© The Author(s), 2021. Published by Cambridge University Press on behalf of NIAB
ISSN 1479-2621

Plant Genetic Resources: Characterization and Utilization (2021) **19**(3); 252–260
doi:10.1017/S1479262121000307

Development of an embryo germination protocol for shy-seeded grape (*Vitis vinifera* L.)

Ashwini P. Benke[1]*, Ram Krishna[1], Roshni R. Samarth[2]*, Shweta S. Dhumal[1], Waquar A. Ansari[3], Poonam V. Shelke[1], Somnath S. Dukare[1] and Major Singh[1]

[1]*ICAR-Directorate of Onion and Garlic Research, Pune, Maharashtra 410505, India,* [2]*ICAR-National Research Centre for Grapes, Pune, Maharashtra 412307, India and* [3]*Department of Botany, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India*

Received 10 December 2020, revised 10 April 2021; Accepted 12 April 2021 – First published online 22 June 2021

Abstract

Acquisition and germination of seeds are the most desired targets for the improvement of vegetatively propagated crops. In the present study, we developed a potential embryo germination protocol for the Red Globe grape cultivar having a low seed germination rate. Three grape berries at different developmental stages, viz. 50, 60 and 70 days after flowering (DAF), were selected for *in-vitro* embryo germination. Three growth media, namely Emershad and Ramming (ER), Nitsch and Nitsch (NN) and Murashige and Skoog (MS), and plant growth regulators (benzyl amino purine (BA), 0.5, 0.7 and 0.9 mg/l; indole butyric acid (IBA), 1.0, 1.5 and 2.0 mg/l; and gibberellic acid (GA), 0.1, 0.3 and 0.9 mg/l) were screened individually in different combinations with three amino acids, namely cysteine, glutamine and proline (2.0 µmol/l each). The maximum embryos germination percentage recorded at 70 DAF was 63.33, 47.78 and 45.56% in ER, NN and MS media, respectively, supplemented with 0.9 mg/l BA, 2.0 mg/l IBA, 0.9 mg/l GA and 2.0 µmol glutamine. Glutamine was found to have the most significant impact, and it improved the rescued embryos germination. The present study provides a potential recipe for a medium that can facilitate efficient germination of grape embryos.

Keywords: benzyl amino purine, embryo rescue, grape, gibberellic acid, *Vitis vinifera*

Introduction

Grape (*Vitis vinifera* L.) that belong to the family Vitaceae comprise 12 genera and 60 species and are one of the most commercially important fruit crops in the world (Melyan *et al*., 2015). Grape is a medicinally important fruit because studies have reported the presence of several compounds with antiaging, anti-infection, anticarcinogenic, antidiabetes, immune-modulatory, antiobesity, antiatherogenic and neuroprotective properties in grapes (Yadav *et al*., 2009; Yang and Xiao, 2013; Doulati-Baneh *et al*., 2015). Globally, a total of 69,31,353 million hectares of tropical and subtropical area are under grape cultivation, with the overall grape production being 71,781,883 metric tons. In terms of grape production, China stands first, followed by Italy and USA, producing 16.8% (11,868,697), 10.9% (6,504,283) and 9.6% (6,059,278) metric tons of grapes, respectively (FAOSTAT, 2018). India ranks seventh with a total production of 2,650,793 metric tons (FAOSTAT, 2018). Based on their utilization, grapes are classified into two major groups: (1) table grapes, which are used for eating, and (2) wine grapes, which are used for preparing wine. Grapes are rich sources of mineral nutrients, vitamins and antioxidative dietary fibre and have anticancer property (Llobera and Cañellas, 2007; Deng *et al*., 2011). *In-vitro* regenerated plants may show genetic variations because of different media and hormones used during tissue culture (Jiao *et al*., 2018). Currently, European grapes

*Corresponding authors. E-mail:ashwini.ashwini.chavan@gmail.com; sroshnir@gmail.com

