horticulturae

MDPI

Article

Embryo Rescue Breeding of New Cold-Resistant, Seedless Grapes

Yannan Chu [1,2,3,†], Min Li [1,2,†], Ruonan Li [1,2], Kangzhuang Zhang [1,2], Pengpeng Qiu [1,2], Xiaojian Yuan [1,2], Yulei Han [1,2], Xinyu Liu [1,2], Yan Xu [1,2,*] and Guotian Liu [1,2,*]

1 State Key Laboratory of Crop Stress Biology in Arid Areas, College of Horticulture, Northwest A&F University, Yangling 712100, China; cynnx102030@163.com (Y.C.); minmin@nwafu.edu.cn (M.L.)
2 Key Laboratory of Horticultural Plant Biology and Germplasm Innovation in Northwest China, Ministry of Agriculture, Northwest A&F University, Yangling 712100, China
3 Institute of Horticulture, Ningxia Academy of Agricultural and Forestry Sciences, Yinchuan 750002, China
* Correspondence: yan.xu@nwafu.edu.cn (Y.X.); gtliu@nwafu.edu.cn (G.L.)
† These authors contributed equally to this work.

Abstract: Seedlessness in grapes is much appreciated by consumers and especially in cultivars consumed either as table grapes or as raisins. In many parts of the world, low temperature is the main environmental stress limiting grape production. In this study, stenospermocarpic (seed abortion) cultivars were selected as the female parents while seeded cold-resistant cultivars were selected as the male parents to develop new cold-resistant seedless grapes using embryo rescue technology, which has previously been shown to be a highly efficient way of breeding seedless grapes. Here, we report optima in genotype, sampling time, and culture medium for the embryo rescue of 14 hybrid combinations. Our results indicate that the embryo development rate (39.9%) and the seedlings rate (21.5%) were highest among the 14 crosses when 'Ruby Seedless' was used as the female parent and 'Beibinghong' was used as the male parent. The best sampling times for 'Yuehong Wuhe', 'Ruby Seedless', and 'Melissa seedless' were 37, 55, and 52 days after flowering, respectively. Embryo rescue efficiency was highest when the sucrose concentration for seedlings was maintained at about 1.0%. Using molecular markers, we detected 91 hybrids with seedless traits and 18 hybrids with cold resistance traits.

Keywords: grape; embryo rescue breeding; seedless traits; cold resistance; molecular marker

Citation: Chu, Y.; Li, M.; Li, R.; Zhang, K.; Qiu, P.; Yuan, X.; Han, Y.; Liu, X.; Xu, Y.; Liu, G. Embryo Rescue Breeding of New Cold-Resistant, Seedless Grapes. *Horticulturae* **2023**, *9*, 992. https://doi.org/10.3390/horticulturae9090992

Academic Editor: Jérôme Grimplet

Received: 25 July 2023
Revised: 31 August 2023
Accepted: 31 August 2023
Published: 2 September 2023

1. Introduction

The European grapevine (*Vitis vinifera* L.) bears abundant fruit, and thus offers high economic benefit to growers. Its fruit is in strong demand for winemaking but also for fresh consumption and as a dried fruit. Accordingly, grapes are widely grown around the world. The seedless trait, which improves the edibility of both the fresh and the dried product, is much favored by the consumer, and it is therefore a key indicator for evaluating new cultivars selected to meet the growing demand for both fresh and dried grapes. There are many high-quality seedless grapes already in production, including 'Flame seedless', 'Crimson seedless', and 'Ruby Seedless', but seedlessness remains a main focus of breeders of new table and dried grape cultivars because of their increased market value over seeded cultivars.

Prior to 1980, the conventional breeding method for seedlessness in grapes was to use a seeded grape as the female parent and a seedless grape as the male parent. The best hybrids were then backcrossed with their parents or repeatedly crossed to obtain new cultivars [1,2]. This method, in which the female parent is limited to the seeded varieties, involves much time and effort and, thus, a high cost. Meanwhile, the hybrid progeny has a low seedless rate. In vitro culture of embryos was first achieved in cherry by Tukey in

1933 [3]. Some 50 years later, embryo rescue technology was achieved in grapes when, in 1982, two grape seedlings were successfully cultivated from the ovules of a seedless grape by Ramming and Emershad using embryo rescue [4–6]. Experiments show that by using this technology, a seedless grape can be used as the female parent, and the breeding process is sped up by about six years.

Embryo rescue technology involves three stages: immature ovule embryo culture, embryo germination, and embryo seedling formation. Based on large numbers of experimental studies, Li et al. (2001) concluded that the main factors determining the success of embryo rescue are the sampling time, the parental genotype, the culture medium, and conditions [7].

The emergence of molecular markers further sped up the breeding process for seedless grapes [1,8]. The application of biotechnology allows a further speeding up of seedless grape breeding [9,10]. In 1998, Lahogue obtained two RAPD markers (random amplified polymorphic markers) through screening the major genes that control seedless traits [11]. It was found that seedless traits in grapes were controlled by multiple genes, and, among these, a few played major roles in regulating the seedless traits while others, called complementary recessive genes, played auxiliary roles [8,12]. Using the seedless marker SCF27-2000, Mejía and Hinrichsen carried out a preliminarily screening and identified the seedless traits of the hybrid progeny of 'Ruby Seedless' × 'Sultanina' [13], and they reported that VvAGL11 was a candidate gene related to the seedless trait of grapes and that a seedless marker named P3-VvAGL11 was obtained [14,15]. The above markers are of great significance for the initial identification of seedless grapes, and are widely used in the preliminary screening of grape hybrid progenies [16]. In China, based on the RAPD marker UBC-26945 that had been studied earlier, Wang et al. used the hybrid combination of 'Thompson seedless' and 'Muscat' in order to continue to develop a 'GLSP1' marker, which can be used to test the seedless characteristic of grapes [17].

