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Effects of Different Pre-Treatments on Callus Induction from Anther in Loquat

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Abstract: Three cultivars of loquat including Longquan1, Dawuxing, Zaozhong6 were used to study the effects of different pre-treatments on callus induction from anther. The results showed that there were no anther callus induction without any pre-treatment (CK), the anther callus induction rate of bud with pre-treatments was significantly increased. In all the pre-treatments, low temperature (4°C) was the best for anther callus induction and followed by heat shock, high osmotic pressure and centrifugation; 48 h low temperature (4°C) pre-treatment was beneficial as it significantly increased the induction rate of callus and callus induction rate changed with cultivars, reaching the apex of 54.67, 46.33 and 72.00% for cv. Longquan1, Dawuxing and Zaozhong6, respectively.

Key words: Loquat, anther, callus induction, pre-treatment, pressure, heat shock

INTRODUCTION

Loquat (*Eriobotrya japonica* Lindl.), originating in China (Lin *et al*., 2004) is an important perennial fruit crop because of both its economic and ecological attributes. It is cultivated between 20 and 35°C latitude including China, Japan, Spain, Turkey, Italy, Greece, Israel, India, Pakistan, Madagascar, Reunion Island, Mauritius Island, the USA (mainly California and Florida), Brazil, Venezuela and Australia (Badenes *et al*., 2000; Hu *et al*., 2006). Because of heterozygosity and 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. The traditional breeding methods are time-consuming and limited by space required for field experiments. Anther culture allows rapid production of haploids and homozygous diploid plants. It 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). Until recently, only Li *et al*. (2008) have established regeneration system for anther culture successfully. But it was not a sufficiently high frequency regeneration system which limited the research and utilization of anther culture in loquat. There are a number of factors that affect androgenesis including genotype and physiological state of the donor plant, anther age and pollen developmental stage, pre-culture treatment, physical factors and chemical factors (Sopory and Munshi, 1996). Of them, pre-treatments are presented as essential experimental factors. The aim of this study is to investigate the effects of different pre-treatments on callus induction in loquat cv. Longquan1, Dawuxing and Zaozhong6 to establish a high frequency anther culture system.

MATERIALS AND METHODS

Flower buds (5-6 mm in diameter) of loquat cv. Longquan1, Dawuxing and Zaozhong6 were harvested from loquat orchard of Research Center for Horticulture Biotechnology, Sichuan Agricultural University, Yaan, China which contained approximately 80% uninucleate microspores confirmed by periodically microscopically checking the microspore stage. The flower buds were collected in a conical flask with a piece of wet cotton to do various pre-treatments. Four different pre-treatments hot shock 35°C, 6 h, low temperature (4°C, 48 h, high osmotic pressure 0.7% PEG8000, 24 h, centrifugation 2000 r min^{-1}, 12 h and with no any pre-treatment as Control (CK). Different time last of low temperature kept at 4°C in dark for 0 (CK), 24, 48, 96 and 144 h. Before culturing of anthers, the thick floss of the sepals was completely scraped off (otherwise they would make it difficult to sterilize the flower buds) with a surgical scalpel and then washed in distilled water containing 3% (v/v) Tween (detergent) for 5 min. The final step of sterilization and further operations were carried out in a laminar air-flow cabinet under aseptic conditions. The flower buds were surface-sterilized by immersion in 0.1% (w/v) mercuric chloride solution with periodical agitation for 8 min and

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washed with sterile distilled water for 5 times. After removing filaments, the intact anthers were plated horizontally with the connective tissues contacting the medium in the conical flasks (150 mL) containing 30 mL of medium. Callus induction media consisted of Murashige and Skoog (MS) mineral salts and vitamins, 7% (w/v) sucrose, 0.6% (w/v) agar, supplemented with various types and concentrations of plant growth regulators including 2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (6-BA). Each treatment was applied to 300 anthers with 15 replicates. The researchers count data after 4 weeks.

The data were analyzed statistically using the SPSS statistical package for Windows (release 11.0, SPSS INC). Significance between means was tested by Duncan's multiple range test.