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

(*V. vinifera* L.) are the leading cultivars that contribute to a major share in the global market. However, the disease susceptibility and cracking nature of the fruits create hurdles in their cultivation in South and East Asia (Morinaga, 2001; Ergül *et al.*, 2011). Therefore, breeders develop hybrids of *V. labrusca* and *V. vinifera* to improve disease and cracking tolerance (Ban *et al.*, 2016). Over 1500 years, *V. labruscana*-derived Bailey cultivars have been developed and introduced in East and South Asia and have become the leading cultivars (Ban *et al.*, 2016). Thus, interspecific hybrids of grapes are routinely developed for high-quality berries. The essential criteria for quality are large berry size, firm flesh, low acidity, crackless nature, striking appearance, disease resistance and high concentration of soluble solids. Furthermore, hybrid grape exhibit a lack of seed propagation because of the lower quality of seedlings than those of parent vine; however, the seeds are important for breeding and development of new varieties. Seeds from fresh grapes remain dormant and thus are tough to germinate (Selim *et al.*, 2016; Uzun *et al.*, 2019). Many approaches have been used to break the dormancy of seeds, including long-term storage of seeds at low temperature under a moist condition or in the presence of chemicals such as acetic acid, hydrogen peroxide, lactic acid, hydrogen cyanamide and plant growth regulators (PGRs), such as benzyl amino purine (BA), indoleacetic acid, indole butyric acid (IBA), gibberellic acid (GA), thiourea, kinetin and ethephon (Ledbetter and Shonnard, 1990; Ergenoglu *et al.*, 1996; Celik, 2001; Akkurt *et al.*, 2013; Uzun *et al.*, 2019). All these approaches are time-consuming and have less potential, and hence they cause a great loss of shy seeds (Akkurt *et al.*, 2013). To overcome this issue and improve the breeding efficiency, breeders generally use the embryo rescue or immature embryo culture technique (Li *et al.*, 2020) as this technique can be performed rapidly and results in a high germination rate. Embryo rescue is a technical practice for facilitating embryos recovery from abortion and speeding up the breeding programme by breeders for crop improvement. The success of embryos rescue depends on several factors such as the genotype of parents, composition of the culture medium, sampling time and age of the embryos (Tian and Wang, 2008; Li *et al.*, 2020). At an early developmental stage, embryos are not suitable to rescue because of their partial development; however, embryos at the 40–60 days after flowering (DAF) stage are suitable for embryo rescue (Li *et al.*, 2020). The efficiency and germination percentage of embryos can be improved by changing the medium composition and exogenous application of PGRs (Tian *et al.*, 2008). In India, downy mildew causes approximately 30–40% of grape loss. This problem triggers the need for developing a disease-resistant grape cultivar by using interspecific hybridization. Thus, standardization of the *in-vitro* seed germination protocol is a prerequisite for hybrid development. In the present study, three media (ER: Emershad and Ramming, 1984; MS: Murashige and Skoog, 1962; NN: Nitsch and Nitsch, 1969) with a combination of different PGRs and amino acids (AAs) were screened for *in-vitro* embryos germination. The present study provides a suitable and efficient medium combination for *in-vitro* embryos germination of grape and will help accelerate the breeding programme for grape improvement.

Materials and methods

Plant material

The berries were collected from the orchard of the Indian Council of Agriculture Research-National Research Centre for Grape, Pune, Maharashtra, India. For sample collection, the dates of anthesis for the Red Globe grape variety were recorded and the berries were collected after 50, 60 and 70 days of anthesis. The berries were washed under running tap water and treated with 0.5% bavistin (TATA, Mumbai, India) and then stored at 4°C with proper tagging for embryos rescue.

Media preparation and sterilization

Three media, namely, ER, NN and MS (Ducefa Biochemie, Haarlem, the Netherlands), supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Hi-Media, Mumbai, India) were used as the base culture media. The pH of the media was set to 5.8 ± 0.2 by using 1 N NaOH. Agar was added into the media after adjusting the pH and autoclaving the media for 15 min at 121°C. PGRs such as BA (0.5, 0.7 and 0.9 mg/l), IBA (1.0, 1.5 and 2.0 mg/l) and GA (0.1, 0.3 and 0.9 mg/l) and three AAs, namely cysteine, glutamine and proline (2.0 µmol/l each), were added as per their specification either before or after autoclaving. Cultures were grown at 25 ± 2°C under 16 h photoperiod and white fluorescent tube lights (40 W, Philips, India) emitting the light intensity of 50 µmol/m^2/s, with 60–70% relative humidity.

Sterilization of berries, embryo rescue and germination

The berries were washed thoroughly under running tap water for 30 min and then treated with 0.5% bavistin (TATA) and 0.1% Tween 20 (Hi-Media) solution for 15 min with continuous shaking, followed by washing with 70% ethanol for 10 min and washing twice with sterile distilled water. The berries were further treated with 0.1% mercuric chloride (Hi-Media) for 5 min, followed by washing with sterile distilled water four times. For embryo germination, 36 medium combinations were prepared with three different PGRs (BA, IBA and GA_3) and three AAs (cysteine,

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

Table 1. The response of grape embryo germination under different media composition

