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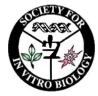

EMBRYO CULTURE

Improved *in vitro Vitis vinifera* L. embryo development of F_1 progeny of 'Delight' × 'Ruby seedless' using putrescine and marker-assisted selection

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Abstract

Production of seedless *V. vinifera* L. cultivars is one of the major goals of table grape breeding. Traditional methods result in only a low percentage of seedless progeny. Modern techniques, such as embryo rescue, are used to improve production efficiency of seedless cultivars. In this study, the effects of various putrescine concentrations (0, 1, 2, 3, 4, and 5 mmol L^{-1}) and different stages of embryo development (globular-, heart-, torpedo-, and cotyledon-shaped embryo) on the success rate of embryo rescue were assessed in three lines of the F_1 *Vitis vinifera* L., cross 'Delight' × 'Ruby Seedless,' 'DR2,' 'DR3,' and 'DR6' (with small seed traces). The plant development rates of 'DR2,' 'DR3,' and 'DR6', which contain small seed traces, as female parents, were significantly higher than those of seedless varieties. The rates of embryo development on woody plant medium (WPM) of 'DR2' × 'Thompson Seedless,' 'DR3' × 'Thompson Seedless,' and 'DR6' × 'Monukka' were highest with 2, 2, and 4 mmol L^{-1} putrescine, respectively. The germination rates of torpedo- and cotyledon-shaped embryos were significantly higher than those of globular- and heart-shaped embryos. Characteristics of the embryo germination progress of 'DR2' × 'Thompson Seedless' at different developmental stages were recorded. The Grape Seedless gene Probe 1 (GSLP1) marker was used to help identify the seedless descendants; 14 plantlets showed a corresponding electrophoresis band at 569 bp and were preliminarily defined as seedless.

Keywords *Vitis vinifera* L. · Embryo rescue · Muscat aroma · Putrescine · Molecular marker for seedlessness

Introduction

Seedless fruits of *Vitis vinifera* L. (grapes) are either stenospermocarpic or parthenocarpic, with most seedless cultivars being stenospermocarpic. With stenospermocarpy, fertilization occurs, but embryos frequently abort during development (Stout 1936). Since it was first reported by Ramming and Emershad (1982) that stenospermic grapes can be used for *in vitro* embryo rescue *via* ovule culture, many breeders have employed this technique in seedless grape breeding.

Many factors affect the efficiency of embryo rescue, including genotype, embryo rescue time, medium composition, the presence of growth regulators, and initial embryo developmental stage. Research that allows optimization of these factors has considerable potential value. The success of embryo rescue is variable and is related to cultivar genotype (Cain *et al.* 1983; Goldy and Amborn 1987; Emershad *et al.* 1989; Ramming *et al.* 1990; Gribaudo *et al.* 1993; Burger and Goussard 1996; Ponce *et al.* 2000; Liu *et al.* 2003; Tian and Wang 2008; Singh *et al.* 2011; Ji *et al.* 2013; Razi *et al.* 2013). The formation and development of female embryos differ between genotypes. It has been shown that only stenospermocarpic grapes and only embryos developing to the advanced global embryo stage can be saved successfully and with relative ease (Cain *et al.* 1983; Ramming *et al.* 1990; Singh *et al.* 2011). Hence, the survival and development of maternal material are important factors in the successful culture of seedless hybrid embryos. Cain

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et al. (1983) generated 13 self-pollinated, seedless grape cultivars and found that only seven of the 13 seedless grape cultivars produced viable embryos. However, the embryo formation capacity differed significantly among those seven seedless grape varieties. Ji and Wang (2013) found that the production of hybrid plants varied significantly among genotypes, ranging from 21.1% ('Ruby Seedless' × 'Beichun') to only 1.1% ('Pink Seedless' × 'Beichun'). The male parent genotype affected both the capacity for embryo formation and the germination of rescued grape embryos (Spiegel-Roy *et al.* 1985, 1990; Gray *et al.* 1990; Garcia *et al.* 2000; Qi and Ding 2002; Ebadi *et al.* 2004; Liu *et al.* 2008; Li *et al.* 2013). In addition, genotypes with larger seed traces tend to be more easily rescued and tend to produce larger numbers of ovules with viable embryos (Pommer *et al.* 1995). Some researchers have reported that the embryo formation, germination, and plant development rates of crosses made between 'Delight' and 'Ruby Seedless' (DR) are higher than those of most other crosses (Tian *et al.* 2008; Ji *et al.* 2013; Ji and Wang 2013; Li *et al.* 2014). The DR lines are F_1 strains of 'Delight' × 'Ruby Seedless' with small seed traces. When DR lines are used as the female parents, the plant development rate of embryos is higher than that of seedless cultivars (Li *et al.* 2015).

Polyamines are growth regulators present in all plant tissues and are important for cell division, signal transduction, and protein synthesis (Tiburcio *et al.* 1993; Kusano *et al.* 2008). They can speed up or slow down micro-shoot rooting, depending on their type and concentration. In some cases, polyamine inhibition leads to rooting (Arena *et al.* 2005). A number of studies have reported that in some plant species, polyamines are also important for somatic embryogenesis and regeneration (Kumar and Rajam 2004). In *V. vinifera*, the main polyamines are putrescine, spermidine, and spermine; their effects at different concentrations have been determined on different organs and at different stages of development (Geny *et al.* 1997). Ponce *et al.* (2002) pointed out that putrescine has a role in promoting the development of seedless grapes in embryo rescue. A culture medium with 2 mmol L^{-1} putrescine significantly enhanced the embryonic development rate and seedling recovery rate of the seedless cultivar 'Perlón' (Ponce *et al.* 2002).

Embryo development stage and size are important factors in embryo rescue. Embryogenesis includes nine major stages: zygote, two-celled, four-celled-stage, octant-stage, dermatogen-stage, globular-shaped, heart-shaped, torpedo-shaped, and large cotyledon-shaped embryo (Mansfield and Briarty 1990). The last four stages of embryogenesis can develop into plantlets following embryo rescue. Therefore, the ovule excision time in embryo rescue must be based on the stage of embryogenesis. When embryos are sampled too soon, embryo rescue is difficult; however, when sampled too late, embryos abort.

