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REVIEW

Embryo rescue technique and its applications for seedless breeding in grape

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Abstract Grape (*Vitis vinifera* L.) is one of the most important fruit crops in the world and is subject to intense breeding efforts to develop new seedless cultivars. To overcome inherent obstacles associated with crossing seedless selections, *in ovulo* embryo rescue was developed and successfully utilized by plant breeders to rescue inherently weak, immature and/or abortive embryos, in order to obtain progeny from seedless × seedless crosses. To date, embryo rescue has been utilized in grape breeding for more than three decades. Genotype, sampling/inoculation time and medium are the most crucial and well-studied factors affecting the success of grape embryo rescue. Besides, other factors, such as the culture methods and utilization of plant growth regulators are also important for grape embryo rescue. Thus far, embryo rescue was extensively applied in rescuing inherently weak grape embryos, breeding seedless grapes and triploid grapes, and distant hybridization between different *Vitis* species. Although grape embryo rescue has been widely investigated, the development of improved cultivars is few. Breeding novel grape cultivars through embryo rescue is still a challenging and long-term task, which requires persistent effort of grape breeders. This review provides updated and comprehensive information concerning factors and applications of embryo rescue in grape.

Keywords Grape embryo rescue · Genotype · Medium · Seedless grapes · Triploid grapes · Inter-specific hybridization

Abbreviations

ABA	Abscisic acid
BA	Benzyladenine
BAP	Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
DAF	Days after flowering
GA	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole butyric acid
NAA	Naphthalene acetic acid
NOA	Naphthoxyacetic acid
PGRs	Plant growth regulators

Introduction

The embryo rescue technique has been used by plant breeders probably since the work done in *Phaseolus* and *Fagopyrum* by Charles Bonnet in the eighteenth century (Schopfer 1943; Sharma et al. 1996), while the first successful systematic culturing of plant embryos under aseptic conditions was performed in the early nineteenth century by Hannig (1904) using two crucifers (Narayanaswami and Norstog 1964; Ramming 1990). Laibach (1925, 1929) subsequently emphasized the potential applications of

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embryo culture in rescuing embryos from interspecific hybrids. The culturing of cherry embryos in 1933 by Tukey (1933) represents a milestone in the embryo culturing of fruit crops. To date, embryo rescue has been widely used in many fruit crops, including apple (Dantas et al. 2006; Druart 2000), banana (Bakry 2008; Uma et al. 2011), citrus (Viloria et al. 2005; Xie et al. 2013, 2014), mango (Krishna and Singh 2007), muskmelon (Ezura et al. 1994; Nuñez-Palenius et al. 2006), peach (Anderson et al. 2002; Pinto et al. 1994), persimmon (Hu et al. 2013; Leng and Yamamura 2006; Yamada and Tao 2007), and watermelon (Taşkın et al. 2013), etc. for various purposes such as seedless breeding, triploid breeding and interspecific breeding. With regard to grape, since the first application of *in ovulo* embryo culture reported in 1982 (Emershad and Ramming 1982), embryo rescue technique has been extensively utilized in grape breeding.

In ovulo embryo rescue typically involves aseptically removing and culturing ovules, excising the embryos from the developing ovules, and continually culturing them in vitro until plantlet formation. However, in some cases it has not been technically possible to remove the embryos from the ovules and in order to rescue these embryos, whole ovules are usually cultured without embryo excision (Sharma et al. 1996). The success in rescuing weak and immature plant embryos largely depends on their stage of maturity and the composition of the growth medium (Sharma et al. 1996). Moreover, studies specifically using grape have shown that in this species the main factors influencing *in ovulo* embryo rescue are genotype (Cain et al. 1983; Gribaudo et al. 1993; Liu et al. 2003; Ponce et al. 2000; Ramming et al. 1990b; Singh et al. 2011; Tian and Wang 2008), the age of the ovule upon removal (Gray et al. 1990; Ji et al. 2013b; Liu et al. 2003; Notsuka et al. 2001; Pommer et al. 1995; Singh et al. 2011; Spiegel-Roy et al. 1985; Yang et al. 2007) and the nature of the culture medium (Amaral et al. 2001; Cain et al. 1983; Gray et al. 1990; Hiramatsu et al. 2003; Nookaraju et al. 2007; Sun et al. 2011; Tang et al. 2009; Yamashita et al. 1995, 1998), as well as other variables (Agüero et al. 1996; Bharathy et al. 2003, 2005; Bouquet and Davis 1989; Fernandez et al. 1991; Korpás and Hradilík 2009; Singh et al. 2011). These are summarized in Table 1.

One of the main reasons for using *in ovulo* embryo rescue is to regenerate inherently weak, immature or hybrid embryos that otherwise would not develop into viable mature plants (Bridgen 1994; Fathi and Jahani 2012; Sharma et al. 1996). While in grape, the most widely used application is in seedless grape breeding. Seedless table and raisin grapes are preferred by consumers worldwide and are developed mainly through two approaches: parthenocarpy and stenospermocarpy (Pratt 1971; Stout 1936). While large berried seedless grapes are usually generated from stenospermocarpy rather than parthenocarpy and many seedless grape cultivars are stenospermocarpic (Stout 1936). One difficulty of cultivating stenospermocarpic seedless grapes is that their embryos frequently abort during development, rendering conventional breeding approaches ineffective. However, this problem can be addressed through *in ovulo* embryo rescue, thus making the breeding of stenospermocarpic seedless grapes more efficient (Tsolova and Atanassov 1994; Bouquet and Davis 1989; Burger et al. 2003; Cain et al. 1983; Emershad and Ramming 1982; Gray et al. 1990; Gribaudo et al. 1993; Ji et al. 2013b; Liu et al. 2003, 2008; Notsuka et al. 2001; Perl et al. 2000; Pommer et al. 1995; Ramming 1990; Singh et al. 2011; Tang et al. 2009; Valdez 2005). Besides the use of the technique in breeding seedless cultivars, it has also been applied to the breeding of early ripening grapes (Ramming 1990; Ramming and Emershad 1984) and triploid grapes (Hiramatsu et al. 2003; Ji et al. 2013b; Okamoto et al. 1993; Park et al. 1999; Sun et al. 2011; Xu et al. 2005; Yamashita et al. 1993, 1995, 1998; Yang et al. 2007), as well as for hybridization between distantly related *Vitis* species (Goldy et al. 1988, 1989; Guo et al. 2010, 2011a; Ramming et al. 1991, 2000, 2009; Tian et al. 2008).

This review provides an overview of the development and applications of embryo rescue in grape and the factors that affect influence its efficiency.

Factors affecting the efficiency of grape embryo rescue

Several determinants have been proposed to influence the efficiency of embryo rescue in grape. These include the genotype of the grape cultivars used, the time point of removing ovules from grape berries, medium utilized for culturing ovules, embryos and plantlets, culture method and condition, application of plant growth regulators, etc. (Table 1).

Genotype

The effects of grape genotypic variation on the efficiency of embryo rescue have been extensively studied and linked to embryo development (Burger and Goussard 1996; Cain et al. 1983; Emershad et al. 1989; Goldy and Amborn 1987; Gribaudo et al. 1993; Ji et al. 2013a; Liu et al. 2003; Ponce et al. 2000; Ramming et al. 1990b; Razi et al. 2013; Singh et al. 2011; Tian and Wang 2008). Ovule and embryo development in seedless grapes is strictly controlled by the cultivar genotype. Parthenocarpic seedless grapes, such as Black Pearl and White Corinth, are able to fruit without pollination, fertilization and embryo formation. They are therefore not suitable as female parents used