					Media used		
	Hormone/concentration				ER	NN	MS
Age of the seeds	BA mg/l	IBA mg/l	GA mg/l	Amino acid (2.0 µ mole/l)	Plant germination %		
50 days	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Cysteine	6.67 ± 0.47^{a}	3.33 ± 0.47^{c}	4.44 ± 0.27^{b}
	0.7	1.5	0.3	Cysteine	7.78 ± 0.55^{a}	4.44 ± 0.27^{c}	5.56 ± 0.27^{b}
	0.9	2	0.9	Cysteine	10.00 ± 0.00^{a}	6.67 ± 0.47^{b}	6.67 ± 0.47^{b}
	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Glutamine	7.78 ± 0.27^{a}	2.22 ± 0.27^{c}	3.33 ± 0.00^{b}
	0.7	1.5	0.3	Glutamine	8.89 ± 0.27^{a}	5.56 ± 0.72^{c}	6.67 ± 0.47^{b}
	0.9	2	0.9	Glutamine	11.11 ± 0.27^{a}	6.67 ± 0.47^{c}	8.89 ± 0.55^{b}
	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Proline	4.44 ± 0.27^{a}	2.22 ± 0.27^{b}	2.22 ± 0.27^{b}
	0.7	1.5	0.3	Proline	6.67 ± 0.47^{a}	5.56 ± 0.55^{b}	4.44 ± 0.27^{c}
	0.9	2	0.9	Proline	6.67 ± 0.47^{a}	5.56 ± 0.27^{b}	6.67 ± 0.48^{a}
60 days	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Cysteine	21.11 ± 0.54^{a}	16.67 ± 0.47^{b}	15.56 ± 0.27^{b}
	0.7	1.5	0.3	Cysteine	26.67 ± 0.47^{a}	18.89 ± 0.27^{b}	18.89 ± 0.72^{b}
	0.9	2	0.9	Cysteine	27.78 ± 0.27^{a}	20.00 ± 0.47^{b}	21.11 ± 0.27^{b}
	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Glutamine	22.22 ± 0.72^{a}	17.78 ± 0.72^{b}	15.56 ± 0.72^{b}
	0.7	1.5	0.3	Glutamine	27.78 ± 0.55^{a}	20.00 ± 0.47^{b}	17.78 ± 0.27^{bc}
	0.9	2	0.9	Glutamine	28.89 ± 0.27^{a}	22.22 ± 0.55^{b}	20.00 ± 0.95^{bc}
	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Proline	16.67 ± 0.47^{a}	14.44 ± 0.27^{a}	13.33 ± 0.49^{ab}
	0.7	1.5	0.3	Proline	20.00 ± 0.47^{a}	15.56 ± 0.27^{b}	14.44 ± 0.33^{b}
	0.9	2	0.9	Proline	21.11 ± 0.54^{a}	16.67 ± 0.47^{b}	15.56 ± 0.77^{b}
70 days	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Cysteine	55.56 ± 0.98^{a}	44.44 ± 0.72^{b}	45.56 ± 1.44^{b}
	0.7	1.5	0.3	Cysteine	56.67 ± 1.42^{a}	47.78 ± 1.19^{b}	46.67 ± 0.47^{b}
	0.9	2	0.9	Cysteine	63.33 ± 0.94^{a}	48.89 ± 0.72^{b}	47.78 ± 0.54^{b}
	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Glutamine	58.89 ± 0.98^{a}	46.67 ± 0.82^{b}	42.22 ± 0.72^{c}
	0.7	1.5	0.3	Glutamine	62.22 ± 0.72^{a}	45.56 ± 0.72^{b}	43.33 ± 0.82^{b}
	0.9	2	0.9	Glutamine	63.33 ± 0.94^{a}	47.78 ± 0.72^{b}	45.56 ± 0.27^{b}
	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	1	0.1	Proline	34.44 ± 0.72^{a}	32.22 ± 0.54^{a}	31.11 ± 0.82^{a}
	0.7	1.5	0.3	Proline	35.56 ± 0.72^{a}	33.33 ± 0.94^{a}	32.22 ± 0.73^{a}
	0.9	2	0.9	Proline	38.89 ± 0.72^{a}	34.44 ± 0.27^{ab}	33.33 ± 0.55^{b}

BA, benzylaminopurine; IBA, indole butyric acid; GA, gibberellic acid; ER, Emershad and Ramming; NN, Nitsch and Nitsch; MS, Murashige and Skoog.
The figure followed by the same letters within the rows is not significantly different according to Duncan's multiple range test at $P < 0.01$.

glutamine and proline) (Table 1). Immature seeds were collected from the sterilized berries; their seed coats were partially removed and were plated into Petri dishes (Tarsons, Kolkata, India) containing 40 ml of medium with different concentrations of PGRs. The cut sides were kept facing the media (Fig. 1(a)) and incubated at 25 ± 2°C in the dark.

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

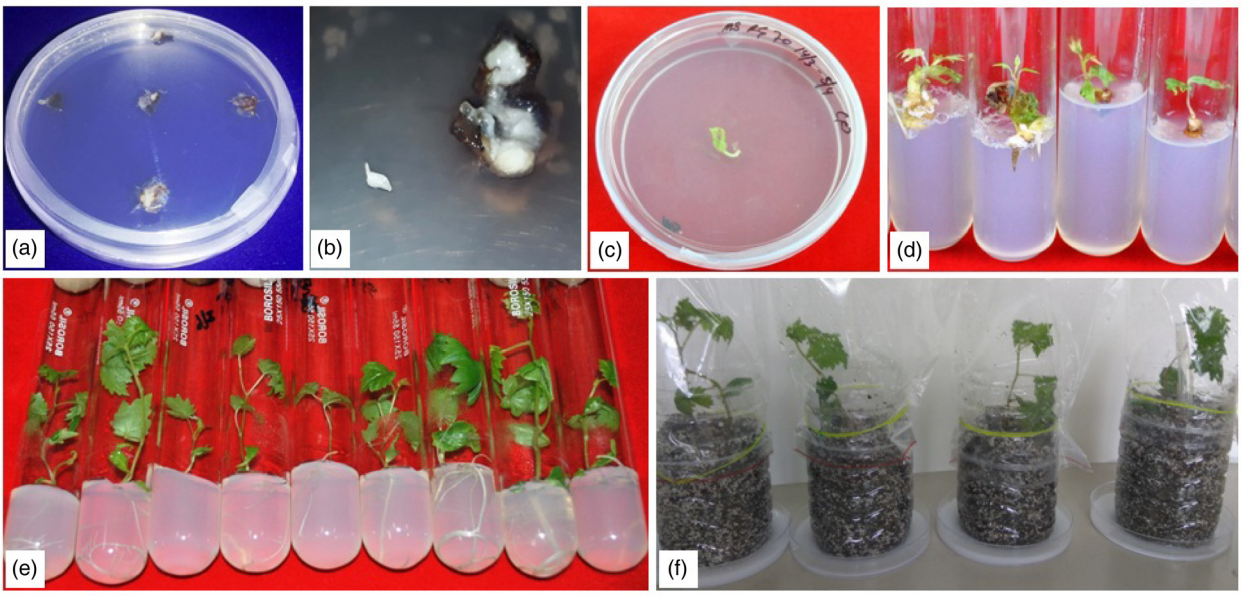


Fig. 1. Developmental stages of rescued embryo; (a) inoculation of partially dissected seeds on the medium; (b) dissection of developed embryos; (c) sub-culturing of fully germinated embryo; (d) transfer of grown embryo in respective medium; (e) maintenance of plantlets in MS medium; hardening of the plantlets.