In recent years, marker-assisted selection (MAS) has been employed in breeding studies, with markers linked to the seedless trait (Lahogue *et al.* 1998; Adam-Blondon *et al.* 2001; Mejía and Hinrichsen 2003; Fatahi *et al.* 2004; Cabezas *et al.* 2006). However, each reported molecular marker has its own range of applications; none are universal. Fatahi *et al.* (2004) proposed that the co-dominant sequence characterized amplified region (SCAR) SCC8 marker could be used to test progenies from different crosses for seedlessness, because this marker showed the expected distribution ratio in the progenies they studied. However, Mejía and Hinrichsen (2003) found that only 42% of the F_1 hybrids of 'Ruby Seedless' x 'Sultanina' amplified a band with the probe; therefore, the SCC8 marker was considered to be inadequate for early selection of seedless grapes. Furthermore, Mejia and Hinrichsen (2003) found a correlation of 81%, between the seedlessness trait and the amplification of a band in hybrid progenies from the examined crosses, by a second SCAR marker, SCF27; the usefulness of this marker for MAS requires confirmation with a higher number of additional lines.

The micro-satellite marker developed by the Vitis Microsatellite Consortium (VMC), VMC7F2, was found to be closely associated with the seedless characteristics linked to a major quantitative trait locus (QTL) on grape chromosome 18 (Adam-Blondon *et al.* 2001; Karaagac *et al.* 2012). The sequence tagged site (STS) marker p3_VvAGL11 is located in the promoter region of the *AGAMOUS-like 11* gene of *V. vinifera* L. (*VvAGL11*). It is able to identify lines that are definitely not seedless but is unable to discriminate between the different seedless classes. The markers VMC7F2 and p3_VvAGL11 were all in the promoter region of *VvAGL11* gene, but there was a region (~430 bp) between them. This region might contain the causative genetic variation of the seedless phenotype (Mejía *et al.* 2011; Bergamini *et al.* 2013).

The 18-bp Grape Seedless gene Probe 1 (GSLP1) produces a 569-bp band in seedless grapes and was used to identify seedlessness by Wang and Lamikanra (2002). Since then, it has been used to distinguish seedless individuals in many studies. Ji *et al.* (2013) used the GSLP1 probe to identify 45 lines obtained from four cross-combinations and found 11 lines with the 569-bp specific band and tentatively considered these to be seedless. A little later, Ji and Wang (2013) identified 17 seedless lines out of 115 progenies of seven hybrids using GSLP1. Li *et al.* (2015) used three molecular markers (SCC8, SCF27, and GSLP1) to analyze 15 parents used in the study and found that only GSLP1 could distinguish between the three types of parents used. There was a 569-bp band in all the seedless parents tested, whereas this band was absent in the seeded and DR parents. Therefore, GSLP1 was used to screen the progeny for seedlessness in that study.

The primary aim of the present study was to improve embryo rescue methods by defining optimal conditions for

embryo development, based on genotype of the female, putrescine concentration, and molecular markers for selecting new seedless lines, along with observations of embryo morphogenesis at different developmental stages. It is hoped that the improved technique will be useful as a tool for breeding new seedless grape cultivars.

Materials and Methods

Plant materials The plants in this study were 4–5-yr-old and all were grown in a vineyard of the Xinjiang Development and Research Center of Grapes and Melons in Shanshan County, Xinjiang Uygur Autonomous Region of China. The eight female parents were ‘Crimson Seedless,’ ‘Heshi Seedless,’ ‘Autumn Royal,’ ‘Kunxiang Seedless,’ ‘Zixiang Seedless,’ ‘DR2,’ ‘DR3,’ and ‘DR6’. Lines ‘DR2,’ ‘DR3,’ and ‘DR6’ are soft-seeded F_1 progeny of ‘Delight’ × ‘Ruby Seedless’ and the others are seedless grapes. ‘Delight’ and ‘Ruby Seedless’ are both *V. vinifera* L. and were derived from ‘Thompson Seedless’. ‘Delight’ is a descendant of ‘Queen of vineyard’ × ‘Kiralynoie’ released by the University of California in 1948, and ‘Ruby Seedless’ is a descendant of ‘Emperor’ × ‘Pirorano,’ also selected by the University of California (Liu 2003).

The six male parents were: ‘Thompson Seedless,’ ‘Monulka,’ ‘Guifeimeigui,’ ‘Fenhongmeigui,’ ‘Shengdanmeigui,’ and ‘Kunxiang Seedless’. Ten crosses were performed, based on the characteristics of each cultivar (Table 1). These were ‘Crimson Seedless’ × ‘Kunxiang Seedless,’ ‘Heshi Seedless’ × ‘Kunxiang Seedless,’ ‘Heshi Seedless’ × ‘Guifeimeigui,’ ‘Autumn Royal’ × ‘Kunxiang Seedless,’ ‘Autumn Royal’ × ‘Fenhongmeigui,’ ‘Kunxiang Seedless’ × ‘Guifeimeigui,’ ‘Zixiang Seedless’ × ‘Shengdanmeigui,’ ‘DR2’ × ‘Thompson Seedless,’ ‘DR3’ × ‘Thompson Seedless,’ and ‘DR6’ × ‘Monukka’.

Hybridization Prior to artificial pollination, pollen was collected from the male parents and held at 4°C with desiccation for up to 1 mo. Inflorescences were emasculated 3 d before anthesis (Fig. 1*A*), then washed and bagged immediately. When the mucus started to appear on the stigma of the emasculated female parents, artificial pollination was carried out with a piece of absorbent cotton wool loaded with the previously stored pollen (Fig. 1*B*). The inflorescences were again bagged and marked to record the pollination date (Fig. 1*C*; Liu *et al.* 2016).

