Plant Cell, Tissue and Organ Culture (PCTOC) (2020) 140:551–562
https://doi.org/10.1007/s11240-019-01751-y

ORIGINAL ARTICLE

New cold-resistant, seedless grapes developed using embryo rescue and marker-assisted selection

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Received: 24 August 2019 / Accepted: 17 December 2019 / Published online: 23 December 2019

Abstract

One of the major objectives of table grape breeding is to produce seedless grapes of *Vitis vinifera* L. Of high importance among these objectives is the development of new cold-resistant seedless grapes for the colder regions of the world. Embryo rescue is an effective tool in breeding seedless grapes. Here, we report on nine cross-combinations between seedless cultivars and cold-resistant seedless cultivars or seeded hybrids. We carried out embryo rescue and molecular marker-assisted selection to create new cold-resistant seedless grape germplasm. We also examined the effects of different parents and the use of exogenous hormones on the success of embryo rescue as well as the optimal sampling time for a new seedless cultivar Qinxiu. The results show that a total of 473 new grape genotypes were obtained by embryo rescue using double-phase MM3 (modified ER) as the medium for embryo development and solid phase WPM as the medium for embryo germination and plantlet formation. We found the seedless cultivars Perlette, Qinhong No.2, Ruby Seedless and Qinhong No.10 were the most suitable female parents for embryo rescue, and the cold-resistant seedless cultivar Jupiter (a *V. vinifera* × *V. labrusca* hybrid) as the male parent. This was better than the seeded hybrid 0-1-5 (*V. vinifera* × *V. amurensis*). The best embryo development medium was MM3 with 500 mg/L CH, 1 mmol/L serine, 0.5 mg/L GA_3, 1.0 mg/L IAA and 0.5 mg/L 6-BA. The best embryo germination and plantlet formation medium was WPM with 0.2 mg/L 6-BA and 0.1 mg/L IAA. The best sampling time for Qinxiu for embryo rescue was 42 days after flowering. We obtained a total of 440 hybrids by embryo rescue using the seedless molecular marker SCF27-2000.

Key message

Hybridization between grape seedless cultivars and cold-resistant cultivars and embryorescue were performed. We obtained 473 new germplasms and performed markerassisted-selection using SCF27-2000. The embryo rescue system was optimized.

Keywords Seedless grapes · Cold resistance · Embryo rescue · Marker-assisted selection

Communicated by Klaus Eimert.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11240-019-01751-y) contains supplementary material, which is available to authorized users.

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Abbreviations

CH	Casein hydrolysate
IAA	Indole-3-acetic acid
6-BA	6-Benzyladenine
GA_3	Gibberellic acid, GA_3
CCC	Chlorocholine chloride
CPPU	Forchlorfenuron
PGR	Plant growth regulator
MS	Murashige and Skoog
ER	Emershad and Ramming
NN	Nitsch and Nitsch
WPM	Woody plant medium
BD	Bouquet and Davis
MAS	Marker-assisted selection
DAF	Days after flowering

Introduction

Seedless grapes are popular fresh, as table fruit, or preserved, as raisins or canned, because consumers do not have to deal with the seeds. Based on fruit development processes, seedless grapes are of two types: (1) parthenocarpic—where ovaries develop directly into fruits without fertilisation and (2) stenospermocarpic—where the endosperm and embryo abort soon after fertilisation and the ovules are retained only as tiny seed traces (Stout 1936). It is difficult to use parthenocarpy in breeding seedless grapes. In contrast, stenospermocarpic genotypes are commonly used as parental material in breeding (Ramming and Emershad 1982; Ramming et al. 1990). Most commonly, a seeded cultivar is used as the female parent and a seedless one as the male. Unsatisfactory, the incidence of seedless hybrids among the offspring from such a cross is very low, so breeding new seedless cultivars is slow and expensive (Ramming and Emershad 1982, 1984; Spiegle-Roy et al. 1985). Ramming and Emershad (1982) first reported that stenospermic seedless grapes can be used to generate new plants using in vitro culture of the excised ovules. The ovules must be excised before they abort. After embryos had developed into young, fruiting vines, seedless individuals could be identified in the field. Since that time, embryo rescue has been used in seedless grape breeding across the world, including in Israel (Agüero et al. 1996; Valdez 2005), Japan (Notsuka et al. 2001), Australia (Liu et al. 2008), China (Tian et al. 2008; Li et al. 2014), Spain (Carreo et al. 2009), India (Singh et al. 2011), France (Reynolds 2015) and Iran (Khoshandam et al. 2017). With the resulting expansion of the range of potential female parents, the rate of introduction of new seedless hybrids has increased. Embryo rescue has also been used by breeders of other perennial fruit crops (Viloria et al. 2005; Uma et al. 2011; Mansvelt et al. 2015; Ren et al. 2019).

The key to embryo rescue, is to be able to promote the ongoing development of immature embryos in vitro. Previous studies have shown that a number of factors affect in vitro embryo development (Li et al. 2015a). These include, the genotypes of the female and male parents (Valdez 2005; Nicole et al. 2006; Liu et al. 2008; Niu et al. 2012), the sampling time (Gray et al. 1990; Xu et al. 2005), the pre-flowering application of plant growth regulators (PGR) (Nookaraju et al. 2007; Razi et al. 2013; Khoshandam et al. 2017), the nature of the growth medium (Gray et al. 1990; Burger and Goussard 1996; Tang et al. 2009a, b; Singh et al. 2011; Razi et al. 2013; Li et al. 2014; Ebadi et al. 2016) and whether the medium is liquid or solid or liquid–solid (i.e. two-phase) (Emershad and Ramming 1984; Spiegle-Roy et al. 1985; Gray et al. 1990; Okamoto et al. 1993; Tang et al. 2009b; Ebadi et al. 2016). Also involved are additions of exogenous hormones (Emershad and Ramming 1994; Burger and Goussard 1996; Tian et al. 2008; Singh et al. 2010; Razi et al. 2013; Li et al. 2014; Liu et al. 2016) and of other organic substances (Bharathy et al. 2005; Ji et al. 2013; Jiao et al. 2018; Li et al. 2014; Ebadi et al. 2016; Li et al. 2018) and the conditions under which the culture is held (Agüero et al. 1996; Singh et al. 2010; Zhang and Niu 2013). Due to the complexity of, and interactions between, these numerous factors, an optimal embryo rescue system has not yet been developed for grapes. Hence, it is worth while seeking to optimise this system, so as to increase the efficiency of seedless grape breeding (Ramming et al. 1990; Emershad and Ramming 1994; Nicole et al. 2006; Nookaraju et al. 2007; Tian et al. 2008; Tang et al. 2009a, b; Liu et al. 2016).

