V. vinifera* L.). (**a**) Berry collection at 40 DAP. (**b**) Immature ovule isolation and embryo development on embryo culture medium (3rd week). (**c**) Shoot formation after 6-week culture on WPM germination medium. (**d**) Shoot development after 2-week culture on rooting medium. (**e**) Shoot rooting after 4-week culture on rooting medium. (**f**) Eighteen-week old plantlets during acclimation on perlite:peat:soil substrate in pots in the greenhouse.

4. Conclusions

Embryo rescue is a very powerful tool to overcome some technical limitations of conventional breeding for seedlessness; however, its effectiveness is strictly dependent on several variable factors. In the present work, both genetic and methodological issues were addressed to optimize an in vitro protocol for the regeneration of viable hybrids from crosses between stenospermocarpic table grape cultivars. The following important conclusions were drawn, and we think they could be helpful to maximize the efficiency of embryo recovery in future breeding programs for grape seedlessness. The rescue protocol was successfully applied to all of the hybridization events, as it led to some plantlet obtainment, but sampling time (berry ripening stage for immature ovule isolation) and crossing genotypes were crucial factors affecting the efficiency of ovule fertilization, embryo formation and germination, shoot rooting, and plantlet formation. Among the three rescue steps—immature ovule culture (embryo formation), embryo germination, and plantlet development—the first two were fundamental and critical in determining the recovery rate of viable plants. Moreover, the extent of embryo induction could also be opportunely tuned (prolonged not over 8 weeks) to optimize space and time for gaining the best recovery efficiency. Finally, the ovule fertilization rate increased in berries collected in a more advanced developmental stage (50 DAP), but the most viable embryos came from immature ovules isolated at 40 DAP.

In conclusion, both genetic and technical factors should be carefully addressed to establish the best in vitro protocol for the regeneration of seedless grape hybrids. Based on the evidence collected in this study on some stenospermocarpic cultivars and given the consistency with similar works in the literature, our findings could help improve future breeding programs for grape seedlessness and make embryo rescue a more efficient and predictable method compared to conventional breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8020121/s1, Table S1: Composition of ER medium.

Author Contributions: Conceptualization, G.F.; Data curation, A.G. (Agata Gadaleta) and G.F.; Formal analysis, A.G. (Angelica Giancaspro) and G.F.; Funding acquisition, G.F.; Investigation, A.G. (Angelica Giancaspro), A.M. and A.C.; Methodology, A.G. (Angelica Giancaspro), S.S., A.M. and A.C.; Project administration, G.F.; Resources, S.S. and G.F.; Supervision, G.F.; Validation, A.G. (Angelica Giancaspro) and G.F.; Visualization, A.G. (Angelica Giancaspro) and A.G. (Agata Gadaleta); Writing—original draft, A.G. (Angelica Giancaspro); Writing—review and editing, A.G. (Angelica Giancaspro), A.G. (Agata Gadaleta) and G.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by Grape & Grape Group S.r.l.—agreement n.11 of 22 September 2017.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available within the article and its Supplementary Material. The data are available on request from the corresponding author.

Acknowledgments: This work was partly supported by Grape & Grape Group S.r.l. The authors wish to thank Antonio Carlomagno for the support in the vineyards (pollen collection and crosses) and Stefano Somma for the technical support in the pollination activities.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Li, S.; Geng, X.; Chen, S.; Liu, K.; Yu, S.; Wang, X.; Zhang, C.; Zhang, J.; Wan, Y.; Luo, Q.; et al. The co-expression of genes involved in seed coat and endosperm development promotes seed abortion in grapevine. *Planta* **2021**, *254*, 87. [CrossRef] [PubMed]
2. Singh, N.V.; Singh, S.K.; Singh, A.K. Standardization of embryo rescue technique and bio-hardening of grape hybrids (*Vitis vinifera* L.) using Arbuscular mycorrhizal fungi (AMF) under subtropical conditions. *Vitis* **2011**, *50*, 115–118.
