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Genetics/Genética

Establishment of efficient adventitious shoots induction system and *ex vitro* rooting in *Vaccinium corymbosum* (Ericaceae)

Enraizamiento *ex vitro* y establecimiento de un sistema eficiente de inducción de brotes adventicios en *Vaccinium corymbosum* (Ericaceae)

Ye Wang[1,2], Xian Dong[1], Heng-Yu Huang[1]*, and Yuan-Zhong Wang[2]

[1] Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese medicinal materials, Yunnan University of Chinese Medicine, Kunming, China
[2] Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, Kunming, China

* Corresponding author: hhyhhy96@163.com

Abstract
Background: The extension of the blueberry industry in China is restricted by the low performance of proliferation coefficient, transplanting survival rate and the long cycle production.
Hypothesis: We hypothesized the suitable medium with optimal concentration and type combination would improve the survival rate of *Vaccinium corymbosum.*
Species studied: *Vaccinium corymbosum* L. cultivar 'O'Neal.
Study site and years of study: Our study was conducted in Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese medicinal materials, Yunnan University of Chinese Medicine since November 2015.
Methods: Efficient protocol of plant regeneration about Southern highbush blueberry (cultivar 'O'Neal) was established using annual shoots via single factor and orthogonal experiments.
Results: Olive medium with 2.0 mg·L^{-1} zeatin was found to be most suitable for initiation culture. The highest callus induction and bud formation rate were determined with 93.67 % and 95.57 %, respectively. Furthermore, as the germination of axillary buds, numerous shoots were formed with the proliferation coefficient (> 60). Well-developed shoots were obtained using rejuvenation culture on half-strength Olive medium supplemented with combination of 2.0 mg·L^{-1} indole-3-butyric acid, 1.0 mg·L^{-1} naphthaleneacetic acid, 0.01 mg·L^{-1} chlormequat chloride and 0.5 g·L^{-1} activated charcoal. The rooting rate could reach to 100 % using 2 cm length of well-developed shoots transferred into the 5:1 sterilized peat:perlite, which was enhanced from 27.61 up to 95 % after transplanting to the field.
Conclusions: An efficient system for plant regeneration from bud induction to transplanting to the field was established to maintain the characteristics of southern highbush blueberry varieties.
Key words: Adventitious shoot, blueberry, callus, orthogonal experiments, regeneration, rooting.

Resumen
Antecedentes: La ampliación de la industria del arándano en China está restringida por bajo rendimiento del coeficiente de proliferación, tasa de supervivencia del trasplante y ciclos de producción largo.
Hipótesis: Un medio adecuado adicionado con tipos y concentración óptima de auxina mejorará la tasa de supervivencia de *Vaccinium corymbosum*.
Especies estudiadas: *Vaccinium corymbosum* L. cultivar 'O'Neal.
Sitio y años de estudio: Centro de Investigación y Desarrollo de Reproducción y Cultivo de Materiales Medicinales Chinos en Peligro de Extinción y "Daodi" de Yunnan, Universidad de Medicina China de Yunnan, desde noviembre de 2015.
Métodos: Se estableció un protocolo eficiente de regeneración de plantas de arándano azul del sur (cultivar 'O'Neal) utilizando brotes anuales vía factor único y experimentos ortogonales.
Resultados: El medio de oliva con 2.0 mg de L^{-1} de zeatina fue más adecuado para iniciar el cultivo. Aumentó en 93.67 y 95.57 % la inducción de callo y tasa de formación de brotes, respectivamente. La germinación de los brotes axilares provocó numerosos brotes con un coeficiente de proliferación > 60. Se obtuvo brotes bien desarrollados usando cultivos de rejuvenecimiento con medio de oliva adicionado con 2.0 mg·L^{-1} de ácido indol-3-butírico, 1.0 mg·L^{-1} de ácido naftalenacético, 0.01 mg L^{-1} de cloruro de clormequato L^{-1} y 0.5 g L^{-1} de carbón activado. La tasa de enraizamiento alcanzó 100 % utilizando brotes bien desarrollados de 2 cm de longitud y transferidos a turba-perlita 5:1, aumentando de 27.61 a 95 % después del trasplante.
Conclusiones: Se estableció un sistema eficiente de regeneración a partir de inducción de yemas hasta su establecimiento en el campo, para mantener las características de las variedades de arándanos del sur.
Palabras clave: Brotes adventicios, arándanos, callos, experimentos ortogonales, regeneración, enraizamiento.

Blueberry (Ericaceae, *Vaccinium* spp.) is a perennial small berry fruit rich in flavonoids, unsaturated fatty acids and trace elements (Wang *et al*. 2017). It was reported that daily dietary consumption of blueberries could improve endothelial function over six weeks in subjects with metabolic syndrome, which was not linked to lowering low-density lipoprotein cholesterol (Chen *et al*. 2008), but also slowed the aging of motor skills and memory function (Krikorian *et al*. 2010, Meng 2011). Because of its high antioxidant capacity, anti-tumor and anti-inflammatory activities, it is considered as a health-promoting food and is known as the "King Berry" (Rowland & Ogden 1993, Zifkin *et al*. 2012). The production and consumption of blueberry have increased sharply in the world nearly for ten years because of its high economic value and beneficial effect on human health condition. Thirty-five varieties rabbiteye blueberry, and 7 varieties of half highbush blueberry have been produced respectively. According to the statistics of the Food and Agriculture Organization, blueberry cultivation area and output increased by 255 % since from 2008.