After 3–5 days, the seeds were redissected and embryos were rescued (Fig. 1(b)) and inoculated on a fresh medium. After 1-week germination, the embryos were subcultured on the respective medium. Fully grown embryos (approximately 3 cm) (Fig. 1(c)) were transferred to test tubes containing 25 ml of the respective medium without any PGRs or AAs (Fig. 1(d)). For further growth, development and maintenance, the plants were cultured on basal MS medium (Fig. 1(e)).

Hardening of plantlets

Plantlets that grew well were taken out from the test tubes, and plant roots were washed with sterile distilled water and transplanted into bottom holed plastic cups containing autoclaved soil, cocopeat, borokit and perlite in a 1:1:1:1 ratio. The plants treated with 0.5% (w/v) bavistin were covered with transparent plastic bags for tissue culture initially for 3 days (Fig. 1(f)). Then, the plants were shifted to the natural environment for hardening. The plants were regularly irrigated on a requirement basis. Then, the bags were removed, and the plants were transplanted in the field after 2 weeks.

Assessment of genetic fidelity

The molecular analysis of regenerated plants was performed using the inter simple sequence repeat (ISSR) analysis. DNA was isolated from 5 g of young leaves of the mother plant and seven randomly selected embryo-rescued plants according to the method described by Doyle and Doyle (1987). Leaf tissues were crushed in liquid nitrogen, and fine powder was used for DNA isolation. A total of 13 ISSR primers (Table 2) were assessed for the study by using polymerase chain reaction (PCR). PCR amplification was performed in a 15 µl volume of reaction mixture containing 1 µl of template DNA, 1.5 µl of 10 × PCR buffer, 0.35 µl of dNTPs (25 mM), 0.3 µl of $MgCl_2$ (1.5 mM), 1.2 µl of random primer (10 pM), 0.25 µl of Taq-DNA polymerase and 10.4 µl of sterile Milli-Q water. PCR was performed using the ProFlex™ PCR System (Thermo Fisher Scientific, Mumbai, India). The temperature cycle was set as follows: initial denaturation at 94°C for 4 min and 1 min for cyclic denaturation for 36 cycles; annealing at 55°C for 1 min; and extension at 72°C for 1 min (with a final extension for 10 min at 72°C). PCR amplicons were analysed using agarose (1.5%) gel electrophoresis. One kilobase of DNA ladder (Fermentas, Mumbai, India) was used to determine the size of amplicons. The amplified products were visualized using a gel documentation system (AlphaImager™ 3400, USA). The amplification was repeated thrice with each primer to verify the reproducibility of results.

Statistical analysis

The experiment was set up in a completely randomized block design with three replications per treatment, and each replicate consisted of 30 explants. Data are

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

Table 2. ISSR details screened for the genetic stability analysis

ISSR primer	Nucleotide sequence(5′-3′)	Annealing temperature (°C)	Total number monomorphic band	Total number polymorphic band
UBC-812	GAGAGAGAGAGAGAGAA	50.9	3	0
UBC-815	CTCTCTCTCTCTCTCTG	55.0	6	0
UBC-822	TCTCTCTCTCTCTCTCA	55.0	0	0
UBC-834	AGAGAGAGAGAGAGACYT	55.0	0	0
UBC-835	AGAGAGAGAGAGAGAGYC	55.0	2	0
UBC-836	AGAGAGAGAGAGAGACYA	55.0	3	0
UBC-840	GAGAGAGAGAGAGAGAY	58.0	0	0
UBC-842	GAGAGAGAGAGAGAGAYG	59.1	1	0
UBC-843	CTCTCTCTCTCTCTCTRA	53.2	1	0
UBC-844	CTCTCTCTCTCTCTCTRG	50.9	3	0
UBC-850	GTGTGTGTGTGTGTGTCTC	60.0	3	0
UBC-853	TCTCTCTCTCTCTCTCRT	55.0	4	0
UBC-854	TCTCTCTCTCTCTCTCAGG	58.0	2	0
Total			27	0

represented as mean ± standard deviation. Analysis of variance was performed using SPSS 11.0, and differences between the means were compared by performing the Tukey's range test at a probability level of 5%.

Results

Effect of media containing different combinations of PGRs and AAs on embryo germination

The germination efficiency of embryos under different medium combinations was found to vary (Table 1). The seeds of 50, 60 and 70 days of age were grown in three different media, viz. ER, NN and MS under 36 different concentrations of BA, IBA and GA along with cysteine, glutamine and proline. The germination percentage of 50-day seeds was lesser than that of 70-day seeds. The germination percentage of 50-day seeds grown in ER, NN and MS media supplemented with 0.5, 1 and 0.1 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 6.67, 3.33, 4.44; 7.78, 2.22, 3.33; and 4.44, 2.22, 2.22, respectively. Similarly, the germination percentage of 50-day seeds grown in ER, NN and MS media supplemented with 0.7, 1.5 and 0.3 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 7.78, 4.44, 5.56; 8.89, 5.56, 6.67; and 6.67, 5.56, 4.44, respectively. The germination percentage of 50-day seeds grown in ER, NN and MS media supplemented with 0.9, 2 and 0.9 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 10, 6.67, 6.67; 11.11, 6.7, 8.89; and 6.67, 5.56, 6.67, respectively. However, without PGRs and AAs, no germination was recorded in all the three media.

