

Full Length Research Paper

# Morphological and histological *observations* on the induction of anther calluses and embryos in loquat (*Eriobotrya japonica* Lindl.)

Juan Yan, Yong-qing Wang\*, Jun-qiang Li, Lian Tao, Qun-xian Deng and Xiu-lan Lv

College of Horticulture, Sichuan Agricultural University, Ya'an 625014, China.

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Morphological and histological studies were conducted systematically for the first time on the induction of anther derived calluses and embryos in loquat (*Eriobotrya japonica* Lindl.). The results showed that the calluses were derived from microspores and both microspores and capillaments, when capillaments were not completely scraped off. Upon transfer onto the embryo differentiation medium, two types of calluses were formed within 5 to 7 days: 90% non-embryogenic calluses (NECs) and 10% embryogenic calluses (ECs). The NECs and ECs differed significantly in morphological and histological features. NECs were compact with a smooth surface and there was no evidence of the formation of adventitious buds or embryos, while ECs were uneven with a crisp texture and loose structure and were beginning to form salient multicellular structures on the surface. NECs cells had little cytoplasm that stained only lightly, few large vacuoles, no or only a small nucleus and wide intercellular spaces. NECs had a very low cell division capability and turned brown and eventually became necrotic. EC cells were smaller, globular and abundant in cytoplasm, with one or two big nuclei located in the centre of tightly aligned heavily stained cells. They had a high capability for cell division and continued to divide and produced somatic pro-embryos which could develop further through the typical globular, heart, torpedo and cotyledon stages. Although only 10% of ECs were induced they had a high differentiation rate and produced 12.7 globular, 8.1 heart-shape, 3.8 torpedo and 10.7 cotyledonary embryos per EC after 48 days of induction.

**Key words:** *Eriobotrya japonica* Lindl., non-embryogenic callus (NEC), embryogenic callus (EC), morphology, histology.

## INTRODUCTION

Loquat (*Eriobotrya japonica* Lindl.), originated in China (Lin et al., 2004) and is an important perennial fruit crop because of both its economic and ecological attributes. It is cultivated between 20 and 35° latitude including China, Japan, Spain, Turkey, Italy, Greece, Israel, India, Pakistan, Reunion Island, Mauritius Island, the USA (mainly California and Florida), Brazil, Venezuela, Australia, Madagascar, New Zealand and South Africa

(Badenes et al., 2000; Vilanova et al., 2001).

Because of heterozygosity, and the long juvenile period of the plant, only a limited number of genetic studies have been performed and no long-term breeding program has ever been established for this crop. Anther culture allows the rapid production of haploids and homozygous diploid plants and has become one of the major techniques in plant breeding programs (Bajaj, 1983). Compared with conventional inbreeding, the *in vitro* androgenesis technique enables a faster generation of virtually fully homozygous lines (Aulinger et al., 2003). Following the successful establishment of a regeneration system for anther culture in loquat (Li et al., 2008), a series of experiments have been carried out to improve the system (Wang et al., 2011) and to investigate the effects of ethyl

\*Corresponding author. E-mail: [huanhuan2700@163.com](mailto:huanhuan2700@163.com) or [yqw14@sicau.edu.cn](mailto:yqw14@sicau.edu.cn).

**Abbreviations:** NEC, Non-embryogenic callus; EC, embryogenic callus.

methane sulfonate (EMS) as an *in vitro* mutation mutagen using the anther-derived embryos (Qin et al., 2011). However, the research and utilization of anther culture in loquat has been limited by the low frequency of the embryo induction.

Morphological and histological studies of callus and embryo induction of plant are important for increasing the incidence of callus production and induction of embryogenic callus (Feng et al., 2007; Tan et al., 2009). In the present study, the morphology and histology of the callus and embryos produced from the anthers were systematically studied in order to clarify the origin of calluses and the differences in morphological and histological characteristics between the non-embryogenic callus (NEC) and embryogenic callus (EC) in loquat.