As already noted, the seedless trait among the embryo rescue progeny has had to be determined in the field, and this cannot be done until the young vines have come into bearing. Obviously, this greatly restricts the speed of breeding, but it can be greatly accelerated by using molecular markers which allow key target traits to be identified at plantlet stage, greatly shortening the breeding cycle. There are now five molecular markers associated with the grape seedless gene. These are: SCAR markers SCC8-1018 (Lahogue et al. 1998), SCF27-2000 (Mejía and Hinrichsen 2003) and GSLP1-569 (Wang and Lamikanra 2002), microsatellite markers VMC7F2-198 (Cabezas et al. 2006) and p3-VvAGL11-216 (Bergamini et al. 2013). These markers have already been used in seedless grape breeding—marker assisted selection (MAS) (Akkurt et al. 2012; Li et al. 2015a, b; Liu et al. 2016; Li et al. 2018).

However, there is a special problem in the production of new seedless grape cultivars—most of today's seedless grapes belong to *V. vinifera*. These are characterised by good eating quality but suffer low resistance to cold. In conventional breeding, severe inbreeding depression was observed (Kandel et al. 2016). This severely limits the production of seedless grapes for use in the colder regions of the world.

China is an important centre of origin for *Vitis* spp. Some of the Chinese wild *Vitis* species are strongly resistant to cold, these include *V. amurensis* and *V. yenshanensis*. The native American species *V. labrusca* and *V. riparia* also show good cold resistance (Zhang et al. 2012). But due to their dioecism, direct crosses with the seedless *V. vinifera* cultivars are a problem. Also, the hybrids suffer poor eating quality and some are staminiferous.

In contrast, the superior F1 genotypes emerging from various combinations of *V. vinifera* × the Chinese wild *Vitis* species, share half their genetic composition with *V. vinifera* and so enjoy good affinity with the *V. vinifera* seedless cultivars. The hybrids so obtained by embryo rescue strongly reduce the inferior traits of their wild-grape parentage. This

arrangement greatly improves the efficiency of breeding high-quality, cold-resistant, seedless grapes by embryo rescue (Niu et al. 2012).

Based on previous research (Niu et al. 2012; Zhang and Niu 2013; Liu et al. 2016), this paper aims to reduce the dearth of cold-resistant seedless grapes for commercial production by developing new cold-resistant seedless germplasm by crossing stenospermocarpic seedless cultivars with cold-resistant hybrids and by optimising the embryo rescue system. We consider the effects of different parents, exogenous hormones, embryo development media and plantlet formation media, along with identifying optimal sampling times for the new seedless cultivar Qinxiu. This work lays a foundation for more efficient breeding of new cold-resistant seedless grape cultivars.

Material and methods

This work was carried out from April 2017 to May 2018. The parents included nine stenospermocarpic seedless grapes and two cold-resistant hybrids. These were grown in the repository of Northwest A&F University, Yangling, Shaanxi, China. The grapevines were 10 years old or more, they were trained to a T-trellis with a planting density of 1.0 × 2.5 m and were managed to local standards in the same vineyard. Embryo rescue was carried out in the State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A&F University, Yangling, Shaanxi, China.

Parent characteristics

A total of nine cross combinations were used: Qinxiu × Jupiter, Perlette × 00-1-5, Flame Seedless × 00-1-5, Qinhong No.10 × 00-1-5, Qinhong No.2 × Jupiter, Crimson Seedless × 00-1-5, Ruby Seedless × 00-1-5, Jupiter × Su-67 and Flame Seedless × Jupiter. Jupiter is a cold-resistant, seedless hybrid (*V. vinifera* × *V. labrusca*) introduced from the USA, while 00-1-5 is a cold-resistant, seeded hybrid (*V. vinifera* × *V. amurensis*) obtained by our research group. The detailed characteristics of the female and male parents used in this study are in Tables 1 and 2.

Hybridisation

In early flowering (about 5% of flowers open) male parent plants with well-developed inflorescences were prepared to produce fresh pollen. After collection, drying, sifting,

Table 1 Female parents and their characteristics

Female parent	Species or hybrids	Characteristics
Ruby Seedless	*V. vinifera* L.	Stenospermocarpic, large cluster, long cone type, oval fruit, bright red or purplish red, flesh crisp, late mature, poor disease and cold resistance
Perlette	*V. vinifera* L.	Stenospermocarpic, large cluster, big berries, cone type, yellow/green oval fruit, soft flesh, mid-maturation, poor disease and cold resistance
Flame Seedless	*V. vinifera* L.	Stenospermocarpic, large cluster, cone type, near circle fruit, bright red or purplish red, flesh crisp, early-maturation, poor disease and cold resistance
Crimson Seedless	*V. vinifera* L.	Stenospermocarpic, large cluster, cone type, bright red, flesh crisp, pale yellow fruit, late maturing, poor disease and cold resistance
Jupiter	*V. vinifera* × *V. labrusca* hybrid	Stenospermocarpic, small cluster, dark red berry, bigger berry, soft flesh, with rose scent, early-maturation, disease and cold resistance
Qinhong No.2	*V. vinifera* L.	Stenospermocarpic, a cross between Delight and Ruby Seedless, near spherical fruit, medium size, cone type, red, flesh crisp, mid-maturation, poor disease and cold resistance
Qinhong No.10	*V. vinifera* L	Stenospermocarpic, a cross between Delight and Ruby seedless, flesh crisp, near spherical fruit, medium size, cone type, red, poor disease and cold resistance
Qinxiu	*V. vinifera* L.	Stenospermocarpic, a cross between Jingxiu and Zhengguodawuhe, large cluster, cone type, big berries, flesh crisp, early-maturation, poor disease and cold resistance