The germination percentage of 60-day seeds grown in ER, NN and MS media supplemented with 0.5, 1 and 0.1 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline, was 21.11, 16.67, 15.56; 22.22, 17.78, 15.56; and 16.67, 14.44, 13.33, respectively. Similarly, the germination percentage of 60-day seeds grown in ER, NN and MS media supplemented with 0.7, 1.5 and 0.3 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 26.67, 18.89, 18.89; 27.78, 20, 17.78; and 20, 15.56, 14.55, respectively. The germination percentage of 60-day seeds grown in ER, NN and MS media supplemented with 0.9, 2 and 0.9 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 27.78, 20, 21.11; 28.89, 22.22, 20; and 21.11, 16.67, 15.56, respectively. However, without PGRs and AAs, no germination was recorded in all the three media.

The germination percentage of 70-day seeds grown in ER, NN and MS media supplemented with 0.5, 1 and 0.1 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 55.56, 44.44, 45.56; 58.89, 46.67, 42.22; and 34.44, 32.22, 31.11, respectively. Similarly, the germination percentage of 70-day seeds grown in ER, NN and MS media supplemented with 0.7, 1.5 and 0.3 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 56.67, 47.78, 46.67; 62.22, 45.56, 43.33; and 35.56, 33.33, 32.22, respectively. The germination percentage of 70-day seeds grown in ER, NN and MS media supplemented with 0.9, 2 and 0.9 mg/l of BA, IBA and GA, respectively, and cysteine, glutamine and proline was 63.33, 48.89, 47.78; 63.33, 47.78, 45.56; and 38.89, 34.44, 33.33, respectively. However, without PGRs and AAs, no germination was recorded in all the three media (Table 1).

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

The maximum germination efficiency recorded was 11.1% with ER media supplemented with 0.9 mg/l BA, 2 mg/l IBA, 0.9 mg/l GA and 2 µmol/ L-glutamine. No germination was recorded in any of the three media not supplemented with PGRs and AAs at any stage of the seed development. The germination percentage of 60-day seeds was in between the germination percentage of 50- and 70-day seeds. The maximum embryo germination percentage was 28.9 and 22.2% with ER and NN media supplemented with 0.9 mg/l BA, 2 mg/l IBA, 0.9 mg/l GA and 2 µmol/l glutamine as well as in MS media supplemented with 0.9 mg/l BA, 2 mg/l IBA, 0.9 mg/l GA and 2 µmol/l cysteine. However, the maximum germination percentage was observed for 70-day seeds in all the three media supplemented with different combinations of PGRs and AAs. The maximum germination percentage of 63.3, 48.9 and 47.8% was observed in ER, NN and MS media, respectively, supplemented with 0.9 mg/l BA, 2 mg/l IBA, 0.9 mg/l GA and 2 µmol/l cysteine (Table 1).

Assessment of genetic uniformity

To assess genetic uniformity, randomly selected regenerated plants and the mother plant were analysed using the ISSR analysis under optimized PCR conditions. ISSR markers were used for a broad analysis of the genetic stability and variations in plants. The gel profile produced resolvable, clear and reproducible amplification products during the analysis; a unique set of amplified product was obtained from each primer. Among the 13 ISSR primers used in this analysis, 10 primers produced distinct and scorable bands ranging from 300 to 2500 base pairs, which varied from one to six in number (Table 2; Fig. 2(a) and (b)). No ISSR polymorphism was observed between the regenerated plants and the mother plant.

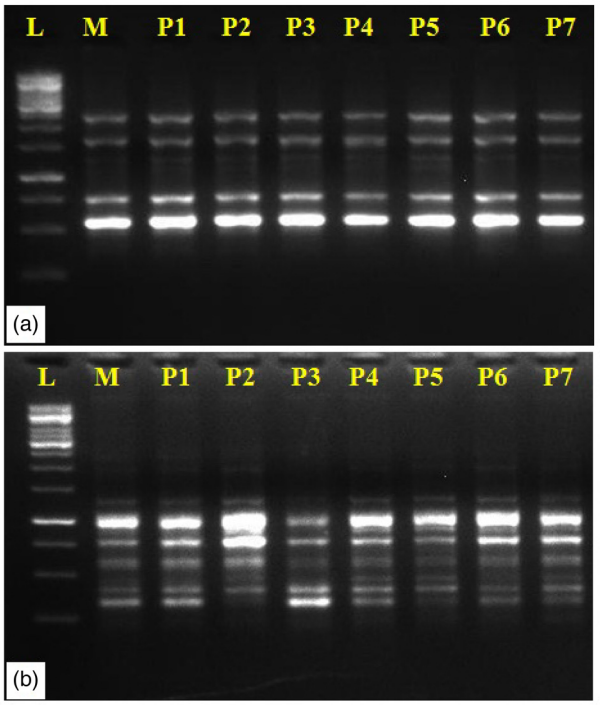


Fig. 2. ISSR amplification of regenerated plantlets and mother plant; L, ladder 1 kb GeneRular; M, mother plant; P1 to P7 embryo rescued plants. (a) PCR amplification pattern of UBC-853 and (b) UBC-815.

