INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0653/2018/20–12–2835–2841
DOI: 10.17957/IJAB/15.0842
http://www.fspublishers.org

Full Length Article

Improved Embryogenic Callus Induction and Histological Analysis of the Embryogenesis in Callus Culture from Loquat Anther (*Eriobotrya japonica*)

Li Junqiang[1,2], Lin Lihua[2], Qin Hongmei[1], Wang Yongqing[1], Deng Qunxian[1], Wang Bo[1] and Hua Zong[3*]
[1]*College of Forestry and horticulture, Sichuan Agricultural University, Ya'an 625014, Sichuan, China*
[2]*Department of Bioengineering, Yibin Vocational & Technical College, Yibin 644003, Sichuan, China*
[3]*Department of Landscape, Southwest Jiaotong University, Chengdu 610031, Sichuan, China*
[*]For correspondence: huangjiaqiutian@aliyun.com

Abstract

In this study, three loquat cultivars (*Eriobotrya japonica* L.) cultivars *viz*. 'Longquan1', 'Dawuxing' and 'Zaozhong6' were selected for *in vitro* anther culture. To improve the induction rate of microspore-derived callus, different pretreatments and carbohydrates were evaluated. Results showed that cold pretreatment significantly enhanced callus formation from anther microspores compared to other pretreatments. The average callus induction rate was 61.11% out of 450 anthers with cold pretreatment. The inoculation density of anthers had no significant effect on the survival of anthers and callus induction, but contacted faces of the anther brought different influence on the callus induction. Sucrose was more advantageous for embryonic callus induction than glucose. The capacity of dedifferentiation and calli derived from anther wall were seriously inhibited by the increased concentrations of sucrose. Media with more than 5% sucrose ensured most of calli to originate from microspores and inhibited anther wall to form calli. However, glucose failed to inhibit anther-wall-derived callus. Under the optimal concentration of sucrose the callus induction rate of 'Zaozhong6'anthers was the highest (74%), followed by 'Longquan 1'(50.67%), and was the lowest in 'Dawuxing' (29.33%). Histological observations manifested great differences between the embryogenic and non-embryonic calli. The embryonic cells were arranged compactly with a larger karyoplasm and thick cytoplasm, and showed a vigorous division. The development of loquat anther embryos was similar with zygotic embryo, through the globular, the heart-shaped, the torpedo and the cotyledonary embryos stages. Between the heart-shaped and the torpedo-shaped stage, vascular tissue initially formed. At the torpedo stage, the V-shaped vascular bundle appeared. Then at the cotyledonary stage, the V-shaped vascular bundle was clearly observed.

Keywords: *Eriobotrya japonica* L; Anther culture; Microspore; Origin of callus

Introduction

Biotechnological methods are well known as efficient tools for obtaining new variability, which can improve the efficiency and increase breeding speed. Anther culture, originally developed by Guha and Maheshwari (1964) allowed rapid production of haploids, and subsequently chromosome doubling could lead to completely homozygous diploid plants (Bajaj, 1990). Compared with conventional inbreeding, the *in vitro* androgenesis technique enables a faster generation of virtually fully homozygous lines (Aulinger *et al*., 2003). Nowadays this technique has been used for more than 250 species, belonging to 100 genera and more than 40 families, but most of them are herbs, such as cabbage (Cilingir *et al*., 2017), wheat and rice (Eshaghi *et al*., 2015; Naik *et al*., 2016). Compared with herbs, many woody plants are heterozygous and rather recalcitrant, thus only a few of them efficient *in vitro* protocols to induce anther embryogenesis have been reported (Bueno *et al*., 2005). In fruit crops, anther culture has been successfully applied in *Eriobotrya japonica* L., *Citrus madurensis* L., *Musa balbisiana* Colla, *Poncirus trifoliata* L., *Annona squamosa* L. and *Carica papaya* L. However, all of them were not stone fruit species.

Anther culture is popularly used to produce haploid, but sometimes plants regenerated from cultured anthers show changes in chromosome numbers and get polyploidization. That is because there are a number of factors that affect androgenesis, including plant age, genotype and physiological state of the donor plant, anther age and pollen developmental stage, pre-culture treatment, physical factor including temperature, light, atmospheric condition, anther density, orientation, pH and chemical factor such as culture medium, sugar and plant growth regulator (Tyankova and Zagorska, 2008).

To cite this paper: Junqiang, L., L. Lihua, Q. Hongmei, W. Yongqing, D. Qunxian, W. Bo and H. Zong, 2018. Improved Embryogenic Callus induction and histological analysis of the embryogenesis in callus culture from loquat anther (*Eriobotrya japonica*). *Int. J. Agric. Biol*., 20: 2835–2841

Pretreatment is a key point in the system of microspore embryo formation. Stress pretreatments is usually applied to the isolated microspores, which must be obtained from buds, spikes and whole explants with strong viability (Würschum *et al*., 2014). That is because stress pretreatment could switch isolated microspores development from the gametophytic pollen pathway to a sporophytic pathway (Touraev *et al*., 1997). So far, a variety of stress pretreatment, such as cold shock (Moraes *et al*., 2004), heat shock (Abdollahi *et al*., 2005, Eshaghi *et al*., 2015), carbon source (Yadollahi *et al*., 2011), nitrogen starvation (Cimò *et al*., 2017), salt (Liu *et al*., 2016), and colchicine (Mohammadi *et al*., 2011), had been proved to bring effect on microspore embryogenesis induction. For many plant species with anther culture, cold pretreatment was very effective to induce embryogenesis callus (Maheshwari, 1996).