Table 1 Factors affecting grape embryo rescue

Genotype	Sampling/inoculation time	Medium	Culture approach and condition	Other factors	Reference
Several genotypes and cross combinations	3–101 days after anthesis (DAA)	10 medium combinations			Cain et al. (1983)
		Cain's (1983) and SH (1977) media; indole butyric acid (IBA), Ergostim, tartaric acid, L-glutamine and L-cysteine			Emershad and Ramming (1984)
Several genotypes		Amino acids			Emershad et al. (1989)
Pollen parent, reciprocal cross	10–70 days after pollination (DAP)	NN (1969) and MS (1962) media; gibberellic acid (GA_3), indole-3-acetic acid (IAA), benzyladenine (BA), sucrose			Gray et al. (1990)
				Plant growth regulators (PGRs: chlormequat (CCC), uniconazole)	Ledbetter and Shonnard (1990)
29 Genotypes					Ramming (1990)
Pollen parent					Ramming et al. (1990a)
Reciprocal cross	52 and 66 DAA	NN medium with IAA, GA_3 and active charcoal (AC)			Tsolova (1990)
			Seedcoat manipulation		Fernandez et al. (1991)
	10–40 DAA	MS, White's (1954) and NN media; PGRs, amino acids			Singh et al. (1992)
	10–40 DAA				Gray and Hanger (1993)
14 Cultivars		NN medium; GA_3, IAA, naphthalene acetic acid (NAA), IBA, AC			Gribaudo et al. (1993)
Reciprocal cross		MS, NN media; GA_3, IAA, AC			Okamoto et al. (1993)
Reciprocal cross		MS medium with malt extract			Yamashita et al. (1993)
				PGRs	Bordelon and Moore (1994)
336 Cross combinations		ER (1984), WP (1980), MS media; phase (liquid, agar)			Emershad and Ramming (1994)
Upon the induction of polyembryony and secondary embryogenesis					Tsolova and Atanassov (1994)
				Growth retardants (CCC, paclobutrazol and XE 1019)	Agüero et al. (1995)
18 Genotypes differing in ripening season and in seed trace size	6–22 weeks after bloom (WAB)				Pommer et al. (1995)
		GA_3	Cold and wounding		Agüero et al. (1996)

Table 1 continued

Genotype	Sampling/inoculation time	Medium	Culture approach and condition	Other factors	Reference
4 Cultivars	4–10 WAB	NN and BD (1989) media; PGRs, AC		Ovule size	Burger and Goussard (1996)
Reciprocal cross			Ovule manipulation		Valdez and Ulanovsky (1997)
5 Cross combinations	Veraison	IAA and GA_3			Yamashita et al. (1998)
				Uniconazol and GA_3 treatments	Agüero et al. (2000)
			Ovule manipulation		Burger and Trautmann (2000)
32 Cross combinations					Garcia et al. (2000)
Several genotypes	Harvest date			Proceeding place	Ponce et al. (2000)
			Seed coat manipulation		Valdez et al. (2000)
	Growth stage of rescued embryos				Amaral et al. (2001)
Several genotypes and 20 cross combinations	Veraison				Notsuka et al. (2001)
	Ovule age (post pollination)				Midani et al. (2002)
				Putrescine	Ponce et al. (2002a)
		Putrescine			Ponce et al. (2002b)
5 cross combinations	30–55 DAP	B5 (1968), NN and MS media; GA_3, IAA, NAA, BA			Qi and Ding (2002)
				Pre-bloom sprays of BA	Bharathy et al. (2003)
	6–48 DAP; Veraison	PGRs			Hiramatsu et al. (2003)
Sunmuscat, Meibein Seedless and Marroo Seedless	30–70 days after flowering (DAF)	BD, NN, ER and WP media; GA_3, IAA and AC			Liu et al. (2003)
Reciprocal cross		PGRs, malt extract, casein hydrolysate (CH)			Wakana et al. (2003)
Male parent				Application of boric acid	Ebadi et al. (2004)
	35–45 DAA	NN, ER, MS media; BA, IBA and GA_3			Guo et al. (2004)
Pollen parent					Sahijram and Kanamadi (2004)
Pollen parent				BA	Bharathy et al. (2005)
			Extended period of seed trace culture		Valdez (2005)
12 cross combinations	4–11 weeks after pollination (WAP)	MS medium; IAA, GA_3, BA, NAA			Xu et al. (2005)
				Pre-bloom sprays of CPPU and BA	Nookaraju et al. (2007)

Table 1 continued

Genotype	Sampling/inoculation time	Medium	Culture approach and condition	Other factors	Reference
Several genotypes	Explant age	PGRs			Roichev et al. (2007)
Several genotypes	Explant age	PGRs			Yancheva and Roichev (2007)
9 Cross combinations; reciprocal cross	20–70 DAP	NN, B5, MS media; AC, CH			Yang et al. (2007)
Pollen parent		$CaCl_2$ and FeEDTA; CH, AC		Year of cross	Liu et al. (2008)
8 Cross combinations		Different amino acids			Tian and Wang (2008)
12 cross combinations		Liquid, solid and double-phase media; ER, NN and MS media; PGRs	Culture duration: 8–16 weeks		Tian et al. (2008)
				Pre-bloom antigibberellin treatments	Korpás and Hradilík (2009)
		PGRs: CCC, BA, ethephon and putrescine		Ovule size	Tang et al. (2009)
	Best inoculation time: 60–70 DAP	Medium states: solid, liquid, semi-solid and liquid over solid double layer media			Guo et al. (2011a)
5 Cross combinations	Best inoculation time: 55–80 DAP	MS, B5, ER, modified NN, White's media; phytohormones			Guo et al. (2011b)
4 Cross combinations	16–28 DAP	MS, B5, NN media; phytohormones	Chilling treatment; hardening strategy, Arbuscular mycorrhizal fungi		Singh et al. (2011)
10 Cross combinations; reciprocal cross	4–12 WAP	MS, NN media; PGRs; 3 medium combinations			Sun et al. (2011)
12 Cross combinations	From 28 June to 3 August	MM4 and ER media; GA_3, IAA, mashed banana			Ji and Wang (2013)
6 Cross combinations		Different embryo development and germination media with PGRs, $ZnSO_4$ or banana	Pre-chilling treatment	Polyembryony	Ji et al. (2013a)
12 Cross combinations	Best sampling times: 39–72 DAF	ER, MM4, WP, MS media; PGRs and banana			Ji et al. (2013b)
4 Cross combinations	31–65 DAP	WP medium with different PGRs: 7 types			Li et al. (2013)
3 Cross combinations		3 Different concentrations of IAA		Sprays with BA	Razi et al. (2013)
7 Cross combinations	25–75 DAP	Double-phase media with five different solid media; PGRs, amino acids			Li et al. (2014)

for breeding by embryo rescue. Conversely, stenospermocarpic grapes, in which pollination and fertilization occur and embryos form but abort, are widely used as female parents (Cain et al. 1983; Ramming et al. 1990b; Singh et al. 2011). Finally, the ability to form zygotic embryos has also been shown to vary greatly among grape cultivars, and this has a major impact on the embryo rescue success rates (Emershad et al. 1989; Gribaudo et al. 1993; Ji et al. 2013a; Liu et al. 2003; Razi et al. 2013).

Cain et al. (1983) noticed a clear influence of the genotype on the capacity to form viable embryos in culture in seedless variants. In this study, 13 seedless grape cultivars were self-pollinated and four crosses between one seedless and four seeded/seedless cultivars were made. Ovules were excised and placed on White's medium containing 5 % sucrose; however, only seven of the 13 seedless grape cultivars produced viable embryos from cultured self-pollinated ovules. The embryo formation capacity differed significantly among the seven seedless grapes, with the most successful cultivar producing viable embryos in nearly half of the ovules. With regard to the four hybridizations, crosses resulting from the different male parents produced a significantly different number of viable embryos when crossed to the same seedless cultivar. Specifically, more embryos were produced when a seeded cultivar or a seedless cultivar which has large abortive seeds was used as male parent, compared to the results obtained from two seedless cultivars which have very small abortive seeds (Cain et al. 1983). It is well known however, that both female and male parent genotypes can have great impact on the capacity for embryo formation, embryo germination and plant development in the context of grape embryo rescue (Ebadi et al. 2004; Garcia et al. 2000; Gray et al. 1990; Ji et al. 2013b; Li et al. 2013; Liu et al. 2008; Qi and Ding 2002; Spiegel-Roy et al. 1985, 1990; Tian et al. 2008). In one report it was found that, using identical conditions, the rates of embryo germination and seedling formation obtained from ovule culture were much higher when Flame Seedless was used as female parent than when Perlette or Sultanina were used as female parents (Spiegel-Roy et al. 1985). While the identity of the male parent can significantly influence both embryo and plant recovery at certain sampling times (Gray et al. 1990) as well as affect both ovule blackening and ovule germination (Ebadi et al. 2004), it does not influence callus formation of the ovule, ovule growth or ovule collapse (Ebadi et al. 2004). Furthermore, the effects of seeded male parents were reported to be different in crosses with different female parents (Ebadi et al. 2004). Recently, Ji et al. (2013b) obtained seven hybrids from 12 cross-combinations and the production of hybrid plants ranged from 23 % (Ruby Seedless × Black Olympia) to only 1 % (Pink Seedless × Beichun). The effect of genotype on the success rate of the crosses in this study might reflect differences in hybridization affinity and or genetic compatibility between different parental germplasm.