RESULTS AND DISCUSSION

Effect of different pre-treatments on callus induction from anther: The growth chart of callus induction from cultured anther in loquat was shown in Fig. 1, forming a large number of cullas after 4 weeks. The results of different pre-treatments on callus induction from anthers were shown in Table 1. There were no anther callus induction without any pre-treatment (CK), the anther callus induction rate of bud with pre-treatments was significantly increased. In all the pre-treatments, low temperature (4°C) is the best for anther callus induction and followed by heat shock, high osmotic pressure and centrifugation and the effects of pre-treatments on callus induction rate changed with cultivars.

Different low temperature (4°C) pre-treatment time on callus induction from anther: The results of different low temperature (4°C) pre-treatment time on callus induction from anthers shown in Table 2. Prolonged low temperature (4°C) pre-treatment (24-48 h) can significantly improve the cv. Longquan1, Dawuxing and Zaozhong6 loquat anther callus induction rate; prolonged low temperature (4°C) pre-treatment (96-144 h) dramatically decreased the potential of anthers to produce calluses while it increased the frequency of anthers to turn brown and then become necrotic and the anthers turn brown soon after inoculated into the medium and become necrotic within 1 week; 48 h low temperature (4°C) pre-treatment was beneficial as it significantly increased the induction rate of callus, reaching the apex of 54.67, 46.33 and 72.00% for cv. Longquan1, Dawuxing and Zaozhong6, respectively.

Pre-treatment plays a key role for anther callus induction, it can effectively increase the frequency of isolated male reproductive of anther culture (Nitsch, 1974).

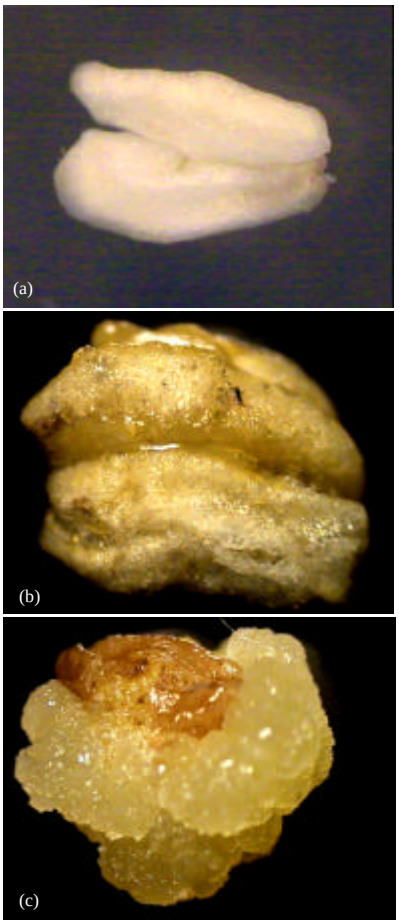

Fig. 1: Growth chart of callus induction from cultured anther in loquat. a) an individual anther whose filament was completely removed; b) anther after 1 week of culture, anther swelling; c) anther after 4 weeks of culture, forming a large number of cullas

Table 1: Effect of different pre-treatment on callus induction from anthers after 4 weeks of culture in loquat

	Anthers forming callus					
	cv. Longquan1		cv. Dawuxing		cv. Zaozhong6	
Pre-treatment	No.	%	No.	%	No.	%
CK	0	0.00^{d}	0	0.00^{d}	0	0.00^{d}
Hot shock	72	14.00^{a}	45	15.00^{b}	72	18.33^{b}
Low temperature	169	56.33^{a}	159	53.00^{a}	222	74.00^{a}
High osmotic	15	5.00^{e}	40	13.33^{bc}	20	6.67^{c}
Centrifugation	14	4.67^{e}	22	7.33^{c}	23	7.67^{c}

Means following the same letter within columns were not significantly different according to Duncan's multiple range test ($p = 0.05$)