Table 2 Male parents and their characteristics

Male parent	Species or hybrids	Characteristics
00-1-5	*V. vinifera* × *V. amurensis*	Bisexual flower, cross between Muscat Hamburg and Heilongjiang seedling (*V. amurensis*), seeded, small berry, cone type cluster, with rose scent, mid-maturation, resistant to cold and downy mildew
Su-67	*V. vinifera* L	Origin Soviet Union, large cluster, long cone type, oval fruit, purplish red berry, big berry, flesh crisp, mid-maturation, poor disease and cold resistance
Jupiter	*V. vinifera* × *V. labrusca*	Stenospermocarpic, small cluster, soft seed, dark red berry, big berry, soft flesh with rose scent, early maturation, disease and cold resistance

bottling and sealing, the fresh pollen was kept at 4 ℃ with desiccation.

On the female plants, similar-stage flowers were selected and emasculation was carried out 3–4 days before opening (Fig. 1a). After emasculation, inflorescences were immediately rinsed twice with distilled water and bagged. About 2 days later at the stage when mucus had appeared on the stigma, they were hand pollinated using a soft brush, loaded with dry pollen. Pollination was repeated three times on each of three consecutive days, followed by bagging the whole inflorescence.

Optimal sampling time for the seedless cv. Qinxiu

The optimal sampling time for the seedless hybrid cultivar, Qinxiu, was determined by the staged-sampling method. Green berries of Qinxiu were taken on 36, 38, 40, 42, 44 and 46 days after flowering and the ovules excised and cultured on solid–liquid, double-phase medium MM3 + 500 mg/L CH + 1.0 mmol/L serine + 0.5 mg/L GA_3 + 1.0 mg/L IAA + 0.5 mg/L 6-BA + 60 g/L sucrose (pH 5.8). After 8–9 weeks, the young embryos were stripped and cultivated on solid medium WPM + 0.2 mg/L 6-BA + 20 g/L sucrose + 1.5 g/L activated carbon + 7 g/L agar + 0.1 g/L inositol (pH 5.8). The optimal sampling time was determined

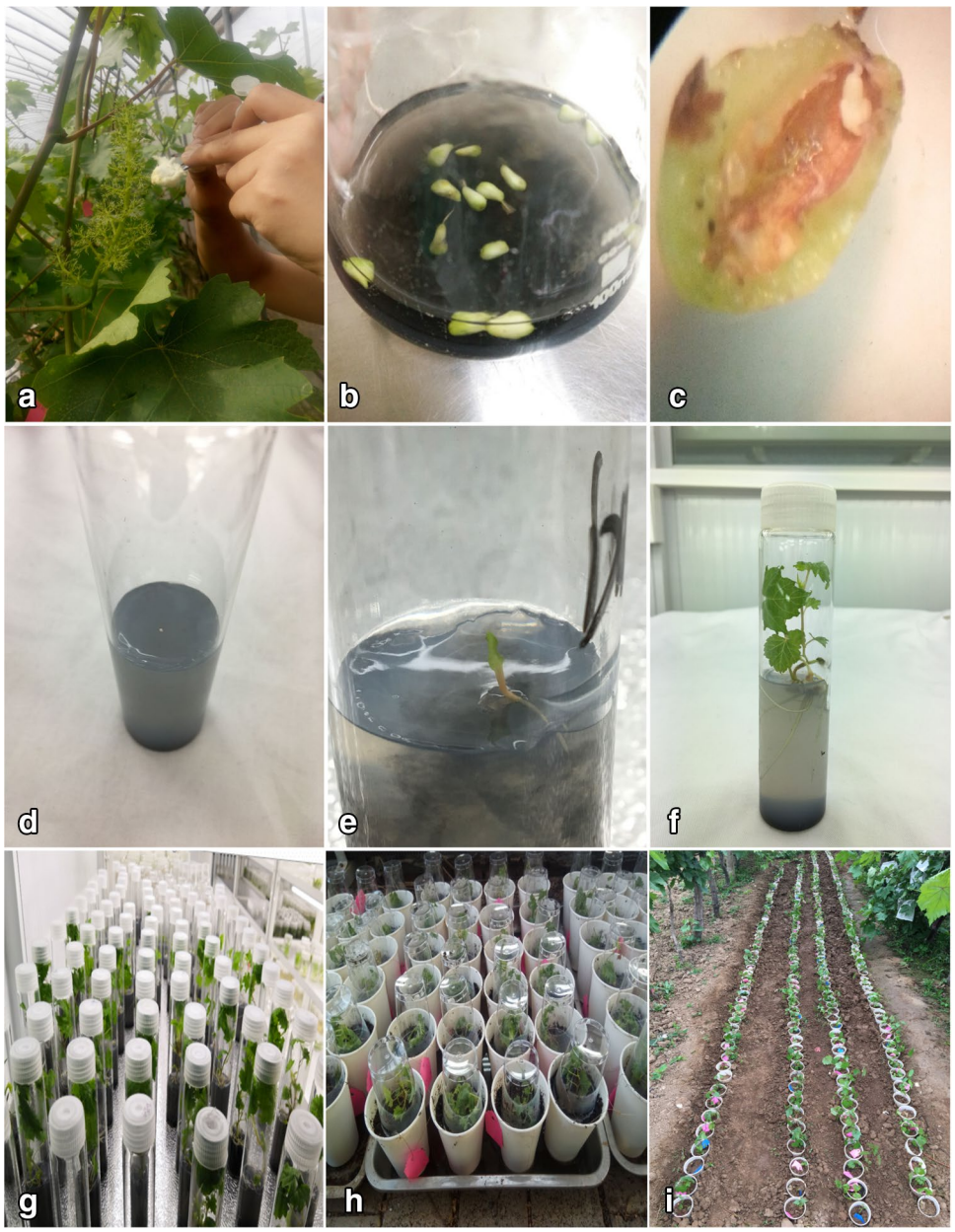

Fig. 1 Embryo rescue protocol for hybrid progenies from stenospermocarpic seedless grapes (*V. vinifera* or Euro-American hybrid) × cold-resistant grapes (*V. amurensis* hybrid or Euro-American hybrid). **a** Pollination after emasculation; **b** ovules cultured in double-phase medium; **c**, **d** an excised embryo from ovules and embryo cultured on WPM medium; **e–g** germinated embryo developed to plantlet; **h** domestication and transplantation of hybrid seedlings; **i** plants established in the soil

by recording the embryo development rate, germination rate and plantlets formation rates.