There were conventional methods of vegetative propagation by softwood and hardwood cuttings, which is arduous for variable results differ in genotypes, age of stock plant and growing seasons (Kaldmae *et al*. 2006). Moreover, conventional propagation methods are not particularly effective in the propagation and production of healthy, pathogen-free plant material by homogeneous progeny (Meiners *et al*. 2007), which in turn do not allow the conservation of important germplasm of plant species, rare and endangered species in particular. These limitations can be overcome by the utilization of *in vitro* culture techniques, and the most effective propagation system for blueberries is micropropagation due to the rapid and continuous production of a large number of plants. Over the last three decades, *in vitro* culture propagation on the various basal medium using axillary bud proliferation and adventitious shoot regeneration with different explants has been achieved with varying success (Gajdošová *et al*. 2006, Li *et al*. 2006). The culture of blueberries in North America has been more than one hundred years up to now where is the origin place for blueberry. Highbush blueberries were first cultivated by the pioneering researchers of Coville and Elizabeth in the early 1900s (Eck & Childers 1966). Up to now, several basal medium supplemented with different types and concentrations of plant growth regulators (PGRs) such as N^6-[2-isopentenyl] adenine (2ip), zeatin (ZT), zeatin riboside, thidiazuron or 3-indolyl acetic acid and indole-3-butyric acid (IBA), were tested to induce adventitious shoot regeneration of highbush blueberry. In previous reports, protocols of blueberry micropropagation have proceeded successfully (Cao *et al*. 2002, Debnath 2009). However, there have been significant differences between the researchers such as the best basal medium and the most effective PGRs. *In vitro* establishment of propagation of blueberry is not only strongly influenced by the physiological condition of the donor plant serving as the explant source, including the harvest time, collecting parts and age of the stock plant, but also the genotypes of the mother plant (Gonzalez *et al*. 2000, Kaldmae *et al*. 2006).

Many analyses of rooting on blueberry has been successfully investigated by many researchers, including rooting *in vitro* or *ex vitro* and cuttings of field-grown plants. *In vitro* shoots planted directly in the field was rooted significantly better than the cuttings of mature field-grown plants and acclimatized tissue culture-derived plants that root formation was increased from 28.3 % to 92.6 % (Meiners *et al*. 2007). According to Ostroluckà *et al*. (2004), micro-shoot rooting was achieved *in vitro* with IBA (0.8 $mg \cdot L^{-1}$) or *ex vitro* after dipping of shoots into IBA with suitable percentage. Similarly, IBA with suitable concentration found effective for root induction of highbush blueberry (Sedlak & Paprstein 2009). Testsumura *et al*. (2008) found that the superiority in rooting percentage varied with the cultivar and the multiplication medium by a comparison of Murashige and Skoog medium (MS), Woody plant medium (WPM) and a mixture of equal parts of above two media.

The extension of blueberry industry in China is restricted by the low performance of proliferation coefficient, transplanting survival rate and the long cycle production. As far as Yunnan Province is concerned, it has not been able to provide industrialized test tube seedlings to meet the needs of its blueberry industry development. In this study, we used annual branch derived from the juvenile plant as explant with different basal medium and various PGRs to establish an effective regeneration system based on adventitious shoot induction as well as an effective rooting procedure for the southern highbush blueberry cv. 'O'Neal, which is to provide a theoretical basis and technical support of industrialization breeding. This study could also reduce the cost of blueberry planting in Yunnan and offer a realistic basis for promoting the expansion of blueberries industry in this region.

Materials and methods

Preparation of explants. The mother plant southern highbush blueberry (*Vaccinium corymbosum* L.) cultivar (*cv*.) 'O'Neal was cultivated in a greenhouse of Yuxi Xiangxin Agricultural Plantation in Yunnan Province of China. Nodal segments were carefully excised from juvenile individuals then were soaked in 10 % (w/w) detergent solution (common laundry detergent) for 10 min and washed 30 min under current water. The washed segments were surface disinfected with 75 % (v/v) ethanol for 15 seconds followed by 6 min incubation in 0.1 % (w/w) mercury bichloride containing 0.02 % (v/v) Tween 20 along with three rinses in sterile deionized water. Single-node explants were excised about 1.5 to 2 cm in length and cultured in 500 mL culture vessel containing 100 ml nutrient medium mentioned below.