Embryo rescue Four to 8 wk after pollination, hybrid fruits were collected and placed into a glass container. Fruits were surface sterilized with 75% (*v/v*) ethanol for 30 s, then soaked in 2.5% (*w/v*) NaClO (Xilong Scientific, Guangdong, China) for 30 min, and washed with sterilized, distilled water four to five times under aseptic conditions, until all bubbles disappeared (Fig. 2*A*). Ovules were excised from the fruits and inoculated onto solid ER medium (Emershad and Ramming 1994) with 500 mg L^{-1} casein hydrolysate (CH, Solarbio, Beijing, China), 1 mmol L^{-1} serine, 3 g L^{-1} activated carbon (AC, Xilong Scientific), 60 g L^{-1} sucrose (Jinhuada, Guangzhou, China), 7 g L^{-1} agar (Solarbio), and adjusted to pH 5.8 with 0.1 M NaOH or 0.1 M HCl, then autoclaved at 120°C for 25 min (Fig. 2*B*; Li *et al.* 2015). Twenty-five to 30 ovules were inoculated per Erlenmeyer flask (100 mL) containing 25–30 mL ER medium, covered with a piece of black cloth, and placed in a room at 25 ± 2°C. After 8 wk of *in vitro* culture (Fig. 2*C*), embryos were excised under a ×40 magnification stereomicroscope (Olympus, CX21FS1, Tokyo, Japan) (Fig. 2*D*) and embryo formation rate was recorded. Each embryo was cultured on woody plant medium (WPM, Lloyd and McCown 1980) with 0.2 mg L^{-1} 3-indolebutyric acid (IBA, Sigma-Aldrich®, St. Louis, MO), 0.2 mg L^{-1} 6-benzylaminopurine (BAP, Sigma-Aldrich®), 20 g L^{-1} sucrose, 3 g L^{-1} AC, and 7 g L^{-1} agar and adjusted to pH 5.8 with 0.1 M NaOH or 0.1 M HCl, then autoclaved at 120°C

Table 1. Characteristics of *Vitis vinifera* cultivars used for crosses

Cross-combinations	Female characteristics	Male characteristics
‘Crimson Seedless’ × ‘Kunxiang Seedless’	Stenospermic, red, no aroma	Stenospermic, golden yellow, aroma of Muscat
‘Heshi Seedless’ × ‘Kunxiang Seedless’	Stenospermic, yellow green, no aroma	Stenospermic, golden yellow, aroma of Muscat
‘Heshi Seedless’ × ‘Guifeimeigui’	Stenospermic, yellow green, no aroma	Seeded, golden yellow, aroma of Muscat
‘Autumn Royal’ × ‘Kunxiang Seedless’	Stenospermic, fuchsia, no aroma	Stenospermic, golden yellow, aroma of Muscat
‘Autumn Royal’ × ‘Fenhongmeigui’	Stenospermic, fuchsia, no aroma	Seeded, pink, aroma of Muscat
‘Kunxiang Seedless’ × ‘Guifeimeigui’	Stenospermic, golden yellow, aroma of Muscat	Seeded, golden yellow, aroma of Muscat
‘Zixiang Seedless’ × ‘Shengdanmeigu’	Stenospermic, atropurpureus, aroma of Muscat,	seeded, fuchsia, no aroma
‘DR2’ × ‘Thompson Seedless’	Small seed trace, red, no aroma	Stenospermic, yellow green, no aroma
‘DR3’ × ‘Thompson Seedless’	Small seed trace, red, no aroma	Stenospermic, yellow green, no aroma
‘DR6’ × ‘Monukka’	Small seed trace, fuchsia, no aroma	Stenospermic, atropurpureus, no aroma

DR ‘Delight’ × ‘Ruby Seedless’

Figure 1 . Depiction of the *Vitis vinifera* hybridization process in the field. (*A*) Emasculation of inflorescence at 3 d before anthesis. (*B*) Artificial pollination with pollen on cotton wool. (*C*) Bagged and tagged inflorescence post-pollination.

for 25 min. The embryos were placed at 25°C under fluorescent light (L58W/77, OSRAM, Munich, Germany, 40 μmol m^{-2} s^{-1}) with 16-h photoperiod (Ji *et al.* 2013; Li *et al.* 2015). After 1 mo, the number of germinated embryos was recorded, and plantlets were sub-cultured on MS (Murashige and Skoog 1962) rooting medium (0.2 mg L^{-1} IBA + 0.2 mg L^{-1} BAP + 30 g L^{-1} sucrose + 1.5 g L^{-1} AC, 7 g L^{-1} agar) (Fig. 2*E*). The number of well-rooted plantlets (Fig. 2*F*) was recorded before such plantlets were transplanted to pots containing a 1:1:3 (*v/v/v*) vermiculite/perlite/peat-soil mixture (Liu *et al.* 2016) for hardening (Fig. 2*G*) in a greenhouse at 20–30°C under natural light conditions. At first, the pots were covered with transparent plastic cups to maximize humidity (Fig. 2*H*). Later, the cups were gradually removed. After 4–6 wk, the surviving plants were planted in a field (Fig. 2*I*). The numbers of embryos formed, their germination, and plant development rates were recorded. For each cross, approximately 54–60 ovules were inoculated with three replicates.

Effect of putrescine concentration on DR embryo rescue efficiency Three combinations 'DR2' × 'Thompson Seedless,' 'DR3' × 'Thompson Seedless,' and 'DR6' × 'Monukka' were selected to investigate the effects of putrescine on embryo rescue. Semi-solid, basal WPM with 0 mmol L^{-1} (control), 1 mmol L^{-1}, 2 mmol L^{-1}, 3 mmol L^{-1}, 4 mmol L^{-1}, or 5 mmol L^{-1} of putrescine (Sigma-Aldrich®) was used as the culture medium. Each putrescine treatment was inoculated with 20–25 ovules, with three replicates per treatment.