3. Liu, S.M.; Sykes, S.R.; Clingeleffer, P.R. Improved in ovulo embryo culture for stenospermocarpic grapes (*Vitis vinifera* L.). *Aust. J. Agric. Res.* **2003**, *54*, 869–876. [CrossRef]
4. Zhu, P.; Gu, B.; Li, P.; Shu, X.; Zhang, X.; Zhang, J. New coldresistant, seedless grapes developed using embryo rescue and marker-assisted selection. *Plant Cell Tissue Organ Cult.* **2020**, *140*, 551–562. [CrossRef]
5. Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.W.; Fong, H.H.S.; Farnsworth, N.R.; Kinghorn, A.D.; Mehta, R.G.; et al. Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. *Science* **1997**, *275*, 218–220. [CrossRef] [PubMed]
6. Nivelle, L.; Hubert, J.; Courot, E.; Jeandet, P.; Aziz, A.; Nuzillard, J.-M.; Renault, J.-H.; Clément, C.; Martiny, L.; Delmas, D.; et al. Anti-Cancer Activity of Resveratrol and Derivatives Produced by Grapevine Cell Suspensions in a 14 L Stirred *Bioreactor*. *Molecules* **2017**, *22*, 474. [CrossRef]
7. Annunziata, G.; Maisto, M.; Schisano, C.; Ciampaglia, R.; Narciso, V.; Tenore, G.C.; Novellino, E. Resveratrol as a Novel Anti-Herpes Simplex Virus Nutraceutical Agent: An Overview. *Viruses* **2018**, *10*, 473. [CrossRef]
8. Kumar, S.; Chang, Y.-C.; Lai, K.-H.; Hwang, T.-L. Resveratrol, a molecule with anti-inflammatory and anti-cancer activities: Natural product to chemical synthesis. *Curr. Med. Chem.* **2021**, *28*, 3773–3786. [CrossRef]
9. Li, G.R.; Ji, W.; Wang, G.; Zhang, J.X.; Wang, Y.J. An improved embryo-rescue protocol for hybrid progeny from seedless *Vitis vinifera* grapes x wild Chinese Vitis species. *In Vitro Cell. Dev. Biol.-Plant.* **2014**, *50*, 110–120. [CrossRef]
10. Akkurt, M.; Tahmaz, H.; Veziroglu, S. Recent developments in seedless grapevine breeding. *S. Afr. J. Enol. Vitic.* **2019**, *40*, 1. [CrossRef]
11. Ramming, D.W.; Emershad, R.L.; Tarailn, R.A. stenospermocarpic, seedless *Vitis vinifera* × *Vitis rotundifolia* hybrid developed by embryo rescue. *Hort. Sci.* **2000**, *3*, 732–734. [CrossRef]
12. Notsuka, K.; Tsuru, T.; Shiraishi, M. Seedless-seedless grape hybridization via *in-ovulo* embryo culture. *J. Jpn. Soc. Hort. Sci.* **2001**, *70*, 7–15. [CrossRef]
13. Angulo, O.; Fidelibus, M.W.; Heymann, H. Grape cultivar and drying method affect sensory characteristics and consumer preference of raisins. *J. Sci. Food Agric.* **2007**, *87*, 865–870. [CrossRef]
14. Aujla, K.M.; Shah, N.A.; Ishaq, M.; Farooq, A. Post-harvest losses and marketing of grapes in Pakistan. *Sarhad J. Agric.* **2011**, *27*, 485–490.
15. Li, S.; Li, Z.; Zhao, Y.; Zhao, J.; Luo, Q.; Wang, Y. New disease-resistant, seedless grapes are developed using embryo rescue and molecular markers. *3 Biotech* **2020**, *10*, 4. [CrossRef]
16. Stout, A.B. Seedlessness in grapes. *Hortic. Rev.* **1936**, *11*, 159–184.
17. Lahogue, F.; This, P.; Bouquet, A. Identification of a codominant scar marker linked to the seedlessness character in grapevine. *Theor. Appl. Genet.* **1998**, *97*, 950–959. [CrossRef]

18. Gray, D.J.; Mortensen, J.A.; Benton, C.M.; Durham, R.E.; Moore, G.A. Ovule culture to obtain progeny from hybrid seedless bunch grapes. *J. Am. Soc. Hortic. Sci.* **1990**, *115*, 1019–1024. [CrossRef]
19. Agüero, C.B.; Riquelme, C.; Tizio, R. Embryo rescue from seedless grapevines (*Vitis vinifera* L.) treated with growth retardants. *Vitis* **1995**, *34*, 73–76.