Ovule culture

The hybridised fruits were surface-sterilised in 75% (v/v) ethanol for 30 s. The ethanol was then poured off and replaced with 1% (w/v) NaClO for 30 min and this followed by three washes in sterile water. Fruits were dissected and ovules cultured in vitro under aseptic conditions in Erlenmeyer flasks (100 mL) containing a solid and liquid double-phase embryo development medium (Fig. 1b). Solid and liquid phases were all MM3 medium adding 500 mg/L CH, 1 mmol/L serine, 0.5 mg/L GA_3, 1.5 mg/L IAA, 60 g/L sucrose, 1.5 g/L activated carbon, 0.1 g/L inositol and 7 g/L agar or not (pH 5.8). A total of 20 ovules were inoculated in each flask for recording.

Optimisation of embryo development medium

A cross combination, Ruby Seedless × 00-1-5 with sufficient numbers of ovules was selected to investigate the effects of IAA, 6-BA and GA_3 on embryo rescue in a range of concentration ratios. A total of nine random ratios of the three hormones were designed (Table S1).

Embryo germination and plant development culture

After 8–9 weeks of ovule culture in the dark, all embryos developed were stripped and cultured on solid WPM + 0.2 mg/L 6-BA + 20 g/L sucrose + 1.5 g/L activated carbon + 7 g/L agar + 0.1 g/L inositol (pH 5.8) (Fig. 1c–g). Frequencies of embryo development, embryo germination and plantlet formation were evaluated.

Optimisation of embryo germination medium

The combination Ruby Seedless × 00-1-5 was selected for this study, after 8–9 weeks of ovule culture in the dark, all developed embryos were stripped and cultured on solid medium WPM + 0.2 mg/L 6-BA and WPM + 0.2 mg/L 6-BA + 0.1 mg/L IAA (pH 5.8), to study the effects of the two kinds of media on germination and plantlet growth.

Identification of seedless trait using seedless molecular marker SCF27-2000

The genomic DNA of grapevines was extracted by the CTAB method (Wang and Lamikanra 2002). The parents and their progeny from embryo rescue were detected by the seedless gene molecular marker SCF27-2000 (F: 5′CAG GTGGGAGTAGTGGAATG3′; R: 5′CAGGTGGGAGTA AGATTTGT3′).

PCR reactions were carried out according to the corresponding references. Amplification products were separated on 1.5% agarose and photographed (GeneGenius Bio Imaging System GeneSnap, SynGene Co).

Statistical analyses

The numbers of embryos developing and germinating and of plantlets forming for each embryo combination were counted and the rates of development, germination and plantlet establishment were recorded.

$$\text{The embryo development rate (\%)} = \text{number of embryos excised from ovule/number of ovules} \times 100$$

$$\text{Embryo germination rate (\%)} = \text{number of germinated embryos/number of ovules} \times 100$$

$$\text{Plantlet formation rate (\%)} = \text{number of plantlets/number of ovules} \times 100$$

Significance analysis of the developmental rate, germination rate and plantlet formation rate of the three biological replicates in the same cross-combination was carried out using SPSS 22.0 software ($P < 0.05$).

Acclimatisation and transplanting of tube plantlets

In early March, after exposure to natural daylight for one week in the greenhouse, the tube plantlets were transplanted to a paper cup (250 mL) filled with a synthetic soil mix (vermiculite:peat-soil:coconut husk, 1:4:1) and watered with distilled water. Each plantlet was covered with a larger transparent plastic cup and kept in the greenhouse for acclimation under natural daylight (Fig. 1h). The distilled water and 1% carbendazim solution were poured alternately every four days. About two weeks later, the plastic cup was gradually removed. Finally, surviving plantlets were transplanted to the field in spring (Fig. 1i).

Table 3 Effect of different female parent genotype on embryo rescue

Crossing combination	No. of ovules cultured	No. of embryos developed	No. of embryos germinated	No. of normal plantlets	Proportion of embryos developed (%)	Proportion of embryos germinated (%)	Proportion of embryos forming plantlets (%)
Ruby Seedless × 00-1-5	222	32	29	16	14.4 ± 0.8^{b}	13.1 ± 1.6^{b}	7.2 ± 0.8^{b}
Qinhong No.10 × 00-1-5	225	33	16	16	14.7 ± 1.3^{b}	7.1 ± 2.0^{c}	7.1 ± 2.0^{b}
Perlette × 00-1-5	229	59	41	33	25.8 ± 0.7^{a}	17.9 ± 2.6^{a}	14.4 ± 1.2^{a}
Flame Seedless × 00-1-5	266	28	13	9	10.5 ± 0.5^{c}	4.9 ± 1.7^{c}	3.4 ± 0.0^{c}
Crimson Seedless × 00-1-5	354	52	16	12	14.7 ± 0.5^{b}	4.5 ± 0.5^{c}	3.4 ± 0.9^{c}
Flame Seedless × Jupiter	401	51	36	20	12.8 ± 0.8^{a}	9.0 ± 0.8^{b}	5.0 ± 1.6^{b}
Qinxiu × Jupiter	794	44	34	21	5.5 ± 0.2^{b}	4.3 ± 0.9^{c}	2.6 ± 0.4^{c}
Qinhong No.2 × Jupiter	861	118	95	89	13.7 ± 3.9^{a}	11.0 ± 0.5^{a}	10.3 ± 0.5^{a}