Effect of initial embryo developmental stage on germination rate Embryos of 'DR2' × 'Thompson Seedless' were excised under a stereomicroscope and grouped according to developmental stage. The stage of embryogenesis was categorized as one of four key stages: globular-, heart-, torpedo-, and large cotyledon-shaped embryo (Fig. 3). Embryos in the same development stage were selected as a cohort and inoculated on semi-solid WPM, with each cohort consisting of 26–45

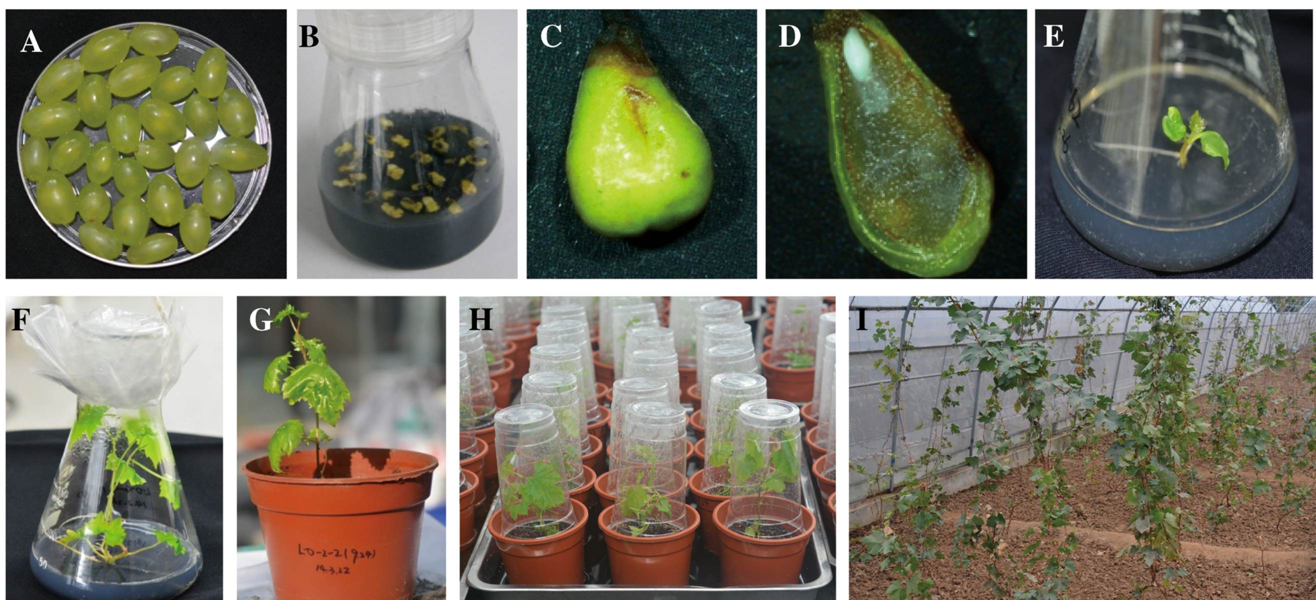

Figure 2. Embryo rescue procedure for *Vitis vinifera*, seedless grapes. (*A*) Immature fruits after surface sterilization. (*B*) Ovules cultured on ER medium (Emershad and Ramming 1994) in dark. (*C*) An ovule after 8-wk culture. (*D*) An embryo excised from an ovule. (*E*) Germinated embryo on rooting medium. (*F*) Well-developed and -rooted plantlet. (*G*) Potting of hybrid plantlets. (*H*) Hardening of plantlets in a greenhouse. (*I*) Plants established in the field.