Initiation culture. Six media were investigated to select the optimal basal medium: a) WPM; b) improved Woody plant medium (IM WPM); c) Gamborg's B_5 medium (B_5); d) MS; e) Olive medium (OM), and; f) White medium (White). All these media contained 2 % sucrose (except of 3 % sucrose in MS) and 0.46 % agar supplemented with 2.0 $mg \cdot L^{-1}$ ZT. pH value of all media above mentioned was adjusted to 5.0 and then autoclaved at 121 °C for 22 min. Explants cultured in

these media were incubated in a growth room under continuous light provided by cool-white fluorescent tubes (60 µmol m^{-2} s^{-1} light intensity) at 22 ± 1 °C under a 16 h photoperiod. Single factor experiments were conducted to investigate the effect of PGRs on the growth of blueberry plantlets *in vitro*. All PGRs were filtered through sterilized 0.22 µm Millipore filtering film and added to the medium cooling to 50 to 60 °C after autoclaving.

Callus induction and adventitious shoot regeneration. Aseptic shoots obtained from the initiation cultures were used as explants and a $L_{16}(4^5)$ orthogonal experiment was designed to test the effect of combinations of MS, OM, WPM and IM WPM with different concentration of ZT, naphthaleneacetic acid (NAA) and Kinetin (KT). The performance of different combination was evaluated by determining the callus induction rate after 30 days, the adventitious shoot formation frequency and the shoot regeneration rate after 60 days (Table 1).

Rejuvenation culture and in vitro rooting. Explants 2 cm length excised from multiple shoots were incubated on rejuvenation rooting medium. The rooting media were designed based on a $L_9(3^4)$ orthogonal experiment consisted of different concentration of IBA, NAA and chlormequat chloride (CCC). All of these combinations were added on basal 1/2 OM medium with 0.5 $g \cdot L^{-1}$ activated charcoal (AC) was designed (Table 2). The rooting rate and the well-developed/weak degree of the shoot were determined after 60 days.

Ex vitro rooting. Plantlets 6-8 cm long were transferred into the greenhouse keeping the transparent plastic film closed for three days, which were followed by opening the cover for one day for acclimatization. Stem about 2 cm in length excised from acclimatized plants were then planted in a bubble chamber, which was filled with peat and perlite (5:1) with 90 % relative humidity. The chamber was covered with transparent plastic film and cultured at 25 °C for 15 days, and then the cover was opened progressively in the next 15 days. During the culture period, the plantlets were watered every other day. Percentage of rooting was recorded after 60 days and the survived rates were scored 90 days later.

Statistical analysis. All experiments were conducted three replications with a minimum of 30 explants per treatment.

Table 1. $L_{16}(4^5)$ orthogonal design for callus induction, adventitious shoots induction and proliferation in Southern highbush blueberry cultivar 'O'Neal.

Levels	Factors			
	A (Medium)	B (ZT/mg·L^{-1})	C (NAA/mg·L^{-1})	D (KT/mg·L^{-1})
1	MS	0.5	0.1	0.05
2	OM	1.0	0.5	0.1
3	WPM	2.0	1.0	0.5
4	IM WPM	3.0	2.0	1.0

Table 2. $L_9(3^4)$ orthogonal design for rejuvenation culture in Southern highbush blueberry cultivar 'O'Neal.

Levels	Factors		
	A (IBA/mg·L^{-1})	B (NAA/mg·L^{-1})	C (CCC/mg·L^{-1})
1	1.0	0.5	0.01
2	2.0	1.0	0.05
4	3.0	2.0	0.1

Statistical analysis of the results data obtained in every chapter was subjected to analysis of variance (ANOVA) using SPSS (version 19.0) software. The difference among every treatment method was determined using Least Significant Differences Test at 5 % probability ($P \leq 0.05$), the mean values were further separated using analysis of range.

Results

Initiation cultures. Shoots cultures of *cv*. 'O`Neal' were established successfully from nodal segments with 100 % survival rate on three media including OM (Figure 1A), IM WPM (Figure 1B) and MS (Figure 1C) after 60 days, and the callus with no differentiating ability were generated from the base of explants. The shoot growth of *cv*. 'O`Neal' was various on different basic media (Table 3). In terms of growth rate, shoots cultured on OM was superior to IM WPM and MS. B_5 (Figure 1D), White (Figure 1E) and WPM (Figure 1F) have proved to not be suitable for the culture of *cv*. 'O`Neal'.

Based on the results, single factor experiments were conducted to investigate the effect of various PRGs on the growth of *cv*. 'O`Neal'. The results were revealed that sensitive concentration levels of PGRs when used alone were: NAA 1.0 mg·$L^{-1}$ (Figure 2A), KT 0.1 mg·$L^{-1}$ (Figure 2B) and IBA 1.0 mg·$L^{-1}$ (Figure 2C). 6-Benzyladenine (BA) was not suitable for vigorous growth of *cv*. 'O`Neal' nodal segments (Figure 2D).

Adventitious shoot regeneration and callus induction. Different media and PGRs combinations were investigated to determine the highest performance on callus induction and adventitious shoot regeneration. There were visible differences among different combinations from the $L_{16}(4^5)$ orthogonal experiment (Table 4). In terms of callus induction rate, the range (R_1) of different medium and PGRs sorted in descending order was A (18.353) > B (13.484) > C (4.005) > D (2.398). It was indicated that the medium type and ZT treatment played a vital importance on callus induction while NAA also has weak influence. The type of medium, ZT and NAA were greater than blank control (R_1 = 2.891), which meant a reliable positive regulation on callus induction except for KT. Based on the results of ANOVA analysis in Table 5, NAA and KT treatments did not significantly affect the callus induction rate, whereas the medium type and ZT significantly influenced the callus induction ($P < 0.01$ in medium type and $0.01 < P < 0.05$ in ZT). In light of the statistical analysis above, the effect of KT could be ignored.