Values represent means ± SD. Different lowercase letters within a column indicate significant ($P \leq 0.05$) differences according to Duncan's multiple range test

Results

Effect of different female parents on embryo rescue

Table 3 shows paternal genotype significantly influences embryo rescue efficiency. The five female cultivars were each hybridised with the same male parent, 00-1-5. Of these, the progeny with Perlette showed the highest percentages of embryo development, and the highest rates of germination and plantlet formation. For Ruby Seedless and Qinhong No.10, the embryo development rates were not significantly different but the embryo germination rate of the two cultivars was significantly different and Ruby Seedless was higher than Qinhong No.10; the plantlet formation rates of them were not significantly different ($P < 0.05$). However, the embryo development rate and germination rate of Flame Seedless were the lowest of the five combinations, with the embryo development rate and plantlet formation rate being only 10.5% and 3.4%; similarly, Crimson Seedless had the lowest embryo germination rate and plantlet formation rate.

When Jupiter was used as the male parent, embryo rescue with Qinhong No.2 as the female parent was best. Here, the embryo development rate, germination rate and plantlet formation rates were: 13.7%, 11.0% and 10.3%, respectively. Next best female parent was Flame Seedless, but embryo rescue with Qinxiu as female parent was the worst, with the embryo development rate, germination rate and plantlet formation rates of 5.5%, 4.3% and 2.6%, respectively.

We conclude that Perlette, Qinhong No.2, Ruby Seedless and Qinhong No.10 are the best female parents for embryo rescue.

Effect of different male parents on embryo rescue

The effect of different male parents on embryo rescue is shown in Table 4. When Flame Seedless was chosen as the female parent and Jupiter as the male parent, the development rate, germination rate and plantlet formation rate of the

Table 4 Effect of different male parent genotype on embryo rescue

Cross combination	No. of ovules cultured	No. of embryos developed	No. of embryos germinated	No. of normal plantlets	Proportion of embryos developed (%)	Proportion of embryos germinated (%)	Proportion of embryos forming plantlets (%)
Flame Seedless × Jupiter	401	51	36	20	12.8 ± 0.8^{a}	9.0 ± 0.8^{b}	5.0 ± 1.6^{b}
Flame Seedless × 00-1-5	266	28	13	9	10.5 ± 0.5^{c}	4.9 ± 1.7^{c}	3.4 ± 0.0^{c}

Values are means ± SD. Different lowercase letters within a column indicate significant ($P \leq 0.05$) differences according to Duncan's multiple range test

Table 5 Determination of optimal sampling time for the seedless Qinxiu

Sampling time (DAF)	No. of ovules cultured	No. of embryos developed	No. of germinated	No. of normal plantlets	Proportion of embryos developed (%)	Proportion of embryos germinated (%)	Proportion of embryos forming plantlets (%)
36	129	4	2	1	3.1 ± 0.1^{c}	1.6 ± 0.1^{c}	0.8 ± 0.1^{b}
38	156	6	5	1	3.9 ± 0.2^{bc}	3.2 ± 0.1^{c}	1.3 ± 0.1^{b}
40	175	9	7	5	5.4 ± 0.2^{bc}	4.2 ± 0.3^{bc}	3.0 ± 0.1^{b}
42	101	11	9	9	10.8 ± 0.3^{a}	8.9 ± 0.0^{a}	8.9 ± 0.0^{a}
44	141	10	9	4	7.1 ± 0.1^{b}	6.4 ± 0.0^{ab}	2.8 ± 0.3^{b}
46	92	2	2	1	2.2 ± 0.2^{c}	2.2 ± 0.2^{c}	1.0 ± 0.2^{b}

Values are means ± SD. Different lowercase letters within a column indicate significant ($P \leq 0.05$) differences according to Duncan's multiple range test. Days after flowering, DAF

embryos were 12.8%, 9.0% and 5.0%, respectively. All these gave significantly ($P < 0.05$) better results than with 00-1-5 as the male parent.

Optimal sampling time for Qinxiu

The results for embryo rescue at different sampling times show that when the seedless cv. Qinxiu was sampled on DAF 42, the embryo development rate, germination rate and plantlet formation rate were higher than for the other sampling times; these rates were 10.8%, 8.9% and 8.9%, respectively (Table 5). We conclude Qinxiu is best sampled at DAF 42.

Exogenous hormones and embryo rescue of Ruby Seedless × 00-1-5

Different hormone additions to the MM3 medium effected embryo rescue (Table S2). The embryo development rate, germination rate and plantlet formation rate were highest (26.5%, 21.4% and 19.3%, respectively) on medium No.7 which contains 0.5 mg/L GA_3, 1.0 mg/L IAA and 0.5 mg/L 6-BA.

Embryo germination rate and plantlet formation rate were highest on solid WPM with 0.1 mg/L IAA and 0.2 mg/L 6-BA, reaching 86.3% and 57.6%, respectively.

Detection of parents by the seedless molecular marker SCF27-2000

The PCR analyses show the primer SCF27 amplifies a 2000 bp band in all the seedless female parents, but not in the seeded male parent, 00-1-5. Thus, SCF27 is likely able to identify any seedless progeny (Fig. S1).

Detection of seedless progeny with SCF27-2000

A total of 473 new genotypes obtained from nine cross combinations were screened by PCR using the primer SCF27. The results show that 440 of these carried the seedless marker SCF27-2000 and the seedless rate in hybrids from seedless cultivar × seedless cultivar was 89.9–100%, the rate of seedless in hybrids from seedless cultivar × seeded hybrid reached 83.3–96.2%. These genotypes are tentatively identified as seedless (Table 6; Figs. 2, 3, 4).