The degree of difficulty in using embryo rescue of different grape genotypes can also vary with ripening seasons or seed trace sizes. Compared with early and mid-season ripening genotypes, the use of those that are late maturing has been shown to result in fewer rescued embryos, germinated embryos and transplantable plants. In addition, genotypes with a larger ratio of seed trace weight to length tend to provide a larger number of ovules with embryos, more germinated embryos and more transplantable plants (Pommer et al. 1995).

Reciprocal crossing is widely utilized in grape breeding and has a great impact on the success rate of grape embryo rescue (Park et al. 1999; Tsolova 1990; Valdez and Ulanovsky 1997), especially in the recovery of triploid grapes resulting from crosses between diploids and tetraploids (Okamoto et al. 1993; Sun et al. 2011; Wakana et al. 2003; Yamashita et al. 1993; Yang et al. 2007). When making such crosses it has proven easier to obtain hybrid progeny using the diploid as a female parent (Okamoto et al. 1993; Sun et al. 2011; Wakana et al. 2003; Yang et al. 2007). In one study, "Muscat of Alexandria" (2X) and its 4X mutant were crossed reciprocally in order to breed new seedless grape cultivars, and the immature ovules and embryos were cultured in vitro (Okamoto et al. 1993). After culturing for 55 days, normally developed embryos were obtained from 50–70 to 20–30 % of the ovules from 2X × 4X and 4X × 2X, respectively. Finally, 10–20 % of embryos from the 2X × 4X crosses successfully grew to rooted plantlets which were confirmed to be triploid by measuring chromosome number, while embryos from 4X × 2X failed to root (Okamoto et al. 1993). Similarly, it was recently reported that while only a few seeds from 2X × 4X crosses exhibited a high rate of germination, those from 4X × 2X had entirely lost their ability to germinate (Sun et al. 2011). However, in the recovery of triploid grapes, Yamashita et al. (1993) thought it was better to use tetraploid grapes as the female parents than to use diploid female parents. In addition, one study has suggested that although it is easier to obtain plants using diploid grapes as the female parents, triploid progenies are only obtained when the tetraploid variants are used as the female parents (Yang et al. 2007).

The formation of somatic embryos within ovules can be continuously observed during the culturing process of the immature zygotic embryos in many cultivars (Emershad and Ramming 1994; Gray 1992; Ji et al. 2013a; Margosan et al. 1994; Ramming 1990; Yancheva and Roichev 2007). The formation and development of somatic embryos was also found to be influenced by genotype (Emershad and Ramming 1994; Ramming 1990; Roichev et al. 2007; Yancheva and Roichev 2007). In one study of 336 seedless × seedless crosses using 108 different female grape

genotypes, it was found that 12 % of the zygotic embryos formed somatic embryos and that the number of female parent genotypes that formed somatic embryos was approximately 89 % (Emershad and Ramming 1994). More recently, Yancheva and Roichev (2007) indicated that the frequency of somatic embryo formation was higher using hybrid genotypes than in the inbred treatments whereas more zygotic embryos were produced in the inbred treatments.

Sampling/inoculation time

The developmental time point at which ovules are isolated is another important factor in the success rate of grape embryo rescue. Although the early development stage of stenospermocarpic seedless grapes is similar to that of seeded cultivars, only a few of the zygotic embryos of stenospermocarpic seedless grapes are able to grow into mature embryos. Investigating the abortion process of embryos and determining the best inoculation time for culturing ovules is therefore highly advisable to increase the likelihood of success of the technique.

To date, many studies have investigated the importance of sampling/inoculation time (Burger and Goussard 1996; Emershad et al. 1989; Gray and Hanger 1993; Gray et al. 1990; Guo et al. 2004, 2011a, b; Ji et al. 2013b; Kebeli et al. 2003; Li et al. 2013; Liu et al. 2003; Midani et al. 2002; Notsuka et al. 2001; Pommer et al. 1995; Ponce et al. 2000; Qi and Ding 2002; Roichev et al. 2007; Singh and Brar 1992; Singh et al. 2011; Spiegel-Roy et al. 1985; Xu et al. 2005; Yancheva and Roichev 2007; Yang et al. 2007). Gray et al. (1990) cultured ovules from pollinated berries of the Orlando Seedless variety sampled at 10, 20, 40, and 60 days after pollination, and found that more embryos and plants were recovered from cultured ovules sampled at 40 or 60 days. It has also been reported that more embryos can be obtained from ovules cultured at 60 and 70 days after flowering (DAF), although the most vigorous growth in this study was observed from ovules cultured at 30 and 43 DAF. Here the ovules were cultured from berries of several self-pollinated seedless grape varieties and sampled at 30, 43, 60 and 70 DAF (Liu et al. 2003). More recently, Guo et al. (2011b) performed five crosses between diploid and tetraploid grapes and investigated the effect of inoculation time of ovules from the progeny on the success rate of embryo rescue. They found that the optimal inoculation time ranged from 55 to 80 days after pollination, resulting in an emergence rate from 39 to 73 %, and diploid, triploid and tetraploid plantlets accounted for 53.62, 43.48 and 2.90 % of rescued progenies, respectively.

Generally, the optimal inoculation time for this technique is when ovules are in the latest developmental stage while not aborting and so sampling ovules at a relatively late stage can boost the success rate, as confirmed by multiple reports (Burger and Goussard 1996; Burger and Trautmann 2000; Emershad et al. 1989; Gray and Hanger 1993; Gray et al. 1990; Guo et al. 2004, 2011a; Liu et al. 2003; Notsuka et al. 2001; Spiegel-Roy et al. 1985; Tsolova and Atanassov 1994). In one such study, Amaral et al. (2001) assessed the influence of the growth stage of rescued embryos on the number of plants obtained using crosses between seedless grapes. Four classes of embryo stages, including globular, heart, torpedo and undefined were identified. The globular stage had the lowest capacity for producing plants while the torpedo stage was the most efficient for recovering plants, supporting the view that the use of embryos in an advanced developmental stage can promote the production of mature plants.

Veraison, or the onset of berry ripening, represents an important developmental stage in grape and some researchers have used veraison as a reference point for the sampling time of ovules/embryos when performing embryo rescue. For instance, Yamashita et al. (1998) and Notsuka et al. (2001) recovered viable embryos from ovules sampled at veraison, while Hiramatsu et al. (2003) suggested that the best sampling time for efficient production of triploid grapes through embryo rescue was 1–2 weeks prior to veraison.

Numerous studies indicate that the genotype also influences the optimal ovule sampling time (Ji et al. 2013b; Liu et al. 2003; Midani et al. 2002; Xu et al. 2005; Yang et al. 2007). It has been demonstrated that the best sampling time for crosses between diploid and tetraploid grape varieties can be determined by the seasonal maturation time of the female parent varieties. For example, the best sampling periods were 6–9, 7–10 and 9–12 weeks after pollination for early-, mid- and late-maturing varieties, respectively (Xu et al. 2005). Recently, Ji et al. (2013b) demonstrated the value of assessing different sampling times for different grape crossing combinations in order to obtain the highest ovule germination rates, and that they varied from 39 to 63 DAF for 11 different crosses between grapes of different ploidy levels.

Culture medium

The culture medium is the source of nutrition for grape embryos developing in vitro as part of the embryo rescue process and, as such, represents a critical factor in determining the success rate.