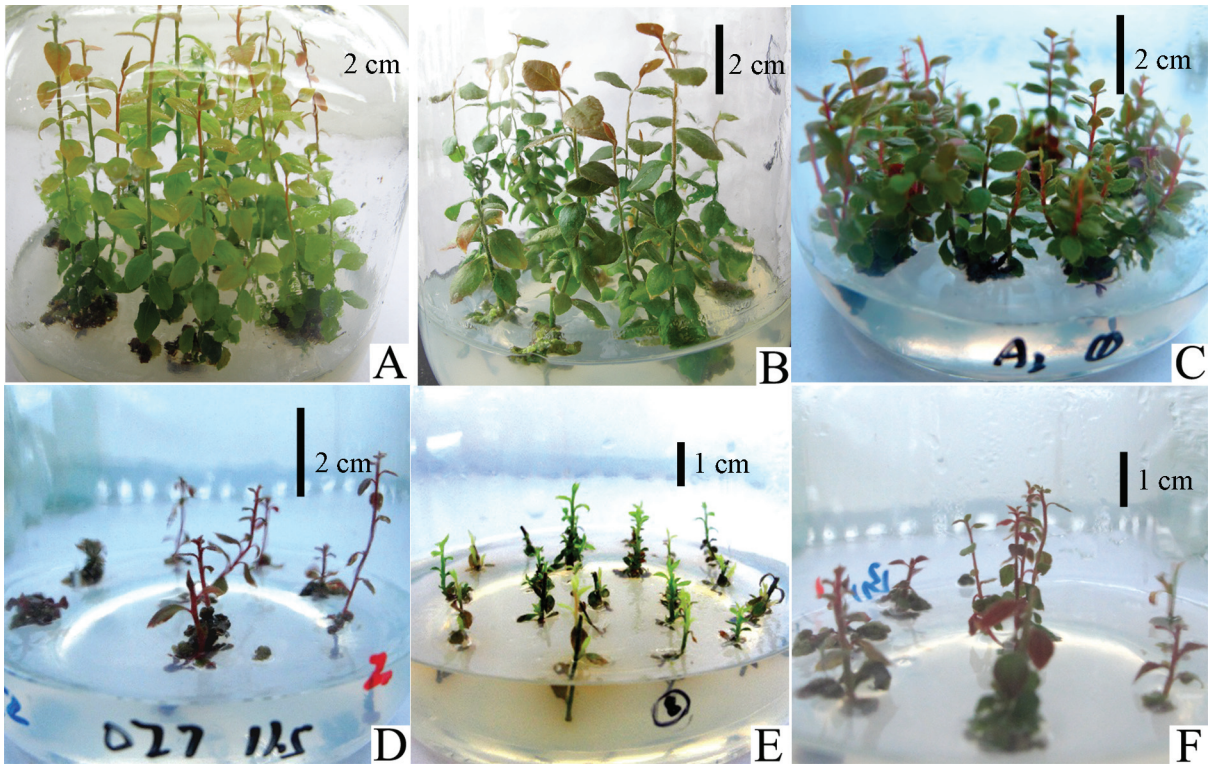

Figure 1. Growth of Southern highbush blueberry cultivar 'O'Neal explants cultured on different basic medium after 60 days. A. OM medium; B. IM WPM medium; C. MS medium. D. B_5 medium. E. White medium. F.WPM medium.

Therefore, the optimal combination for callus induction of *cv*. 'O`Neal' was OM equipped with 2.0 mg·L^{-1} ZT and 2.0 mg·L^{-1} NAA (A2B3C4 group).

However, the performance of different media and PGRs on adventitious shoot regeneration rate were different from that of callus induction, which range (R_2) sorted in descending order was A (48.700) > D (11.945) > B (2.312) > C (1.672) in Table 4. Similar to the results callus induction, the medium still contributed the greatest impact on adventitious shoot induction, after that was the minimum effect of KT. The results showed a reliable effect obviously in adventitious shoot induction because of a higher range of medium and KT for compared with the contrast group (R_2 = 3.418). Furthermore, the adventitious shoot induction was significantly influenced by medium ($P < 0.01$) and KT ($0.01 < P < 0.05$) based on the result of ANOVA analysis in Table 5. The other two PRGs (ZT and NAA) may have a negative impact on adventitious shoot induction not only in the range whose range was higher than the blank contrast group but the significant evaluation ($P > 0.05$). Therefore, the optimal combination for adventitious shoot induction of *cv*. 'O`Neal' was OM coupled with 0.05 mg·L^{-1} KT (A2D1 group).