Low temperature is one of the most severe abiotic stresses that limits grape growth and geographical distribution. Extreme low temperatures in winter can cause serious damage to both buds and branches [18,19], and the grapevines should thus be buried in soil when the temperature falls below −15 °C in order to cope with the cold environment in winter, which not only increases the production cost of the vineyard but also causes a series of problems, such as tree body damage, soil erosion, and soil horizon destruction, that considerably limit the development of grape production [20,21]. Thus, it is of great importance to breed new cold resistant cultivars. The Chinese wild grape Vitis amurensis is extremely cold tolerant, and can survive in temperatures as low as −40 to −50 °C [22]. It is therefore an excellent germplasm resource for cold-resistance breeding [23,24]. Two RAPD markers, S238-854 and S241-717, can be used to identify the cold resistant traits that were obtained by Zhang et al. in their 2010 experimental study on the cold resistance characteristics of Chinese wild grapes [25].

In our present study, based on embryo rescue technology, using high quality Chinese grape varieties with good cold resistance as male parents and seedless grapes as female parents, new germplasm combining good cold resistance with seedlessness was developed. The cold resistance and the seedless characteristics of their hybrid offspring were preliminarily screened using molecular marker-assisted selection.

2. Materials and Methods

2.1. Plant Materials

Fourteen hybrid crosses were conducted in the Xinjiang Development and Research Centre of Grapes and Melons, Shanshan County, Xinjiang Uygur Autonomous Region, China (42°53′ N, 90°13′ E), in the Pigeon Mountain Demonstration area of Qingtongxia wine producing area, eastern foot of Helan Mountain, Ningxia (38°25′ N, 105°97′ E), and in the Grape Repository of Northwest A&F University, Yangling, Shaanxi (34°27′ N, 108°08′ E) during 2019–2020.

2.2. Reagents and Instruments

Regents were 75% ethanol solution, 1% NaClO, agar, IAA, IBA, 6-BA, casein, and activated carbon.

Instrument were tweezers, scalpel, filter paper, scissor, inoculating device sterilizer (MM-2), autoclave (MLS-3751L-PC, Panasonic, Osaka, Japan), alcohol burner, dissecting microscope (SMZ-140, Motic, Xiamen, China), and gel imaging system (Alpha imager HP, ProteinSimple, San Jose, CA, USA).

2.3. Pollen Collection, Emasculation and Hybridization

The best time to collect pollen is to choose grapevines whose growth is vigorous and when the inflorescences are yellow and blooming in a small area. Emasculation, which means removing the anthers from female parents, was performed 3~4 days before the flowering of the female parents. On the second day after emasculation, pollination started when a transparent mucus was seen to be secreted by the stigma. The whole pollination procedure lasted for 2–3 days (Figure 1).

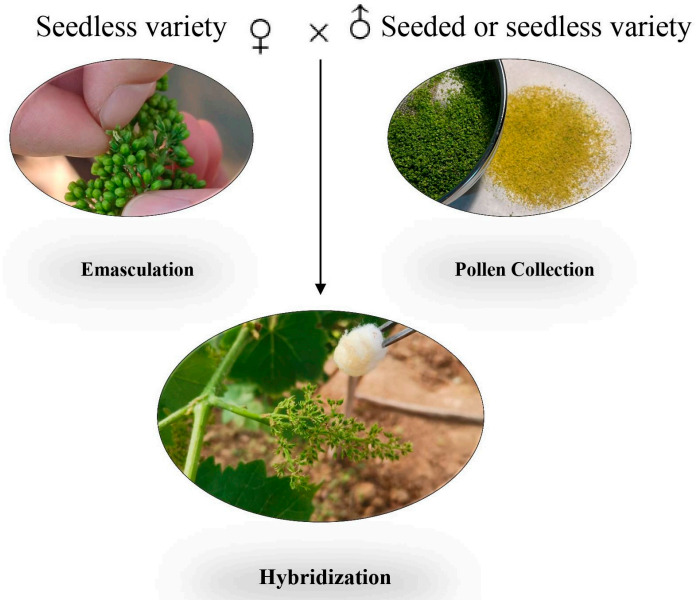

Figure 1. Field Hybridization.

2.4. Sampling

In this experiment, three hybrid combinations were selected for continuous sampling, and different sampling times were selected according to the different phenological phases (the different phase of grape development during the whole growth period in a year) between the male parent and the female parent. 'Melissa seedless' × 'Xinyu' was sampled at 52 days after full bloom (DAF), 54 DAF and 56 DAF; 'Ruby Seedless' × 'Shine-Muscat' was sampled at 53 DAF, 55 DAF, and 57 DAF; and 'Yuehong Wuhe' × 'SP740' was sampled at 33 DAF, 35 DAF, and 37 DAF. These hybrid fruits were put into an ice box, and were then brought to the laboratory for embryo rescue. The embryo development rate and the seedling rate for different sampling times were recorded and compared, and the best sampling times of each combination were determined.