Medium type

Typically, the embryo rescue technique consists of three phases: embryo development in the ovules, embryo

germination and plantlet formation. Since embryos vary in their physiological and developmental characteristics at different stages, different types of growth media, including different basal media and media with different phases (liquid, solid or both) are used for various phases of the embryo rescue.

Embryo development in the ovules is the first stage in embryo rescue, and represents the stage that largely determines whether the embryos will become viable and develop further. To date, many different types of basal culture media have been used by researchers for embryo development, including: White's medium (White 1954); MS medium (Murashige and Skoog 1962); B5 medium (Gamborg et al. 1968); NN medium (Nitsch and Nitsch 1969); Smith's medium (Smith et al. 1969); SH medium (Stewart and Hsu 1977); Cain's medium (Cain et al. 1983); ER medium (Emershad and Ramming 1984) and BD medium (Bouquet and Davis 1989). Of these, NN medium, MS medium and ER medium are the most widely used basal media. However, in the majority of cases, the same basal medium is used by researchers in the subsequent embryo germination and plantlet formation stages of the protocol (Agüero et al. 1995, 2000; Amaral et al. 2001; Bharathy et al. 2003, 2005; Gray et al. 1990; Hiramatsu et al. 2003; Liu et al. 2003, 2008; Nookaraju et al. 2007; Park et al. 1999; Pommer et al. 1995; Roichev et al. 2007; Sun et al. 2011; Tang et al. 2009; Tian et al. 2008; Yamashita et al. 1995, 1998; Yancheva and Roichev 2007), with some exceptions (Guo et al. 2004, 2011a; Ji et al. 2013b; Tian and Wang 2008). Compared with the initial culturing of the embryo development stage, fewer types of basal media are generally used in these two stages, and the most popular are MS medium and woody plant (WP) medium (Lloyd and McCown 1980). In addition, other basal media that have been used include B5 medium, NN medium and BD medium. The basal media used in different stages of grape embryo rescue are summarized in Table 2.

Studies comparing different media to determine the most suitable for embryo rescue have been performed. As one example, ovules from several seeded and seedless grape cultivars were cultured using five different media, including MS medium, 1/2 MS medium, Smith's medium, White's medium and Stewart and Hsu's medium (Cain et al. 1983). All ovules eventually produced prolific callus; however, ovules grown on MS and 1/2 MS media produced more callus and survived longer than those grown on the other media. Similarly, Singh et al. (1992, 2011) also found that the MS medium was more suitable for culturing ovules and for embryo germination than other media. In contrast, other studies have reported higher embryo recovery rates of several grape cultivars when ovules were excised and cultured in ether BD or NN media (Liu et al. 2003), or when ovules of the seedless grape Venus were cultured on ER basic medium supplemented with 6-benzyladenine (6-BA, 0.5 mg/L), indole butyric acid (IBA, 2 mg/L) and gibberellic acid (GA_3, 0.5 mg/L) compared to NN and MS medium (Guo et al. 2004).

Both solid media (Burger and Trautmann 2000; Cain et al. 1983; Guo et al. 2011b; Hiramatsu et al. 2003; Ji et al. 2013b; Liu et al. 2008; Park et al. 1999; Ramming et al. 1990a; Spiegel-Roy et al. 1985; Tian et al. 2008; Yang et al. 2007) and liquid media (Emershad and Ramming 1984, 1994; Gray et al. 1987; Okamoto et al. 1993; Roichev et al. 2007; Singh et al. 1992; Tang et al. 2009; Tian et al. 2008) have been extensively utilized for grape embryo rescue; however, solid media are more widely accepted and used. Semi-solid media and liquid over solid double layer media can also be used and double-phase ER medium has been shown to be superior to both liquid and solid ER media when crossing Emerald Seedless × Beichun in terms of embryo formation, germination and plantlet formation rates (Tian et al. 2008). More recently, a higher germination percentage of embryos rescued in liquid over solid double layer medium was reported, compared with solid, liquid or semi-solid media (Guo et al. 2011a).

Medium composition

Media used for embryo rescue contain essential components, such as mineral salts, sugars and other growth promoting substances, the ratios of which affect the success rate of rescue to different degrees. Most basal media contain sufficient mineral salts for embryo development, germination and plantlet formation and so there are few reports describing the effects of mineral salt levels. However, Liu et al. (2008) reported that increasing the concentration of $CaCl_2$ in the culture medium promoted embryo recovery.

Apart from offering the necessary energy as a carbon source for the development of young embryos, sugar also serves as an osmotic stabilizer in culture media (Sharma et al. 1996). Sucrose is the most commonly used sugar for grape embryo cultures, with a concentration ranging from 10 to 60 g/L, and the most widely being 20–30 g/L. A high osmotic potential of the medium prevents precocious germination of young embryos and supports normal embryonic growth, and a higher concentration of sucrose is therefore often utilized for culturing immature plant embryos (Sharma et al. 1996). A relatively high concentration of sucrose (60 g/L) is often used to culture the initial stage of immature grape embryos (Bharathy et al. 2003, 2005; Emershad and Ramming 1994; Guo et al. 2004, 2011b; Ji et al. 2013b; Nookaraju et al. 2007; Pommer et al. 1995; Tang et al. 2009; Tian et al. 2008), but as the young embryos develop the osmotic potential of the

Table 2 Different basal media that have been used in different stages of grape embryo rescue

Basal medium	Embryo development (references)	Embryo germination and plantlet formation (references)
White's medium (1954)	Cain et al. (1983), Guo et al. (2011b), Ramming and Emershad (1982), Ramming et al. (1990a) and Singh et al. (1992)	
Murashige and Skoog medium (1962)	Amaral et al. (2001), Cain et al. (1983), Celik and Ilbay (2003), Fernandez et al. (1991), Guo et al. (2004, 2011b), Okamoto et al. (1993), Roichev et al. (2007), Singh et al. (1992, 2011), Sun et al. (2011), Wakana et al. (2003), Xu et al. (2005), Yamashita et al. (1998) and Yang et al. (2007)	Agüero et al. (1995, 2000), Cain et al. (1983), Gray et al. (1990), Hiramatsu et al. (2003), Park et al. (1999), Roichev et al. (2007), Sun et al. (2011), Tang et al. (2009), Yamashita et al. (1995, 1998) and (Yancheva and Roichev 2007)
B5 medium (1968)	Guo et al. (2011b), Qi and Ding (2002), Singh et al. (2011) and Yang et al. (2007)	Guo et al. (2011a)
Nitsch and Nitsch medium (1969)	Agüero et al. (1995, 2000), Burger and Goussard (1996), Ebadi et al. (2004), Gray (1992), Gray et al. (1990), Gribaudo et al. (1993), Guo et al. (2004), Hiramatsu et al. (2003), Liu et al. (2003), Park et al. (1999), Ponce et al. (2002b), Qi and Ding (2002), Singh et al. (1992, 2011), Sun et al. (2011), Tsolova and Atanassov (1994), Tsolova (1990), Valdez (2005), Valdez and Ulanovsky (1997) and Yang et al. (2007)	Burger and Goussard (1996) and Tsolova and Atanassov (1994)
Smith's medium (1969)	Cain et al. (1983)	
Stewart and Hsu's medium (1977)	Cain et al. (1983) and Emershad and Ramming (1984)	
Woody plant medium (1980)		Amaral et al. (2001), Bharathy et al. (2003, 2005), Kebeli et al. (2003), Liu et al. (2003), Nookaraju et al. (2007), Pommer et al. (1995) and Tian et al. (2008)
Cain's medium (1983)	Emershad and Ramming (1984), Emershad et al. (1989) and Pommer et al. (1995)	
Emershad and Ramming medium (1984)	Amaral et al. (2001), Bharathy et al. (2003), Emershad and Ramming (1994), Guo et al. (2004, 2011b), Ji et al. (2013b), Kebeli et al. (2003), Liu et al. (2003), Nookaraju et al. (2007), Tang et al. (2009) and Tian et al. (2008)	
Bouquet and Davis medium (1989)	Burger and Goussard (1996), Korpás and Hradilík (2009) and Liu et al. (2003, 2008)	Burger and Goussard (1996) and Liu et al. (2008)

medium is less important (Amemiya 1964; Niederwieser et al. 1990; Pecket and Selim 1965; Sharma et al. 1996) and the concentration of sucrose used for subsequent embryo germination and plantlet formation can be reduced (Bharathy et al. 2003, 2005; Emershad and Ramming 1994; Guo et al. 2004, 2011b; Nookaraju et al. 2007; Pommer et al. 1995; Tang et al. 2009; Tian et al. 2008; Valdez 2005; Valdez and Ulanovsky, 1997; Wakana et al. 2003; Xu et al. 2005).