Table 6 Detection of seedless progeny with SCF27-2000

Cross combination	No. of hybrids obtained	No. of hybrids with marker SCF27-2000	Proportion of hybrids with marker SCF27-2000 (%)
Ruby Seedless × 00-1-5	153	140	91.5
Qinhong No.10 × 00-1-5	79	76	96.2
Perlette × 00-1-5	33	30	90.9
Flame Seedless × 00-1-5	42	37	88.1
Crimson Seedless × 00-1-5	12	10	83.3
Flame Seedless × Jupiter	20	20	100
Qinxiu × Jupiter	21	21	100
Qinhong No.2 × Jupiter	89	80	89.9
Jupiter × Su67	24	24	100

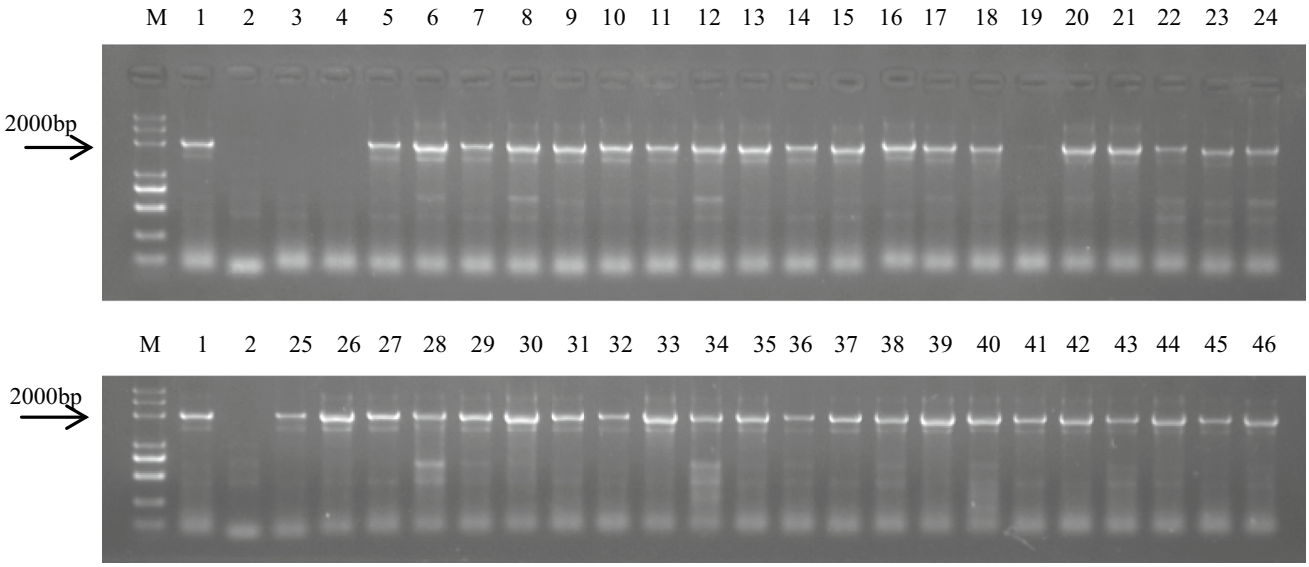

Fig. 2 Detection of the seedless marker SCF27-2000 to the hybrids of the combination Ruby Seedless × 00-1-5. M: Marker (Trans2K Plus); 1: Ruby Seedless; 2: 00-1-5; 3–46: hybrid seedlings from the cross combination Ruby Seedless × 00-1-5

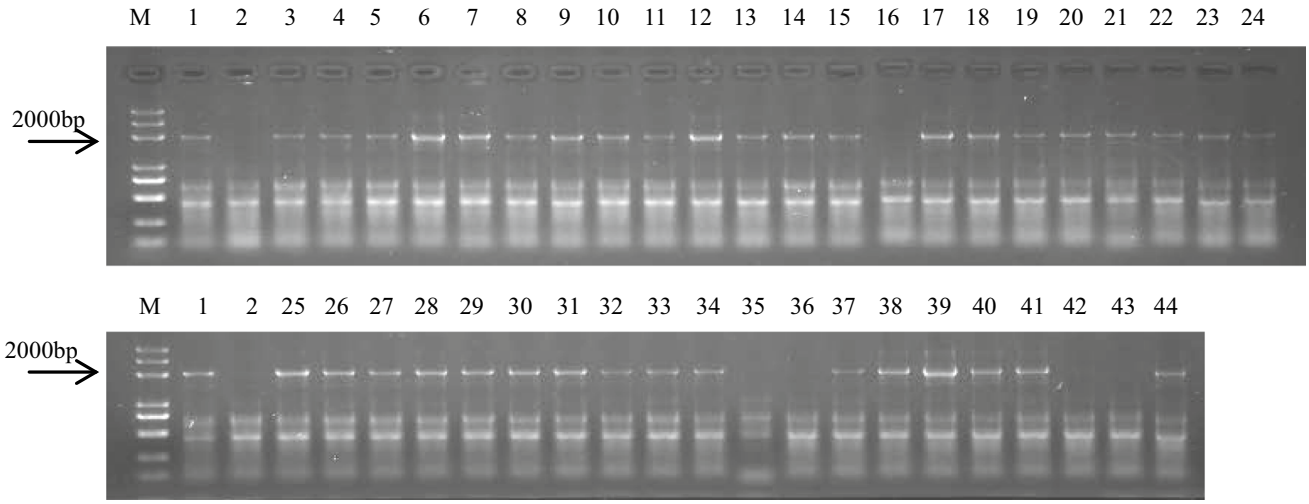

Fig. 3 Detection of the seedless marker SCF27-2000 to the hybrids of the combination Flame Seedless × 00-1-5. M: Marker (Trans2K Plus); 1: Flame Seedless; 2: 00-1-5; 3–44: hybrid seedlings from the cross combination Flame Seedless × 00-1-5

Discussion

The main factors influencing embryo rescue in seedless grapes include: the genotype of both parents, the culture medium, the exogenous hormones, the sampling time and the culture conditions. However, the rates of embryo development, germination and plantlet formation from embryo rescue are still in comparatively low which severely reduces breeding efficiency for seedless grapes. In line with our results, a large number of researchers have reported that parental genotype is important in embryo rescue (Gray et al. 1990; Bharathy et al. 2005; Ji et al. 2013; Li et al. 2014; Li et al. 2015a; Liu et al. 2016). In seedless grape embryo rescue, embryo development rate is determined mainly by the female parent (Zhang and Niu 2013). Different seedless cultivars have different ratios of zygotic embryos, and the zygotic embryos have different abortion times. Previous studies have found that some seedless cultivars are more suitable than others for embryo rescue work. Examples are, Ruby Seedless, Perlette, Red Seedless, Blush Seedless, Delight and Dawn Seedless (Valdez 2005; Nicole et al.