2.5. Ovule Culture and Embryo Development

Embryo rescue. The harvested hybrid fruits were placed on a 'clean bench' for disinfection, as in a previous study [26]. In brief, the fruits (Figure 2a) were soaked in 75% ethanol solution for 30 s, then washed with sterile water twice, and then soaked in 1% NaClO for 20 min. The bottle was shaken every five mins in order to make it fully soaked and disinfected. After NaClO was poured out, the bottle was cleaned with sterile water 5 times

until there was no foam in order to complete the disinfection of the grape fruit. Then, the sterilized fruits were cut open in order to strip out all of the ovules aseptically (Figure 2b) and rescue the ovules, as per our previous study [26]. The ovules were inoculated into embryo development medium (MM3 + sucrose 60 g/L + hydrolyzed casein 0.5 g/L + inositol 0.1 g/L + agar 7 g/L + activated carbon 3 g/L) (Figure 2c). Ovules were then cultured in the dark for 60 days at 25 ± 2 °C. At the end of this time, the ovules were excised under a dissecting microscope (Figure 2d,e), and they were then placed in an embryo germination medium (WPM + 0.2 mg/L 6-BA + 20 g/L sucrose (2% sucrose) + 1.5 g/L activated carbon + 0.1 g/L inositol + 7 g/L agar) (Figure 2f,g). From the records and the counts of the numbers of embryos developing and germinating and the seedlings formed, we calculated the rate of ovule development and seedling formation. For 'Ruby seedless' × 'Shine Muscart' and 'Yuehong Wuhe' × 'Sp740', we used embryo gemination medium with different concentrations of sucrose (1%, 1.5%, 2%, and 3%) in order to evaluate the effectors of sucrose concentration on embryo rescue.

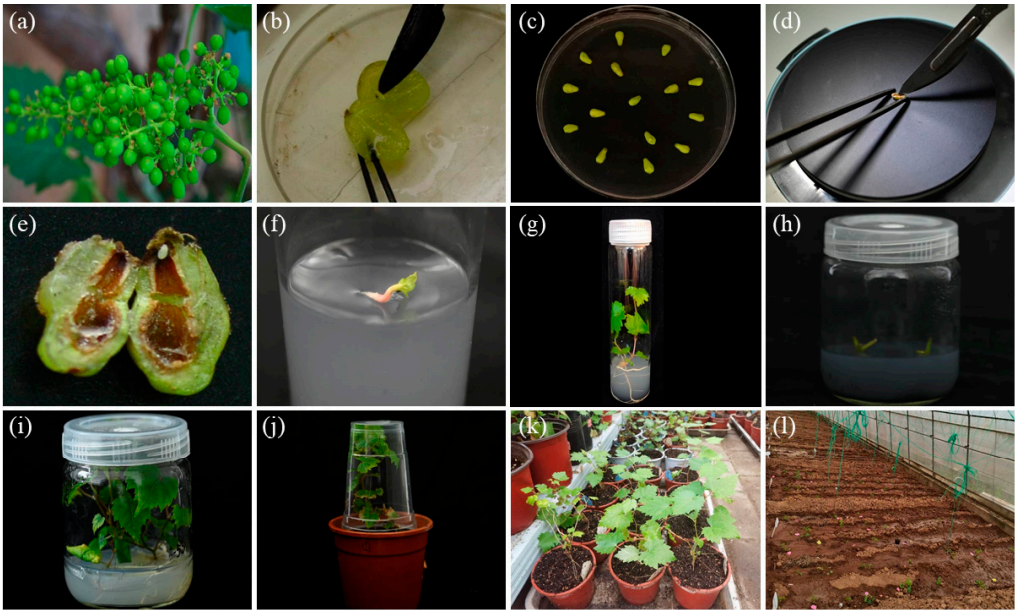

Figure 2. The embryo rescue process: (**a**) hybrid fruits; (**b**) ovule collection; (**c**) ovule inoculation; (**d**) embryo excision; (**e**) excised embryo; (**f**) immature embryo germination; (**g**) plantlet from germinated embryo; (**h**) subculture; (**i**) plantlet from secondary culture; (**j**) seedlings harden; (**k**) seedling in greenhouse; (**l**) seedlings in field.

Subsequent transplantation of hybrid seedlings. When the seedlings grown from the germinated embryos had grown close to the top of the bottle, a sub-culture medium was used for propagation, which was $\frac{1}{2}$Ms + 30 g/L sucrose + 7 g/L agar. The developed stem segments were cut off and packed into covered transparent glass bottles to continue growing (Figure 2h). The growth temperature was controlled at about 25 °C. During this period, seedling formation was continuously observed and recorded. The tissue culture seedlings with a good root system were selected (Figure 2i) for hardening (Figure 2j,k) and were transplanted into the field after 35 days (Figure 2l). The survival rate of the hybrid plants was recorded.

2.6. Analysis of Seedlessness and Cold Resistance by Molecular Markers

The DNA of the test material was extracted from the young leaves of the parents and the progenies. GSLP1-569 and S241-717 were used as molecular markers for seedless trait and cold resistance markers, respectively. The primer sequence and the reaction procedure refer to a previous study [13]. Amplification products were separated on 2% agarose and

photographed (Alpha imager HP, ProteinSimple, San Jose, CA, USA) in order to identify potentially seedless and cold-resistant progenies.

2.7. Data Analyses

The growth and development of the different genotype combinations, the number of ovules germinating, and the number of seedlings developing were observed and recorded. From this data, the ovule development rate and the seedling rate of hybrid progenies were calculated:

The embryo formation rate = the number of embryos developed/the number of ovules cultured.

The embryo germination rate = the number of embryos germinated/the number of embryos developed.

The plant development rate = the number of seedling recovered/the number of ovules cultured.

3. Results

3.1. Preliminary Screening of the Best Combination

In this study, 14 combinations were conducted. and a total of 5807 hybrid ovules and 427 hybrid seedlings were obtained, as shown in Table 1. Among them, precocious means that the ripening time is earlier than the ripening period of the same type of fruit.

Table 1. Configuration of hybrid combination.

