Establishment of a rapid and highly effective recycling system for production embryos in 'Chardonnay'

xinyi hao (✉ xinyihao@nxu.edu.cn)

Ningxia University <https://orcid.org/0000-0001-6531-1414>

Rong Ya

Ningxia University

Tianyue Zhang

Ningxia University

Benyu Kang

Ningxia University

Ruocen Zhao

Ningxia University

Weirong Xu

Ningxia University

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Abstract

Somatic embryo regeneration is an important pathway for mass propagation of grape plants and the basis for grape transgenic technology. The aim of the present study was to establish of a rapid and highly effective recycling system for production embryos in 'Chardonnay'. The white grapevine cultivars 'Chardonnay' was used for the research. Cultured on different media, found that MC and PIV media were beneficial to callus formation of embryonic with 8.60% and 39.52% induction rates, respectively. For cyclic induction of somatic embryos, the CIM medium was conducive to callus formation with 8.33% induction rates. The different concentrations of melatonin on the induction efficiency in somatic embryogenesis and found only MEL2 medium appeared somatic embryos with 5.06% induction rate. Scanning electron microscopy (SEM) revealed that the round spherical granules with full texture and structure were mostly beneficial to callus tissue formation. The study on the cyclic induction of cotyledons, hypocotyls and radicles of somatic embryos showed that the callus induction rate of over 80% of cotyledons and hypocotyls. The reuse abilities of normal embryo (NE), embryo axis elongation embryo (ECE), vitrification embryo (VE) and cotyledon fusion embryo (FCE) were different on somatic embryo cycle induction. Both NE and FCE were induced to form embryonic callus tissue with 8.42% and 4.44% of induction rates, respectively. The results have developed a rapid and highly effective protocol for establishment of a recycling system for production of cyclic embryos in 'Chardonnay'.

Key Message

We optimized the somatic embryo regeneration system of wine grape 'Chardonnay' by regulating plant hormones, judging the type of callus tissue by SEM, and exploring the recycling of malformed embryos by somatic embryo cycle induction system.

Introduction

Grapevine (*Vitis* spp.) is one of the most economically important fruit crops worldwide, which has been extensively used for table, juice, seed oil and wine and become the focus of molecular studies in diverse areas (Song et al., 2021). The traditional breeding via combination of genes responsible of abiotic and biotic resistance is greatly constrained due to the long juvenile period and a highly heterozygous genome in grape (Zhou et al., 2014). A rapid and highly efficient regeneration and transformation system is urgently required for improving abiotic and biotic tolerance of grapes (Li et al., 2008; Vidal et al., 2010). Somatic embryo regeneration is an important pathway for rapid propagation of grape plants and the basis for grape transgenic technology. Since the first report of somatic embryogenesis in grapes (Mullins and Srinivasan, 1976), the suspensions cell, anthers, ovaries, tendrils, immature leaves, immature whole flowers and immature zygotic embryos have been reported to induce somatic embryos from various grapevine genotypes (Morgana et al., 2004; Wang et al., 2005; Gambino et al., 2007; Dhekney et al., 2009; Zhou et al., 2014; Dai et al., 2015). The grape flower, as the material widely used to induce somatic embryos, blooms only once a year and keeps for a week or two. This greatly limits the opportunity for induction and proliferation of embryogenic cultures as well as for transformation. Therefore, it is critically

important to establishment of a recycling system for regeneratively embryogenic tissues efficiently and to maintain them over long periods, especially in the process of genetic transformation, and embryonic callus can be closely combined with *Agrobacterium tumefaciens*, which greatly improves the transformation efficiency and also avoids the problem of chimerism formation induced by the organogenesis pathway, and the system is suitable as a stable and efficient regeneration pathway. Therefore, the establishment of a stable and efficient somatic embryogenesis pathway is the basis for achieving molecular breeding in grapevines (Berres et al., 1992; Dhekney et al., 2008).