Loquat (*Eriobotrya japonica* L.), originated in China (Zhang *et al*., 1990) is a widely grown fruit and ornamental tree in subtropical and Mediterranean climatic area. Nowadays more than 300 Loquat varieties are cultivated in China. However, due to the large loquat seed, the thin flesh and the low edible rate, it is imperative to cultivate small-core, low-nuclear or non-nuclear loquat varieties. Conventional methods were difficult to achieve the seedless loquat varieties. Haploid plants are good breeding materials, and triploid plants can produce seedless fruits. But anther culture *in vitro* has been limited and insufficient for the recalcitrance of loquat. Germanà *et al*. (2006) established a protocol for the formation of multicellular pollen only. In our former researches, we built a complete technique system for androgenesis, plant regeneration and haploid production from anther culture in loquat for the first time (Li *et al*., 2008). However, the callus induced rate was from 3.33% to 10.00% with 14% diploids regenerants (Li *et al*., 2008). Moreover, later researches were focused on the recognition of the best pollinizers (Sharafi *et al.*, 2011) and the exploration of the various levels of growth regulators in the anther culture medium (Blasco *et al*., 2015). Thus, the aim of the present work was to adjust the pretreatment methods and carbohydrates to improve the calli induction rate and ensure microspore derived calli from the cultured anthers.

Materials and Methods

Plant Material and Pretreatment

The buds (0.3-0.8 mm diameter) of three six years old healthy loquat cultivars (*Eriobotrya japonica* L）. cv. 'Longquan1', cv. 'Dawuxing', cv. 'Zaozhong6' were obtained from Ya'an City, Sichuan Province, China, which were used for *in vitro* anther culture. The buds were washed under running tap water and pretreated with cold shock (4℃ for 24-240 h), hot shock (35℃ for 6 h), osmoticum (0.7% PEG800 for 24 h), and centrifugation (2000 r/min for 12 h). Scraped off the thick floss on the sepal, and the buds were sterilized by immersion in 70% (v/v) ethanol for 30 sec followed by immersion in 0.5% (w/v) mercuric chloride solution for 8 min and rinsed 5-6 times with sterile distilled water. Finally, the petals were removed and dissected. Quickly removed the filament, incomplete and abnormal anthers were discarded. The anthers were randomly picked out for the microscopic examination.

Anther Density and Inoculation Method

The number of anthers per Erlenmeyer flask was varied by culturing 5, 10, 15 or 20 anthers, five replicates for each anther density were carried out. Anthers were contacted with medium by front face (Fig. 1A), back face (Fig. 1B) and side faces. The explants were observed and subcultured at four weeks interval. All experiments were conducted at 25℃ in the darkness.

Carbohydrates and Concentrations on Callus Induction Media

After 48 h of cold shock, MS medium containing different carbohydrates (sucrose and glucose; 3, 5, 7 and 9%), supplemented with 6-BA at 2.0 mg/L, 2,4-D at 0.50 mg/L and solidified with 0.6% (w/v) agar were used for callus induction. After 4 weeks culture, embryogenic callus was transferred to differentiation MS medium containing ZT at 0.05 mg/L, NAA at 0.01 mg/L and IBA 0.02 mg/L. The pH was always adjusted to 5.5 before autoclaving (121ºC/20 min).

Histological Observations

The anther culture sometimes showed changes in chromosome numbers and gets polyploidization. Histological studies were used to confirm callus origination. Cultured anthers and callus were collected at one week interval and fixed in FAA (70% ethanol:formalin:acetic acid, 18:1:1 v/v/v), rinsed, dehydrated from 50% to 90% ethanol, Ehrlich's hematoxylin stained (Ehrlich, 1886) for 2 days (until it becomes a deep purple red) and embedded in paraffin wax (56-58ºC) using classical techniques(Li, 1987). Sections (10 μm thick) were cut using a rotary microtome, and deparaffinized in 2-3 changes of xylene, hydrated in 2 changes of 100% ethanol for 3 min each, 95% and 80% ethanol for 1 min each, then rinsed in distilled water. At last, the sections were observed under a light microscope and photographed by a digital camera (Motic BA200).

Statistical Analysis and Collection of Picture

Pictures of buds, anthers and callus were from Motic stereoscopic microscope. Histological character of callus was observed under Motic BM200. All data were statistically analyzed using Duncan's multiple range tests implemented in SPSS (ver.11.0).