In conclusion, these results suggested that OM containing 2.0 mg·L^{-1} ZT, 2.0 mg·L^{-1} NAA and 0.05 mg·L^{-1} KT (C07 medium) can be successfully used for callus induction and adventitious shoot regeneration of *cv*. 'O`Neal'.

Because of its strong regenerated ability, stem with a bud was determined as an optimal explant on C07 medium. The first shoots occurred after five days and a vigorous growth was observed after 15 days (Figure 3A). Meanwhile, light

Table 3. The choice of basic media for initiation culture in Southern highbush blueberry cultivar 'O'Neal.

No.	Medium	Plantlet growing
S01	OM + 2.0 mg·L^{-1} ZT	Growth fastest, plant with green color, 100 % shoot regeneration rate, callus formation from the base
S02	IM WPM + 2.0 mg·L^{-1} ZT	Growth fast, plant with green color, 100 % shoot regeneration rate, callus emergence from the base
S03	MS + 2.0 mg·L^{-1} ZT	Growth slowly, plant in green color, 100 % shoot regeneration rate, a few callus formation from the base
S04	B_5 + 2.0 mg·L^{-1} ZT	Growth slowly, plant in red color, 60 % shoot regeneration rate, a few callus formation from the base
S05	White + 2.0 mg·L^{-1} ZT	Growth slowly, plant in yellowish green color, 80 % shoot regeneration rate, no callus
S06	WPM + 2.0 mg·L^{-1} ZT	Growth slowly, plant in red color, 60 % shoot regeneration rate, a few callus formation from the base

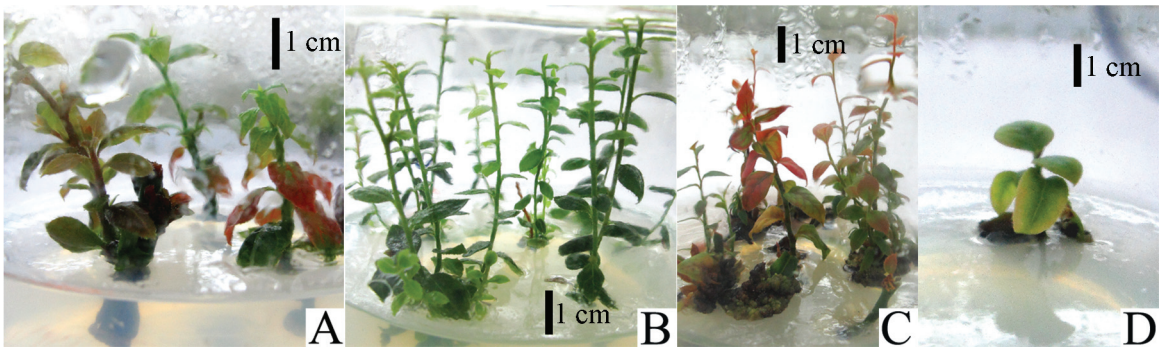

Figure 2. The growth of blueberry on OM medium with different PGRs. A. NAA (1.0 mg·L^{-1}); B. KT (0.1 mg·L^{-1}); C. IBA (1.0 mg·L^{-1}); D. BA.

green callus was generated at the base of shoots (Figure 3B) and differentiated into adventitious buds after five days (Figure 3C and 3D). Propagation coefficient was more than 60.0 of clustered shoots after 60 days (Figure 3E-F). In contrast, stem tip maintained on the same medium grew significantly (Figure 3G) after 15 days. Meanwhile, callus was induced

Table 4. Results of callus induction and adventitious shoots formation of Southern highbush blueberry cultivar 'O'Neal by $L_{16}(4^5)$ orthogonal test.

No.		A Medium	B ZT/mg·L^{-1}	C NAA/mg·L^{-1}	D KT/mg·L^{-1}	Error	Callus induction rate (%)	Adventitious buds formation (%)
C 01		MS	0.5	0.1	0.05	1	68.87	86.67
C 02		MS	1.0	0.5	0.1	2	71.33	82.86
C 03		MS	2.0	1.0	0.5	3	80.33	80.67
C 04		MS	3.0	2.0	1.0	4	76.23	77.36
C 05		OM	0.5	0.5	0.5	4	80.33	85.28
C 06		OM	1.0	0.1	1.0	3	82.65	88.67
C 07		OM	2.0	2.0	0.05	2	93.67	95.57
C 08		OM	3.0	1.0	0.1	1	81.26	92.25
C 09		WPM	0.5	2.0	1.0	2	60.25	33.40
C 10		WPM	1.0	1.0	0.5	1	66.67	38.23
C 11		WPM	2.0	0.1	0.1	4	72.33	45.34
C 12		WPM	3.0	0.5	0.05	3	65.25	50.00
C 13		IM WPM	0.5	2.0	0.1	3	72.20	78.67
C 14		IM WPM	1.0	1.0	0.05	4	78.92	83.33
C 15		IM WPM	2.0	0.5	1.0	1	85.25	68.45
C 16		IM WPM	3.0	0.1	0.5	2	75.00	72.60
	K_1	74.190	69.411	74.713	76.678	76.514		
	K_2	84.478	75.894	75.540	74.280	74.061		
Callus induction rate	K_3	66.125	82.895	74.189	76.584	75.108		
	K_4	77.843	74.435	78.194	75.094	76.952		
	R_1	18.353	13.484	4.005	2.398	2.891		
	K_1	81.890	70.983	73.320	78.893	71.423		
	K_2	90.443	73.295	71.648	74.780	71.085		
Adventitious buds formation	K_3	41.743	72.508	72.390	69.217	74.503		
	K_4	75.763	73.053	72.480	66.948	72.827		
	R_2	48.700	2.312	1.672	11.945	3.418		

Table 5. Variance analysis of callus induction, adventitious shoots and proliferation of Southern highbush blueberry cultivar 'O'Neal.