Up to now, European grape (*V. vinifera*) (Gambino et al., 2007), muscadine grape (*V. rotundifolia*) (Robacker et al., 1993), sandy grape (*V. rupestris*) and riparian grape (*V. riparia*) (Dhekney et al., 2009), Chinese wild grape (*V. pseudoreticulata*) (Dai et al., 2015) and European and American hybrids (Martinelli et al., 2001a) have all established somatic embryo regeneration systems. However, the frequency of primary somatic embryos production in many important *V. vinifera* cultivars is very low (Martinelli et al., 2001b). Somatic embryogenesis from stem and leaf explants of *Quercus robur* showed that it is also a genotype-dependent process (Cuenca et al., 1999), thereby making efficiency of the somatic embryogenesis system limited. Moreover, the efficient regeneration system of grapevine somatic embryos is influenced by several factors such as exosomes, medium composition, and culture environment. These factors led to the low efficiency, poor reproducibility and large differences in regeneration frequency of somatic embryos induced from the same or different varieties, especially for the generation of a large number of malformed seedlings. This seriously hinders the development and application of grapevine tissue culture technology (Oláh et al., 2009; Zhou et al., 2014). The generation of malformed embryos is an important constraint to the seedling formation of grapevine somatic embryos (Ji et al., 2017). Therefore, reduction or recycling of malformed embryos is also one of the concerns in the regeneration pathway of grapevine somatic embryos.

It has previously been observed that, morphogenetic responses such as secondary embryogenesis and organogenesis may occur during somatic embryogenesis (Nakano et al., 2000; Cadavid et al., 2008; Dai et al., 2017). Also, secondary embryogenesis has been reported to allow the establishment of cycling cultures, thus improving the frequency of somatic embryo production in plants recalcitrant to somatic embryogenesis (Ji et al., 2017). Accordingly, it is important to establish a rapid and routine protocol for somatic embryogenesis and efficient recycling system of different morphological somatic embryos in grapevine. For this purpose, wine grape variety of *Vitis vinifera* cv. 'Chardonnay' was used for the tested material to explore the effect of different medium on the efficiency of embryonic callus induction. Moreover, fragmentations from the normal mature cotyledon were compared to investigate their embryonic callus-forming capacities. The reuse abilities of NE, ECE, VE and FCE formed during germination of grapevine somatic embryos were further tested. To this end, we established a recycling system for production of cyclic embryogenesis in 'Chardonnay' which can be used to scale up the frequency of embryo multiplication through repeated subculture of matured embryos. The findings presented here would be conducive to mass propagation and genetic manipulation associated with somatic embryogenesis, thereby holding great promise for its improvement in grapevine.

Materials And Methods

Plant materials and pretreatments

The 7-year-old *V. vinifera* L. cv. 'Chardonnay', grown at the Chateau located in Yinchuan, Ningxia Province (106°15' E, 38°49' N). The unopened flower buds were collected at about 16:00 h on May 19, 2019 (7-12 d before grapevine flowering) and stored in 4 °C. The immature flower buds were washed in running water for 8 h, surface disinfected in 70% ethanol for 30 s on the clean bench, washed 3 times in sterile water, then immersed in 1.5% NaClO for 20 min, washed 5 times in sterile water, placed in sterile triangular bottles for 24 h at 4 °C, and then sterilized for a second time.

Induction of embryonic callus tissue

The unopened flower buds were excised from the flower stalk and inoculated on the six media (Table 1), and cultured at 26 °C in the dark, and subculture were performed once every 4 weeks. Each medium was added with 0.5 g/L activated carbon, solidified using 3.0 g/L phytigel, pH was adjusted to 5.8-6.0 with NaOH, and sterilized at 121 °C for 25 min. The inoculation contamination rate was counted after 1 week, and the embryonic tissue induction rate was counted after 5 months. Embryonic callus induction rate (%) = number of embryonic callus tissues/total number of inoculated explants × 100. All experiments were repeated three times, with no less than 100 inoculation replicates each time.