The effects of specific amino acids, such as L-asparagine, L-glutamine, L-serine, L-cysteine, in the culture media on the efficiency of embryo rescue have also been investigated (Emershad and Ramming 1984; Emershad et al. 1989; Park et al. 1999; Singh et al. 1992; Tian and Wang 2008) and in most cases their addition promoted ovule growth. For example, Emershad et al. (1989) reported that P60-58 and Thompson Seedless ovules cultured on Cain's basal medium supplemented with L-serine, L-glutamine, L-cysteine or L-asparagine, increased the number of enlarged embryos. A higher plantlet formation rate has also been reported to result from adding asparagine, glycine, arginine and glutamine (2.0 mmol/L) to the basal medium, with the most prominent effect resulting from supplementation with asparagine, which resulted in 55 % of the ovules giving rise to plantlets (Tian and Wang 2008).

Several types of natural adjuvants derived from endosperm tissues, such as coconut water, casein hydrolysate and malt extract, have been used as additives to plant embryo culture media (Sharma et al. 1996). In the case of grape, the most widely used are casein hydrolysate (Liu

Table 3 Plant hormones that have been widely used in grape embryo culture media

Phytohormone	References
Gibberellic acid (GA_3)	Agüero et al. (1996), Burger and Goussard (1996), Ebadi et al. (2004), Gribaubo et al. (1993), Guo et al. (2004), Liu et al. (2003), Koh and Oh (2013), Okamoto et al. (1993), Qi and Ding (2002), Rendón et al. (2013), Singh et al. (2011), Sun et al. (2011), Tsolova (1990), Wakana et al. (2003), Yamashita et al. (1998) and Yang et al. (2007)
Indole-3-acetic acid (IAA)	Burger and Goussard (1996), Ebadi et al. (2004), Gribaudo et al. (1993), Guo et al. (2004), Liu et al. (2003), Okamoto et al. (1993), Qi and Ding (2002), Rendón et al. (2013), Singh et al. (2011), Sun et al. (2011), Tsolova (1990), Wakana et al. (2003), Yamashita et al. (1998) and Yang et al. (2007)
Indole butyric acid (IBA)	Gribaubo et al. (1993), Ji et al. (2013b) and Singh et al. (2011)
Naphthalene acetic acid (NAA)	Gray (1992), Gribaubo et al. (1993), Singh et al. (2011), Spiegel-Roy et al. (1990), Sun et al. (2011) and Wakana et al. (2003)
Benzyladenine (BA)	Cain et al. (1983), Emershad and Ramming (1994), Gray (1992), Gray et al. (1990), Guo et al. (2004), Liu et al. (2003), Park et al. (1999), Qi and Ding (2002), Sun et al. (2011), Wakana et al. (2003) and Yamashita et al. (1995)
Abscisic acid (ABA)	Agüero et al. (1996)

et al. 2008; Park et al. 1999; Wakana et al. 2003; Yamashita et al. 1995; Yang et al. 2007) and malt extract (Horiuchi et al. 1991; Wakana et al. 2003; Yamashita et al. 1993). Various effects have been obtained by the addition of these natural adjuvants, ranging from no obvious effect resulting from adding malt extract (25–1,600 mg/L) or casein hydrolysate (20–1,500 mg/L) to MS medium (Wakana et al. 2003), to a report that supplementing with casein hydrolysate improved embryo recovery, emergence and germination (Liu et al. 2008). In addition, Ji et al. (2013b) determined that the addition of 500 mg/L mashed banana to MM4 medium generated the highest rates of embryo formation (13 %) and plant development (90 %) when ER or MM4 medium with different supplements including indole-3-acetic acid (IAA), GA_3 and mashed banana were used.

Phytohormones, including GA_3, auxins such as IAA, IBA and naphthalene acetic acid (NAA), cytokinin hormone benzyladenine (BA) and abscisic acid (ABA), have also been widely tested in grape embryo culture media (Table 3). The addition of IAA (2.0 mg/L) and GA (0.4 mg/L) to 1/2 MS medium can enhance the formation and growth of triploid grape embryos (Yamashita et al. 1998), while in vitro embryo survival and plantlet formation is largely improved in BA-supplemented WP medium containing 3 g/L activated charcoal (Liu et al. 2003). In a recent study, it was found that 4 mg/L IAA, in addition to 0.5 mg/L GA_3, provides the most effective combination of phytohormone supplements for embryo germination, while 1.0 mg/L IBA and 1.5 mg/L NAA is superior for promoting shooting and rooting (Singh et al. 2011). Although the addition of phytohormones can be beneficial for grape embryo rescue and development, they are not strictly necessary. For example, triploid seedlings can be obtained from immature, mature and abnormal embryos that have been aseptically excised from ovules and cultured on MS medium without phytohormones (Yamashita et al. 1995), and there are reports that some phytohormones, such as GA_3, IAA and BA, have no obvious effect on grape embryo recovery (Burger and Goussard 1996; Wakana et al. 2003). Indeed, a recent study indicated that embryo formation, germination and plantlet formation rates can actually be reduced by adding 0.5 mg/L GA_3 and 1.5 mg/L IAA to ER or MM4 medium (Ji et al. 2013b).

The effects of several other plant growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, benzylaminopurine (BAP), naphthoxyacetic acid (NOA) and putrescine, on embryo rescue have also been tested. When culturing ovules of the seedless grape cultivar Perlette on three basal media (MS medium, White's medium and NN medium) supplemented with various combinations and concentrations of 2,4-D, NAA, IAA, kinetin, BAP and gibberellin A_4/A_7, the maximum survival rate was found to result from using MS medium fortified with 2 mg/L IAA and 0.5 mg/L BAP (Singh et al. 1992). In addition, two reports have stated that including putrescine in the medium of immature grape seeds increased the percentages of embryo formation, germination, polyembryos and normal plantlets (Ponce et al. 2002b), as well as promoted the development and germination of grape ovules (Guo et al. 2009).

Due to the high concentration of phenolic compounds in grape ovules, which can oxidize or brown during culturing, the addition of antioxidants or adsorbents, such as activated charcoal, to the medium can help remove inhibitory substances, thereby aiding embryo recovery from excision injury and stimulating in vitro growth. To this end, activated charcoal, at a concentration of 1–3 g/L has been extensively and effectively used for grape culturing (Burger and Goussard 1996; Cain et al. 1983; Emershad and Ramming 1994; Gribaubo et al. 1993; Korpás and Hradilík 2009; Liu et al. 2003; Okamoto et al. 1993; Tian et al. 2008; Tsolova 1990; Yang et al. 2007).

Culture approaches and conditions

While grape embryos used for subsequent rescue are most often initially excised from ovules and then cultured, in order to germinate, embryos remaining in the ovule are also able to germinate in vitro and grow into plantlets, although with a lower success rate. The effect of ovule manipulation has been well characterized (Burger and Trautmann 2000; Fernandez et al. 1991; Valdez 2005; Valdez and Ulanovsky 1997) and in one study three approaches were compared (Fernandez et al. 1991): intact ovules cultured with subsequent embryo excision; ovules with approximately one quarter of the seed coat and adjacent endosperm removed; and intact ovules. The highest number of both germinated embryos and plantlets formed were obtained using the embryo excision approach, whereas the lowest number was obtained when embryos grew from intact ovules. Embryo excision therefore seems to be optimal for the embryo culture approach, a result that has subsequently been verified by others (Valdez 2005). However, it should be noted that there may be a difference in how well an embryo performs in culture depending on whether it was derived from a variety with a soft or a hard seed coat (Valdez and Ulanovsky 1997). It was reported that for one variety with a very soft seed coat (Superior), the germination rate for seed coat ruptured embryos was slightly higher than for directly germinated or excised embryos. In contrast, for a variety with a harder seed coat (Ruby), the germination rates for excised and seed coat ruptured embryos were much higher than those that were directly germinated (Valdez and Ulanovsky 1997). Finally, Burger and Trautmann (2000) reported that an effective embryo culture approach is to remove part of the ovule before culture and then use ovule portions with the cut surface in contact with the solid medium.