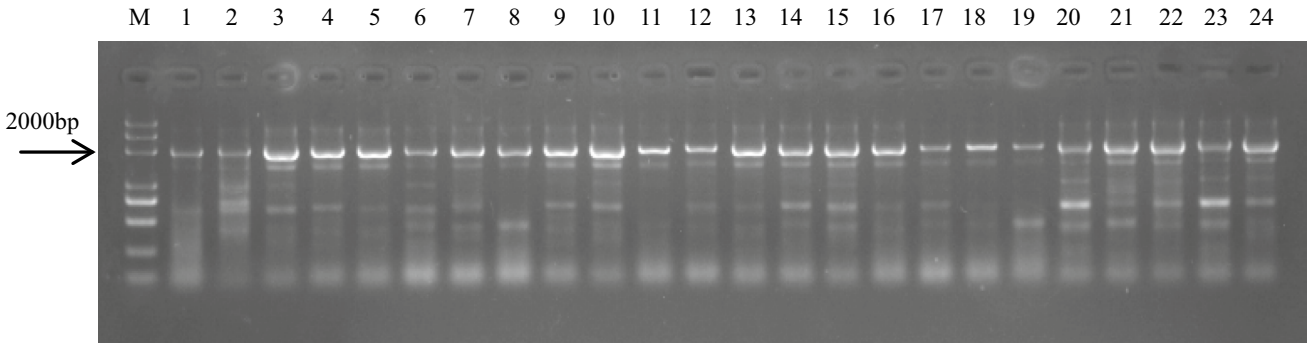

Fig. 4 Detection of the seedless marker SCF27-2000 to the hybrids of the combination Jupiter × Su-67. M: Marker (Trans2K Plus); 1: Jupiter; 2: Su-67; 3–24: hybrid seedlings from the cross combination Jupiter × Su-67

2006; Niu et al. 2012; Zhang and Niu 2013). Some studies also show the male parent influences embryo rescue (Gray et al. 1990; Niu et al. 2012; Li et al. 2018). In our study, the results show the seedless cultivars Perlette, Qinhong No.2, Ruby Seedless and Qinhong No.10 are more suitable as female parents, which agrees with earlier results (Valdez 2005; Niu et al. 2012; Ji et al. 2013; Liu et al. 2016). The seedless cultivar Crimson Seedless we found to be less effective as the female parent for embryo rescue. This agrees with Valdez (2005) but not with Liu et al. (2016). As for the effects of the male parent on embryo rescue, our result show that when Flame Seedless was the female parent, the difference between the two male parents Jupiter and 00-1-5, was significant. Niu et al. (2012) considered the embryo development rate and plantlet formation rate were different with F1 hybrids (e.g. the *V. vinifera* cv. Muscat Hamburg × *V. amurensis*) and the wild *V. amurensis* accessions as male parents. The former were higher than the latter.

The development of seedless grape embryos goes through a critical stage when embryos tend to abort. Obviously, dealing with this is important for the success of embryo rescue. The sampling time can be determined by referring to the maturity of the female parent (Xu et al. 2005). Gray et al. (1990) suggest the best inoculation time for ovules is before veraison. According to Notsuka et al. (2001), the best sampling time is 50 ± 10 DAF. Xu et al. (2005) suggest the best sampling time for early-, mid- and late-ripe cultivars is 6–9, 7–10 and 9–12 weeks after pollination, respectively. There are two reliable ways to determine best sampling time. One is by cytological observation for embryonic development (Ebadi et al. 2001), and another is stage sampling (Khoshandam et al. 2017). Here, for the first time, the seedless Qinxiu (Jingxiu × Zhengguodawuhe) has been used, adopting the staged sampling method. The results show the development, germination and plantlet formation rate of embryos were best at DAF 42, so this is best for Qinxiu.

Spraying PGRs on the vine before flowering promotes embryo development in situ. Some studies report that PGR sprays before flowering are effective. These include, CCC (Tang et al. 2009a), CPPU (Nookaraju et al. 2007), and 6-BA (Nookaraju et al. 2007; Razi et al. 2013). However, Tang et al. (2009a) believes 6-BA sprays before flowering have no effect on embryo development.

The embryo development medium provides rich nutrients and the exogenous hormones required for the further development of immature embryos in vitro. The availability of sufficient nutrients is the basis fundamental for normal growth of any plant (Li et al. 2014). Embryo development medium and germination plantlet medium have been much studied (Burger and Goussard 1996; Niu et al. 2012; Li et al. 2014; Ebadi et al. 2016). Some researchers suggest Bouquet and Davis (BD) medium, NN medium and MM3 medium (modified ER, patent No. ZL02139330.3) work best (Gray et al. 1990; Burger and Goussard 1996; Tang et al. 2009a, b; Singh et al. 2011; Raziet al. 2013; Li et al. 2014; Ebadi et al. 2016). Our previous research shows MM3 medium is most suitable for embryo rescue with the Chinese wild grapes as male parents (Tian et al. 2008; Li et al. 2014; Liu et al. 2016). In this study, hybrid ovules of Ruby Seedless × 00-1-5 were inoculated onto MM3 medium (no exogenous hormones) with the higher development rate, germination rate and plantlet formation rate, reaching 14.4%, 13.1% and 7.2%, respectively. MM3 medium has higher concentrations of Mg^{2+} and K^{+} than ER medium. It is speculated that high Mg^{2+} and K^{+} concentrations may be beneficial to embryo development and germination. Some studies suggest solid-phase media are more effective than liquid-phase (Spiegle-Roy et al. 1985; Gray et al. 1990), while others consider liquid-phase is better (Emershad and Ramming 1984; Okamoto et al. 1993). However, some other studies found that solid–liquid, double-phase media are best (Tang et al. 2009b; Ebadi et al. 2016).