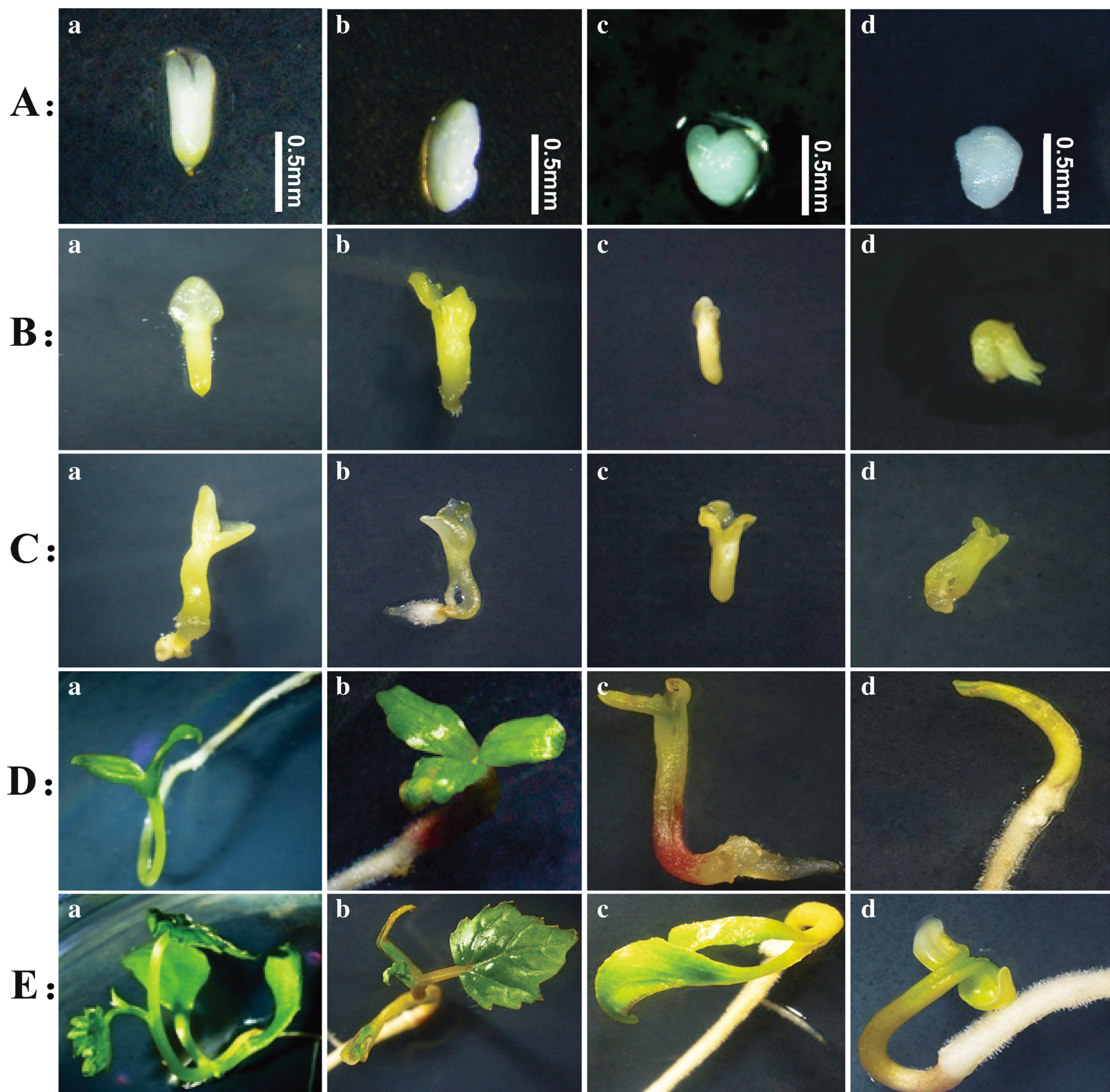

Figure 3. Embryo germination progress of *Vitis vinifera* hybrid 'DR2' × 'Thompson seedless' for different initial developmental stages (*columns*): (*a*) cotyledon-shaped; (*b*) torpedo-shaped; (*c*) heart-shaped; and (*d*) globular embryo after inoculation on Woody Plant medium for (*rows*) (*A*) 0 d, initial morphology of embryos; (*B*) 3 d, start of germination; (*C*) 5 d, emergence of *root* in (*a*) and (*b*); (*D*) 15 d, *greening* of *cotyledons* in (*a*) and (*b*), emergence of *root* in (*c*) and (*d*); and (*E*) 20 d, emergence of true leaves in (*a*) and (*b*). *Scale bars* 0.5 mm.

embryos, with three replicates. These were photographed at 0, 3, 5, 15, and 20 d after inoculation, to record the morphogenesis of embryos at different developmental stages.

Marker-assisted selection The GSLP1 marker (Wang and Lamikanra 2002) was used to identify the parents and their progeny obtained through embryo rescue. Deoxyribonucleic acid was extracted from 0.2 to 0.3 g of young leaves of all parents and a total of 259 descendants from the 10 combinations, using a modified cetyltrimethylammonium bromide (CTAB) method of Wang and Lamikanra (2002), and stored at −80°C. The polymerase chain reaction (PCR) parameters were 94°C for 1 min, 35°C for 2 min, 72°C for 2 min, for a total of 40 cycles, followed by 72°C for 8 min, with the primer 5′CCAGTTCGCCCGTAAATG3′. The amplification products of PCR were separated on 2% (*w/v*) agarose (HydraGene, Piscataway, NJ) and photographed (Wang and Lamikanra 2002; Li *et al.* 2015).

Statistical analyses All experiments were repeated three times and the data were expressed as the means ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). The treatment means were tested for difference from the controls by Duncan's multiple range test at $P \leq 0.05$ using IBM® SPSS® Statistics v. 19.0 (IBM, New York, NY).

Results

Effect of female genotype on embryo rescue efficiency The success of embryo rescue was strongly affected by the female parent cultivar. Ten different cross-combinations were examined to explore the embryo rescue efficiency for different female genotypes (Table 1). When seedless cultivars were used as the female parents, the percentage of embryo formation rate ranged from 18.9 to 42.0% and the plant development rate ranged from 0.6 to 15.5% (Table 2). However, when 'Kunxiang Seedless' was used as the female parent, the embryo formation rate was significantly higher than that of the other four seedless cultivars: 'Crimson seedless,' 'Zixiang Seedless,' 'Heshi Seedless,' and 'Autumn Royal'. Both 'Kunxiang Seedless' and 'Zixiang Seedless' had similar plant development rates, when used as female parents. Plants were not obtained for 'Heshi Seedless' as female parent. When using DR lines as female parents, plant development rates were significantly higher (between 39.2 and 55.0%), than for any of the seedless cultivars (Table 2). Due to the low (less than 6.0% plant development) success rate of embryo rescue for 'Crimson Seedless,' 'Heshi Seedless,' and 'Autumn Royal' (Table 2), these were deemed not suitable for use as female parents. The embryo rescue efficiency of 'Zixiang Seedless' and 'Kunxiang Seedless' was moderate at 14.9 and 15.5%, respectively (Table 2). Interestingly, when the soft-seeded DR strains were used as female parents, plant development rates were much higher, above 39% (Table 2). Therefore, DR lines were judged more suitable for use as female parents.

Effect of putrescine concentration on DR embryo rescue efficiency An optimal concentration of putrescine accelerated embryo development. The plant development rates of 'DR2' × 'Thompson Seedless,' 'DR3' × 'Thompson Seedless,' and 'DR6' × 'Monukka' on WPM with different concentrations of putrescine varied significantly (Fig. 4). The highest plant development rates for 'DR2' × 'Thompson Seedless' and 'DR3' × 'Thompson Seedless' were 56.7 and 40.9%, respectively, both on WPM with 2 mmol L^{-1} putrescine. However, plant development rates for ovules of 'DR6' × 'Monukka' were highest (45.0%) on WPM with 4 mmol L^{-1} putrescine (Fig. 4). Thus, the optimal putrescine concentration varied with genotype.

Table 2. Effect of *Vitis vinifera* female parent genotype on embryo rescue efficiency