Results

Effect of Pretreatment on Callus Induction in Loquat Cultivars

As shown in Table 1, no calli were formed without pretreatment, while pretreatment was the key factor to restrict callus formation. Calli showed more significant responsiveness (61.11%/450) to cold pretreatment than others for callus formation. The browning rate of callus was increased with the extended time of cold pretreatment. When the cold pretreatment time was longer than 7 day (168 h), all the anthers became brown in the next week and died in 3 weeks later. In addition, average 15.78% of 189 anthers pretreated with hot shock formed callus. Less calli formation was observed with osmoticum and centrifugation pretreatment. Above all, 48 h of cold shock was the best pretreatment for loquat anther callus induction.

Effect of Anther Density and Inoculation Mode on Callus Induction

The results showed that the inoculation density of anthers had no significant effect on the survival of anthers and callus induction, but contacted faces of the anther brought different influence on the callus induction. The callus induction was from high to low as follows: anther front contact medium > anther back contact medium > anther lateral contact medium. The time of callus induction was shortest from anther front contacted medium. But in the later culture, the callus from anther front contacted medium grew lower than which was from anther back contacted medium.

Effect of Carbohydrates and Concentrations on Callus Induction

The callus induction rate of ‘Zaozhong6’ anthers was the highest (74.00%), followed by 'Longquan 1' (50.67%), and 'Dawuxing' lowest (29.33%), which was obviously higher than callus induction rates (3.33% -10.00%) as previously reported. The loquat cultivars showed a consistent effect of different carbohydrates and their concentrations on callus formed (Table 2). Anthers inoculated on induction medium supplemented with sucrose became light brown in 2-3 days, and then light yellow callus began to form in 5-10 days. Kinds of calli with different textures were observed as follows: crisp callus from medium supplemented with 3% sucrose (Fig. 1C), soft callus from 5% sucrose (Fig. 1D), delicate callus from 7% sucrose (Fig. 1E), compact callus from 9% sucrose (Fig. 1F). In addition, high concentration of sucrose showed a capacity of inhibiting redifferentiation from anther wall. Such as on the medium added 3% sucrose, both anther wall and microspores formed the callus simultaneously. However, on the medium added with 5% sucrose, most of the calli were from microspores and grew fast, which partially inhibited anther wall to develop into callus (arrows in Fig. 1C).

Table 1: Effects of different pretreatments on callus induction from anthers after 4 weeks culture

Pretreatment	Callus from anther					
	cv. ‘Longquan1’		cv. ‘Dawuxing’		cv. ‘Zaozhong6’	
	No.	%	No.	%	No.	%
CK (no pretreatment)	0	0 d	0	0d	0	0 d
Hot shock	72	14.00 b	45	15.00 b	72	18.33 b
Cold shock	169	56.33 a	159	53.00 a	222	74.00 a
Osmoticum	15	5.00 c	40	13.33 bc	20	6.67 c
Centrifugation	14	4.67 c	22	7.33 c	23	7.67 c

Results are means ± standard deviations (SD). Values followed by letter are significantly different at P≤0.05 according to Duncan’s tests

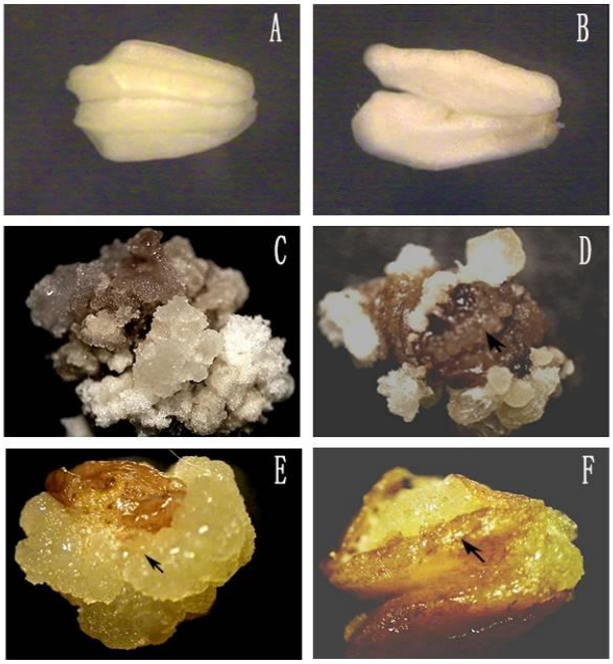

Fig. 1: A. The front of the loquat anther; B. The back of the loquat anther; C. Callus derived from anther wall and microspores (MS containing 0.5 mg·L^{-1} 2,4-D and 3% sucrose) after 3 week of culture (the arrowhead indicated the anther wall) ; D. Callus derived mainly from microspores (MS containing 0.5mg·L^{-1} 2,4-D and 5% sucrose); E. Callus induction from cultured anther (MS containing 0.5mg·L^{-1} 2,4-D and 7% sucrose) after 3 week of culture (the arrowhead indicated the anther wall); F: Callus induction from cultured anther (MS containing 0.5mg·L^{-1} 2,4-D and 9% sucrose) after 3 week of culture (the arrowhead indicated the anther wall)

On the medium added with 7% sucrose, callus derived from anther wall was restricted completely (arrows in Fig. 1D), then the inhibited anther wall became brown. When the sucrose concentration was higher than 7%, both the anther wall and microspores callus formation was inhibited, which became brown later on. A white callus was observed on the medium with glucose in 14-17 days (taking a longer time than sucrose).