Discussion

Embryo rescue is the most widely used technique to remove pre and post-transcriptional barriers, which hinder the proper development of embryos in intervarietal or interspecific crosses or distant breeding programmes. In case of grapes, seedlessness is the most common phenomenon important for table grapes and raisin preparation. In advanced breeding techniques, potential breeding varieties act as a crucial parenting material to target the issues related to biotic and abiotic stress. In grape breeding, to rescue inherently weak embryos, embryo rescue is performed for cultivars with seedless fruits/seeds with low germination capacity because of physiological and/or nutritional scarcity. This technique helps in developing such embryos into a normal plant, and it involves excising seeds/ovules and culturing on various compositions of the culture medium. The successful development of an embryo into a plant depends on various factors such as the genotype and developmental age of berries, season of berry collection, culture medium and PGRs, and additional supplements including AAs (Ji *et al.*, 2013). The developmental stage of embryos is a crucial factor; in the early developmental stage, a few embryos develop into mature embryos and then into plants (Li *et al.*, 2013; Wang *et al.*, 2016). In the present study, a large number of embryos were obtained from the grape berries at the 70-DAF stage. The lowest embryo survival rate was found in the grape berries at 50 DAF (Table 1), and these results are in accordance with those of a study by Guo *et al.* (2011), who reported embryo germination rates of 39–73% in grape berries from 55 to 80 DAF, which was the most suitable stage. In the early stage, embryos remain globular, which has the lowest germinating capacity, whereas during the late germination stage, embryos remain in the torpedo stage, which has the highest germination power (Li *et al.*, 2015).

Growth medium and its composition are the most important factors that determine the proper growth and development of embryos. In the present study, different medium combinations were screened for successful embryos rescue (Li *et al.*, 2015). Among all the tested combinations, ER medium supplemented with 0.9 mg/l BA, 2.0 mg/l IBA,

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

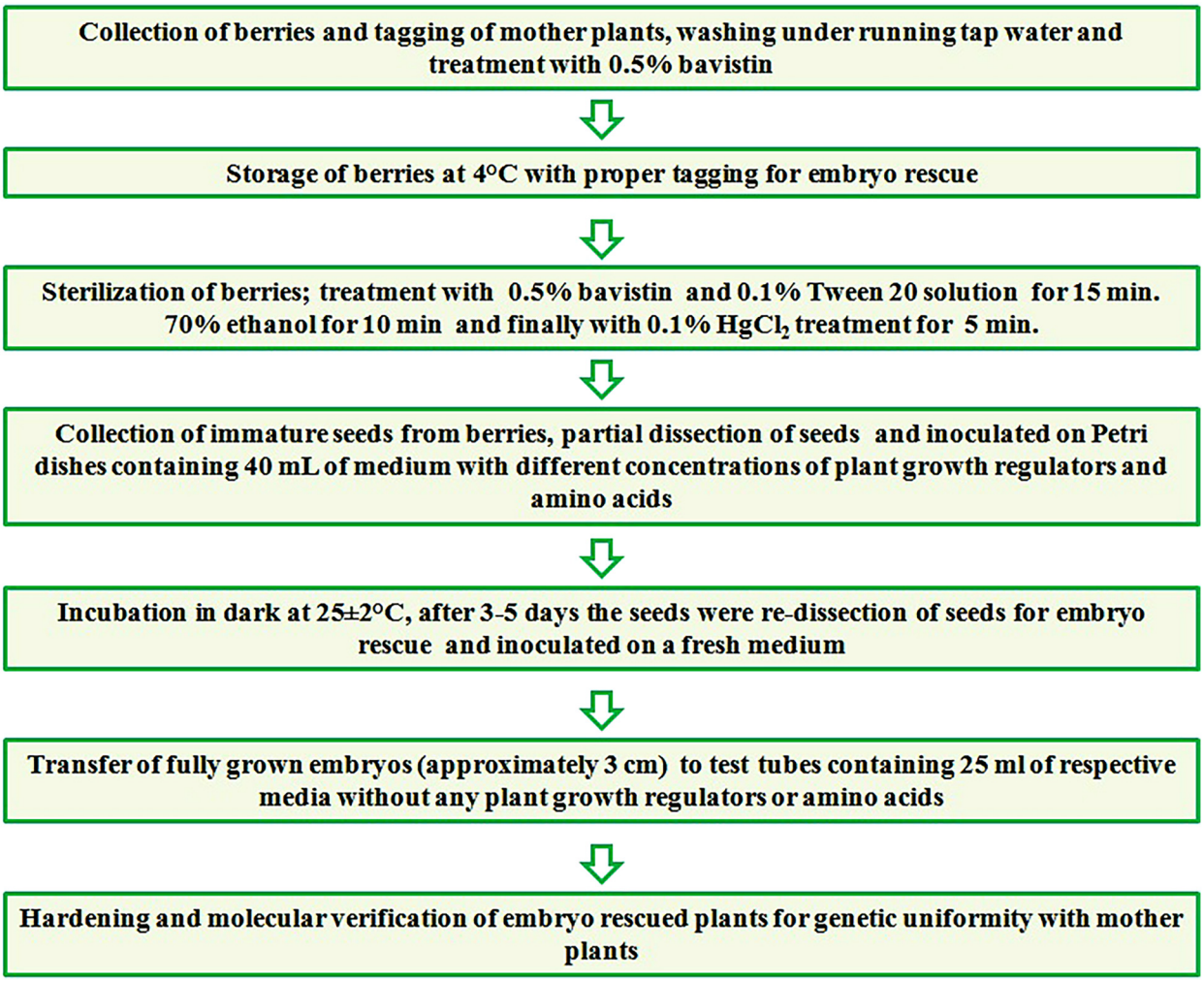


Fig. 3. Flow chart showing embryo germination protocol step by step.