Cross-combinations	Number of ovules cultured	Number of embryos developed	Number of embryos germinated	Number of plantlets	Embryo formation rate (%)	Embryo germination rate (%)	Plant development rate (%)
'Crimson Seedless' × 'Kunxiang Seedless'	60	11 ± 2.1^{a}	4 ± 1.0^{bc}	2 ± 0.6^{ab}	18.9 ± 3.4^{a}	35.0 ± 3.0^{d}	2.8 ± 0.9^{ab}
'Zixiang Seedless' × 'Shengdanmeigui'	56	20 ± 1.5^{c}	12 ± 1.0^{e}	8 ± 0.6^{c}	36.3 ± 2.7^{c}	59.0 ± 1.1^{e}	14.9 ± 1.0^{c}
'Kunxiang Seedless' × 'Guifeimeigui'	58	24 ± 1.5^{d}	15 ± 1.0^{f}	9 ± 2.0^{c}	42.0 ± 2.6^{d}	61.6 ± 0.8^{ef}	15.5 ± 3.5^{c}
'Heshi Seedless' × 'Kunxiang Seedless'	54	15 ± 1.5^{b}	1 ± 0.6^{a}	0 ± 0.6^{a}	27.2 ± 2.8^{b}	4.3 ± 3.8^{a}	0.6 ± 1.1^{a}
'Heshi Seedless' × 'Guifeimeigui'	56	14 ± 1.5^{b}	2 ± 0.6^{ab}	1 ± 0^{ab}	25.6 ± 2.7^{b}	16.2 ± 2.3^{b}	1.8 ± 0^{ab}
'Autumn Royal' × 'Kunxiang Seedless'	50	17 ± 1.5^{b}	5 ± 0.6^{c}	3 ± 0.6^{ab}	33.3 ± 3.1^{c}	28.0 ± 1.4^{c}	5.3 ± 1.2^{b}
'Autumn Royal' × 'Fenhongmeigui'	50	17 ± 1.0^{b}	7 ± 1.5^{d}	3 ± 1.0^{b}	34.0 ± 2.0^{c}	39.0 ± 6.8^{d}	6.0 ± 0^{b}
'DR2' × 'Thompson Seedless'	60	52 ± 1.1^{g}	41 ± 1.5^{i}	33 ± 1.7^{e}	86.1 ± 2.6^{g}	80.1 ± 5.1^{g}	55.0 ± 2.9^{e}
'DR3' × 'Thompson Seedless'	60	41 ± 1.2^{e}	28 ± 2.0^{g}	24 ± 1.5^{d}	68.3 ± 1.7^{e}	68.4 ± 6.5^{f}	40.5 ± 2.5^{d}
'DR6' × 'Monukka'	60	49 ± 1.3^{f}	31 ± 0.6^{h}	24 ± 2.5^{d}	81.7 ± 1.7^{f}	62.6 ± 1.8^{ef}	39.2 ± 4.2^{d}

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

Embryo formation rate (%) the number of embryos developed/the number of ovules cultured × 100; *Embryo germination rate (%)* the number of embryos geminated/the number of embryos developed × 100; *Plant development rate (%)* the number of plantlets recovered/the number of ovules cultured × 100; *DR* 'Delight' × 'Ruby Seedless'

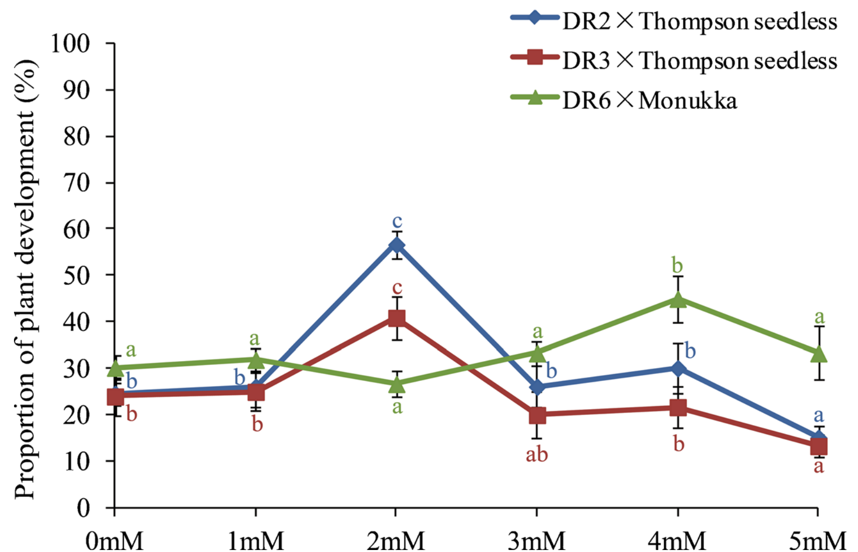

Figure 4. Effect of putrescine concentrations on plant development of three *Vitis vinifera* cross-combinations. Values represent means ± SD. Different *lowercase* letters in a *line* indicate significant differences according to Duncan's multiple range test ($P \le 0.05$). The *lowercase* letters in different *gray-levels* and *lines* marked by *shapes* represent the different cross-combinations (*medium gray diamond* 'DR2' × 'Thompson Seedless,' *dark gray square* 'DR3' × 'Thompson Seedless,' *light gray triangle* 'DR6' × 'Monukka,' see *legend*). *DR* 'Delight' × 'Ruby Seedless'.

Effect of initial embryo developmental stage on germination rate The initial developmental stage of embryos rescued by *in vitro* culture significantly affected the efficiency of embryo rescue. The four embryo developmental stages of 'DR2' × 'Thompson Seedless' used in this study were cotyledon embryo, torpedo embryo, heart embryo, and globular embryo (Fig. 3*A*: *a*, *b*, *c*, *d*, respectively). The rates for embryo germination, plant development, and plant morphogenesis differed among the four embryo developmental stages. For the cotyledon-shaped embryo, the embryo germination rate (79.2%) and plant development rate (65.9%) were the highest, followed by those of the torpedo-shaped embryo (63.5 and 44.4%, respectively) (Table 3). The germination rates of torpedo embryos and cotyledon embryos were significantly higher than those of globular embryos (12.8 and 6.4%) and heart embryos (18.2 and 10.6%) (Table 3). Embryo germination started 3 d after inoculation onto solidified WPM, as seen in Fig. 3*B*: *a*, *b*, *c*, *d*. The torpedo embryo and cotyledon embryo began rooting after 5 d (Fig. 3*C*: *a*, *b*), cotyledons turned green by 15 d (Fig. 3*D*: *a*, *b*), true leaves had emerged by 20 d, and the leaves were dark green and grew strongly (Fig. 3*E*: *a*, *b*). However, true leaves were not found in the globular embryo and heart embryo at 20 d after inoculation (Fig. 3*E*: *c*, *d*). Their cotyledons had not fully expanded nor turned green 20 d after inoculation. The plants developed abnormally (Fig. 3*E*: *c*, *d*). In fact, rooting was delayed for the globular embryo and heart embryo until 15 d (Fig. 3*D*: *c*, *d*). It was clear that the developmental stage of the embryo affected the embryo development rate: the more advanced the developmental stage of the embryo, the higher the embryo development rate and the better the quality of the resulting plantlet.