Table 2: The effects of different carbohydrates and concentrations on callus induction of anthers after 4 weeks culture

Carbohydrate (%)	No. of anthers cultured	Days of callus formation	Anthers forming calli						Origin of callus	Callus characteristics
			cv. 'Longquan1'		cv. 'Dawuxing'		cv. 'Zaozhong6'			
			No.	%	No	%	No.	%		
Not added	300	---	0	0C	0	0	0	0D	---	---
Sucrose (3)	300	5—7	116	38.67 AB	60	20.00 ab	201	69.00 A	+ =	granular
Sucrose (5)	300	5—7	152	50.67 A	74	24.67 ab	236	73.67 A	+	soft
Sucrose (7)	300	5—7	151	50.33 A	54	18.00 ab	224	74.00 A	+	delicate
Sucrose (9)	300	7—10	104	34.67 AB	44	14.67 b	212	70.67 A	+	compact
Glucose (3)	300	14—17	80	26.67 B	72	24.00 ab	69	23.00 C	+ =	granular
Glucose (5)	300	14—17	108	36.00 AB	88	29.33 a	139	46.33 B	+ =	soft
Glucose (7)	300	14—17	121	40.33 AB	72	27.33 ab	216	72.00 A	+ =	soft
Glucose (9)	300	14—17	95	31.67 AB	69	23.00 ab	43	14.33 C	+ =	soft

Results are means ± standard deviations (SD). Values followed by letter are significantly different at $P \leq 0.05$ or $P \leq 0.01$ according to Duncan's tests. ** +: microspore; + =: anther wall and microspore

The callus induction rate decreased with an increased glucose concentration, while the callus structure became denser. However, different concentrations of glucose failed to inhibit anther-wall-derived callus (Table 2).

Histological and Investigations of Induced Calli

In our research, the differences between non-embryonic callus and embryogenic callus were observed. The embryonic cells (Fig. 2A) were arranged compactly with a larger karyoplasm and thick cytoplasm, showed a vigorous division than non-embryonic cells (Fig. 2B). Moreover, embryonic cells volume was smaller than the non-embryonic cells, and its shape was more regular. Some of embryonic cells could be observed with 1-2 nucleoli. If the filaments were not completely removed, calli were originated from both micropores and filaments, which could result in diploid regenerants (Fig. 2C). It was also confirmed that callus from anther walls formation was not inhibited on the medium containing glucose. Most of calli were originated from microspores on the medium with 5% sucrose (Fig. 2D). After transferring for 12-14 days on differentiation culture, a series of smooth-surface multicellular proembryos were formed (Fig. 3A, B, C and D). After 18-21 days of culture, the multicellular proembryos further developed the globular embryo (Fig. 4A). A cluster of globular embryos (Fig. 4B and C) were observed after 25-28 days; and eventually formed a cluster of cotyledonary embryos (Fig. 4D). Histological studies revealed the development of embryogenic callus: globular embryos (Fig. 3E①), heart-shaped embryos (Fig. 3E②), torpedo-shaped embryos (Fig. 3G), and cotyledonary embryos (Fig. 3H). Between the heart-shaped and the torpedo-shaped stage, vascular tissue was initially formed (arrow in Fig. 3F). Then at the torpedo stage, the V-shaped vascular bundle appeared (arrow in Fig. 3G). At the cotyledonary stage, the V-shaped vascular bundle was clearly observed (arrow in Fig. 3H).

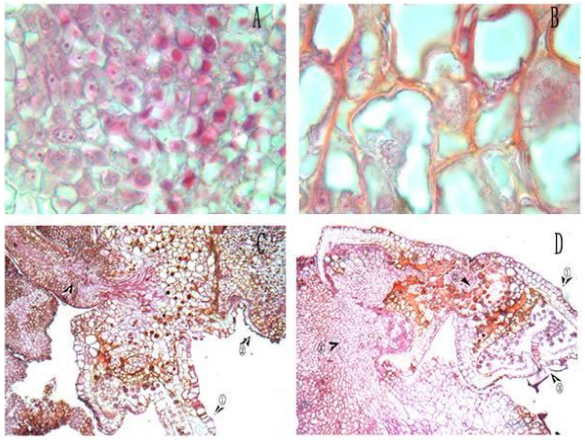

Fig. 2: A. Embryogenic cell (1000×); B. Non-embryogenic callus (1000×); C. calli derived from microspores and filament (①:anther wall; ②:calli derived from microspores; ③: callused derived form filament, 100×); D. Origination of anther derived callus (①: anther wall; ②: vascular bundle; ③: The opening of anther; ④: calli of microspores origination, 100×)

Discussion

In our study, it was observed that no callus formed without any pretreatment, which was proved that stress pretreatment was an indispensable protocol for loquat anther embryogenesis induction. In this research, cold shock for 2 days was achieved with a highest frequency of callus induction from anther microspores. Sato *et al.* (2002), Gu *et al.* (2004) figured out that cold pretreatment for 2-4 days initiated the most microspore embryogenesis than any other treatment used. However, the mechanism of beneficial effect on anther microspore embryogenesis by cold pretreatment is not clear. It is speculated that cold pretreatment delays anther senescence to ensure prolonged viability of microspore induction (Keller and Armstrong, 1979; 1983; Xu and Sunderland, 1983). Nitsch and Norreel (1973) suggested cold shock repositioned the spindles, which may change the microspore developmental pathway, or increase the number of inducible microspores (Duncan and Heberle, 1976). Vasil and Nitsch (1975) revealed that cold pretreatment could reduce the total level of metabolism in the anthers, which helped to accumulate large amounts of pollen grains suitable for the developmental period.