SEM observation of callus, embryonic callus and somatic embryos

Observation of microstructure for different types of tissues was performed in the SEM according to the method described previously (Liao et al., 2011). The callus tissues, embryonic tissues, brown stain tissues, vitrification tissues, PEMs and somatic embryos in healthy growth were selected and placed in 2.0 mL centrifuge tubes, fixed, dehydrated, dried and gold sprayed with reference to the method of (Liao et al., 2011). The typical cell structures were photographed and recorded using a SEM (HITACHI Regulus 8100, Japan).

Somatic embryo re-induction of embryonic callus

Somatic embryos with healthy growth were selected for embryonic callus reinduction. The 0.5 cm of cotyledon and embryo axe were cut from the healthy somatic embryos (about 1.0-1.5 cm), and inoculated onto MC, PIV, and CIM media and cultured at 26°C in the dark. The culture time and status of embryonic callus tissue development were recorded, and the rate of embryonic tissue induction was counted after three months of induction. Each experiment was repeated three times, with at least 50 explants inoculated each time.

Cotyledons, embryonic axes, and radicles induce embryonic callus

The healthy and normal somatic cotyledon embryo of approximately 1.0-1.5 cm were selected and cut into cotyledons, hypocotyls and radicles, respectively, and cultured on CIM medium. Embryogenic callus

induction was carried out under dark conditions at 26°C. The occurrence time and callus induction rate was recorded three months later. Each experiment was repeated three times.

Different types of somatic embryos for induction of embryonic callus tissue

Somatic cotyledon embryos formed in different status during germination were phenotypically classified into NE with normal development of the cotyledonary embryo axis, ECE with rapid development of the embryo axis, VE with high water content and easy browning at a later stage, and fused FCE with deformed cotyledons and embryo axis, respectively. These four types of cotyledon embryos were cut and cultured on CIM medium, and the time of embryonic callus tissue development was recorded and counted after three months. Embryonic callus tissue induction rate was recorded and counted after three months. Each experiment was repeated three times.

Statistical analysis

All experiments were repeated three times, and the data were analyzed by one-way ANOVA with Duncan's multiple comparisons with LSD test using SPSS 24.0 software (IBM, Chicago IL, USA). Descriptive statistical values were expressed as mean $\pm$ standard deviation.

Results

Screening medium for embryonic callus tissue induction

To explore the effect of different medium on embryonic callus induction of 'Chardonnay', PIV, MC, CIM and MEL1, MEL2, MEL3 media were selected for the test. As shown in Fig. 1, the initial callus tissues induced on the six media had no significant difference in status. Our statistical analysis of the callus tissue induction rates demonstrated that the induction rates on MC, MEL1 and MEL2 media were 85%, higher than the others (70%). After 6 months of induction, MC and PIV medium were observed to be benefit for the formation of golden yellow granular embryonic callus tissues with the induction rates of 9.52% and 8.63%, respectively. In contrast, CIM medium with picloram did not induce the formation of embryonic callus tissues and dried-up with browning at a later stage. Among the three melatonin media, the medium supplemented with 2.0 mg/L melatonin was beneficial to somatic embryogenesis and growth, with an induction rate of 5.06% (Table 2). This data suggests that the induction of 'Chardonnay' embryonic material varied greatly among different media. The results demonstrate that the suitable media is important for induction of 'Chardonnay' embryonic material.

SEM observation of primary callus tissue, embryonic callus tissue and somatic embryo

To further evaluate the ultrastructural differences, SEM observations of grape tissue cultures were made to further document the developmental events occurring during somatic embryogenesis of 'Chardonnay'. Results from SEM observations indicated that the unopen flower buds could be induced to produce yellowish initial callus tissues (Fig. 2A); and during successive cultures, two types of non-embryonic callus tissues were formed, namely loosely organized water-stained callus tissues and browning callus