Cross Combinations	Female Characteristics	Male Characteristics
'Wuhe Cuibao' × 'Shine-Muscat'	Seedless; Precocious; Rose fragrance	Seeded; Late maturing; Rose fragrance
'Wuhe Cuibao' × 'Xinyu'	Seedless; Precocious; Rose fragrance	Seeded; Precocious; great quality
'Flame Seedless' × 'Shuangyou'	Seedless; Precocious; great quality	Seeded; Medium ripe; Strong Cold resistance
'Heshi Seedless' × 'Shuangyou'	Seedless; Medium-ripe; Poor disease resistance	Seeded; Medium ripe; Strong Cold resistance
'Flame Seedless' × 'Munake'	Seedless; Precocious; great quality	Seeded; Late maturing; Poor Cold resistance
'Kunxiang Seedless' × 'Beichun'	Seedless; Medium ripe; Rose fragrance	Seeded; Late maturing; Strong Cold resistance
'Jingzaojing' × 'Beichun'	Seedless; Medium ripe; Rose fragrance	Seeded; Late maturing; Strong Cold resistance
'Sultanina Rose' × 'Beichun'	Seedless; Medium ripe	Seeded; Late maturing; Strong Cold resistance
'Huozhou Hongyu' × 'Beibinghong'	Seedless; Precocious; Poor disease resistance	Seeded; Medium ripe; Strong disease resistance
'Centennial Seedless' × 'Beibinghong'	Seedless; Precocious	Seeded; Medium ripe; Strong disease resistance
'Ruby Seedless' × 'Beibinghong'	Seedless; Late maturing; great quality	Seeded; Medium ripe; Strong disease resistance
'Melissa seedless' × 'Xinyu'	Seedless; Precocious; Rose fragrance	Seeded; Precocious; Strong Cold resistance
'Yuehong Wuhe' × 'SP740'	Seedless; Precocious; Moderate resistance	Seeded; Late maturing
'Ruby Seedless' × 'Shine-Muscat'	Seedless; Late maturing; great quality	Seeded; Late maturing; Rose fragrance

Among the 14 combinations, the average development rate of embryos was 19.6%, and the average seedling rate of combinations was 8.1%, as shown in Table 2.

The effects of the genotype of both parents on embryo rescue efficiency of seedless grapes were compared and analyzed, and it was found that the highest seedling rate was 21.5% when 'Beibinghong' was used as the male parent and when 'Ruby Seedless' was used as the female parent.

In the combination of 'Flame Seedless' as the female parent with different genotypes as the male parent, the embryo development rate and the seedling rate of 'Shuangyou' as the male parent were the highest, reaching 14.3 and 11.3%, respectively. In the combination of 'Wuhe Cuibao' as the female parent with different genotypes as the male parent, the embryo development rate and the seedling rate of 'Xinyu' as the male parent were the highest, reaching 21.3 and 9.8%, respectively.

3.2. Effect of Sampling Time on Embryo Rescue

The time of maturity of the different parent cultivars varied, and this affected the sampling times. For the combination 'Yuehong Wuhe' × 'SP740', the embryo development rate and the seedling rate were highest when sampled on 37 DAF, reaching 27.5 and 7.8%, respectively. The embryo development rate and the seedling rate of 'Melissa

seedless' × 'Xinyu' were highest on 52 DAF, reaching 42.6 and 26.2%, respectively. The embryo development rate and the seedling rate of 'Ruby Seedless' × 'Shine-Muscat' were highest on 55 DAF, reaching 67.9 and 17.9%, respectively (Table 3).

Table 2. Embryo rescue of hybrid combination.

Cross Combinations	Pollination Time	Sampling Time	No. of Ovules Cultured	No. of Embryos Developed	No. of Germinated		No. of Normal Seedlings	
					No.	%	No.	%
'Flame Seedless' × 'Shuangyou'	5.14	6.25	664	595	85	14.3	67	11.3
'Flame Seedless' × 'Munake'	5.22	6.27	213	248	44	17.7	12	4.8
'Heshi Seedless' × 'Shuangyou'	5.16	7.18	600	180	15	8.3	11	1.8
'Kunxiang Seedless' × 'Beichun'	5.17	6.28	332	101	6	5.9	4	1.7
'Jingzaojing' × 'Beichun'	5.17	7.14	1022	941	161	17.1	8	0.8
'Sultanina Rose' × 'Beichun'	5.2	7.19	594	396	17	4.3	15	3.8
'Huozhou Hongyu' × 'Beibinghong'	5.17	7.14	151	84	24	28.6	11	13.1
'Centennial Seedless' × 'Beibinghong'	5.16	6.24	520	131	12	9.2	6	4.6
'Ruby Seedless' × 'Beibinghong'	5.16	7.14	640	1088	434	39.9	234	21.5
'Wuhe Cuibao' × 'Xinyu'	5.22	7.15	110	61	13	21.3	6	9.8
'Wuhe Cuibao' × 'Shine-Muscat'	6.1	7.21	188	40	4	10	2	5
'Ruby Seedless' × 'Shine-Muscat'	5.27	7.2	170	65	39	60	8	12.3
'Melissa seedless' × 'Xinyu'	5.22	7.15	363	140	31	22.1	23	16.4
'Yuehong Wuhe' × 'Sp740'	5.22	6.28	240	299	45	15.1	20	6.7
∑			5807	4369	930	19.6	427	8.1

Table 3. The effect of sampling time on embryo rescue.