In the study, there were no anther callus induction without any pre-treatment (CK), the anther callus induction rate of bud with pre-treatments was significantly increased. The main pre-treatment methods successfully used in anther culture are low-temperature, centrifugation, hot shock, water culture and so on

Table 2: Effect of low temperature (4°C) pre-treatment on the callus induction of anthers after 4 weeks of culture in loquat

	Anthers forming callus					
	cv. Longquan1		cv. Dawuxing		cv. Zaozhong6	
Time (h)	No.	%	No.	%	No.	%
0	0	0.00^{C}	0	0.00^{D}	0	0.00^{D}
24	88	29.33^{B}	46	15.33^{B}	72	24.00^{C}
48	164	54.67^{A}	139	46.33^{A}	216	72.00^{A}
96	53	17.67^{C}	61	20.33^{B}	132	44.00^{B}
144	27	9.00^{D}	23	7.67^{C}	95	31.67^{C}

Means following the same letter within columns were not significantly different according to Duncan's Multiple Range test (p = 0.01)

(Sangwan-Norreel, 1977; Wilson *et al*., 1978; Keller *et al*., 1983). The researchers studied the effects of different pre-treatments including hot shock 35°C, 6 h, low temperature (4°C, 48 h, high osmotic pressure (0.7% PEG8000, 24 h) and centrifugation (2000 r min^{-1}, 12 h) on callus induction from anther. The results showed that low temperature (4°C) is the best and followed by heat shock, high osmotic pressure and centrifugation.

Shock treatment of anthers or whole flowers including high or low temperature for various lengths of time prior to culture has been applied to a variety of plant species (Immonen and Robinson, 2000). Cold pre-treatment is easy to practise and has been used to induce embryogenesis from isolated anthers as it disrupts the cytoskeleton in microspores in the initial phase (Ferrie *et al*., 1995). Application of cold pre-treatment has become an essential measure to increase the effciency of androgenesis in many species (Pechan and Smykal, 2001).

The mechanism of low temperature is unclear. Many researchers considered that the low temperature pre-treatment reposited the spindle, resulting in the pollen produces two similar nuclear during first mitosis and changes developmental pathways of microspore or ensure the long survival of microspores through delaying anther senescence (Keller and Armstrong, 1979). Vasil and Nitsch (1975) said that low temperature pre-treatment can lower the general level of metabolism to accumulate large amounts of suitable pollen grains which can be induced succesfully. Low temperature pre-treatment play an active effecte for the anther culture in most cases. However, some studies have shown that the negative effect of low temperature pre-treatment (Keller *et al*., 1983). In the experiment of Marsolais *et al*. (1984) of four genotypes wheat anther culture, cold pre-treatment (5°C) lead to premature death of microspores within anthers and turned white after 1 week. In the study, the researchers found that in loquat the cold pre-treatment of flower buds was necessary for subsequent callus induction from anthers and 48 h of cold (4°C) pre-treatment proved to be optimal as it gave the best callus induction. However, the callusing potential of loquat anthers was impaired by prolonged cold pre-treatment. When the cold pre-treatment was up to 144 h, the anthers turned brown a few minutes after being removed from the buds. Early anther browning was associated with low callus induction rate and the anthers became necrotic within 1 week. Therefore, cold pre-treatment was one of key factors for callus induction in loquat anther culture.

The results of the research also showed that based on the same pre-treatment, medium formulation and culture condition, the callus induction were different in different cultivars. Many studies have shown that the genotype of donor plants plays an important role in anther culture (Prakash and Giles, 1992). Kiefer *et al*. (1993) reported anther culture of kale (*Brassica oleracea*) determined by genotypes; Lu *et al*. (1991) reported callus induction of different genotypes of Brassica varied between 0 and 35%.

CONCLUSION

In this study, loquat genotype has a obvious impact on callus induction rate of anther culture, callus induction rate of cv. Zaozhong6 is highest followed by cv. Longquan1 and cv. Dawuxing. Therefore, the researchers should select the genotype which can produce high frequency of callus induction firstly in anther culture of loquat.

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