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REGENERATION OF PLANTS FROM ANCESTRY TREE OF *CITRUS CHANGSHAN-HUYOU* Y. B. CHANG *VIA* TISSUE CULTURE

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Abstract

Tissue culture with young leaves, stems, and embryos of the commonly grown cultivars of *Citrus changshan-huyou* Y. B. Chang was carried out. The results indicated that the young embryo of *C. changshan-huyou* cultivars was the best explant for regeneration of plants *via* tissue culture. Based on this result, young fruit of "Ancestry Tree" of *Citrus changshan-huyou* was selected. After being stored at 4°C for 6 days, the embryos were cultured on MS medium containing 0.5 mg/l BA + 0.5 mg/l NAA to induce the formation of callus. The induced callus were cultured on MS medium containing 1 mg/l BA and induced to differentiate into cluster buds, which were cut and cultured on MS medium containing 0.4 mg/l IAA to induce the formation of roots. Finally, a technological system for regeneration of plants from the "Ancestry Tree" of *C. changshan-huyou via* tissue culture was successfully established. This study has paved the foundation for protection and further utilization of "Ancestry Tree" of *C. changshan-huyou*, the precious and rare germplasm resource.

Introduction

Citrus changshan-huyou Y. B. Chang (Fam.: Rutaceae) was originally generated in Changshan County in Zhejiang Province, China. It was formally named as *Citrus changshan-huyou* Y. B. Chang by Zhang (1991) and became a new species of genus *Citrus reticulate* (Chen *et al.* 2002). Its fruits are plump, have a bright-yellow appearance and are rich in juicy fluid with strong fragrant odor and moderately acidic-sweet taste as well as slight sweetness. In addition to serving as ordinary fruit, its fruit also possess medicinal values. They contain a large number of essential amino acids and vitamins required for human body and have many pharmacological functions, including cooling, refreshing and reducing internal heat, relieving cough and reducing sputum, soothing throat and dispelling the effects of alcohol, and reducing blood sugars (Jiang 1994, Zhong and Tian 1995, Xu *et al.* 2014, Tang *et al.* 2014).

According to the textual research on the recorded literature, Dipu village and Chentang village in Qingshi township, Changshan County, Zhejiang Province, China were the original places, where *C. changshan-huyou* was artificially cultivated. In 1983 when The Agricultural Bureau of Changshan County conducted a survey on natural citrus resources, a 70-year-old *C. changshan-huyou* plant was found in Chentang village, Qingshi township (Yu *et al.* 2006). This *C. changshan-huyou* plant currently growing in Chentang village is the oldest citrus seedling-plant and aged (as of 2017) 111 years. The local farmers thought that all the *C. changshan-huyou* plants were originally reproduced and developed from this tree. Thus, they considered this oldest plant as the "Ancestry Tree" (Zhong 2002). The Forest Bureau of Quzhou City and The Government of Changshan County listed this old tree as the "Ancient and Rare Trees".

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Few studies indicated that *C. changshan-huyou* was originally generated by natural hybrid between *Citrus maxima* and sweet acidic citrus or between *Citrus maxima* and sweet citrus or *Citrus reticulata* Blanco. It might also be generated by multi-hybrid and this possibility can not be eliminated (Zhong 2002, Chen *et al.* 2002, Chen *et al.* 2006). Because it is the progeny of natural hybrid, when it is sexually reproduced with seeds, after reproduction for multiple generations, some good traits of its parents are hardly preserved. Especially, the economically important traits of their fruits displayed the mixture of good and bad traits. Currently, after being reproduced for many generations, the *C. changshan-huyou* is facing a number of problems, such as variety deterioration, quality deterioration and reduced resistance capability, etc. (Zhong 2004, Ye and Bei 2000, Chen *et al.* 2011, Li *et al.* 2011). Some farmers frequently applied grafting to conduct asexual reproduction. However, the quality of the scion used for grafting directly affects the quality of the bearing fruit. Cuttage propagation was also applied by some farmers to conduct reproduction and propagation. However, the reproduction rate with cuttage propagation is quite slow and the survival rate is also low. Thus, its propagation by this way hardly meets the requirement for the production and development of the *C. changshan-huyou* (Gong *et al.* 1989, Zhong 2000). Therefore, this study aimed at establishing a biotechnological system for rapid reproduction and propagation of this "Ancient Trees" of *C. changshan-huyou*, in order to use the precious and rare germplasm resource for citrus breeding.