0.9 mg/l GA and 2.0 µmol glutamine showed the highest embryos germination capacity. The ER medium with the same PGRs and 2.0 µmol cysteine showed similar embryos germination capacity. Similar results were reported in a study by Guo *et al.* (2004), wherein the embryos germination capacity of seedless grape was found to be higher in ER medium supplemented with 0.5 mg/l BA, 2 mg/l IBA and 0.5 mg/l GA3 than in NN and MS media; the same media with a higher concentration of PGRs showed higher embryos germination capacity. Furthermore, MS medium among all the tested media showed the highest callus formation and successful growth of embryos. Similarly, callus formation and durable embryos survival efficiency of MS medium compared with those of ER and NN media were reported by Singh and Brar (1992) and Singh *et al.* (2011).

Addition of specific AAs such as L-cysteine, L-glutamine and proline into the culture media improves embryo germination by supporting the maturation of developing embryos. In the present study, the addition of AAs significantly improved embryos germination, which may be because of the growth-supporting property of AAs; L-glutamine showed the highest effect on embryo germination among all the three AAs (Table 1, Fig. 3). Similar results were reported by Emershad *et al.* (1989) and Tian and Wang (2008), who cultured the ovules of Thompson Seedless on Cain's basal medium fortified with L-asparagine, L-cysteine, L-glutamine and L-serine.

Genetic stability is the most desired characteristic during *in-vitro* plant development as different PGRs induce genetic changes. In this study, we also assessed the genetic stability of embryos by using ISSR markers. ISSR markers are frequently used to assess the genetic stability and diversity of plants and microbes (Srivastava *et al.*, 2017; Verma *et al.*, 2018). The ISSR analysis showed no noticeable variations among the embryo-rescued germinated plantlets and the mother plant from which the berries were collected.

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

Similar to our results, unique and monomorphic banding patterns were reported by Srivastava *et al.* (2017). The genetic stability analysis confirmed the genetic uniformity and true-to-type nature of the *in-vitro* regenerated plants.

Conclusion

The present study provides a potent embryo-rescue protocol for the cultivation of grape cultivars having low seed-germination capacity. The screening of the three most commonly used media, ER, NN and MS, revealed that ER medium supplemented with 0.9 mg/l BA, 2.0 mg/l IBA and 0.9 mg/l GA is potent for rescuing embryos of the Red Globe grape cultivars. This study also showed that the grape berries at the 70-DAF stage are most suitable for rescuing embryos from the immature seed. Furthermore, L-glutamine was found to be the most potent supplement for embryo-rescue germination. This study provides an efficient medium recipe consisting of PGRs and AAs for embryo-rescue germination without affecting the genetic stability of plants, and the medium composition can also be efficient for the germination of other grape cultivars.

Acknowledgements

The authors are thankful to the Director, ICAR-Directorate of Onion and Garlic Research, Rajgurunagar, Pune, and ICAR-National Research Centre for Grape, Pune, Maharashtra, India for generously providing laboratory facilities.

References

Akkurt M, Keskin N, Shidfar M, Shidfar AC and Çakir A (2013) Effects of some treatments prior to strati cation on germination in Kalecik Karası (*Vitis vinifera* L.) seeds. *Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi* 3: 9–13.

Ban Y, Mitani N, Sato A, Kono A and Hayashi T (2016) Genetic dissection of quantitative trait loci for berry traits in interspecific hybrid grape (*Vitis labruscana*×*Vitis vinifera*). *Euphytica* 211: 295–310.

Celik H (2001) Effect of bottom heating, germination medium and gibberellic acid treatments on germination of Isabella (*Vitis labrusca* L.) grape seeds. *Pakistan Journal of Biological Sciences* 4: 953–957.

Deng Q, Penner MH and Zhao Y (2011) Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins. *Food Research International* 44: 2712–2720.

Doulati-Baneh H, Mohammadi SA, Labra M, De Mattia F, Bruni I, Mezzasalma V and Abdollahi R (2015) Genetic characterization of some wild grape populations (*Vitis vinifera* subsp. sylvestris) of Zagros mountains (Iran) to identify a conservation strategy. *Plant Genetic Resources* 13: 27.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Emershad RL and Ramming DW (1984) In-ovulo embryo culture of *Vitis vinifera* LCV 'Thompson seedless'. *American Journal of Botany* 71: 873–877.

Emershad RL, Ramming DW and Serpe MD (1989) In ovulo embryo development and plant formation from stenospermic genotypes of *Vitis vinifera*. *American Journal of Botany* 76: 397–402.

Ergenoglu F, Tangolar SGKS and Gök S (1996) The effects of some pre-treatments for promoting germination of grape seeds. *Acta Horticulturae* 441: 207–212.

Ergül A, Perez-Rivera G, Söylemezoglu G, Kazan K and Arroyo-Garcia R (2011) Genetic diversity in Anatolian wild grapes (*Vitis vinifera* subsp. sylvestris) estimated by SSR markers. *Plant Genetic Resources* 9: 375.