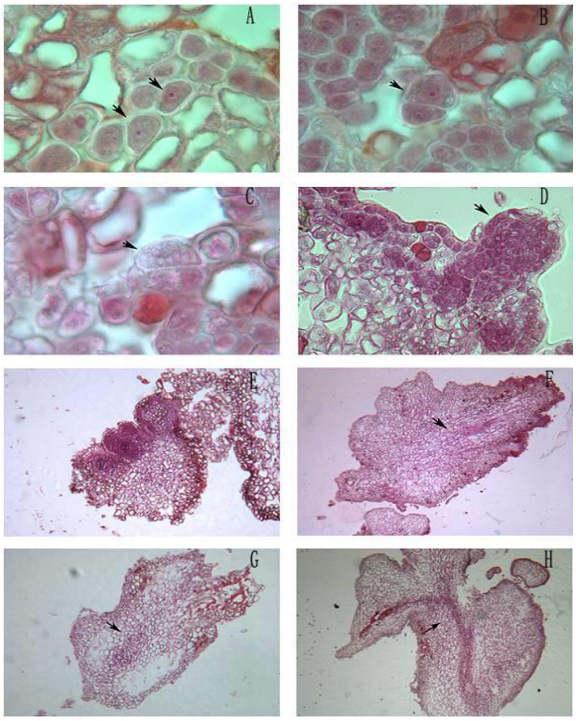

Fig. 3: Histological observation of the embryogenesis origin from embryogenic callus. A. One-cell proembryo (arrowheads, 1000×); B. Two-cell proembryo (arrowhead, 1000×); C. Four-cell proembryo (arrowheads, 1000×); D. Multi-cell proembryo (arrowheads, 1000×); E. Globular (①) and heart-shape embryos (②); F. Embryo developed between heart-stage and torpedo-stage with vascular bundle (arrowheads, 100×); G. A torpedo embryo with vascular bundle (arrowheads, 100×); H. A cotyledonary embryo with vascular bundle (arrowheads, 40×)

Mårtensson and Widell (1993) found an increase in specific peroxidase activity after cold pretreatment for tobacco. It was also reported the induction effect of osmotic stress under cold pretreatment conditions would be a possible reason for increasing microspore embryogenesis potential (Kiviharju and Pehu, 1998).

Cultured anthers release endogenous hormones and other metabolites that may affect embryogenesis, while the density of anthers in the culture vessel could play an important role in embryogenesis (George *et al*., 2008). An optimal callusing density was 6 anthers for *B. Campestris*, while a relatively high culture density (60 per mL) was crucial for *B. Napus* (Xu and Sunderland, 1983) and barley (Dunwell and Thurling, 1985). The number of calli developed was significantly greater at a density of 10 anthers per mL for coconut (Perera *et al.*, 2008). In our research, loquat anther density did not significantly affect androgenesis. In this regard, Arnison *et al.* (1990), Zhao *et al.* (2006) reported the number of embryos formed per responsive anther was independent of anther density.

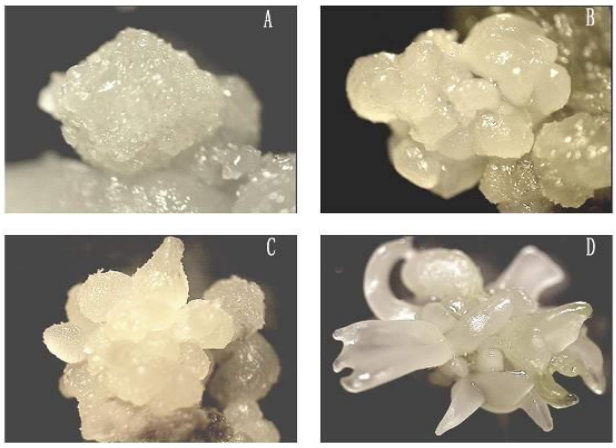

Fig. 4: Embryogenesis from cultured anther-derived callus of loquat (*E. japonica* L. cv. 'Dawuxing')
A. Embryogenic calli with proembryos; B and C. The development of a cluster of globular embryos; D. A cluster of cotyledonary embryos

Some reports showed that the inoculation method of anthers had a significant effect on embryogenesis. In the case of barley, when only the anther side was in contact with the solid medium, embryogenic callus was induced (Hunter, 1985; Shannon *et al*., 1985; Powell, 1988). In our study, anther front contact medium was faster to produce callus. The reason could be when the anthers were in contact with the medium in the front, the microtubule bundles were exposed to the medium, which helped the anthers to absorb the nutrients and hormones.