Materials and Methods

Young stem, leaves, buds and fruits of the commonly grown cultivar of *Citrus changshan-huyou* and the young fruit of the "Ancestry Tree" were selected as the explants. The young stems, leaves and buds were cut from the seedlings. The young fruits were about three months old fruits after flowering. The diameters of the young fruits were about 1 - 2 cm.

The explants were soaked in detergent solution for 5 min, washed with tap-water to clean and then soaked in 5× diluted sodium hypochlorite solution containing 100 - 140 g/l effective chloride for 5-10 min. After disinfection, the solution was poured away, the explants were washed with sterile water three times and the excess water on the surface of explants was absorbed with sterile filter paper. Prior to disinfection, young fruit were stored at -4°C in refrigerator for 0 - 8 days.

Young leaves were cut into 0.5 cm small square pieces with anatomy knife. The young stems were cut into 0.5 cm small sections with scissor. Young embryos were shucked out of the young fruit with anatomy knife and a pair of forceps. MS was used as the basic culture medium containing 6 g/l agar and 30 g/l sucrose. pH of the medium was maintained at 5.8. Plant hormones at different concentrations were added to formulate the induced culture medium, differentiation medium and rooting-medium, respectively. The explants were transferred to different culture media and 3-5 explants were implanted in each culture bottle. The explants were cultured at 22 ± 2°C under a light intensity of 1000 - 1200 lux. The formation of callus was induced in dark. Calli were transferred onto differentiated-medium to induce shoots. Shoot clusters were cut off and then transferred onto root-induced medium. All subculture were conducted every 30 days.

Results and Discussion

After being cultured, three stages occurred on the cultured leaves: yellowing death, or shriveled but with green color, or formation of callus. When being cultured with MS + 1.0 mg/l BA+1.5 mg/l NAA medium, the efficiency of the induced formation of callus was the highest one (Table 1).

At 20 - 30 days after being cultured, a few young stems expanded while a majority of young stem died. No obvious callus was formed (Table 2).

Table 1. Results of the induction of callus from young leaves of *Citrus changshan-huyou*.

BA (mg/l)	NAA (mg/l)	Leaf shrivel (N)	Yellowing death (N)	Induced callus (N)	Inoculated explants (N)
0	0.5	0	50	0	50
	1.0	3	47	0	50
	1.5	4	44	2	50
	2.0	3	45	2	50
0.5	0.5	4	44	2	50
	1.0	10	37	3	50
	1.5	12	35	3	50
	2.0	15	29	6	50
1.0	0.5	13	35	2	50
	1.0	15	25	10	50
	1.5	23	10	**17**	50
	2.0	19	18	13	50
1.5	0.5	26	21	3	50
	1.0	29	15	6	50
	1.5	28	14	8	50
	2.0	24	22	4	50

Table 2. Results of the induced formation of callus from young stems of *Citrus changshan-huyou*.

BA (mg/l)	NAA (mg/l)	Expanded young stems (N)	Dead stems (N)	Callus (N)	Inoculated young stem (N)
0	0.5	0	50	0	50
	1.0	0	50	0	50
	1.5	0	50	0	50
	2.0	0	50	0	50
0.5	0.5	4	46	0	50
	1.0	5	45	0	50
	1.5	2	48	0	50
	2.0	1	49	0	50
1.0	0.5	3	47	0	50
	1.0	2	48	0	50
	1.5	5	45	0	50
	2.0	4	46	0	50
1.5	0.5	8	42	0	50
	1.0	6	44	0	50
	1.5	3	47	0	50
	2.0	5	45	0	50

For those young embryos without being stored at low temperature, when they were cultured on medium, they needed to take 2 months to germinate and the germination time was long. Therefore, we picked up young fruit and treated them with low temperature at 4°C for 0 - 8 days. The germination time was significantly shortened and germination rate was significantly

increased. Among them, the germination rate of the young embryos being treated at 4°C for 6 days was the highest one (Table 3).

Table 3. Effects of different cold-storage times on the germination rate of young fruit.