## MATERIALS AND METHODS

Flower buds (5 to 6 mm in diameter) of loquat cv. 'Dawuxing', which contained approximately 80% uninucleate microspores, confirmed by periodically microscopically checking the microspore development stages, were harvested from the loquat orchard of the Research Center for Horticulture Biotechnology, Sichuan Agricultural University, Ya'an, China.

The flower buds were kept at 4°C for 48 h, then scraped off the thick floss of the sepals and washed in distilled water containing 3% (v/v) Tween (detergent) for 5 min. The flower buds were then surface-sterilized by immersion in 0.1% (w/v) mercuric chloride solution with periodical agitation for 8 min and washed five times with sterile distilled water. After removing the filaments 180 intact anthers were plated horizontally, with the connective tissues contacting the medium. Callus induction media consisted of Murashige and Skoog (1962) mineral salts and vitamins, 7% (w/v) sucrose and 0.6% (w/v) agar supplemented with 0.5 mg·L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 mg·L<sup>-1</sup> benzyladenine (6-BA). After 4 weeks, the induced calluses were separated from the anther walls and transferred to an embryo differentiation medium consisting of MS mineral salts and vitamins, 3% (w/v) sucrose, 0.6% (w/v) agar, and 0.05 mg·L<sup>-1</sup> zeatin (ZT) in combination with 0.20 mg·L<sup>-1</sup> α-naphthaleneacetic acid (NAA) and 0.20 mg·L<sup>-1</sup> indolebutyric acid (IBA). Number of callus cultured was 180.

The media *in this study* were adjusted to pH5.6 prior to addition of agar and sterilized at 122°C and 104 kPa pressure for 17 min. For callus induction, cultures were maintained at 25 ± 1°C in the dark; for embryo differentiation, cultures were cultured in a culture chamber at 25 ± 1°C and illuminated for 16 h daily with 2000 lux fluorescent light. The morphology was monitored throughout the initiation, growth and differentiation of calluses and photographs were taken using a stereoscopic microscope (Motic DM143) and a digital camera (SONY F717). Changes in the histology of the calluses and embryos were followed after samples were fixed in FAA (70% ethanol: formalin: acetic acid, 18:1:1, v/v/v), rinsed, dehydrated and embedded in paraffin at 56 to 58°C standard techniques. 10 mm sections were cut using a rotary microtome, stained with haematoxylin and observed under a light microscope and documented using a digital camera (Motic BA200).

## RESULTS

Anthers expanded and their walls began to crack after 1 week in culture (Figure 1A). Microspores produced

calluses after 2 weeks of culture, and these burst out through the cleft of anther wall (Figure 1B). After 3 weeks the anther walls burst open and callus grew out (Figure 1C). After 4 weeks of culture, histological observations revealed that when the capillament was completely scraped off anther walls, vascular bundles, stomium, and calluses could be detected originating from the microspores (Figure 1D). In contrast, when the capillament was not completely scraped off, the calluses developed from both the microspores and capillament (Figure 1E).