The hormone levels in berries of seedless grapes differ from those in seeded ones. Hence, some studies have suggested adding IAA (Burger and Goussard 1996; Singh et al. 2010; Razi et al. 2013; Li et al. 2014), 6-BA (Emershad

and Ramming 1994; Tian et al. 2008; Li et al. 2014), GA_3 (Burger and Goussard 1996; Singh et al. 2010; Li et al. 2014) to the medium promotes embryo development. Tang et al. (2009b) considered the best ratio of exogenous hormones in Nitsch embryo development medium was 0.5 mg/L GA_3 and 1.5 mg/L IAA. However, our study shows with MM3 except the addition of GA_3 at the same concentration as Tang et al. (2009b), the concentration of IAA and 6-BA should be 1.0 mg/L and 0.5 mg/L. These are most suitable for embryo development in cold-resistant seedless grape embryo rescue. Singh et al. (2010) showed that when Murashige and Skoog (MS) medium was used as the basic medium for embryo rescue, adding 4 mg/L IAA and 0.5 mg/L GA_3 were the most effective for embryo germination, and the germination rate reached 13.8%. However, our study shows adding 0.1 mg/L IAA and 0.2 mg/L 6-BA to WPM medium significantly improves embryo germination rate with Ruby Seedless × 00-1-5, indicating IAA plays an important role in promoting embryo germination.

The embryo development rate is thought to increase significantly if suitable amounts of polyamines (Jiao et al. 2018; Ebadi et al. 2016; Li et al. 2018), amino acids (Li et al. 2014) and other components (Bharathy et al. 2005; Ji et al. 2013) are also added to the culture medium. Culture conditions such as temperature and light also effect the success of embryo rescue. We speculate that the benefit of in vitro culturing of ovules in darkness simulates the natural light level in vivo, in the developing seed (Zhang and Niu 2013). Low temperature treatments of ovules and the addition of GA_3 to the culture medium helped break embryo dormancy (Agüero et al. 1996; Singh et al. 2010).

It is worth commenting that evolution 'designed' fruits to distribute seeds. A seedless fruit goes against all evolutionary selection pressures since the appearance of fruit-eating animals. The inheritance of seedless traits seems very complicate in grapes. Current theories are unable to fully explain the genetics of the seedless trait (Bouquet and Danglot 1996; Striem et al. 1996; Roytchev 1998). Despite this, seedless genes can be detected by molecular markers linked to the seedless gene at the molecular level. In general, the accuracy and reliability of a molecular marker depends on the degree of linkage between the marker and the gene of interest. Of the five molecular markers linked to the seedless genes of grapes, one of them (GLSP1-569) was obtained from Thompson Seedless (Wang and Lamikanra 2002) and was effective in detecting Thompson Seedless and closely-related seedless genotypes (Li et al. 2015b; Liu et al. 2016; Li et al. 2018). However, this marker is not useful for detecting genotypes of the 'DR' series of hybrids (Delight × Ruby Seedless) (Li et al. 2015b; Li et al. 2018). Another seedless gene molecular marker, SCF27-2000, has been shown to have broader applicability to detect seedlessness across a number of grape genotypes (Li et al. 2015b; Liu et al. 2016). Hence, we employed SCF27-2000 here to help identify the seedless trait in both a range of parents and their hybrid progeny.

Previous studies showed that the ratio of seedless to seeded progeny in a cross population from a seedless cultivar × seedless cultivar can reach above 85% (Cain et al. 1983; Emershad and Ramming 1984; Spiegle-Roy et al. 1985; Gray et al. 1987). Here, molecular marker identification showed the seedless rate in hybrids from seedless cultivar × seedless cultivar was 89.9–100%, which is consistent with the previous studies. However, the seedless rate of the hybrids from seedless cultivar × seeded cultivar reached 83.3–96.2%, which is higher than our previous results (Liu et al. 2016). It is presumed the heritability of the seedless trait is different between different cross combinations. Also, the seedless trait may be related to maternal cytoplasmic inheritance (Cosmides and Tooby 1981). Therefore, identification of seedless individuals from embryo rescue, should also be combined with traditional (slow) field identification to screen-out the 'false positive' and to screen-in the 'false negative' seedless hybrids.

Conclusion

The parental genotype has a significant effect on embryo rescue. Perlette, Qinhong No.2, Ruby Seedless and Qinhong No.10 are fit to serve as seedless female parents. The cold-resistant seedless Jupiter is suitable as the male parent. The medium MM3 + 0.5 mg/L GA_3 + 1 mg/L IAA + 0.5 mg/L 6-BA is most suitable for ovule culture in vitro.

Adding an optimal concentration of IAA to the embryo germination medium promotes the plantlet formation rate of the embryos. WPM + 0.1 mg/L IAA + 0.2 mg/L 6-BA is most suitable as the embryo germination medium.

Sampling time is also critical, greatly affecting the results of embryo rescue. The best embryo rescue results with the seedless Qinxiu were with a sampling time of 42 DAF.

In this study, 440 hybrid genotypes contained the molecular marker SCF27-2000 linked to the seedless character. However, their true seedless character still requires conformation in the field.

Acknowledgements This work received financial support from 'The Shaanxi Province Key Project (Agriculture) of the People's Republic of China' (Grant Number 2017ZDXM-NY-026).

Author contributions JZ designed the experiment and revised the manuscript. PZ wrote the manuscript. All authors took part in the crossing work. PZ, XZ and PL carried out the work seedless grape embryo rescue. BG, PL, PZ and XS carried out the marker-assisted selection.

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