4°C Treatment time (d)	Number of young embryos (N)	Number of germinated young embryos (N)	Germination rate of young embryos (%)
0	50	5	10
2	50	12	24
4	50	18	36
6	**50**	**26**	**52**
8	50	14	38

The embryos and flesh pulp of young fruit were taken out and cultured, respectively. Among them, the formation of callus can be induced from only a few flesh pulps and the growth of callus was very slow. The formation of callus can be induced from all young embryos under different culture conditions. Among which, the induction rate of callus in the culture medium of MS+0.5 mg/l NAA+0.5 mg/l BA was the highest one, which was 78% (Table 4).

Table 4. The effects of the proportions of different plant hormones on the induction of callus from young embryos.

BA (mg/l)	NAA (mg/l)	Number of embryo (N)	Number of induced callus (N)	Rate of callus induction (%)
0	0.5	50	13	26
	1.0	50	27	54
	1.5	50	22	44
0.5	**0.5**	**50**	**39**	**78**
	1.0	50	31	62
	1.5	50	26	52
1.0	0.5	50	25	50
	1.0	50	32	64
	1.5	50	19	38

From the results, it is apparent that out of the induced formation of callus from young leaves, young stem and young embryos, the rate of the induced formation of callus from young embryos was the highest one. Thus, the calli formed from the young embryos were selected to further induce differentiation. When being cultured on the basic MS medium, a majority of calli grew slowly and became brown. Only a few number of calli could differentiate to small buds. When different concentrations of BA were added to the basic MS medium, the differentiation rate of callus to small buds in MS medium containing 0.5 - 1.0 mg/l BA was higher. Whereas, at higher 6-BA concentration, the differentiation rate decreased to certain extend. All differentiated buds can grow leaves further (Table 5).

Table 5. The results of differentiation of the induced callus from young embryos of *Citrus changshan-huyou*.

6-BA concentration (mg/l)	Number of inoculated callus (N)	Number of the differentiated buds from callus	Rate of differentiation (%)
0	50	2	4
0.5	**50**	**35**	**70**
1.0	**50**	**39**	**78**
1.5	50	20	40
2	50	19	38
2.5	50	17	34

The differentiated cluster buds were cut and implanted into the rooting-medium containing different proportions of plant hormones. On the medium without any plant hormones added, only a few buds could generate roots. When being cultured on the medium containing 0.2 - 1.0 mg/l IAA, all buds could form roots. Combination addition of both 0.2 - 1.0 mg/l IAA and 0.5 mg/l 6-BA to the culture medium did not significantly increase the formation of roots. When being cultured on medium containing 0.4 - 0.6 mg/l IAA or 0.4 - 0.6 mg/l IAA + 0.5 mg/l BA, the length of principal root formed was longer with fibrous roots. When being cultured in medium containing NAA, the basis of cluster buds was callus and the root-formation was significantly inhibited (Table 6).

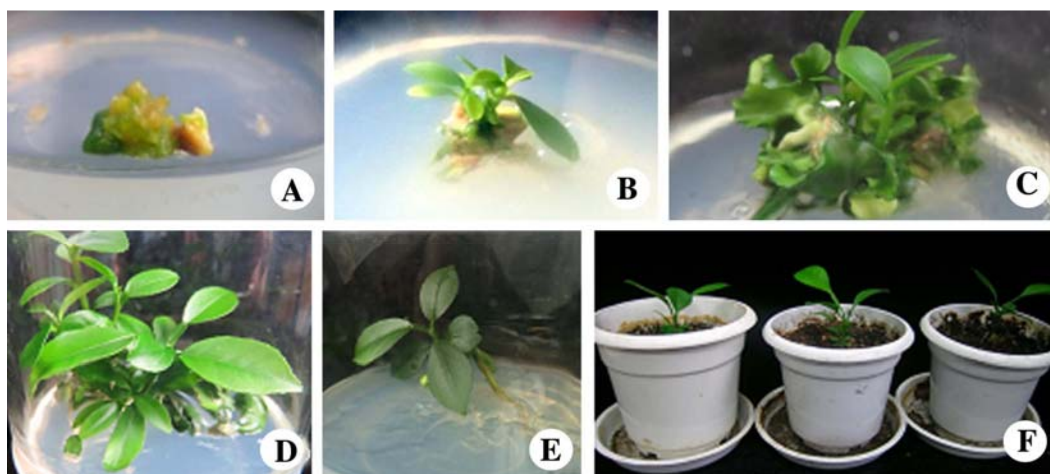

Fig. 1. Culture of embryo of the "Ancestry Tree" of *Citrus changshan-huyou.* A- the induced callus; B, C and D- the differentiation process of cluster buds; E- complete plants with roots; F- the surviving regenerated plants after being transplanted.