There is also evidence that increased success of embryo germination and plantlet formation can result from culturing excised embryos or wounded/intact ovules at low temperatures (Agüero et al. 1996). Singh et al. (2011) demonstrated that a chilling treatment is crucial for embryo maturation, and 60 days of chilling at 4 °C promoted embryo germination, while pre-chilling has also been shown to reduce the number of abnormal plantlets and enhance embryo germination (Ji et al. 2013a).

The importance of the duration of embryo culture was investigated by culturing seed traces of several crosses among seedless grape varieties for 90, 120, 150, 180, 210 or 240 days, without changing the medium. No decline was observed in the number of rescued embryos for any cross within, and up to, 210 days of culture (Valdez 2005), supporting the finding that embryo formation and germination did not changed in the ovules cultured for 8–16 weeks (Tian et al. 2008). In this later study however, a significantly lower rate of plant development was found in the ovules cultured for 16 weeks and extended culture duration may therefore reduce the regeneration ability of zygotic embryos (Tian et al. 2008).

Finally, different hardening strategies for plantlets rescued from grape embryo culture have been investigated, the most effective of which was found to be placing them in a glass jar with a polypropylene cap. In this study it was also found that an effective approach for promoting the field survival of the rescued plantlets was by using a bio-hardening strategy, which is inoculating the roots of rooted grape plantlets with different symbiotic Arbuscular mycorrhizal fungi strains (Singh et al. 2011).

Other factors

Plant growth regulators (PGRs) are not only important for embryo rescue when added to the embryo culture medium, but also exhibit an effect when sprayed onto the plant at the pre-blooming and blooming stages (Agüero et al. 1995; Bharathy et al. 2003; Korpás and Hradilík 2009; Ledbetter and Shonnard 1990; Nookaraju et al. 2007; Razi et al. 2013). The PGRs that have been used for such spraying treatments are mainly anti-gibberellins (Agüero et al. 1995, 2000; Bordelon and Moore 1994; Ledbetter and Shonnard 1990; Korpás and Hradilík 2009; Tang et al. 2009), cytokinins (Bharathy et al. 2003, 2005; Nookaraju et al. 2007; Tang et al. 2009) and putrescine (Ponce et al. 2002a; Tang et al. 2009). Generally, the application of PGRs before or during flowering has proven beneficial for grape embryo rescue, with anti-gibberellins, such as chlormequat and uniconazole, applied before flowering promoting embryo germination (Ledbetter and Shonnard 1990). In a more comprehensive study, the effect on seed trace development and germination of four stenospermic grape cultivars was investigated following the application of several types of PGRs, including anti-gibberellins (mepiquat chloride, uniconazole, ancymidol, daminozide, chlormequat, ethephon, methazole), cytokinins (BAP, kinetin, BTP, 2iP) and ABA. Certain PGRs were able to stimulate seed trace formation in some stenospermic grape cultivars, and the addition of this group of compounds might prove a useful tool in grape breeding programs (Bordelon and Moore 1994). A positive effect on grape embryo recovery, germination and plantlet formation has also been shown by spraying with BA at the pre-bloom and bloom stages; however, this can depend on the type of BA treatment and the genotype of the grape (Bharathy et al. 2003, 2005; Nookaraju et al. 2007). Finally, spraying with putrescine has also been reported to have a beneficial effect on the development of grape ovules, embryos and plantlets (Ponce et al. 2002a; Tang et al. 2009).

Whether or not the ovule size at the time of rescue has an effect on grape embryo rescue is disputed. Some investigators have reported that there is no correlation between the percentage of ovules with embryos and the ovule size (Burger and Goussard 1996; Rarmming 1990), while Bouquet and Davis (1989) indicated that a correlation exists between viable embryos and ovule size within a given cultivar. It has also been observed that the percentage of embryo formation is significantly higher for larger ovules and that there is a significant relationship between embryo development and the proportion of ovules that are longer than 2 mm (Tang et al. 2009). This apparent discrepancy may be explained if the influence of ovule size on embryo culture success is cultivar-specific.

Finally, environmental cues and pruning intensity may also be of some importance, as identical crosses from two different years showed variation in the rescue efficiency (Liu et al. 2008) and the number of plantlets obtained from the cultivar Fantasy Seedless increased with decreasing pruning intensity (Ponce et al. 2009).

Applications for the embryo rescue technique in grape breeding

The major applications of embryo rescue for grape breeders involve rescuing inherently weak grape embryos, breeding seedless grapes and triploid grapes, and hybridization between distantly related variants, references for which are summarized in Table 4.

Rescuing inherently weak grape embryos

The most common application for embryo rescue in grape breeding is rescuing inherently weak embryos. For certain grape cultivars, such as stenospermocarpic grapes, early-ripening grapes and muscadine grapes, seeds exhibit very poor germination efficiency, due to nutritional and/or physiological deficiencies. However, if these embryos are excised from seeds/ovules and cultured, normal grape plants can be obtained through *in ovulo* embryo rescue.

There are many reports describing the application of embryo rescue for stenospermocarpic grapes (Agüero et al. 1995; Burger and Goussard 1996; Emershad and Ramming 1984; Guo et al. 2004; Ledbetter and Shonnard 1990; Ponce et al. 2002a; Ramming et al. 1991; Singh and Brar 1992; Tsolova and Atanassov 1994), since they normally abort during development, which is a major problem when breeding using conventional methods. However, through embryo rescue, the abortive embryos can be cultured to viable embryos and subsequently normal grape plantlets can easily be obtained (Brar et al. 1991; Cain et al. 1983; Emershad et al. 1989; Gribaudo et al. 1993; Liu et al. 2003, 2008; Nookaraju et al. 2007; Pommer et al. 1995; Singh et al. 1992). This has been proven true for several genotypes, including Thompson Seedless and P60-58 (Emershad et al. 1989), and Thompson Seedless, Crimson Seedless, 2A-Clone, Maroo Seedless, Kishmis Chernyi and Mint (Nookaraju et al. 2007).

It has been observed that greater numbers of early ripening grape hybrids can be obtained using early ripening genotypes as the female parents (Ramming 1990). However, these genotypes have a short growing period, the embryos do not develop well and normal seed germination is extremely low, all of which are disadvantageous for conventional breeding, but can be circumvented using rescue techniques. In an initial study, Ramming and Emershad (1984) rescued embryos from early ripening grape varieties and the same group was subsequently able to largely improve both the germination percentage (from 0–16 to 19–24 %) and plant production (up to 32 %) (Ramming 1990; Ramming et al. 1990a).

The embryo rescue technique has also been used to rescue immature zygotic embryos of muscadine grapes (Gray 1992; Gray and Hanger 1993), in a study where zygotic embryos from 11 muscadine grapes were recovered and representative normal plants established in greenhouse pots for all cultivars except one (Gray and Hanger 1993).

Breeding of seedless grape cultivars

The most economically and commercially important application of embryo rescue in grape is the breeding of seedless cultivars, which has long been an important goal for grape breeders. In order to obtain a hybrid seedless grape cultivar with specific characteristics using conventional breeding approaches, a seeded cultivar generally needs to be used as the female parent for the first generation crossing, before intermating or backcrossing of the second generation is performed. Compared with conventional breeding methods, grape embryo rescue is more efficient and economical. Specifically, one generation time, which is approximately 5 years, as well as the land, labor, fertilizer and water required for raising one generation, are saved (Ramming 1990; Sharma et al. 1996). In addition, the genotypes can also be hybridized directly, thereby avoiding a dilution by genes from a seeded female, and so the proportion of seedless progeny is also much higher (Ramming 1990).