tissues (Fig. 2B, C); and tightly organized golden-yellow embryonic callus tissues (Fig. 2D). The embryonic callus tissues differentiated to form granular beige protoembryo clusters and then formed different states of somatic embryos (Fig. 2E, F). Our results showed that the initial callus tissues consisted of smooth spherical granular surfaces with irregularly shaped cell structures (Fig. 2G); the water-stained callus tissues had loose surface structures with broken and irregularly shaped cells; and the browning callus tissues formed in a more scattered manner and eventually turns brown and died. Therefore, from the microstructure, it can be seen that the non-embryonic callus tissues showed rough and highly irregular cell shapes with more surface coverings (Fig. 2H, I); the embryonic callus tissues were tightly bound, with smooth and uniformly sized surfaces, and mostly showed round spherical granular shapes (Fig. 2J). The proembryonic clusters were similar to the embryonic callus tissues in microstructure, but the cells were larger and some of them were elongated, which in turn led to the formation of somatic embryos at various stages, namely, spherical embryo (GE), heart-shaped embryo (HE), torpedo embryo (TE) and cotyledon embryo (CE). This could provide a reference for determining the phenotypic structure of different tissues (Fig. 2K, L).

Effect of different media on the induction of embryonic callus in somatic embryos

To improve the reuse and transformation efficiency of somatic embryos in the regeneration pathway of 'Chardonnay', the cotyledon and embryo axes from mature normal cotyledon embryos were cut and cultured in MC, PIV and CIM to induce embryonic callus, respectively. Among these media, CIM medium was conducive to the formation of golden yellowish embryonic callus tissue with normal structure and tight-firmed tissues, which induced the formation of the callus with faster growth (Fig. 3Ac1-c3). However, MC and PIV medium induced poorly, forming more flocculate, loose non-embryonic callus (Fig. 3Aa1-a3,b1-b3), as indicated by the red arrow, Furthermore, our statistical results show that the induction rate of tight embryonic callus tissues in CIM medium was 49.3% higher than in MC (22.3%) and PIV (8.6%) media after 3 months in culture. These data reveal that CIM medium is potentially useful to further induce embryonic callus tissue when compared to MC and PIV medium, thus selecting for the follow-up experiments.

Effect of different parts of the somatic embryo on embryonic callus reinduction

To further investigate the effect of different parts of normal mature cotyledon embryos on the induction of embryonic callus tissue, we test the abilities of the cotyledons, embryonic axes and embryonic roots of somatic embryos for cyclic regeneration of somatic embryos in CIM medium. As shown in Fig. 4A, cotyledons could be induced to callus tissue, where occurred at the wounds (Fig. 4Bg) and displayed more loosely organized (Fig. 4Bb,e,h). Besides, the cotyledon also expanded and partly become green after 14 day of culture. Notably, the embryonic axis induced callus tissue formation faster than the others, and all can form callus tissues after 14 days of culture (Fig. 4Bi). Similarly, the embryonic root could also generate callus tissues from the position of wounds (Fig. 4B). Our statistical results showed that cotyledons and embryonic axes had the highest induction rate of more than 80%, whereas embryonic roots induced only a smaller number of callus tissues with the induction rate of 16.67%, and all dried up

gradually and even died at the later stage of subculture (Fig. 4C). These results show that both cotyledon and embryonic axis are suitable for re-induction of callus tissues.

Effect of different types of somatic embryos on cycle induction

To investigate the reuse ability of NE, embryo axis ECE, VE and FCE formed during germination of grapevine somatic embryos, the four embryo cuts were placed on CIM medium that has been confirmed as the better somatic embryo reuse to observe and count the induction of embryonic callus tissue. As depicted in Fig. 5, the cotyledon- and axis-cut from normal embryos started to enlargement accompanied by callus formation after 2 weeks of culture. After 1 month of induction, golden yellow embryonic callus was produced faster, some of which could directly produce proembryonic clusters, and some which were browned and then produced proembryonic clusters with the highest induction rate of 8.42%. Conversely, although the embryonic axis elongated embryos could form loosely organized yellowish callus tissues after 1 month of induction culture, but no embryonic callus tissues were observed in the following subculture. We also found that both the cotyledons and embryonic axes from cotyledon fused embryos were deformed and expanded, resulting in the low efficiency induction embryonic callus tissues (4.44%), while the rest of the tissues were mostly dried and browned. Due to the browning and drying in a later stage of culture, the vitrification embryos were observed to be seriously prevented the induction of somatic embryo reinduction (Fig. 6A, B). Interestingly, when they were placed in X6 medium to subculture, the white-color primary embryo mass or secondary somatic embryos were formed directly from the dried and browned tissues with normal morphological structure (Fig. 6C, D). These results show that the efficiency of cotyledons and embryonic axes from four different types of cotyledon embryos in the induction of embryonic callus tissues differs, while providing a theoretical reference for the induction of embryonic callus tissues using malformed embryos.