FAOSTAT (2018) http://www.fao.org/.

Guo YS, Gao XY, Zhao YH and Guo XW (2004) Preliminary report on the embryo rescue technique in Venus Seedless grape. *Sino-overseas Grapevine Wine* 3: 6–8.

Guo Y, Zhao Y, Li K, Liu Z, Lin H, Guo X and Li C (2011) In vitro embryo rescue culture of F1 progenies from crosses between tetraploid grape and *Vitis amurensis Rupr*. *African Journal of Agricultural Research* 6: 4906–4909.

Ji W, Li ZQ, Zhou Q, Yao WK and Wang YJ (2013) Breeding new seedless grape by means of in vitro embryo rescue. *Genetics and Molecular Research* 12: 859–869.

Jiao Y, Li Z, Xu K, Guo Y, Zhang C, Li T, Jiang Y, Liu G and Xu Y (2018) Study on improving plantlet development and embryo germination rates in in vitro embryo rescue of seedless grapevine. *New Zealand Journal of Crop and Horticultural Science* 46: 39–53.

Ledbetter CA and Shonnard CB (1990) Improved seed development and germination of stenospermic grapes by plant growth regulators. *Journal of Horticultural Sciences* 65: 269–274.

Li G, Wang G and Wang Y (2013) Embryo rescue to obtain progeny from seedless grapes (*Vitis vinifera* L.)×wild Chinese *Vitis* species. *Journal of Food Agriculture and Environment* 11: 1058–1062.

Li Z, Li T, Wang Y and Xu Y (2015) Breeding new seedless grapes using in ovulo embryo rescue and marker-assisted selection. *In Vitro Cellular and Developmental Biology Plant* 51: 241–248.

Li S, Li Z, Zhao Y, Zhao J, Luo Q and Wang Y (2020) New disease-resistant, seedless grapes are developed using embryo rescue and molecular markers. *3 Biotech* 10: 4.

Llobera A and Cañellas J (2007) Dietary fibre content and antioxidant activity of Manto Negro red grape (*Vitis vinifera*): pomace and stem. *Food Chemistry* 101: 659–666.

Melyan G, Sahakyan A and Harutyunyan A (2015) Micropropagation of grapevine (*Vitis vinifera* L.) seedless cultivar 'Parvana' through lateral bud development. *Vitis* 54: 253–255.

Morinaga K (2001) Grape production in Japan. In Papademetrious MK and Dent FJ (1st eds) *Grape Production in the Asia-Pacific Region*. Bangkok, Thailand: FAO Regional Office for Asia and the Pacific, pp. 38–52.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologiae Plantarum* 15: 473497.

Nitsch JP and Nitsch C (1969) Haploid plants from pollen grains. *Science (New York, N.Y.)* 163: 85–87.

Selim HH, Ibrahim FA, Fayek MA, El-Deen SS and Gamal NM (2016) Effect of different treatments on germination of Romi red grape seeds. *Vitis* 20: 115.

Singh Z and Brar SS (1992) In vivo development of ovule in seedless and seeded cultivars of grapes (*Vitis vinifera* L.): a particular reference to in ovulo embryo culture. *Vitis* 31: 77–82.

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press

Singh NV, Singh SK and Singh AK (2011) Standardization of embryo rescue technique and bio-hardening of grape hybrids (*Vitis vinifera* L.) using arbuscular mycorrhizal fungi (AMF) under sub-tropical conditions. *Vitis* 50: 115–118.

Srivastava S, Krishna R, Sinha RP and Singh M (2017) TDZ-induced plant regeneration in *Brassica oleracea* L. var. botrytis: effect of antioxidative enzyme activity and genetic stability in re-generated plantlets. *In Vitro Cellular and Developmental Biology Plant* 53: 598–605.

Tian L and Wang Y (2008) Seedless grape breeding for disease resistance by using embryo rescue. *Vitis* 47: 15.

Tian L, Wang Y, Niu L and Tang D (2008) Breeding of disease-resistant seedless grapes using Chinese wild *Vitis* spp.: I. In vitro embryo rescue and plant development. *Scientia Horticulturae* 117: 136–141.

Uzun Hİ, Nuray ÖZER, Akkurt M, Cengiz ÖZER, Aydin S and Akturk B (2019) Crossing of alphonse lavallee and regent grape cultivars for downy mildew resistant genotypes. 1. Seed germination and seedling growth. *Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi* 29: 72–78.

Verma JP, Jaiswal DK, Krishna R, Prakash S, Yadav J and Singh V (2018) Characterization and screening of thermophilic *Bacillus* strains for developing plant growth promoting consortium from hot spring of Leh and Ladakh region of India. *Frontiers in Microbiology* 9: 1293.

Wang L, Hu X, Jiao C, Li Z, Fei Z, Yan X and Wang X (2016) Transcriptome analyses of seed development in grape hybrids reveals a possible mechanism influencing seed size. *BMC Genomics* 17: 1–15.

Yadav M, Jain S, Bhardwaj A, Nagpal R, Puniya M, Tomar R and Yadav H (2009) Biological and medicinal properties of grapes and their bioactive constituents: an update. *Journal of Medicinal Food* 12: 473–484.

Yang J and Xiao YY (2013) Grape phytochemicals and associated health benefits. *Critical Reviews in Food Science and Nutrition* 53: 1202–1225.

https://doi.org/10.1017/S1479262121000307 Published online by Cambridge University Press