Source	Type III Sum of Squares	Df	Mean Square	*F* value	Sig.
Dependent variable: Callus induction rate (%)					
A	702.355	3	234.118	46.736	0.005
B	324.5815	3	108.194	21.598	0.016
C	8.317	3	2.7724	0.553	0.680
D	12.1077	3	4.036	0.806	0.568
Error	15.0287	3	5.009		
Dependent variable:Adventitious buds formation (%)					
A	5467.066	3	1822.355	188.639	0.001
B	8.366	3	2.789	0.289	0.833
C	5.665	3	1.888	0.195	0.893
D	346.225	3	115.408	11.946	0.036
Error	28.982	3	9.661		

on it with extremely low induction of adventitious buds and necrosis was appeared after 60 days (Figure 3H). In addition, leaves were not suitable for callus induction due to its low and time-consuming production, callus emerged after 30 days and 60 days was required to induce adventitious buds with poor quality (Figure 3I).

Although stem with a bud maintained on IM WPM, MS and WPM media could induce the callus production and adventitious shoots multiplication, rates of callus induction and adventitious shoots proliferation coefficient were much lower than OM medium. Furthermore, abnormal characteristics such as red shoots or red leaves were occurred after a period of time (Figure 4A to 4C).

Rejuvenation and rooting in vitro. The rejuvenation culture was conducted when the proliferation shoots grew up to 6 cm height. Table 6 shows the results of rejuvenation and rooting *in vitro*.

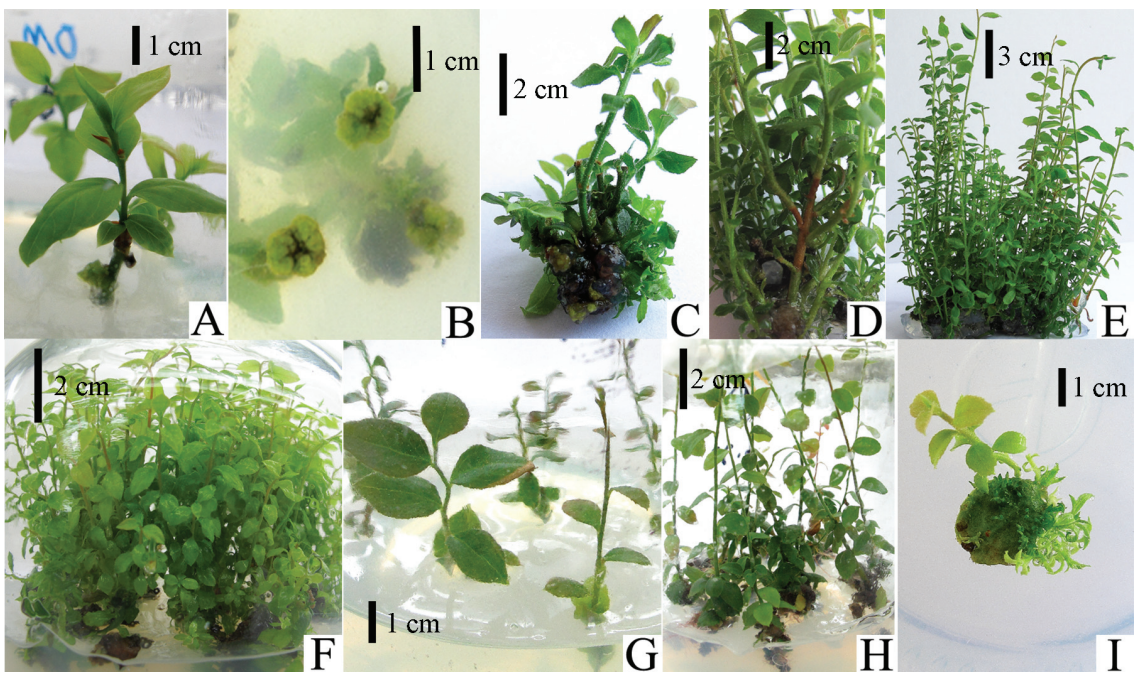

Figure 3. Callus induction, adventitious buds formation and proliferation, and shoots regeneration of Southern highbush blueberry cultivar 'O'Neal on C 07 medium. A**.** Shoots growth after 15 days. B. Callus emerged from the base of the stem with bud segment after 15 days. C. Callus differentiated into adventitious shoots after 20 days. D. Shoots regeneration. E-F. Clustered adventitious shoots formation after 60 days. G. Stem tip cultured for 15 days with no callus formation. H. Stem tip cultured after 60 days. I. Adventitious shoots induction of callus on leaves.