20. Valdez, J.G.; Ulanovsky, S.M. In vitro germination of stenospermic seeds from reciprocal crosses (*Vitis vinifera* L.) applying different techniques. *Vitis* **1997**, *36*, 105–107.
21. Tian, L.L.; Wang, Y.J.; Niu, L.; Tang, D.M. Breeding of disease resistant seedless grapes using Chinese wild *Vitis* spp. I. In Vitro embryo rescue and plant development. *Sci. Hortic.* **2008**, *117*, 136–141. [CrossRef]
22. Gribaudo, I.; Zanetti, R.; Botta, R.; Vallania, R.; Eynard, I. In Ovulo embryo culture of stenospermocarpic grapes. *Vitis* **1993**, *32*, 9–14.
23. Bharathy, P.V.; Karibasappa, G.S. Influence of pre-bloom sprays of benzyladenine on in vitro recovery of hybrid embryos from crosses of thompson seedless and 8 seeded varieties of grape (*Vitis* spp.). *Vitis* **2003**, *42*, 199–202.
24. This, P.; Lahogue, F.; Adam-Blondon, A.F.; Doligez, A. Towards marker-assisted selection for seedlessness in grapevine. *Acta Hort.* **2000**, *528*, 221–229. [CrossRef]
25. Ramming, D.W. The use of embryo culture in fruit breeding. *Hort. Sci.* **1990**, *25*, 393–398. [CrossRef]
26. Liu, S.M.; Sykes, S.R.; Clingeleffer, P.R. Effect of culture medium, genotype, and year of cross on embryo development and recovery from in vitro cultured ovules in breeding stenospermocarpic seedless grape varieties. *Aust. J. Agric. Res.* **2008**, *59*, 175–182. [CrossRef]
27. Narayanaswami, S.; Norstog, K. Plant embryo culture. *Bot. Rev.* **1964**, *30*, 587–628. [CrossRef]
28. Emershad, R.L.; Ramming, D.W. In-ovulo embryo culture of *Vitis vinifera* L. cv. 'Thompson Seedless'. *Hort. Sci.* **1982**, *17*, 576.
29. Ponce, M.T.; Agüero, C.B.; Gregori, M.T.; Tizio, R. Factors aaffecting the development of stenospermic grape (*Vitis vinifera*) embryos cultured in vitro. In *VII International Symposium on Grapevine Genetics and Breeding*; ISHS Acta Horticulturae 528; ISHS: Montpellier, France, 2000; Volume 2, pp. 667–672.
30. Tang, D.; Wang, Y.; Cai, J.; Zhao, R. Effects of exogenous application of plant growth regulators on the development of ovule and subsequent embryo rescue of stenospermic grape (*Vitis vinifera* L.). *Sci. Hortic.* **2009**, *120*, 51–57. [CrossRef]
31. Sharma, D.R.; Kaur, R.; Kumar, K. Embryo rescue in plants-a review. *Euphytica* **1996**, *89*, 325–337. [CrossRef]
32. Li, Z.; Li, T.; Wang, Y.; Xu, Y. Breeding new seedless grapes using in ovulo embryo rescue and marker-assisted selection. *In Vitro Cell Dev. Biol. Plant* **2015**, *51*, 241–248. [CrossRef]
33. Wang, A.L.; Wang, Y.J.; Tang, D.M.; Zhang, C.H. Research on improvement of seedling rate in embryo rescue of seed less grape. *Sci. Agric. Sin.* **2010**, *43*, 4238–4245.
34. Ji, W.; Li, Z.Q.; Zhou, Q.; Yao, W.K.; Wang, Y.J. Breeding new seedless grape by means of in vitro embryo rescue. *Genet. Mol. Res.* **2013**, *12*, 859–869. [CrossRef]
35. Sun, L.; Zhang, G.; Yan, A.; Xu, H. The study of triploid progenies crossed between different ploidy grapes. *Afr. J. Biotechnol.* **2011**, *10*, 5967–5971.