Nitsch (1969) believed that carbohydrate was necessary for microspore embryogenesis. In most cases, 2%-3% sucrose was optimal for embryogenesis. Sharp *et al.* (1971) pointed out that high level of sucrose could enhance the potential capacity of plant morphogenesis. Keller (1984) on *B. capestris* and Sopory (1979) on *Solanum tuberosum*, reported that the medium supplied with higher concentration of sucrose was more effective for embryo and callus induction. In addition, a relatively higher (8.0-9.5%) level of sucrose was improved the differentiation of microspores in the anther culture of maize (Pescitelli *et al*., 1990) and barley (Sorvari and Schieder, 1987). Instead of sucrose, maltose and glucose have been tried with only a few positive results (Trottier *et al*., 1993; Navarro-Alvarez *et al.*, 2010). Some of them were indicated that maltose was the best carbohydrate to induced embryogenic callus, especially in cereal crop (Mendoza and Kaeppler, 2002; Saeed *et al.*, 2017). In our studies, considering the inhibitory effect on pollen wall differentiation, sucrose was more advantageous for callus induction than glucose. In fact, the diploid tissue in the anther culture, including anther wall, filament and connective tissue, always hindered the growth of haploid and produced a more complicate ploidy level of regenerated plantlets. A higher concentration of sucrose seemed to supply an effective way to control the derivation of diploid in loquat anther culture.

The role of the cell wall was to maintain the cell shape to ensure the normal physiological activity (Roberts, 1994). Some reports demonstrated the cell wall was a dynamic structure that changes with the needs of cell growth and development (Showalter, 1993; Cassab and Varner, 2003). The separation of embryonic cells from the surrounding tissue by highly modified cell wall was the first step in embryogenesis (Chapman *et al*., 2000). Our histological investigations observed before the embryonic development, the embryonic cells in embryonic callus were physiologically isolated from the surrounding tissue by thickened cell wall, which supplied an independent environment for the embryonic cell. The developmental pathway of the embryonic cell mass, through globular embryos, heart-shaped embryos, torpedo embryoids, and cotyledonary embryos, was completely similar to that of zygotic embryos.

In addition, high level of management and expertise were required to produce (Perera *et al*., 2008). To obtain a haploid as much as possible, controlling the origin of callus must be a critical step in anther culture. In our study, we found anther wall and filament belong to unexpected diploid tissues, which formed callus easily in the loquat anther culture. Getting rid of filaments before inoculation, discarding wounded anther and using a higher concentration of sucrose (7%) proved to be three effective protocols to control origin of callus during the loquat anther culture.

Conclusion

In this research, the changes of inoculation method, pretreatment method and carbon source greatly improved the callus induction rate of loquat anthers. Histological observation could be helpful for identifying the origin and the process of anther callus and embryogenesis. Further study will focus on the effects of growth regulators on the dedifferentiation of anther callus and build a genetic transformation system based on the loquat anther embryogenesis.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (No.51308464).

References

Abdollahi, M.R., A. Moieni, P. Haddadi and M.J. Javaran, 2005. Effects of heat shock and culture density on the embryo induction in isolated microspore culture of *Brassica napus* L. cv. *Glob*. *Pak. J. Biol. Sci*., 8: 89–91

Arnison, P.G., P. Donaldson, A. Jackson, C. Semple and W. Keller, 1990. Genotype-specific response of cultured broccoli (*Brassica oleracea* var. *italica*) anthers to cytokinins. *Plant Cell Tiss. Org. Cult*., 20: 217–222

Aulinger, I.E., S.O. Peter, J.E. Schmid and P. Stamp, 2003. Gametic embryos of maize as a target for biolistic transformation: comparison to immature zygotic embryos. *Plant Cell Rep*., 21: 585–591

Bajaj, Y.P.S., 1990. *In vitro* production of haploids and their use in cell genetics and plant breeding. *In: Haploids in Crop Improvement*. Springer, Berlin, Heidelberg, Germany

Blasco, M., M.L. Badenes and M.D.M. Naval, 2015. Embryogenic response from anther culture of cultivars of loquat (*Eriobotrya japonica* (Thunb.) Lindl) from different origins. *Euphytica*, 205: 1–12

Bueno, M.A., B. Pintos, M. Höfer and A. Martin, 2005. Pro-embryos induction from *Olea europaea* L. isolated microspore culture. *Acta Physiol. Plant*., 27: 695–701

Cassab, G.L. and J.E. Varner, 2003. Cell wall proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 39: 321–353

Chapman, A.A.S., T. Blervacq, C. Hendriks, J. Slomianny and J.L. Hilbert, 2000. Cell wall differentiation during early somatic embryogenesis in plants. II. Ultrastructural study and pectin immunolocalization on chicory embryos. *Can. J. Bot*., 78: 824–831

Cilingir, A., S.M. Dogru, E.S. Kurtar and A. Balkaya, 2017. Anther Culture in Red Cabbage (*Brassica oleraceae* L. var. *capitata* subvar. *rubra*): Embryogenesis and Plantlet Initiation. *Ekin J*., 3: 82–87