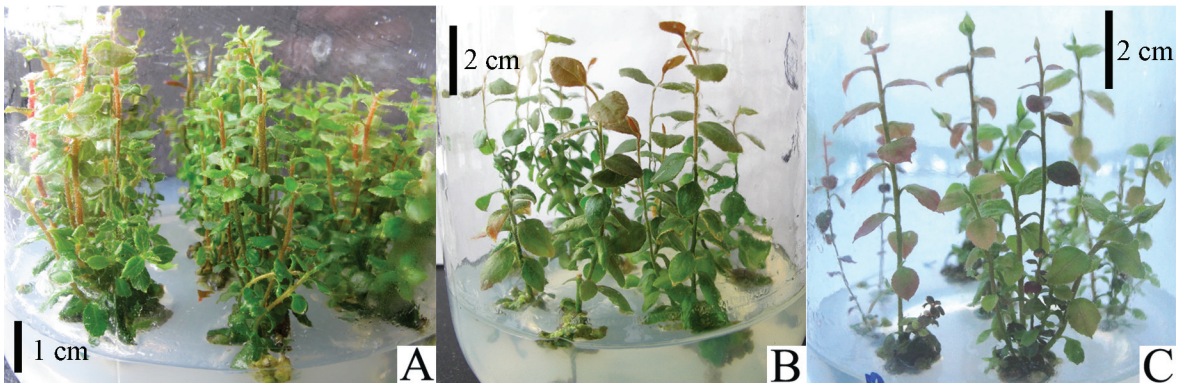

Figure 4. Growth of shoots maintained on IM WPM, MS and WPM medium. A. IM WPM. B. MS. C. WPM.

Since the well-developed/weak degree of seedlings was hard to quantify, the effects of different PGRs (IBA, NAA and CCC) on rejuvenation were visually analyzed in the current study. The results indicated that the well-developed/weak degree of seedlings was mainly influenced by CCC, which promoted the seedlings rejuvenation in low concentrations (0.01 mg·L^{-1}). The well-developed degree of seedlings reduced with the increase of CCC concentration. The rooting rate existed in all combinations media with a relatively low value and the highest rate was obtained in R05 medium (27.61 %). Base on the ANOVA analysis in Table 7, rooting rate was significantly influenced by IBA ($P < 0.01$) and NAA ($0.01 < P < 0.05$) without CCC effect ($P > 0.05$). According to the results of range and ANOVA analysis, combined with the effects of CCC, the optimal medium for rejuvenation and rooting was 1/2 OM + 2.0 mg·L^{-1} IBA + 1.0 mg·L^{-1} NAA + 0.01 mg·L^{-1} CCC + 0.5 g·L^{-1} AC (A2B2C1). It was showed that medium had distinct effects on the weak seedlings for rejuvenation and it was beneficial to the next *ex vitro* rooting (Figure 5A, 5B). In terms of rooting *in vitro*, roots regenerations were observed via either pattern one, where the roots emerged from the callus distributed the base of the plant and it was slender (Figure 5C), or pattern two, where roots directly appeared on the base of plant without callus generation (Figure 5D).

Ex vitro rooting and transplanting. The current study developed to *ex vitro* rooting procedure by micro-cutting with 2 cm length of stems excised from acclimatized plants *in vitro* owing to the low survival rate of transplanting rooting seedlings. Then the stems were planted in bubble chamber, which was filled with peat and perlite (5:1) (Figure 6A) and covered with transparent plastic film to keep a constant temperature and humidity for 15 days. In the following stage, the cover was opened progressively in the next 15 days and watered every other day. 100 % of the rooting percentage was obtained after 60 days culture with irrigation every other day. There were more than 95 % rooting shoots survival after

Table 6. Results of rejuvenation culture and rooting of Southern highbush blueberry cultivar 'O'Neal by $L_9(3^4)$ orthogonal test.

No.	A (IBA mg·L^{-1})	B (NAA mg·L^{-1})	C (CCC mg·L^{-1})	D (Blank)	Well-developed/Weak degree	Rooting rate (%)
R01	1.0	0.5	0.01	1	Thicker	8.35
R02	1.0	1.0	0.05	2	Weak	10.25
R03	1.0	2.0	0.1	3	Weak	6.67
R04	2.0	0.5	0.05	3	Thick	22.33
R05	2.0	1.0	0.1	1	Thick	27.61
R06	2.0	2.0	0.01	2	Thicker	20.25
R07	3.0	0.5	0.1	2	Weak	9.86
R08	3.0	1.0	0.01	3	Thicker	11.95
R09	3.0	2.0	0.05	1	Weak	7.63
K_1	8.423	13.513	13.517	14.530		
K_2	23.397	16.603	13.403	13.453		
K_3	9.813	11.517	14.713	13.650		
R	14.974	5.086	1.010	1.077		

Table 7. Variance analysis of rejuvenation culture and rooting in Southern highbush blueberry cultivar 'O'Neal.