In general, there are two different types of cross combinations that are widely used for *in ovulo* embryo rescue and that have given satisfactory results: seedless × seeded (Bharathy et al. 2003; Cain et al. 1983; Carreno et al. 2009; Ebadi et al. 2004; Ji and Wang 2013; Liu et al. 2008;

Table 4 Applications of grape embryo rescue

Rescuing inherently weak embryos	Breeding seedless grapes	Breeding triploid grapes	Distant hybridization	Reference
Stenospermocarpic grapes	Seedless × seeded; seedless × seedless			Cain et al. (1983)
Stenospermocarpic grapes				Emershad and Ramming (1984)
	Seedless × seedless			Bouquet and Davis (1989)
Stenospermocarpic grapes				Emershad et al. (1989)
Stenospermocarpic grapes	Seedless × seedless			Gray et al. (1990)
Stenospermocarpic grapes, early ripening grapes				Ramming (1990)
Early ripening grapes				Ramming et al. (1990a)
	Seedless × seedless			Ramming et al. (1990b)
Stenospermocarpic grapes	Seedless × seedless			Spiegel-Roy et al. (1990)
	Seedless × seedless			Tsolova (1990)
Stenospermocarpic grapes				Brar et al. (1991)
	Seedless × seedless			Fernandez et al. (1991)
Stenospermocarpic grapes	Seedless × seedless		*Vitis vinifera* × *V. labrusca*	Ramming et al. (1991)
Muscadine grapes				Gray (1992)
Stenospermocarpic grapes				Singh and Brar (1992)
Stenospermocarpic grapes				Singh et al. (1992)
Muscadine grapes				Gray and Hanger (1993)
Stenospermocarpic grapes				Gribaudo et al. (1993)
		2X × 4X; 4X × 2X		Okamoto et al. (1993)
		2X × 4X; 4X × 2X		Yamashita et al. (1993)
Stenospermocarpic grapes				Bordelon and Moore (1994)
	Seedless × seedless			Emershad and Ramming (1994)
Stenospermocarpic grapes				Tsolova and Atanassov (1994)
Stenospermocarpic grapes				Agüero et al. (1995)
Stenospermocarpic grapes				Pommer et al. (1995)
		4X × 2X		Yamashita et al. (1995)
Stenospermocarpic grapes				Burger and Goussard (1996)
	Seedless × seedless			Valdez and Ulanovsky (1997)
		2X × 4X; 4X × 2X		Yamashita et al. (1998)
Stenospermocarpic grapes				Agüero et al. (2000)
	Seedless × seedless			Garcia et al. (2000)
			V. vinifera × *V. rotundifolia*	Ramming et al. (2000)
	Seedless × seedless			Amaral et al. (2001)
Stenospermocarpic grapes	Seedless × seedless			Notsuka et al. (2001)
	Seedless × seeded; seedless × seedless			Midani et al. (2002)
Stenospermocarpic grapes				Ponce et al. (2002a)
	Seedless × seedless			Qi and Ding (2002)

Table 4 continued

Rescuing inherently weak embryos	Breeding seedless grapes	Breeding triploid grapes	Distant hybridization	Reference
	Seedless × seeded		*V. vinifera* × *V. labrusca*, *V. tilifolia*, *V. candicans*, *V. rupestris*	Bharathy et al. (2003)
		4X × 2X		Hiramatsu et al. (2003)
	Seedless × seedless			Kebeli et al. (2003)
Stenospermocarpic grapes				Liu et al. (2003)
		2X × 4X		Motosugi and Naruo (2003)
		2X × 4X; 4X × 2X	*V. vinifera* × *V.* complex	Wakana et al. (2003)
	Seedless × seeded; seedless × seedless			Ebadi et al. (2004)
Stenospermocarpic grapes				Guo et al. (2004)
	Seedless × seeded; seedless × seedless			Sahijram and Kanamadi (2004)
	Seedless × seeded		*V. vinifera* × *V. labrusca*; *V. vinifera* × *V. tilifolia*	Bharathy et al. (2005)
	Seedless × seedless			Valdez (2005)
		2X × 4X; 4X × 2X		Xu et al. (2005)
Stenospermocarpic grapes				Nookaraju et al. (2007)
Stenospermocarpic grapes	Seedless × seeded; seedless × seedless			Roichev et al. (2007)
Stenospermocarpic grapes	Seedless × seeded; seedless × seedless			Yancheva and Roichev (2007)
		2X × 4X; 4X × 2X		Yang et al. (2007)
Stenospermocarpic grapes	Seedless × seeded; seedless × seedless			Liu et al. (2008)
			V. vinifera × wild Chinese *Vitis*	Tian and Wang (2008)
			V. vinifera × wild Chinese *Vitis*	Tian et al. (2008)
			V. vinifera × F8909-08 (*V. rupestris* × *V. arizonica*/*candicans*)	Ramming et al. (2009)
		4X × 2X	Tetraploid grapes × *V. amurensis*	Guo et al. (2011a)
		2X × 4X; 4X × 2X		Guo et al. (2011b)
	Seedless × seedless			Singh et al. (2011)
		2X × 4X; 4X × 2X		Sun et al. (2011)
	Seedless × seeded		*V. vinifera* × wild Chinese *Vitis*	Ji and Wang (2013)
Stenospermocarpic grapes	Seedless × seeded		*V. vinifera* × wild Chinese *Vitis*	Ji et al. (2013a)
	Seedless × seedless	2X × 4X	*V. vinifera* × wild Chinese *Vitis*	Ji et al. (2013b)
			V. vinifera × wild Chinese *Vitis*	Li et al. (2013)
	Seedless × seedless			Razi et al. (2013)
	Seedless × seedless			Uquillas et al. (2013)
	Seedless × seeded; seedless × seedless		*V. vinifera* × wild Chinese *Vitis*	Li et al. (2014)

Midani et al. 2002; Roichev et al. 2007; Sahijram and Kanamadi 2004); and seedless × seedless (Akkurt et al. 2012, 2013; Amaral et al. 2001; Barlass et al. 1988; Bergamini et al. 2013; Bouquet and Davis 1989; Cain et al. 1983; Correa et al. 2014; Ebadi et al. 2004; El-Agamy et al. 2009; Fernandez et al. 1991; Ji et al. 2013b; Liu et al. 2008; Midani et al. 2002; Mullins 1990; Ramming et al. 1990b; Roichev et al. 2007; Sahijram and Kanamadi 2004; Singh et al. 2011; Uquillas et al. 2013; Valdez 2005; Valdez and Ulanovsky 1997).

The application of embryo culture for breeding seedless grapes was first reported by Emershad and Ramming (1982). Since then, the ability to generate seedless grapes by embryo culture has spread worldwide (Bouquet and Danglot 1996; Burger and Goussard 1996; El-Agamy et al. 2009; Emershad and Ramming 1984; Ji et al. 2013b; Ledbetter and Ramming 1989; Mejia and Hinrichsen 2003; Perl et al. 2003; Spiegel-Roy et al. 1990; Uquillas et al. 2013; Valdez et al. 2000). Cain et al. (1983) obtained normal embryos and seedling plants from normally abortive ovules of seedless grapes by embryo rescue and elaborately elucidated the promising application of embryo rescue in breeding of seedless grapes. In another study, *in ovulo* embryo culture of the grape cultivar Thompson Seedless was investigated as an approach to enable the hybridization of stenospermic seedless grapes, although of the thirty embryos recovered, only one grew into a plantlet (Emershad and Ramming 1984). In another report, Bouquet and Davis (1989) obtained more than 1,200 plantlets using 15,610 ovules from 21 different crosses by *in ovulo* and in vitro embryo rescue. Burger and Goussard (1996) in vitro cultured ovules and embryos from stenospermocarpic seedless grapes, and different factors, including plant growth regulators, developmental stage, ovule size and different culture conditions that might affect the success of in vitro culture of seedless grape embryos were investigated. In order to breed seedless grapes for the Australian table and dried grape industries, Liu et al. (2003, 2008) used the *in ovulo* embryo rescue technique to recover new hybrids from stenospermocarpic grapes, and investigated the effects of several factors, such as genotype, medium and ovule removal age, on ovule elongation, embryo recovery, growth and plantlet formation. Finally, in a recent study, Ji et al. (2013b) used embryo rescue to breed new seedless grapes with crosses between *V. vinifera* cultivars and wild Chinese *Vitis* spp, in order to combine desirable traits from different *Vitis* species.