Cimò, G., A. Marchese and M.A. Germanà, 2017. Microspore embryogenesis induced through *in vitro* anther culture of almond (*Prunus dulcis* Mill.). *Plant Cell Tiss. Org. Cult*., 128: 85–95

Duncan, E.J. and E. Heberle, 1976. Effect of temperature shock on nuclear phenomena in microspores of *nicotiana tabacum*, and consequently on plantlet production. *Protoplasma*, 90: 173–177

Dunwell, J.M. and N. Thurling, 1985. Role of sucrose in microspore embryo production in *Brassica napus* ssp. *oleifera*. *J. Exp. Bot*., 36: 1478–1491

Ehrlich, P., 1886. *Die Von Mir Herrührende Hämatoxylinlösung. Z. Wiss. Mikrosk*., 3: 150

Eshaghi, Z.C., M.R. Abdollahi, S.S. Moosavi, A. Deljou and J.M. Seguí-Simarro, 2015. Induction of androgenesis and production of haploid embryos in anther cultures of borage (*Borago officinali*s L.). *Plant Cell Tiss. Org. Cult*., 122: 321–329

Germanà, M.A., B. Chiancone, N.L. Guarda, P.S. Testillano and M.C. Risueño, 2006. Development of multicellular pollen of *Eriobotrya japonica* Lindl. through anther culture. *Plant Sci*., 171: 718–725

George, E.F., M.A. Hall and G.J. De Klerk, 2008. *Plant Tissue Culture Procedure-background*. In Plant propagation by tissue culture, Springer, Dordrecht, The Netherlands

Gu, H.H., P. Hagberg and W.J. Zhou, 2004. Cold pretreatment enhances microspore embryogenesis in oilseed rape (*Brassica napus* L.). *Plant Growth Regul*., 42: 137–143

Guha, S. and S.C. Maheshwari, 1964. *In vitro* production of embryos from anthers of Datura. *Nature*, 204: 497

Hunter, C.P., 1985. The effect of anther orientation on the production of microspore-derived embryoids and plants of *Hordeum vulgare* cv. Sabarlis. *Plant Cell Rep*., 4: 267–268

Keller, W.A., 1984. Anther culture of Brassica. *In: Cell Culture and Somatic Cell Genetic of Plants*, Vasil, I.K. (ed.). Academic Press, Orlando, Florida, USA

Keller, W.A. and K.C. Armstrong, 1979. Stimulation of embryogenesis and haploid production in *Brassica campestris* anther cultures by elevated temperature treatments. *Theor. Appl. Genet*., 55: 65–67

Keller, W.A. and K.C. Armstrong, 1983. Production of haploids via anther culture in *Brassica oleracea* var. italica. *Euphytica*, 32: 151–159

Kiviharju, E. and E. Pehu, 1998. The effect of cold and heat pretreatments on anther culture response of *Avena sativa* and *A. sterilis*. *Plant Cell Tiss. Org. Cult*., 54: 97–104

Li, J., Y. Wang, L. Lin, L. Zhou, N. Luo, Q. Deng and Y. Qiu, 2008. Embryogenesis and plant regeneration from anther culture in loquat (*Eriobotrya japonica* L.). *Sci. Hortic*., 115: 329–336

Li, Z.L., 1987. *Plant Production Technology*, 2nd edition, pp: 138–148. Science Press, Beijing, China

Liu, C.H., R.J. Lu, M.G. Guo, T. He, Y.B. Li and J.H. Huang, 2016. Transcriptome analysis reveals translational regulation in barley microspore-derived embryogenic callus under salt stress. *Plant Cell Rep*., 35: 1719–1728

Maheshwari, S.C., 1996. *The discovery of anther culture technique for the production of haploids. In Vitro Haploid Production in Higher Plants*. Springer, The Netherlands

Mårtensson, B. and S. Widell, 1993. Pollen from cold-treated *Nicotiana tabacum* buds: Embryogenic capacity, peroxidase activity and partitioning in aqueous two-phase systems. *Plant Cell Tiss. Org. Cult.*, 35: 141–149

Mendoza, M.G. and H.F. Kaeppler, 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *In Vitro Cell. Dev. Biol.-Plant*, 38: 39–45

Mohammadi, P.P., A. Moieni, A. Ebrahimi and F. Javidfar, 2011. Doubled haploid plants following colchicine treatment of microspore-derived embryos of oilseed rape (*Brassica napus* L.). *Plant Cell Tiss. Org. Cult.*, 108: 251–256

Moraes, A.P.D., M.H. Bonadese-Zanettini, S.M. Callegari-Jacques and E. Kaltchuk-Santos, 2004. Effect of temperature shock on soybean microspore embryogenesis. *Braz. Arch. Biol. Technol*., 47: 537–544

Naik, N., P. Rout, N. Umakanta, R.L. Verma, J.L. Katara and S. Samantaray, 2016. Erratum to: development of doubled haploids from an elite indica, rice hybrid (bs6444g) using anther culture. *Plant Cell Tiss. Org. Cult.*, 128: 691