Discussion

Somatic embryo regeneration pathway in grapevine is likely influenced by several factors, resulting in the low efficiency of embryonic cell induction, high variation among varieties, difficulty in maintaining and expanding embryonic cells, as well as high frequency of malformed embryo occurrence in this process (Quiroz-Figueroa et al., 2006; Zhou et al., 2014). A large number of studies have been reported on the establishment of grape regeneration systems, targeting for different influencing factors. Currently, the type of explants in the grapevine somatic embryo regeneration pathway largely affects the efficiency of somatic embryo induction. So far, anthers and ovaries are considered to be the most suitable explants for the induction of somatic embryogenesis in many Eurasian *Vitis* species (Nakano et al., 2000; Cadavid et al., 2008). However, the high workload and tedious operation during anther/ovary inoculation, and the short flowering period of grapes largely limit the induction of embryonic callus tissue. It was also demonstrated that the induction of embryonic callus tissues with small flower buds was slightly more efficient than anthers and proved that immature floral organs have a strong potential for embryogenesis, which can be used as better explants for the establishment of somatic embryo regeneration system (Zhou et al., 2014; Dai et al., 2015).

Previous studies have shown that the culture medium with the befitting hormone is essential for induction of somatic embryo (Carimi et al., 2005; Gribaudo et al., 2005; Dai et al., 2015). It was reported that the medium added to 2 mg/ L picloram was superior for embryonic culture growth (Dai et al., 2015), and in (Carimi et al., 2005), the presence of BA and NOA in the medium was essential for induction of somatic embryogenesis. These studies indicated that the medium with hormone was important for induction of somatic embryogenesis. In the present study, we sought to determine whether the effects of six media were different in the induction rate of embryonic callus tissues. Among them, PIV and MC media have been previously shown to be commonly used for grapevine embryonic callus tissue induction and were all effective for different grape varieties (Franks et al., 1998; Gribaudo et al., 2004; Kikkert et al., 2005; Agüero et al., 2006). Our result demonstrated that the unopened flower buds from 'Chardonnay' could be induced to occur golden yellow embryonic callus tissues on MC or PIV medium, which is consistent with the previous report (Dai et al., 2015). It was noted that 'Chardonnay' were induced to form embryonic callus tissues on PIV medium and then secondary cultured in Harst medium (MS + 10 µmol/L 2,2-D + 5 µmol/L TDZ + 30 g/L Sucrose + 3 g/L Phytigel) is more conducive to the production of more proliferative embryogenic callus tissues (Iocco et al., 2001). In our experimental system, the callus tissues formed on CIM medium were mostly dry and brown and gradually died, which was inconsistent with (Dai et al., 2015). Therefore, it was speculated that it might be related to the quality of explants or the geographical characteristics of grape varieties.

It has been well-documented that melatonin has similar physiological functions to IAA, and has a promotional role in plant morphogenesis and somatic embryo regeneration (Kolář et al., 2005; Pelagio-Flores et al., 2012; Sarropoulou et al., 2012). Compared the induction effect of melatonin at different concentrations on callus tissues, we observed that melatonin shortened the time taken to produce callus tissues by bud dedifferentiation, and the induction efficiency of melatonin at different concentrations was high. In particular, the induction rate of 2 mg/L melatonin reached 91.74%, and somatic embryos were formed after 5 months. Embryogenic callus grew faster in this medium than in the other media tested. This result is consistent with a previous report describing in *Brassica juncea* (Chen et al., 2009) and *Coffea canephora* (Ramakrishna et al., 2012). However, the rate of callus tissue and somatic embryogenesis varied considerably under different melatonin concentrations, which is different from the results of a previous study on the effect of melatonin on somatic embryos of 'Thompson Seedless' grapes (Ya et al., 2020). This is most likely related to the large differences in the induction efficiency of different grape varieties under the same medium (Gambino et al., 2005; Li et al., 2006).