Marker-assisted selection Molecular markers associated with the seedless character are helpful for selecting seedless offspring at an early plantlet stage. Hence, marker-assisted selection not only saves time and space but also considerably reduces the cost of production. Here, the GSLP1 marker linked to seedlessness was selected, depending on the amplification results of parents (Fig. 5). When amplified with GSLP1, bands at 569 and 750 bp were found in the seedless parents, 'Crimson Seedless,' 'Heshi Seedless,' 'Autumn Royal,' 'Kunxiang Seedless,' 'Zixiang Seedless,' 'Thompson Seedless,' and 'Monukka,' and two bands of 750 and 1300 bp were seen in the seeded parents, 'Guifeimeigui', 'Fenhongmeigui', 'Shengdanmeigui', 'Red Globe', and 'Beichun' (the last two were used as controls). However, there was only a single 750-bp band observed in 'DR2,' 'DR3,' and 'DR6'. The 569-bp band was amplified only in seedless

Table 3. Embryo rescue success rates for different initial development stages of *Vitis vinifera* hybrid 'DR2' × 'Thompson Seedless' embryos

Stage of development	Number of embryos cultured	Number of embryos germinated	Number of plantlets	Proportion of embryos geminated (%)	Proportion of plants developed (%)
Cotyledon-shaped embryo	45	36 ± 2.5^{d}	30 ± 0.6^{d}	79.2 ± 5.6^{c}	65.9 ± 1.3^{c}
Torpedo-shaped embryo	42	27 ± 1.5^{c}	19 ± 1.5^{c}	63.5 ± 3.7^{b}	44.4 ± 3.6^{b}
Heart-shaped embryo	44	8 ± 2.0^{b}	5 ± 1.2^{b}	18.2 ± 4.6^{a}	10.6 ± 2.6^{a}
Globular-shaped embryo	26	3 ± 1.2^{a}	2 ± 0.6^{a}	12.8 ± 4.4^{a}	6.4 ± 2.3^{a}

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

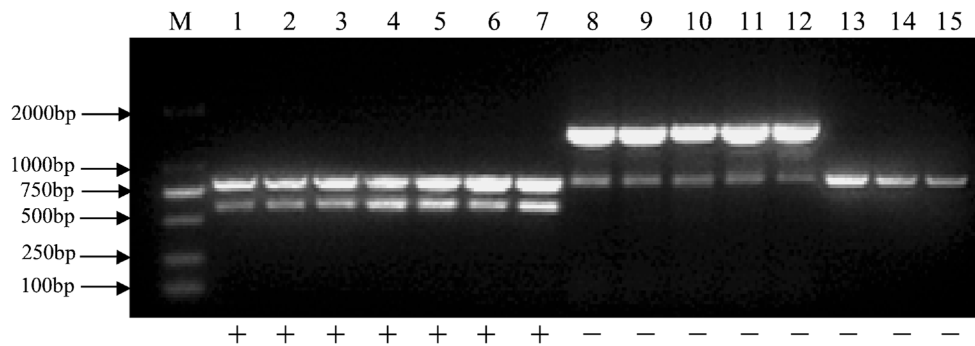

Figure 5. Amplification results of molecular probe Grape Seedless gene Probe 1 (GSLP1) linked to seedlessness gene in *Vitis vinifera* hybrid parents. *Lanes*: *M* Marker (Trans DNA 2K), *1* 'Thompson Seedless,' *2* 'Monukka,' *3* 'Kunxiang Seedless,' *4* 'Zixiang Seedless,' *5* 'Crimson Seedless,' *6* 'Heshi Seedless,' *7* 'Autumn Royal,' *8* 'Guifeimeigui,' *9* 'Shengdanmeigui,' *10* 'Fenhongmeigui,' *11* 'Red Globe' seeded control, *12* 'Beichun' seeded control, *13* 'DR2,' *14* 'DR3,' *15* 'DR6.' "+" indicates the *569-bp specific band* was *present*; "–" indicates the *569-bp specific band was absent.*

parents but not in other genotypes; hence, it was deemed suitable for identifying seedless descendants in this study. The GSLP1 marker was used to identify seedlessness in the 259 descendants of 10 combinations. Fourteen individuals with a 569-bp band were found, and these were preliminarily designated as seedless. These were 'DR2' × 'Thompson Seedless' (2–4, 2–9, and 2–81), 'DR3' × 'Thompson Seedless' (5–18), 'DR6' × 'Monukka' (6–22, 6–25, 6–47, 6–79, 6–90, 6–169, 6–215, and 6–227), and 'Kunxiang Seedless' × 'Guifeimeigui' (7–7 and 7–27) (Fig. 6).

Discussion

In recent years, a main objective of table grape breeding has been to obtain new seedless cultivars with higher fruit quality and good aroma. The DR cultivars used in this study had good clusters and fruit qualities but retained small seed traces. The parents of DR lines were 'Delight' and 'Ruby Seedless'. They are all *V. vinifera* and have relatively large seed traces, compared to many seedless cultivars. However, they are fairly easily rescued by *in vitro* embryo culture (Tian *et al.* 2008; Li *et al.* 2013; Ji *et al.* 2013). Li *et al.* (2015) used them for crosses with the seedless cultivars 'Thompson Seedless' and 'Monukka,' and the embryo development rate was found to be higher than that of seedless grapes. In the present study, 'DR2,' 'DR3,' and 'DR6' were again used as female parents, but for different cross-combinations, and again the embryo rescue efficiency remained high.

Tan *et al.* (2013) pointed out that the Muscat aroma phenotype in the hybrid F_1 showed a trend toward significant weakening. To create new grape cultivars with Muscat aroma, it is better to hybridize between two genotypes with Muscat aroma. Alternatively, at least one parent must have a strong Muscat aroma. In the present study, some new cultivars with strong Muscat fragrance were used as female or male parents for cross-combinations in an attempt to obtain new cultivars with a Muscat aroma. Identification of these descendants will be ongoing, especially for the identified seedless progenies 7–7 and 7–27 of 'Kunxiang Seedless' × 'Guifeimeigui'.