36. Yang, D.; Li, W.; Li, S.; Yang, X.; Wu, J.; Cao, Z. In vitro embryo rescue culture of F1 progenies from crosses between diploid and tetraploid grape varieties. *Plant Growth Regul.* **2007**, *51*, 63–71. [CrossRef]
37. Guo, Y.S.; Li, K.; Zhao, Y.H.; Guo, X.W. Study of embryo rescue on cross between tetraploid table grape (Vitis vinifera L.) and Vitis amurensis Rupr. In Proceedings of the 2nd Conference Horticulture Science Technology, Beijing, China, 18–19 December 2010; pp. 147–149.
38. Ramming, D.W.; Walker, M.A.; Tenscher, A.; Krivanek, A.F. Breeding table and raisin grapes with increased fruit quality while retaining Pierce's disease resistance. *Acta Hortic.* **2009**, *827*, 445–450. [CrossRef]
39. Druart, P. Aneuploids and variants of apple (*Malus domestica* Borkh.) through in vitro culture techniques. *Acta Hortic.* **2000**, *520*, 301–307. [CrossRef]
40. Xie, K.D.; Yuan, D.Y.; Wang, W.; Xia, Q.M.; Wu, X.M.; Chen, C.W.; Chen, C.L.; Grosser, J.W.; Guo, W.W. Citrus triploid recovery based on 2x × 4x crosses via an optimized embryo rescue approach. *Sci. Hortic.* **2019**, *252*, 104–109. [CrossRef]
41. Uma, S.; Saraswathi, M.S.; Akbar, A.; Mustaffa, M.M. Embryo rescue and plant regeneration in banana (*Musa* spp.). *Plant Cell Tissue Organ Cult.* **2011**, *105*, 105–111. [CrossRef]
42. Krishna, H.; Singh, S.K. Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement: A review. *Biotechnol. Adv.* **2007**, *25*, 223–243. [CrossRef]
43. Hu, D.; Tang, X.; Zhang, Q.; Luo, Z. Cross compatibilities of oriental persimmon 'Mopanshi' and 'Luotian-tianshi' with 'Zenjimaru'. *Acta Hortic.* **2013**, *996*, 165–170. [CrossRef]
44. Yamada, A.; Tao, R. Controlled pollination with sorted reduced and unreduced pollen grains reveals unreduced embryo sac formation in *Diospyros kaki* Thunb. *'Fujiwaragosho' J. Jpn. Soc. Hortic. Sci.* **2007**, *76*, 133–138. [CrossRef]
45. Pommer, C.V.; Ramming, D.W.; Emershad, R.L. Influence of grape genotype, ripening season, seed trace size, and culture date on *in ovule* embryo development and plant formation. *Bragantia* **1995**, *54*, 237–249. [CrossRef]
46. Emershad, R.L.; Ramming, D.W.; Serpe, M.D. In Ovulo embryo development and plant formation from stenospermic genotypes of *Vitis vinifera*. *Am. J. Bot.* **1989**, *76*, 397–402. [CrossRef]
47. Burger, P.; Trautmann, I.A. Manipulations of ovules to improve in vitro development of *Vitis vinifera* L. embryos. *Acta Hortic.* **2000**, *528*, 613–619. [CrossRef]

48. Guo, Y.; Zhao, Y.; Li, K.; Liu, Z.; Lin, H.; Guo, X.; Li, C. In vitro embryo rescue culture of F1 progenies from crosses between tetraploid grape and *Vitis amurensis* Rupr. *Afr. J. Agric. Res.* **2011**, *6*, 4906–4909.
49. Valdez, J.G. Immature embryo rescue of grapevine (*Vitis vinifera* L.) after an extended period of seed trace culture. *Vitis* **2005**, *44*, 17–23.