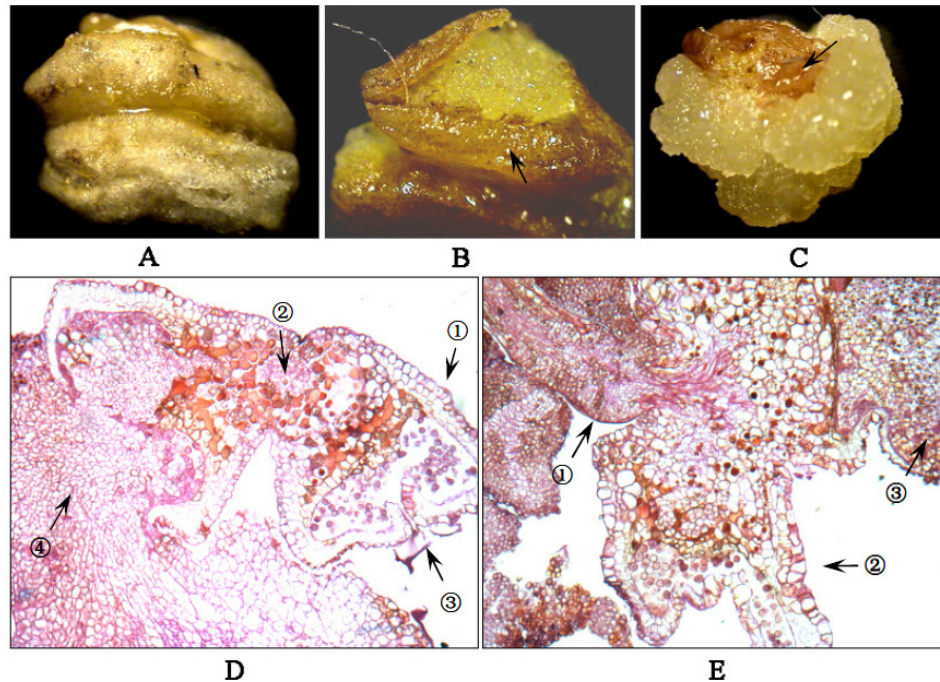
Two types of callus had developed after 5 to 7 days culture on the embryo differentiation medium: 90% were non-embryogenic calluses (NECs) and 10% embryogenic calluses (ECs). The surface of NECs was smooth and compact, and there was no evidence of the formation of adventitious buds or embryos (Figure 2A); ECs were uneven with a crisp texture and loose structure and were starting to form salient multicellular structures on the surface (Figure 2B).

There were also significant histological differences between NECs and ECs. All the NEC cells were large, irregular in shape had little cytoplasm and a few large vacuoles, an average of 1 to 2 per cell. The NEC cells stained only lightly. Most had no nucleus but some had a very small nucleus located near the cell wall. There were wide intercellular spaces between NEC cells and the cells had a very low capability for division (Figure 2C). After being subcultured monthly onto fresh embryo differentiation medium, the NECs turned brown and eventually became necrotic within 7 to 14d.

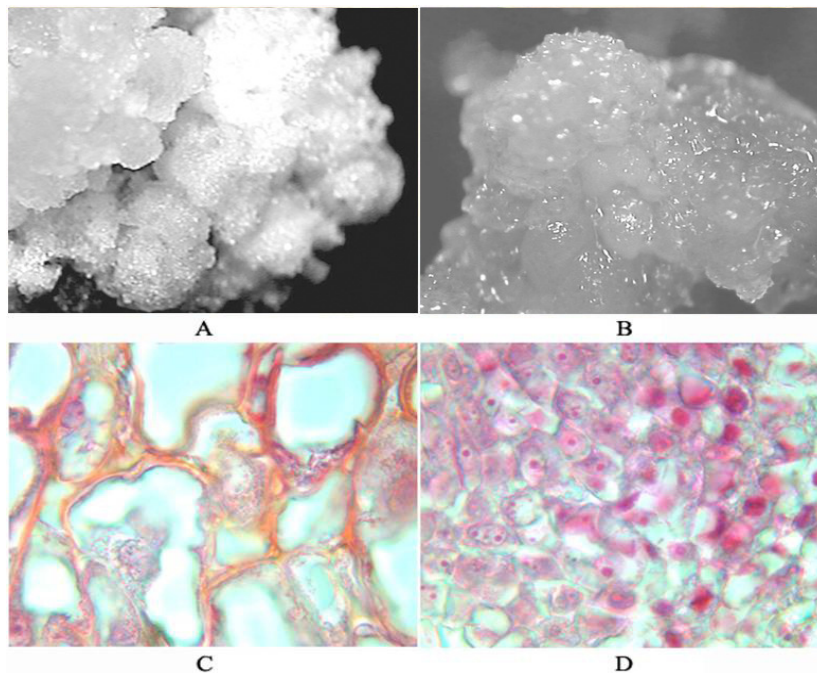
EC cells were smaller than those of NECs, regular and globular in shape had abundant cytoplasm and had a large nucleus located in the centre of the cells, and some had two nuclei. EC cells were tightly aligned, stained heavily and had high cell division capability (Figure 2D). Following transfer to an embryo differentiation medium for 12 to 14 days, the embryogenic cell mass on the surface of ECs continued to divide and develop into multicellular somatic pro-embryos (Figure 3A) that could develop further into globular structures (Figure 3B). Various developmental stages were evident after 25 to 28 days of induction (Figure 3C). When transferred to a fresh embryo differentiation medium the embryos developed through the typical globular, heart, torpedo and cotyledonary stages in the subsequent 14 to 21 days (Figure 3D). Although only 10% of calluses became embryonic they had a high rate of differentiation producing 12.7 globular, 8.1 heart-shapes, 3, 8 torpedo and 10.7 cotyledonary embryos per EC within (counted after 48d of induction). The histological development of globular, heart-shape, torpedo and cotyledonary embryos are showed in Figure 3, E;F;G;H.