Because the "Ancestry Tree" of *C. changshan-huyou* is the protected old tree, collection of its stems, leaves and fruit is under strict control. Therefore, based on the results of the cultures of young leaves, young stems, young embryos of the common citrus cultivars, young fruit of the "Ancestry Tree" was selected as the experimental material. The young fruit were treated with low temperature at 4°C for 6 days. Their embryos were taken out and cultured on MS + 0.5 mg/l NAA

+ 0.5 mg/l BA medium to induce formation of callus and on MS+1.0 mg/l 6 - B medium to induce differentiation into cluster buds and finally on 0.4 mg/l IAA culture medium to induce the formation of roots. Finally, 5 - 10 plantlets can be obtained from each embryo. After their roots were formed, the plants were selected. The cap of the culture bottle was opened and placed in the culture room. The plants were acclimatized for 3-5 days. After being acclimatized, the plants were taken out with forceps and the culture medium in the root system was washed away with water and then transferred to alms bowl containing turf and roseite (2 : 1) and settled in glass-warming room. The survival rate of the cultured plants was over 95% and the plants grew well (Fig. 1).

Table 6. The results of root-formation of cluster buds under different culture conditions.

Proportions of plant hormones	Status of root-formation
0	A few buds formed principle root, the root length was shorter, without fibrous roots, and root color was white.
0.2 mg/ml IAA	The length of principal root was shorter; with fibrous roots, white colored roots.
0.2 mg/ml IAA + 0.5 mg/ml BA	The principal root was shorter; with fibrous roots, the root color was light green.
0.4 mg/ml IAA	The length of principal root was longer, with fibrous roots, the root color was white.
0.4 mg/ml IAA + 0.5 mg/ml BA	The length of principal root was longer, with fibrous roots, the color of fibrous roots was light green.
0.6 mg/ml IAA	The length of principal root was longer, with fibrous roots, the color of root was white.
0.6 mg/ml IAA + 0.5 mg/ml BA	The length of principal root was longer, with fibrous roots, the color of root was light green.
0.8 mg/ml IAA	The length of principal root was longer, protrusions appeared on root surface; with a few fibrous roots, the color of root was white.
0.8 mg/ml IAA + 0.5 mg/ml BA	The length of principal root was longer, protrusions appeared on root surface; with a few fibrous roots, the color of roots was light green.
1.0 mg/ml IAA	The length of principal root was longer, protrusions appeared on root surface; with a few fibrous roots, the color of root was white.
1.0 mg/ml IAA +0 .5 mg/ml BA	The length of principal root was longer, protrusions appeared on root surface; with a few fibrous roots, the color of fibrous roots was light green.
0.2 mg/ml NAA	The base of bud was callus, a few buds formed roots without obvious principal root.
0.2 mg/ml NAA + 0.5 mg/ml BA	The base of bud was callus, a few buds formed roots without obvious principal root.
0.4 mg/ml NAA	No roots were formed, the base of bud was callus.
0.4 mg/ml NAA + 0.5 mg/ml BA	No roots were formed, the base of bud was callus.
0.6 mg/ml NAA	No roots were formed, the base of bud was callus.
0.6 mg/ml NAA + 0.5 mg/ml BA	No roots were formed, the base of bud was callus.
0.8 mg/ml NAA	No roots were formed, the base of bud was callus.
0.8 mg/ml NAA + 0.5 mg/ml BA	No roots were formed, the base of bud was callus.
1.0 mg/ml NAA	No roots were formed, the base of bud was callus.
1.0 mg/ml NAA + 0.5 mg/ml BA	No roots were formed, the base of bud was callus.

Citrus changshan-huyou was originally generated through natural hybrid between pomelo (*Citrus maxima*) and orange or between pomelo and tangerine (Chen *et al.* 2002). It was proposed that the grapefruit of USA was derived from the improvement of *C. changshan-huyou*. In 1830, Portugueses successfully introduced *C. changshan-huyou* in the State of Florida in the USA with the same latitude and named it grape fruit, *i.e.* grapefruit (Min *et al.* 2010, Zhao *et al.* 2010).