Breeding of triploid grapes

Another major application for embryo rescue is the breeding of triploid seedless grapes, which are generally preferred as table grapes due to qualities such as seedlessness and large berries. Triploid grapes are typically bred by hybridizing tetraploid and diploid grapes and their seedlessness results from their unbalanced chromosome sets. However, traditional breeding of triploid grapes is difficult due to the low success rate of crossing tetraploid and diploid grapes: seeds tend to abort due to endosperm degeneration during early embryogenesis (Esen and Soost 1973; Sanford 1983), causing conventional seed sowing to be inefficient. However, if the embryos of the seeds are allowed to develop through embryo culture, triploid grapes may be more efficiently obtained (Yamashita et al. 1993); therefore embryo rescue is a potentially effective strategy for breeding triploid grapes.

To date, breeding of triploid seedless grapes has been performed by a number of groups (Guo et al. 2011a, b; Hiramatsu et al. 2003; Ji et al. 2013b; Motosugi and Naruo 2003; Okamoto et al. 1993; Park et al. 1999; Sun et al. 2011; Wakana et al. 2003; Xu et al. 2005; Yamashita et al. 1993, 1995, 1998; Yang et al. 2007). The application of embryo rescue was first reported in 1993 and subsequently triploid grape seedlings have been obtained from reciprocal crosses between diploid and tetraploid grapes by Okamoto et al. (1993) and Yamashita et al. (1993) through use of the embryo rescue. Park et al. (1999) reported that five aneuploid plants, with chromosome numbers ranging from 51 to 59, were recovered from various crosses among 184 different triploid hybrid grape vines through the use of immature seed culturing and subsequent embryo cultures. Similarly, grapes with different ploidy levels, such as haploid, diploid, tetraploid and aneuploid, were consistently obtained using the same approaches (Guo et al. 2011b; Ji et al. 2013b; Park et al. 2002; Wakana et al. 2003; Yang et al. 2007). In one study, Wakana et al. (2003) obtained 88 triploid grape seedlings through embryo rescue, 38 of which were recovered by secondary embryo formation in vitro, highlighting the importance of this step for increasing the production rate of triploid plants.

Triploid grape rootstocks have also been obtained by embryo rescue and there is a report of one that is highly resistant to the major pest, grape phylloxera (*Daktulosphaira vitifoliae* Fitch) (Motosugi and Naruo 2003). Minernura et al. (2009) characterized a new triploid cultivar, Nagano Purple, selected from a cross between Kyohou (tetraploid) and Rosario Bianco (diploid) using embryo rescue and recently, in order to obtain new cold-resistant triploid grape genotypes, Guo et al. (2011a) used tetraploid grape cultivars with high fruit quality in crosses with the diploid *V. amurensis* Rupr. The rescued hybrid embryos were developed into progeny plantlets, laying the foundation for further breeding of triploid grapes with large berries that are also seedless and hardy. In general, the tetraploid grape parents preferred by grape breeders for

triploid grape breeding are mainly *V. vinifera* × *V. labrusca* hybrids, including Kyoho (Guo et al. 2011a, b; Ji et al. 2013b; Park et al. 1999; Wakana et al. 2003; Yamashita et al. 1993, 1998; Yang et al. 2007), Jingyou (Ji et al. 2013b; Sun et al. 2011; Xu et al. 2005), Yufu (Park et al. 1999; Wakana et al. 2003), Fenghou and Zizhenxiang (Sun et al. 2011; Xu et al. 2005), Fujiminori (Ji et al. 2013b; Yang et al. 2007), Delaware (Sun et al. 2011), Jumeigui (Guo et al. 2011b), Xiangyue and Zuijinxiang (Guo et al. 2011a), Black Olympia (Ji et al. 2013b). In addition, other tetraploid parents that also have been used include Red Pearl (Hiramatsu et al. 2003; Park et al. 1999; Wakana et al. 2003) and some Muscats such as Cannon Hall Muscat (Okamoto et al. 1993; Park et al. 1999; Wakana et al. 2003), Muscat Hamburg (Sun et al. 2011; Xu et al. 2005), and Muscat of Alexandria (Yamashita et al. 1993).

Hybridization between distantly related *Vitis* species

Hybridization between distantly related *Vitis* species is often used by breeders in order to introgress desired traits, such as seedlessness, into genotypes having other important attributes, such as stress-resistance. However, the breeding efficiency of hybridization between distantly related species is low, mainly due to differences in chromosome numbers between different subgenera, which often leads to cross incompatibility and embryo abortion. However, this problem can be addressed by *in ovulo* embryo rescue and the technique has been extensively applied (Bharathy et al. 2003; Goldy et al. 1988, 1989; Guo et al. 2010, 2011a; Ji et al. 2013b; Li et al. 2014; Lu et al. 1993; Ramming et al. 1991, 2000, 2006, 2009, Ramming 2010, Ramming et al. 2012; Ritschel et al. 2010; Tian and Wang, 2008; Tian et al. 2008). Goldy et al. (1988) were the first to use embryo rescue for hybridization between distantly related *Vitis* species and they obtained hybrid plantlets from a cross between *V. vinifera* and *V. rotundifolia*. Subsequently, Ramming et al. (2000) generated the first stenospermocarpic, seedless F1 hybrid of *V. vinifera* × *V. rotundifolia* developed by Goldy et al. (1988) and substantially characterized one derivative seedling, C41-5.

In order to introgress downy mildew resistance into the Thompson Seedless variety, it was crossed with several other *Vitis* species including *V. labrusca*, *V. tilifolia*, *V. candicans* and *V. rupestris*, before hybrid plantlets were obtained through *in ovulo* embryo rescue (Bharathy et al. 2003). Table and raisin grapes with Pierce's disease resistance and increased fruit quality have also been recovered by embryo rescue from *V. vinifera* (seedless) × F8909-08 (*V. rupestris* × *V. arizonica/candicans*) crosses (Ramming et al. 2009). In addition, many attempts have been made by grape breeders to integrate the disease-resistance or cold-resistance traits of wild Chinese *Vitis* species into *V. vinifera* genotypes (Guo et al. 2010, 2011a; Ji et al. 2013b; Li et al. 2013, 2014; Tian and Wang 2008; Tian et al. 2008).

Conclusions and future prospects

In ovulo embryo rescue is widely used by plant breeders to rescue inherently weak, immature or hybrid embryos from a range of crops, including grape, where it has been employed for more than three decades. Many factors, including genotype, sampling/inoculation time, culture medium, culture methodology and utilization of plant growth regulators, can affect the success of the technique. Although many factors influencing embryo rescue have been investigated, the underlying causal basis of some, such as genotypes and cross combinations, medium composition, additional plant growth regulators and nutrients, culture conditions, and the interaction between different factors are not well understood. In addition, efficient standard *in ovulo* embryo rescue protocols need to be established for the most promising grape genotypes or cross combinations.

To date, grape embryo rescue has been extensively applied to recover inherently weak grape embryos, breed seedless and triploid grapes, and for the hybridization of distantly related *Vitis* species. It is likely that the breeding of seedless grapes will still be a major application in the future and more cultivars of different *Vitis* spp. such as *V. rotundifolia*, *V. labrusca* and wild Chinese *Vitis*, which are resistant to various biotic and abiotic stresses, are still encouraged to be used as male parents in hybridizations with *V. vinifera* cultivars in order to obtain stress resistant and high quality seedless grapes. The Combination of distant hybridization and triploid breeding techniques together with embryo rescue will be a promising approach to breed novel seedless grape cultivars.

Although grape embryo rescue has been widely investigated, the development of improved cultivars is few. This might due to that the majority of the hybrid grape plantlets obtained through embryo rescue are still under evaluation in the field. In the future, maker-assisted selection technique, which has already been utilized in grape breeding together with embryo rescue technique (Akkurt et al. 2012, 2013; Bergamini et al. 2013; Mejia and Hinrichsen 2003), will continuously play an important role in the efficient evaluation and selection of the hybrids obtained through grape embryo rescue. Finally, breeding novel grape cultivars through embryo rescue is still a challenging and long-term task, which requires persistent effort of grape breeders.

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