Crosses	Sampling Dates	No. of Ovules Cultured	Embryos Developed		Germinated		Normal Seedlings	
			No.	%	No.	%	No.	%
'Yuehong Wuhe' × 'Sp740'	33	135	30	22.2	22	73.3	8	5.9
	35	113	27	23.9	16	59.3	8	7.1
	37	51	14	27.5	7	50	4	7.8
'Melissa seedless' × 'Xinyu'	52	61	26	42.6	19	73.1	16	26.2
	54	33	11	33.3	4	36.4	2	6.1
	56	46	17	37	8	47.1	5	10.9
'Ruby Seedless' × 'Shine-Muscat'	53	18	7	38.9	3	42.9	2	11.1
	55	28	19	67.9	7	36.8	5	17.9
	57	19	3	34.5	2	66.7	1	3.3

3.3. Effect of Culture Media of Different Sucrose Concentrations on Embryo Rescue

Embryo germination media that had different sucrose concentrations had different effects on the growth of the embryo rescue seedling (Table 4). In this experiment, the seedling rates of 'Ruby Seedless' × 'Shine-Muscat' and 'Yuehong Wuhe' × 'SP740' were highest (15.7 and 25%, respectively) when the sucrose concentration was 1.0%.

3.4. Assisted Selection of Seedless Molecular Markers

The hybrid parents in 2019 were screened using the molecular marker GLSP1-569. The results show that 'Ruby Seedless' and 'Heshi Seedless' had 569 bp bands (Figure 3), which indicated that GLSP1-569 can be used as the molecular marker of seedless trait in order to identify the hybrid progenies of both 'Ruby Seedless' × 'Beibinghong' and 'Heshi Seedless' × 'Shuangyou'. As shown in Figure 4, 63 and 27 hybrids had the positive band, respectively, which indicated that these progenies may possess seedless traits (Figure 4).

Table 4. Effect of Different Sucrose Concentrations on Embryo Rescue.

Cross Combinations	Concentration of Sucrose (%)	No. of Ovules Cultured	Embryos Developed		Germinated	
			No.	%	No.	%
'Ruby Seedless' × 'Shine-Muscat'	1	70	22	31.4	11	15.7
	1.5	80	13	16.3	6	7.5
	2	70	7	10	2	2.9
	3	79	3	3.8	1	1.3
'Yuehong Wuhe' × 'Sp740'	1	16	14	87.5	4	25
	1.5	16	12	75	3	18.8
	2	16	9	56.3	0	0
	3	17	6	35.3	1	5.9

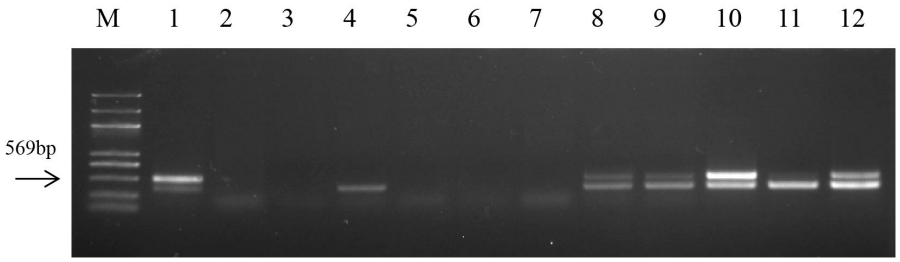

Figure 3. Molecular marker GLSP1-569 on the parental amplification results. M: 2K Plus DNA Maker; (1) Ruby Seedless; (2) Centennial Seedless; (3) Kunxiang seedless; (4) Flame Seedless; (5) Jingzaojing; (6) Sultanina Rose; (7) Shuangyou; (8) Xinyu; (9) Beibinghong; (10) Heshi Seedless; (11) Beichun; (12) Huozhouhongyu.

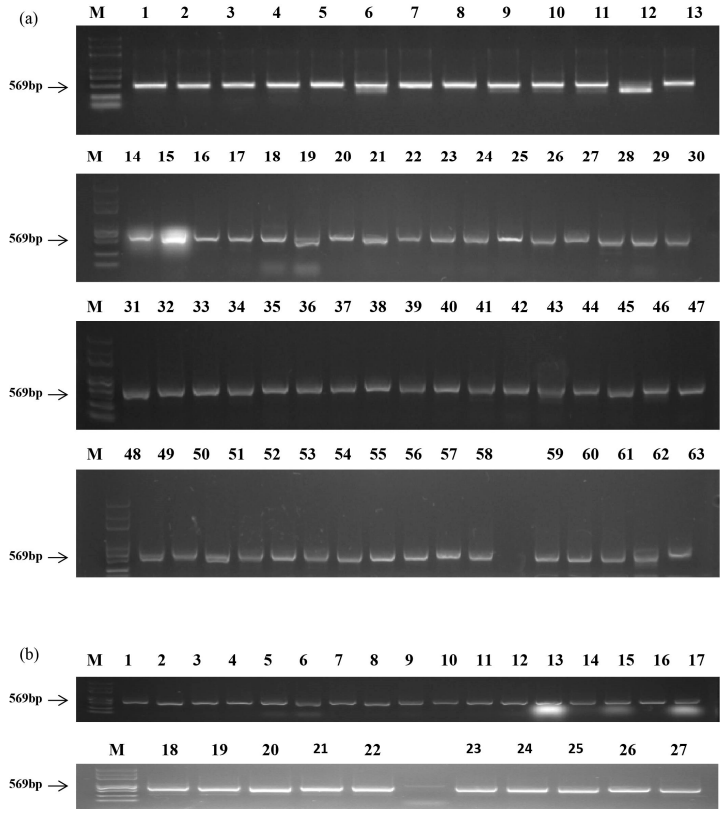

Figure 4. Field Hybridization and in vitro culture of embryo rescue: (**a**) 'Ruby Seedless' × 'Beibinghong'; M: 2K Plus DNA Marker; 1–63 represent progenies of 'Ruby Seedless' × 'Beibinghong'; (**b**) 'Heshi Seedless' × 'Shuangyou'; M: 2K Plus DNA Marker; 1–27 represent progenies of 'Heshi Seedless' × 'Shuangyou'.