Navarro-Alvarez, W., P.S. Baenziger, K.M. Eskridge, D.R. Shelton, V.D. Gustafson and M. Hugo, 2010. Effect of sugars in wheat anther culture media. *Plant Breed*., 112: 53–62

Nitsch, C. and B. Norreel, 1973. *Factors Favoring the Formation of Androgenetic Embryos in Anther Culture*. In Genes, Enzymes, and Populations, Springer, Boston, Massachusetts, USA

Nitsch, J.P., 1969. Experimental androgenesis in *Nicotiana*. *Phytomorphology*, 19: 389–404

Perera, P.I., V. Hocher, J.L. Verdeil, H.D.D. Bandupriya, D.M.D. Yakandawala and L.K. Weerakoon, 2008. Androgenic potential in coconut (*Cocos nucifera* L.). *Plant Cell Tiss. Org. Cult.*, 92: 293–302

Pescitelli, S.M., C.D. Johnson and J.F. Petolino, 1990. Isolated microspore culture of maize: effects of isolation technique, reduced temperature, and sucrose level. *Plant Cell Rep*., 8: 628–631

Powell, W., 1988. Diallel analysis of barley anther culture response. *Genome*, 30: 152–157

Roberts, K., 1994. The plant extracellular matrix: in a new expansive mood. *Curr. Opin. Cell Biol*., 6: 688–694

Saeed, U., I.A. Rana, S. Ijaz and Z. Ali, 2017. Improving regeneration from mature embryo derived callus in wheat (*Triticum aestivum*) by pre- and post-callus induction treatments. *Int. J. Agric. Biol*., 19: 969–975

Sato, S., N. Katoh, S. Iwai and M. Hagimori, 2002. Effect of low temperature pretreatment of buds or inflorescence on isolated microspore culture in *Brassica rapa* (syn. *B. campestris*). *Breed. Sci*., 52: 23–26

Shannon, P.R.M., A.E. Nicholson, J.M. Dunwell and D.R. Davies, 1985. Effect of anther orientation on microspore-callus production in barley (*hordeum vulgare*, L.). *Plant Cell Tiss. Org. Cult*., 4: 271–280

Sharafi, Y., A.R. Motallebbi-Azar and A. Bahmani, 2011. *In vitro* pollen germination, pollen tube growth and longevity in some genotypes of loquat (*Eriobotria japonica* lindl.). *Afr. J. Biotechnol*., 10: 8064–8069

Sharp, W.R., D.K. Dougall and E.F. Paddock, 1971. Haploid plantlets and callus from immature pollen grains of *Nicotiana* and *Lycopersicon*. *Bull. Torr. Bot. Club*, 98: 219–222

Showalter, A.M., 1993. Structure and function of plant cell wall proteins. *Plant Cell*, 5: 9–23

Sopory, S.K., 1979. Effect of sucrose, hormones, and metabolic inhibitors on the development of pollen embryoids in anther cultures of dihaploid *Solanum tuberosum*. *Can. J. Bot*., 57: 2691–2694

Sorvari, S. and O. Schieder, 1987. Influence of sucrose and melibiose on barley anther cultures in starch media. *Plant Breed*., 99: 164–171

Touraev, A., O. Vicente and E. Heberle-Bors, 1997. Initiation of microspore embryogenesis by stress. *Trends Plant Sci*., 2: 297–302

Trottier, M.C., J. Collin and A. Comeau, 1993. Comparison of media for their aptitude in wheat anther culture. *Plant Cell Tiss. Org. Cult.*, 35: 59–67

Tyankova, N. and N. Zagorska, 2008. Factors affecting in vitro androgenesis in cereals. *Int. J. Plant Dev. Biol*., 2: 59–78

Vasil, I.K. and C. Nitsch, 1975. Experimental production of pollen haploids and their uses. *Z. Pflanzenphysiol*., 76: 191–212

Würschum, T., M.R. Tucker and H.P. Maurer, 2014. Stress treatments influence efficiency of microspore embryogenesis and green plant regeneration in hexaploid triticale (×*Triticosecale Wittmack* L.). *In vitro Cell. Dev. Biol.-Plant*, 50: 143–148

Xu, Z. and N. Sunderland, 1983. Induction density in the culture of barley anthers. *Sci. Sin*., 253: 916–968

Yadollahi, A., M.R. Abdollahi, A. Moieni and M. Danaee, 2011. Effects of carbon source, polyethylene glycol and abscisic acid on secondary embryo induction and maturation in rapeseed (*Brassica napus* L.) microspore-derived embryos. *Acta Physiol. Plant*., 33: 1905–1912

Zhang, H.Z., S.A. Peng, L.H. Cai and D.Q. Fang, 1990. The germplasm resources of the genus Eriobotrya with special reference on the origin of *E. japonica* Lindl. *Acta Hortic. Sin*., 17: 5–12

Zhao, F.C., D. Nilanthi, Y.S. Yang and H. Wu, 2006. Anther culture and haploid plant regeneration in purple coneflower (*Echinacea purpurea* L.). *Plant Cell Tiss. Org. Cult.*, 86: 55

(Received 07 May 2018; Accepted 30 July 2018)