## DISCUSSION

Another culture, developed by Guha and Maheshwari

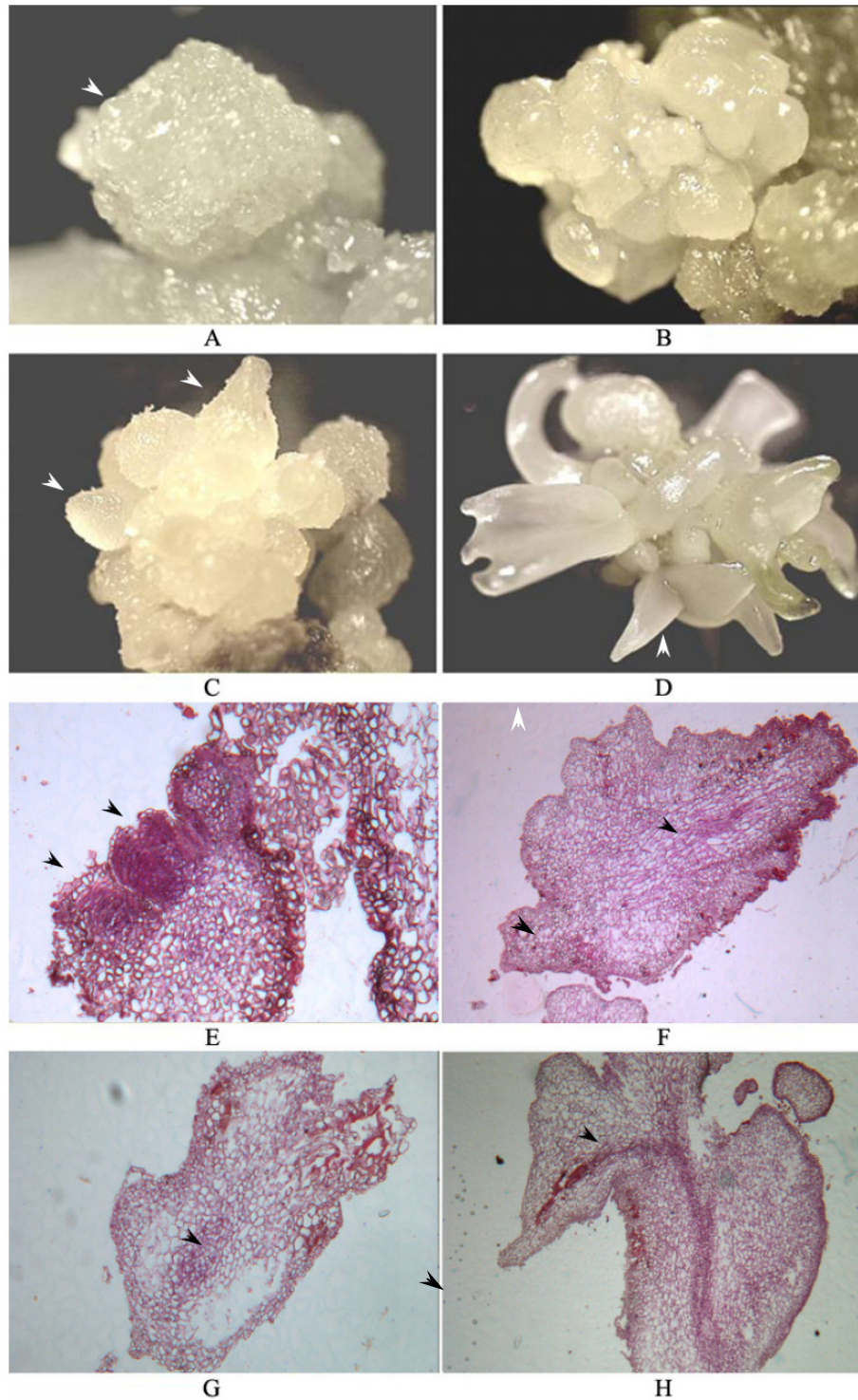


**Figure 1.** Callus induction from cultured anther. Panel A: anther after 1 week of culture; Panel B: anther after 2 weeks of culture (the arrowhead indicated the anther wall); Panel C: anther after 3 weeks of culture (the arrowhead indicated the anther wall); Panel D (100 ×): histological origination of anther derived calluses (①: calluses of anther wall origin; α: calluses of vascular bundle origin; β: calluses of stomium origin; γ: calluses of microspores origin.); Panel E (100×): calluses derived from microspores and capillament (①: calluses derived form capillament; α: calluses derived form anther wall; β: calluses derived from microspores).



**Figure 2.** Types of anther-derived calluses. Panel (A): non-embryogenic callus (NECs); Panel (B): embryogenic callus (ECs); Panel (C): NEC cells (1000×); Panel (D): EC cells (1000×).





**Figure 3.** Embryogenesis from EC of loquat. Panel (A): Embryogenic calluses with proembryos (the arrowhead indicates the protuberant structures with smooth surfaces). Panel (B): A cluster of embryos at the globular stage. Panel (C): Further growth of embryos at different developmental stages (the arrowheads indicate the heart embryo (left) and torpedo embryo (top)). Panel (D): Further development after transfer to a fresh embryo differentiation medium, the arrowhead indicates a cotyledonary embryo). Panel (E): Arrowheads indicate globular and heart-shape embryos (400 $\times$ ); Panel (F): An embryo developed to between the heart- and torpedo- stages with a meristematic region indicated 100  $\times$  Panel (G): A torpedo embryo with meristematic region indicated 100 $\times$ ; Panel (H): Cotyledonary embryo with meristematic region 40 $\times$ .