3.5. Preliminary Screening of Molecular Markers for Cold Resistance

S241-717 with a length of 717bp were used as the cold-resistant marker. Gel electrophoresis shows that 'Beichun' and 'Beibinghong' both have specific bands at 717 bp (Figure 5).

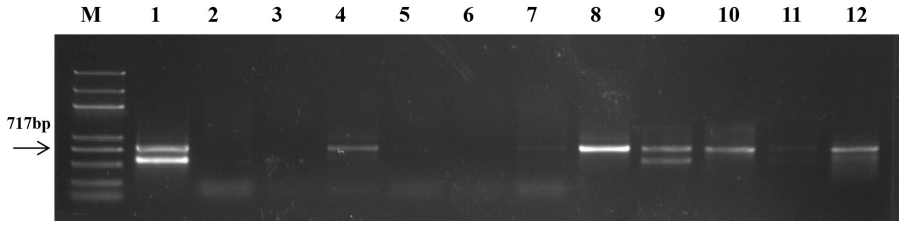

Figure 5. Amplification result of cold tolerance marker S241-717 on parent material. M: 2K Plus DNA Maker. (1) Yuehong Seedless; (2) Shine-Muscat; (3) Sp740; (4) Xinyu; (5) Munake; (6) Munake; (7) Shuangyou; (8) Beichun; (9) Munake; (10) Sultanina Rose; (11) Shine-Muscat; (12) Beibinghong.

Of the 12 progenies of 'Sultanina Rose' × 'Beichun', 11 showed specific bands at 717 bp, which could be preliminarily screened for cold resistance (Figure 6a). Seven of the hybrid progenies of 'Ruby Seedless' × 'Beibinghong' showed the presence of the cold resistant marker S241-717 by specific bands amplified at 717bp (Figure 6b).

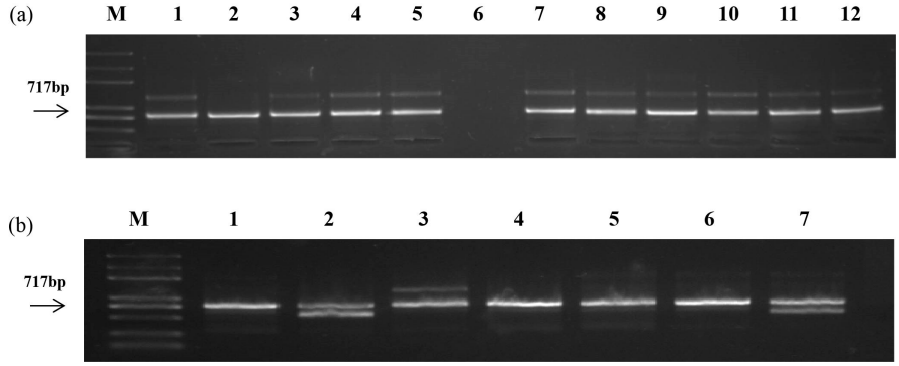

Figure 6. Amplification of the cold resistant molecular marker S241-717 on the hybrid combinations. (**a**) 'Sultanina Rose' × 'Beichun'; M: 2K Plus DNA Marker; 1–12 represent progenies of 'Sultanina Rose' × 'Beichun'; and (**b**) 'Ruby Seedless' × 'Beibinghong'; M: 2K Plus DNA Marker; 1–7 represent progenies of 'Ruby Seedless' × 'Beibinghong'.

4. Discussion

In traditional cross-breeding, a seedless grape cannot be selected as the female parent as the zygotic embryo aborts [27]. Fortunately, this problem can be circumvented by embryo rescue breeding, in which different grape varieties can be selected as parent material in order to make the best combination according to their characteristics. Thus, new germplasm resources expressing superior quality, higher yield, and strong resistance to a range of biotic and abiotic stressors can be produced [8,28–30]. For example, 'Blush Seedless', 'Thompson Seedless', 'Crimson Seedless', 'Emerald Seedless', and 'Flame Seedless' were all used as the maternal parent, while 'Beichun' was used the male parent in order to develop the disease-resistant seedless cultivars [31]. The results indicate that the female parent genotype combination can have a great impact on the developmental rate as well as on the seedling rate. The highest rate of embryo germination and rate of seedling was obtained when the parent material was 'Emerald Seedless' [31]. In addition, a large number of experiments have shown that the embryo development rate and the seedling rate are both high when 'Flame Seedless' and 'Ruby Seedless' are used as the female parents [32–34]. The results of our study show that when 'Ruby Seedless' is used as the female parent, the embryo rescue efficiency is good, rising as high as 39.9%. This makes it highly suitable as female parent material. This finding is consistent with previous results.

Sampling time determines the initial state of embryo growth and development, which plays an important role in embryo rescue. Previous studies have indicated that the germination and the seedling rates of hybrid embryos increase when fruits are in the ripening to softening stages. However, for the fruit in the later stages of the ripening, the ovule adheres strongly to the pulp, making it difficult to peel off the ovule in embryo rescue, and causing contamination of the ovule with the pulp tissue. Therefore, it is suggested that sampling time should be 1~2 days before fruit color change (veraison). Here, we found that the best sampling time for 'Ruby Seedless' × 'Sunshine Rose' was 55 DAF, which is consistent with previous reports. The addition of sucrose to the medium not only provides the carbon and the energy supply for growth but also affects the medium's osmotic potential. Increasing the sucrose concentration of the medium overcomes precocious embryo germination and also contributes to ovule growth. At the time of embryo germination, Ramming considered a medium with 1.5% sucrose concentration as suitable for embryo rescue seedling growth [35]. Our results show that for sucrose concentrations of 1.0–1.5%, the embryo germination rate of the combination was highest for both 'Yuehong Wuhe' × 'Sp740' and for 'Ruby Seedless' × 'Shine-Muscat', reaching 87.5 and 31.4%, respectively.