During the establishment of the grapevine somatic embryo regeneration system, it tends to occur a large number of somatic embryos accompanied by the frequency of morphologically deformed embryos occur in large numbers. Moreover, the use of somatic embryos for secondary embryo regeneration has a significant effect on the induction of the original embryo mass and a high induction efficiency, which can provide a rich new receptor source for grapevine genetic transformation. At present, there are few studies reported on the secondary induction of somatic embryos in *Vitis vinifera*. The torpedo embryos and mid-term cotyledon embryos derived from 'Thompson Seedless' were reported to be induced on the induction medium ECRM1 and ECRM3 medium to produce the highest number of secondary embryonic callus

tissues (Zhou et al., 2014). Another example showed that the germinating somatic embryos in 'Thompson Seedless' were grown in KBN medium could effectively induce secondary embryogenesis and the induction rate was as high as 83% (Cheng et al., 2017).

The occurrence of malformed embryos during somatic embryo germination and seedling establishment seriously hinders the application and development of grape regeneration. We observed four types of somatic cotyledon embryos, including the normal, elongated, vitrification and cotyledon fusion embryos. Taking into account the full use of these different types of cotyledon embryos, we sought to establish a cyclic somatic embryo regeneration system with them. Our results showed that normal and cotyledon fused embryo could be induced to produce embryonic callus tissues and differentiate into proto-embryonic clusters to complete the regeneration and germination of somatic embryos. Similarly, Ji et al. (2017) reported that the 'Thompson Seedless' cotyledon fused embryos derived from 'Thompson Seedless' could be induced to embryonic callus tissues (Ji et al., 2017). For the normal somatic cotyledon embryos, three parts of cotyledons, embryonic axes and embryonic roots could be induced a looser structure of callus tissues with high induction efficiency. The induction rate of embryonic roots was poor and the callus tissues were all located at the wounds of embryonic roots and embryonic axes, which is similar to (Ji et al., 2017). However, we found that the cotyledon elongated embryo failed to induce the formation of embryonic callus tissues. Concurrently, vitrified embryos are hyaline and water-stained and gradually brown and dry at a later stage, which is not suitable for somatic embryo cycle induction studies. When the vitrification embryos were placed in X6 medium for succession culture, it was found that the surface of the dried somatic embryos produced secondary proembryonic clusters or secondary somatic embryos, which may be closely related to the fact that X6 medium without added hormones facilitates the formation of somatic embryos (Franco et al., 2006).

In conclusion, we optimized the somatic embryo regeneration system of wine grape 'Chardonnay' by regulating plant hormones, judging the type of callus tissue by SEM, and exploring the recycling of malformed embryos by somatic embryo cycle induction system. We have developed a rapid and highly effective protocol for establishment of a recycling system for production of cyclic embryos in 'Chardonnay' as the Fig. 7.

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

Abbreviations

MS Murashige and Skoog medium

IAA indoleacetic acid

BA Benzylaminopurine

TDZ Thidiazuron

NE normal embryo

ECE embryo axis elongation embryo

VE vitrification embryo

FCE cotyledon fusion embryo

SEM Scanning electron microscopy

LSD Least-Significant Difference

GE spherical embryo

HE heart-shaped embryo

TE torpedo embryo

CE cotyledon embryo

CIM Callus inducing medium

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Tables

Table 1 Callus induction medium of *V. vinifera* cv. Chardonnay

Medium	Basal salts	2,4-D mg/L	NOA mg/L	Melatonin mg/L	Picloram mg/L	BAP mg/L	4- CPPUmg/L	sucrose g/L
PIV	NN69	1.0	/	/	/	2.0	/	60
MC	NN69	0.55	0.5	/	/	/	1.24	30
CIM	MS	0.5	/	/	3.0	2.0	/	30
MEL1	NN69	/	/	1.0	/	2.0	/	30
MEL2	NN69	/	/	2.0	/	2.0	/	30
MEL3	NN69	/	/	3.0	/	2.0	/	30

Table 2 Statistics of different mediums on growth of embryogenic callus of *Vitis vinifera* cv. 'Chardonnay'.