50. de Menezes, E.F.; de Silva, E.M.; Yano-Melo, A.M.; de Souza Leão, P.C.; de Melo, N.F. Immature embryo rescue and *in vitro* development evaluation of intraspecific hybrids from brazilian seedless Grapevine "Superior × Thompson" clones. *Am. J. Plant Sci.* **2014**, *5*, 1956–1960. [CrossRef]
51. Ji, W.; Li, G.R.; Luo, Y.X.; Ma, X.H.; Wang, M.; Ren, R. In vitro embryo rescue culture of F1 progenies from crosses between different ploidy grapes. *Gen. Mol. Res.* **2015**, *14*, 18616–18622. [CrossRef]
52. Fanizza, G.; Lamaj, F.; Costantini, L.; Chaabane, R.; Grand, M.S. QTL analysis for fruit yield components in table grapes (*Vitis vinifera*). *Theor. Appl. Genet.* **2005**, *111*, 658–664. [CrossRef]
53. Cabezas, J.A.; Cervera, M.T.; Ruiz-Garcia, L.; Carreno, J.; Martínez-Zapater, J.M. A genetic analysis of seed and berry weight in grapevine. *Genome* **2006**, *49*, 1572–1585. [CrossRef]
54. Costantini, L.; Battilana, J.; Lamaj, F.; Fanizza, G.; Grando, M.S. Berry and phenology-related traits in grapevine (*Vitis vinifera* L.): From quantitative trait loci to underlying genes. *BMC Plant Biol.* **2008**, 8–38. [CrossRef]
55. Mejía, N.; Soto, B.; Guerrero, M.; Casanueva, X.; Houel, C.; Miccono, M.; Ramos, R.; Le Cunff, L.; Boursiquot, J.M.; Hinrichsen, P.; et al. Molecular, genetic and transcriptional evidence for a role of *VvAGL11* in stenospermocarpic seedless in grapevine. *BMC Plant Biol.* **2011**, *11*, 57. [CrossRef]
56. Karaagac, E.; Vargas, A.M.; de Andrés, M.T.; Carreño, I.; Ibáñez, J.; Carreño, J.; Martínez-Zapater, J.M.; Cabezas, J.A. Marker assisted selection for seedlessness in table grape breeding. *Tree Gen. Genom.* **2012**, *8*, 1003–1015. [CrossRef]
57. Muñoz-Espinoza, C.; Di Genova, A.; Sánchez, A.; Correa, J.; Espinoza, A.; Meneses, C.; Maass, A.; Orellana, A.; Hinrichsen, P. Identification of SNPs and InDels associated with berry size in table grapes integrating genetic and transcriptomic approaches. *BMC Plant Biol.* **2020**, *20*, 365. [CrossRef]
58. Lloyd, G.; McCown, B. Commercially feasible micropropagation of Mountain Laurel, Kalmia latifolia, by use of shoot-tip culture. *Proc. Int. Plant Prop. Soc.* **1980**, *30*, 421–427.
59. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
60. Emershad, R.L.; Ramming, D.W. Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). *Plant Cell Rep.* **1994**, *14*, 6–12. [CrossRef]
61. Bin, H.; Bird, S.; Kemble, R.; Miki, B.; Keller, W. Plant regeneration from microspore-derived embryos of *Brassica napus*: Effect of embryo age, culture temperature, osmotic pressure, and abscisic acid. *In Vitro Cell. Dev. Biol.* **1991**, *27*, 28–31.
62. Midani, A.R.; Sharma, H.C.; Singh, S.K. Effect of ovule age on ovulo-embryo culture in seeded and seedless grape genotypes. *Ind. J. Hortic.* **2002**, *59*, 359–362.
63. Li, G.R.; Wang, Y.J.; Tang, D.M.; Luo, Q.W. Study on the sampling dates of embryo rescue techniques for seedless grape. *J. Agric. Univ. Hebei* **2004**, *27*, 17–21.
64. Varoquaux, F.; Blanvillain, R.; Delseny, M.; Gallois, P. Less is better: New approaches for seedless fruit production. *Trends Biotechnol.* **2000**, *18*, 233–242. [CrossRef]
65. Premachandran, A.; Dhayasree, K.; Kurien, S. Seedless fruits: Fruits of future. *J. Pharmacogn. Phytochem.* **2019**, *8*, 1053–1059.
66. Liu, S.M.; Sykes, S.R.; Clingeleffer, P.R. Pollen fertility and berry setting behaviour of the grape variety Carina. *Austr. J. Exp. Agric.* **2007**, *47*, 877–882. [CrossRef]