Molecular markers not only increase the efficiency of breeding but also help to select better hybrid combinations in line with breeding objectives. In 2011, Mejía discovered the seedless marker SCF27-2000, which also accurately detects the seedless character in grapes, and this is now widely used [15]. The reports now show that the five main molecular markers for screening and identification of seedless traits in grapes are SCC8-1018, p3-VvAGL11-1200, GSLP1-569, SCF27-2000, and VNCF7f2-198 [36].

In our study, the seedless marker GLSP1-569 was selected to detect seedless trait of our hybrid combinations [34]. Our results show that the hybrid progenies of 'Ruby Seedless' × 'Beibinghong' and 'Heshi Seedless' × 'Shuangyou' were satisfactorily detected, with 91 progeny having obvious specific bands at 569 bp5. Moreover, 17 hybrids were identified to carry cold resistance using the molecular marker S241-717.

5. Conclusions

A total of 14 seedless embryo rescue hybrid combinations were chosen, and a total of 427 hybrids were obtained. Among these, the best combination was 'Ruby Seedless' × 'Beibinghong'. The most suitable sampling times for 'Yuehong Wuhe', 'Ruby Seedless', and 'Melissa seedless' were 37, 55, and 52 DAF, respectively. The optimum sucrose concentration for the ovule growth medium was between 1.0–1.5%. A total of 140 hybrid progenies were screened using seedless markers GLSP1-569. The results show that 91 hybrid progeny had obvious seedless traits and that 18 hybrid progenies had cold resistance, which were identified using the cold-resistant marker S241-717 to detect the progenies of the two hybrid combinations.

Author Contributions: Project design, Y.X. and G.L.; materials collection, Y.C. and M.L.; scientific experiments Y.C., M.L., R.L., K.Z., P.Q., X.Y., Y.H. and X.L.; writing—original draft preparation, Y.C. and G.L.; writing—review and editing, G.L. and Y.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Agricultural Breeding Project of Ningxia Hui Autonomous Region (NXNYYZ202101) and the China Agriculture Research System of MOF and MARA (CARS-29-yc-3).

Data Availability Statement: Data are contained within the article.

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

References

1. Cain, D.W.; Emershad, R.L. In-ovulo embryo culture and seedling development of seeded and seedless grapes (*Vitis vinifera* L.). *Vitis* **1983**, *22*, 9–14.
2. Matsumoto, R.; Kanato, K.; Ozawa, T. New grape cultivar 'Honey Seedless'. *Bull. Fruits Tree Res Stn.* **1993**, *25*, 45–56.
3. Tukey, H.B. Artificial culture of sweet cherry embryos. *J. Hered.* **1933**, *24*, 7–12. [CrossRef]