Medium	No. cultured	Induction rate of callus%	Induction rate of EC/SE%
PIV	336	74.21±4.08 b	8.63 (PEM)
MC	483	87.30±8.04 a	9.52 (PEM)
CIM	147	72.62±5.19 b	0
MEL1	210	86.08±4.98 a	0
MEL2	336	91.74±3.52 a	5.06 (SE)
MEL3	168	73.45±2.46 b	0

Values represent combined results from three independent experiments; a, b represented that the induction rates of different mediums were extremely significant $p < 0.05$.

Figures

Figure 1

Effects of different mediums on growth of embryogenic callus of *Vitis vinifera* cv. 'Chardonnay'. The red linear represents proembryogenic masses (PEM) or somatic embryos (SE), Scale bar=0.5 cm.

Figure 2

Morphological observation of different tissues during the the process of somatic embryo regeneration of *Vitis vinifera* cv. 'Chardonnay'. A. Initial callus (IC); B. Vitrified callus (VC); C: Brown callus (BC); D: Embryonic callus (EC); E. Pro-embryogenic masses (PEM); F. Somatic embryo (SE) (Scale bar = 0.5 cm); G-K. Scanning electron micrographs of IC, critical stages (VC-BC-EC-PEM) (Scale bar=100 μ m) ; L. Scanning electron micrographs of SE. Scale bar=0.5 mm.

Figure 3

Effects of different media on somatic embryo-induced callus of *Vitis vinifera* cv. 'Chardonnay'. A. Somatic embryos were placed in different media (CIM, MC, PIV) to induce callus; B. Different induction rates of three of type somatic embryos inducing callus in different states by different medium. Scale bar=0.5 mm.

Figure 4

Callus induction in different parts of the cotyledon embryo of *Vitis vinifera* cv. 'Chardonnay'. A. Somatic embryo grew normal cotyledonary embryos; B. Callus induction in different parts of somatic embryo; C. The induction rate of callus. (a, d, g) Callus induced by cotyledons of Chardonnay were enlarged and partly greenish after 2 weeks (Fig. d). Callus was formed at the wound site (Fig. g) and was more loosely organized. (b, e, h) Callus induced by hypocotyls formed rapidly, and callus were formed after 2 weeks, and the state was similar to that induced by cotyledons (Fig. h). (c, f, i) Radical-induced callus were all formed at the site of the hypocotyl wound (Fig. f). Scale bar=0.5 mm.

Figure 5

Cotyledon embryo-induced callus of *Vitis vinifera* cv. 'Chardonnay'. The red linear indicated proembryogenic masses or somatic embryos. Scale bar=0.5 mm.

Figure 6

Somatic embryos regeneration of vitrified embryoids. A. Brownd vitrified embryos; B. Vitrified embryos with normal cotyledons and browning hypocotyls; C-D. Proembryogenic masses or somatic embryos grow on the brownd vitrified embryos. The red linear indicated proembryogenic masses or somatic embryos. Scale bar=0.5 cm.

Figure 7

The protocol for recycle the normal embryos of *Vitis vinifera* cv. 'Chardonnay'. Cotyledon of normal embryo from somatic embryo germination placed in CIM medium (A); Pro-embryogenic masses (PEM) formed; Clusters of somatic embryos formed (C); (☐) Global somatic embryos; (☐) Somatic embryo of heart

shape stage; ☐ Somatic embryo of torpedo shape stage; ☐ Hypocotyl elongated somatic embryos); The embryo germinated (D) and converted into plant (E).