Putrescine is a common polyamine in plants and is important for a range of plant growth and development processes. Putrescine added to the culture medium significantly enhances the embryogenic capacity of leaf discs of *Solanum melongena* L. (Yadav and Rajam 1998). In *V. vinifera*, putrescine increases the micropropagation rate, the number of roots and

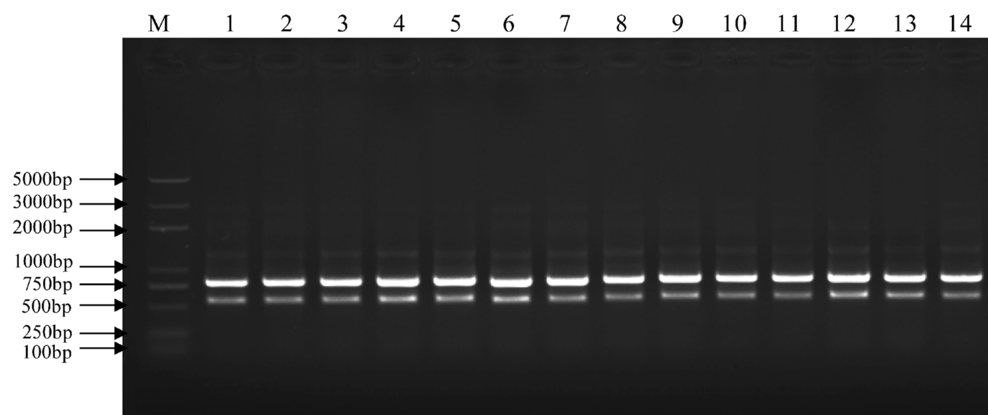

Figure 6. Amplification results of molecular probe Grape Seedless gene Probe 1 (GSLP1) linked to seedless gene in *Vitis vinifera* hybrid descendants. *Lanes*: *M* Marker (Trans DNA 2K Plus); *1* 2–4; *2* 2–9; *3* 2–81; *4* 5–18; *5* 6–22; *6* 6–25; *7* 6–47; *8* 6–79; *9* 6–90; *10* 6–169; *11* 6–215; *12* 6–227; *13* 7–7; *14* 7–27. Progeny were designated as 'seedless', based on the presence of *569 bp band.*

nodes, and the lengths of roots and stems, along with their fresh and dry weights (Martin-Tanguy and Carre 1993). Putrescine can either speed up or slow down plant development, depending on concentration. Tang *et al.* (2009) found that a putrescine spray at 20 mg L^{-1} promoted embryo development of 'Centennial Seedless' and 'Thompson Seedless'. Ponce *et al.* (2002) pointed out that a culture medium with 2 mM putrescine significantly increased embryo development rate and plantlet growth rate of the seedless variety 'Perlón'. In the present study, putrescine was added to the medium at various concentrations, and the optima for 'DR2,' 'DR3,' and 'DR6' were found to be slightly different. It was found that up to 5 mmol L^{-1} putrescine did not inhibit the development of embryos. Thus, it was speculated that even a 5-mmol L^{-1} putrescine concentration might not affect the normal growth of embryos, but that the optimal concentration for different cultivars varied.

The best timing for ovule excision for each cultivar is important to seedless grape breeding, when embryo rescue is used. This timing is best identified based on the development stage of the embryo. Raghavan (1966) identified two phases of embryo development: the heterotrophic phase and the autotrophic phase. In the first, the young embryo depends on the endosperm and the surrounding maternal tissues. During the second phase, embryo development can occur on a simple inorganic medium supplemented with a carbon source, such as sucrose. During embryo rescue, the survival rate of the *in vitro*-cultured ovules in their early stages of development is poor, whereas in the later stages, embryos tend to abort (Singh and Brar 1992). Therefore, embryogenesis and morphogenesis of the various developmental stages must be studied to find the optimal time for ovule excision. By observing and comparing the developmental rate and the developmental level of embryos at four stages, it was noted that the torpedo and cotyledon embryos clearly had higher embryo development rates and produced better quality plantlets. Therefore, ovule excision should be done during either of these two stages to maximize embryo rescue efficiency.

Because of a long juvenile phase, the descendants of embryo rescue-grapes take at least 3 y to yield their first fruit. In recent years, many researchers have reported that genetic markers can greatly assist in the breeding of seedless grapes. A number of markers have been reported for seedlessness, but markers for such quality traits remain scarce. Emanuelli *et al.* (2010) pointed out that there are four missense mutations in the *VvDXS* gene in grapes, and these are closely linked to the Muscat flavor. These researchers provide allele-specific markers for the accurate selection of 242 grapevine accessions (Emanuelli *et al.* 2013). Striem *et al.* (1996) analyzed 'Early Muscat' and its hybrid offspring by random amplification of polymorphic DNA (RAPD), finding 11 markers linked to the Muscat flavor. Unfortunately, each marker has its application limitations and can only be used in specific circumstances. When a marker associated with Muscat aroma (Emanuelli *et al.* 2013) was tried in this present study, it did not produce meaningful results (data not show).

Looking for seedless markers, Ji and Wang (2013) found 17 lines had a 569-bp specific band and they tentatively considered these lines as seedless. Li *et al.* (2015) used SCC8, SCF27, and GSLP1 to amplify hybrid parents and found that only GSLP1 had a specific band in the seedless parents but not in the seeded nor in the DR parents. Hence, GSLP1 was used to identify the seedless strains produced in that study. Liu *et al.* (2016) used both GLSP1 and SCF27 to identify the seedless offspring of five cross combinations. They found five strains had a 569-bp band by using the marker GLSP1 and 43 strains had a 2000-bp band by using the marker SCF27. In the present study, the same result was found, namely that GSLP1 had a 569-bp band only in seedless grapes, but not in seeded and DR grapes. Therefore, it was concluded that GSLP1 could be used to identify the parents and their offspring. Fourteen individuals were found having the same amplification results in common with seedless parents and were putatively defined as seedless individuals. However, further observation is required to ascertain whether they are actually seedless or not.

Conclusion

This study provides a preliminary report on some of the factors affecting embryo rescue. More work should be done to facilitate breeding of seedless grapes using embryo rescue. Further study of the physiological and biochemical parameters of these progenies is in progress. The next stage will be to carry out field studies along with further identification and screening of plants. Meanwhile, developing new molecular markers to assist in early selection of offspring with desired traits, such as seedlessness or Muscat flavor, is urgent.

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