4. 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]
5. Li, Z.Q.; Li, T.M.; Wang, Y.J.; Xu, Y. Breeding new seedless grapes using in ovulo embryo rescue and marker-assisted selection. *Vitr. Cell. Dev. Biol. Plant* **2015**, *51*, 241–248. [CrossRef]
6. Ramming, D.W.; Emershad, R.L. In ovulo embryo culture of seeded and seedless *Vitis vinifera* L. *Hortic. Sci.* **1982**, *17*, 487.
7. Li, G.; Wang, Y.; Tang, D.; Wang, X.; Luo, Q. The studies on embryo rescue techniques of 'Thompson Seedless' grape. *Plant Cell Tissue Organ* **2001**, *21*, 432–436.
8. Garcia, E.; Martinez, A.; Calera, E.; Perez, L.J.; Carreo, J. In Vitro culture of ovules and embryos of grape for the obtention of new seedless table grape cultivars. *Acta. Hort.* **2000**, *528*, 663–666. [CrossRef]
9. 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]
10. Li, J.; Wang, X.; Wang, X.; Wang, Y. Embryo rescue technique and its applications for seedless breeding in grape. *Plant Cell Tissue Organ Cult.* **2015**, *120*, 861–880. [CrossRef]
11. 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]
12. Amato, A.; Cardone, M.F.; Ocarez, N.; Alagna, F.; Ruperti, B.; Fattorini, C.; Velasco, R.; Mejia, N.; Zenoni, S.; Bergamini, C. VviAGL11 self-regulates and targets hormone- and secondary metabolism-related genes during seed development. *Hortic. Res.* **2022**, *9*, uhac133. [CrossRef] [PubMed]
13. Mejía, N.; Hinrichsen, P. A new highly assertive SCAR marker potentially useful to assist selection for seedlessness in table grape breeding. *Acta Hortic.* **2003**, *603*, 559–564. [CrossRef]
14. Bergamini, C.; Cardone, M.F.; Anaclerio, A.; Perniola, R.; Pichierri, A.; Genghi, R.; Alba, V.; Forleo, L.R.; Caputo, A.R.; Montemurro, C.; et al. Validation assay of p3_VvAGL11 marker in a wide range of genetic background for early selection of stenospermocarpy in *Vitis vinifera* L. *Mol. Biotechnol.* **2013**, *54*, 1021–1030. [CrossRef] [PubMed]
15. Mejía, N.; Soto, B.; Guerrero, M.; Casanueva, X.; Houel, C.; de los Angeles Miccono, M.; Ramos, R.; Cunff, L.L.; Boursiquot, J.M.; Hinrichsen, P.; et al. Molecular, genetic and transcriptional evidence for a role of VvAGL11 in stenospermocarpic seedlessness in grapevine. *BMC Plant Biol.* **2011**, *11*, 1–19. [CrossRef] [PubMed]
16. Hur, Y.; Jung, C.; Roh, J.; Jung, S.; Ma, K.; Park, K. Analysis of Genetic Relationship of Seedless Germplasm and Validation Assay of the P3_VvAGL11 Marker Linked to Seedlessness in Grapevines. *Korean J. Breed. Sci.* **2014**, *46*, 28–36. [CrossRef]
17. Wang, Y.; Olusola, L. Application and synthesis on the DNA probe for detecting seedless genes in grapevine. *J. Northwest Sci-Tech Univ. Agric. For.* **2002**, *30*, 41–46.
18. Ma, X.; Zhao, F.; Su, K.; Lin, H.; Guo, Y. Discovery of cold-resistance genes in Vitis amurensis using bud-based quantitative trait locus mapping and RNA-seq. *BMC Genom.* **2022**, *23*, 551. [CrossRef]
19. Ren, C.; Fan, P.; Li, S.; Liang, Z. Advances in understanding cold tolerance in grapevine. *Plant Physiol.* **2023**, *192*, 1733–1746. [CrossRef]
20. Droulia, F.; Charalampopoulos, I. A Review on the Observed Climate Change in Europe and Its Impacts on Viticulture. *Atmosphere* **2022**, *13*, 837. [CrossRef]
21. Gonzalez Antivilo, F.; Paz, R.C.; Tognetti, J.; Keller, M.; Cavagnaro, M.; Barrio, E.E.; Roig Juñent, F. Winter Injury to Grapevine Secondary Phloem and Cambium Impairs Budbreak, Cambium Activity, and Yield Formation. *J. Plant Growth Regul.* **2019**, *39*, 1095–1106. [CrossRef]
22. Zhang, J.; Wu, X.; Niu, R.; Liu, Y.; Liu, N.; Xu, W.; Wang, Y. Cold-resistance evaluation in 25 wild grape species. *Vitis* **2012**, *51*, 153–160.
23. Liu, Q.; Zhang, J.; Wang, Y.; Yu, D.; Xia, H. Breeding for cold-resistant, seedless grapes from Chinese wild Vitis amurensis using embryo rescue. *N. Z. J. Crop Hortic. Sci.* **2016**, *44*, 136–151. [CrossRef]
24. Xu, T.; Guo, Y.; Wang, W.; Yuan, X.; Chu, Y.; Wang, X.; Han, Y.; Wang, Y.; Song, R.; Fang, Y.; et al. Effects of exogenous paclobutrazol and sampling time on the efficiency of in vitro embryo rescue in the breeding of new seedless grape varieties. *J. Integr. Agric.* **2022**, *21*, 1633–1644. [CrossRef]
25. Zhang, J.X.; Xiong, Y.; Wang, Y.J.; Zhu, Z.G. RAPD Marker and sequence Analysis of Cold Resistance Gene in Chinese Wild Grape. *Chin. Agric. Sci. Bull.* **2010**, *26*, 30–37.
26. Li, S.; Yu, S.; Fu, Y.; Luo, Q.; Xu, Y.; Wang, Y. The Embryo Rescue and Molecular Markers are Used to Breed New Seedless, Cold-Resistant Grapes. *Acta Hortic. Sin.* **2022**, *4*, 723–738.
27. Valdez, J.G. Immature embryo rescue of grapevine (*Vitis vinifera* L.) after an extended period of seed trace culture. *Vitis* **2005**, *44*, 17–23.
28. Luo, Y.; Cui, X.; Zhu, P.; Zhang, J. Effects of parental genotypes on embryo rescue of seedless grape hybrid. *N. Z. J. Crop Hortic. Sci.* **2023**, 1–12. [CrossRef]
29. Simin, U.; Kesgin, M.; Dilli, Y. The success of in vitro embryo rescue technique in hybridization of seedless grape varieties. *World Congr. Vine Wine* **2015**, *38*, 01008.
30. Zhu, P.; Gu, B.; Li, P.; Shu, X.; Zhang, X.; Zhang, J. New cold-resistant, seedless grapes developed using embryo rescue and marker-assisted selection. *Plant Cell Tissue Organ Cult. (PCTOC)* **2019**, *140*, 551–562. [CrossRef]

31. Tian, L.; Wang, Y.; 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]
32. 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 × wild Chinese Vitis species. *Vitr. Cell. Dev. Biol.* **2013**, *50*, 110–120. [CrossRef] [PubMed]
33. Ebadi, A.; Aalifar, M.; Farajpour, M.; Moghaddam, M.R.F. Investigating the most effective factors in the embryo rescue technique for use with 'Flame Seedless' grapevine (*Vitis vinifera*). *J. Hortic. Sci. Biotech.* **2016**, *91*, 441–447. [CrossRef]
34. Li, S.S.; Wang, Y.J. Advances in Seedless Gene Researches and Seedless breeding in Grapevine. *Acta Hortic. Sin.* **2019**, *9*, 1711–1726.
35. Ramming, D.W. In ovule embryo culture of early maturing prunus. *Hortscienee* **1985**, *20*, 419–420. [CrossRef]
36. Ocarez, N.; Mejía, N. Suppression of the D-class MADS-box AGL11 gene triggers seedlessness in fleshy fruits. *Plant Cell Rep.* **2016**, *35*, 239–254. [CrossRef]