(1964, 1966), allows the rapid production of haploids and homozygous diploid plants and has become one of the major techniques in plant breeding programs and genetic transformation. Morphological and histological studies of anther culture can provide information useful for increasing the rate of callus and embryo induction (Feng et al., 2007; Tan et al., 2009). In this research, the morphology and histology of the development of loquat anther callus and embryo induction were systematically studied for the first time. During the dedifferentiation phase of tissue culture, there are usually two types of cell clusters, NEC and EC (Francisco et al., 2002; Tan et al., 2009). Morphological and histological comparisons of ECs and NECs in *Coffea arabica* cv. Catura Rojo, indicated a correlation between the morphological features of clusters and their embryogenic competence (Francisco et al., 2002). Moreover, comparisons of the proteomes of ECs and NECs of *Vitis vinifera* L. cv. Cabernet Sauvignon demonstrated that different stress response pathways were activated in ECs and NECs, and a number of differentially expressed proteins involved in various functional categories were also identified (Zhang et al., 2009). In this study, both morphological and histological observations provided experimental evidence of significant differences between NECs and ECs developed from loquat anthers. These results could aid the development of a more efficient means of producing ECs and aid the introduction of genetic transformation systems using agrobacterium or gene gun bombardment (Sakae et al., 2002; Perrin et al., 2004). A number of factors affect EC induction rate in anther culture, including genotype and physiological state of the donor plant, anther age and pollen developmental stage, pre-culture treatment, and physical and chemical factors (Sopory and Munshi, 1996). These need to be optimized in the future studies with loquat.

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