The “Ancestry Tree” of *C. changshan-huyou* contains rich genes with high quality that may have lost during the process of breeding citrus cultivars, and thus, it is extremely precious and rare germplasm resource. Thus, this unique germplasm resource of the “Ancestry Tree” can be utilized to improve the existing cultivars and to breed and develop new cultivars of *C. changshan-huyou* with excellent quality. In this study, on the basis of the result of successful establishment of regenerated plants of the commonly grown varieties of *C. changshan-huyou via* tissue culture, it is finally established the biotechnological system for rapid reproduction and propagation of the “Ancestry Tree” of *C. changshan-huyou*. The successful rapid regeneration of “Ancestry Tree” is beneficial for protection and utilization of “Ancestry Tree”, the precious and rare germplasm resource. These regenerated seedlings and trees not only can serve as the scion for grafting *Citrus* but also can be directly used as the seedlings for production. They can also be used to carry out hybridization with the existing citrus cultivars to improve and breed new cultivars.

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References

Chen L, Hu X and Zhao S 2002. Molecular research on Huyou origin. Acta Hortic. Sin. **29**(3): 276-277 (in Chinese).

Chen S, Yang H, Zheng Y, Chen Y and Qiu Y 2006. Preliminary identification of *Citrus changshan-huyou* elite genotypes by molecular markers. J. Mol. Cell Biol. **39**(6): 502-507.

Chen X, Yu R and Pan F 2011. Preliminary report on new variety breeding of *Citrus changshan-huyou*. J. Zhejiang Forestry Sci. Tech. **31**(4): 62-56 (in Chinese).

Gong J, Zhao J, Miao T and Ye X 1989. The genetic variation in breeding of *Citrus changshan-huyou*. Zhejiang Ganju **5**(1): 31-32 (in Chinese).

Jiang B 1994. Some characteristics of *Citrus changshan-huyou* Y. B. Chang. Zhejiang Ganju **11**(2): 14-15 (in Chinese).

Li Y, Yang B and Huang H 2011. The development status and countermeasures of industry related to *Citrus changshan-huyou.* Zhejiang Ganju **28**(4): 5-8 (in Chinese).

Min P, Zhao Q, Gao Y, Shi G and Yan M 2010. The chemical constituents of *Citrus changshan-huyou* Y. B. Chang. Chinese J. Med. Chem. **30**(2): 146-149 (in Chinese).

Tang R, Lin M, Wu J, Ren S, Zhang Z, Zhang Y and Pan J 2014. The present situation and development of *Citrus changshan-huyou* Y. B. Chang. Sci. Tech. Innov. Herald (28): 250- 252 (in Chinese).

Xu X, Zhao S and Wang L 2014. The composition in peel of *Citrus changshan-huyou* and its function. Zhejiang Ganju **31**(1): 10-12 (in Chinese).

Ye X and Bei Z 2000. Main characteristics and cultivation methods of *Citrus changshan-huyou*. South China Fruits **29**(6): 8-9 (in Chinese).

Yu R, Chen X, Ye Y, Yang X, Bei Z and Zhen G 2006. Study on origin and cultivation of *Citrus changshan-huyou*. J. Zhejiang Forest. Sci. Tech. **26**(3): 83-86 (in Chinese).

Zhang Y 1991. A new species of genus citrus from China. Bull. Bot. Res. **11**(2): 5-7.

Zhao Q, Min P, Shi G and Gao Y 2010. Simultaneous determination of contents of narirutin, naringin and neohesperidin in *Citrus changshan-huyou* Y. B. Chang by HPLC. J. Shenyang Pharm. Univ. **27**(9): 737-740 (in Chinese).

Zhong S 2000. Discussions on sustainable development of *Citrus changshan-huyou* Y. B. Chang, sp. nov. Economic Forest. Res. **18**(4): 59-60 (in Chinese).

Zhong S 2002. The RAPD analysis of *Changshan-huyou* and the research on the varying regualrities of its fruit quality together with the processing technology and utilization of its fruit. Doctoral dissertation of Centr. South Forest Univ. (in Chinese).

Zhong S 2004. The status and tendency of research in *Citrus changshan-huyou*. Non-wood Forest Res. **22**(1): 64-67 (in Chinese).

Zhong S and Tian J 1995. Analysis of nutritional components in fruit of *Citrus changshan-huyou* Y. B. Chang. Non-wood Forest Res. **13**(2): 23-25 (in Chinese).

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