Source	Type III Sum of Squares	Df	Mean Square	*F* value	Sig.
A (IBA)	410.640	2	205.320	208.205	0.005
B (NAA)	39.409	2	19.704	19.981	0.048
C (CCC)	3.161	2	1.580	1.603	0.384
D (error)	1.972	2	0.986	-	-

90 days with obvious growth (Figure 6B-C), which could be transplanted into the nutrition bags (Figure 6D-E) for three months before planting it in the field (Figure 6F).

Discussion

The blueberry has been cultivated for 30 years in China since it was introduced in 1980s. Until the last few years, blueberries have just been introduced to the southwest region of China because of its comfortable climate. Although there were many researchers contributed to the development of the species on various aspects, such as its function (Howell 2009, Stull *et al*. 2015), cultivation and propagation methods (Ozeki & Tamada 2006, Ogden & van Iersel 2009, Fulcher *et al*. 2015). Unfortunately, few literatures reported the respect of adventitious shoot regeneration of blueberry in the southwest of China. Therefore, it's could not be applied mechanically based on the reported culture methods of blueberry due to the variation of climate and soil conditions among places. It is urgent to develop a systemic propagation method to expand blueberry production from tissue culture to field transplantation. Surely, the propagation method is also beneficial for blueberry genetic improvement. To the best of our knowledge, our research is the first report on adventitious shoot organogenesis in southern highbush blueberry *cv*. 'O`Neal'. Two major advantages were displayed in the current study. The one was the propagation coefficient was more than 60 while the other was time-saving for adventitious shoot formation and proliferation simultaneously.

The basal medium was the main factor influencing shoot regeneration of blueberry. The result provided evidence that the basic OM medium increased the *in vitro*-shoot multiplication coefficient of highbush blueberry to more than 60 over eight weeks. OM medium was first to be applied for the culture of blueberry and the basal OM medium supplemented with 2.0 mg·L^{-1} ZT, 2.0 mg·L^{-1} NAA and 0.05 mg·L^{-1} KT was satisfactory for shoot multiplication and further development. Although IM WPM and MS were capable of blueberry proliferation, their performance proved to be inferior to OM medium (Table 4, 5 and Figure 3) compared with previous researchers' study (Rowland & Ogden 1993, Tetsumura *et al*. 2008).

The superior performance of OM medium could be explained that the medium contained a higher level of nitrogen, glutamine and phosphorus than MS and WPM media in terms of the mineral elements of OM, MS and WPM media. The result was the same as Ali (2009) investigation in treatment of olive. Nitrogen in the OM medium presented in the form of NH_4^+ and NO_3^-, which generally believed that both forms of nitrogen were most suited for blueberries (Hanson 2006). Nitrogen also plays an important role in the cell division, differentiation, growth, development and function as a signal molecule of plant growth via increased gene expression for the enzyme responsible for the uptake and utilization of nitrate (Mashayekhi-Nezamabadi 2001). However, the content of reduced nitrogen forms and glutamine (which was also a form of nitrogen) was rare in MS and WPM medium. There was evidence that these amino compounds in the culture medium can improve cell division, differentiation, growth and development of multiple shoots *in vitro* (Sotiropoulos *et al*. 2005). It was reported that phosphorus could be consumed rapidly by shoot forming explants including both the

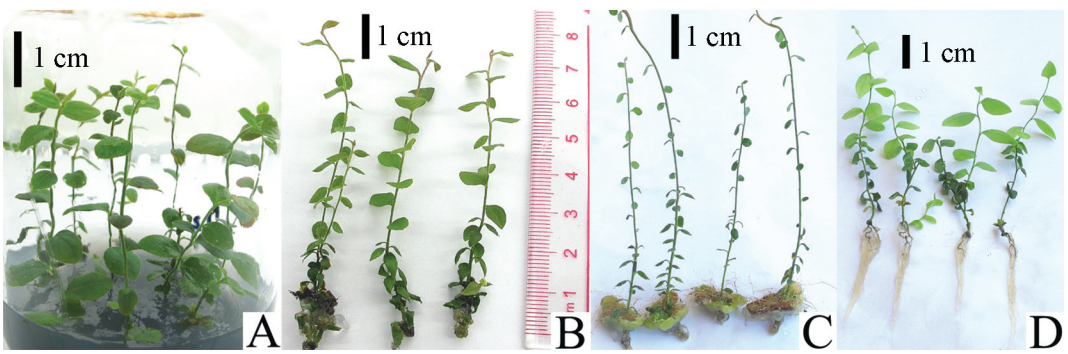

Figure 5. Rejuvenation and rooting *in vitro* in Southern highbush blueberry cultivar. 'O' Neal. A-B. Rejuvenation shoots cultured on combination of 1/2 OM + 2.0 mg·L^{-1} IBA + 1.0 mg·L^{-1} NAA + 0.01 mg·L^{-1} CCC + 0.5 g·L^{-1} AC. C. Adventitious roots differentiated from callus of the stem with bud segment. D. Adventitious roots directly emerged from the shoots base.

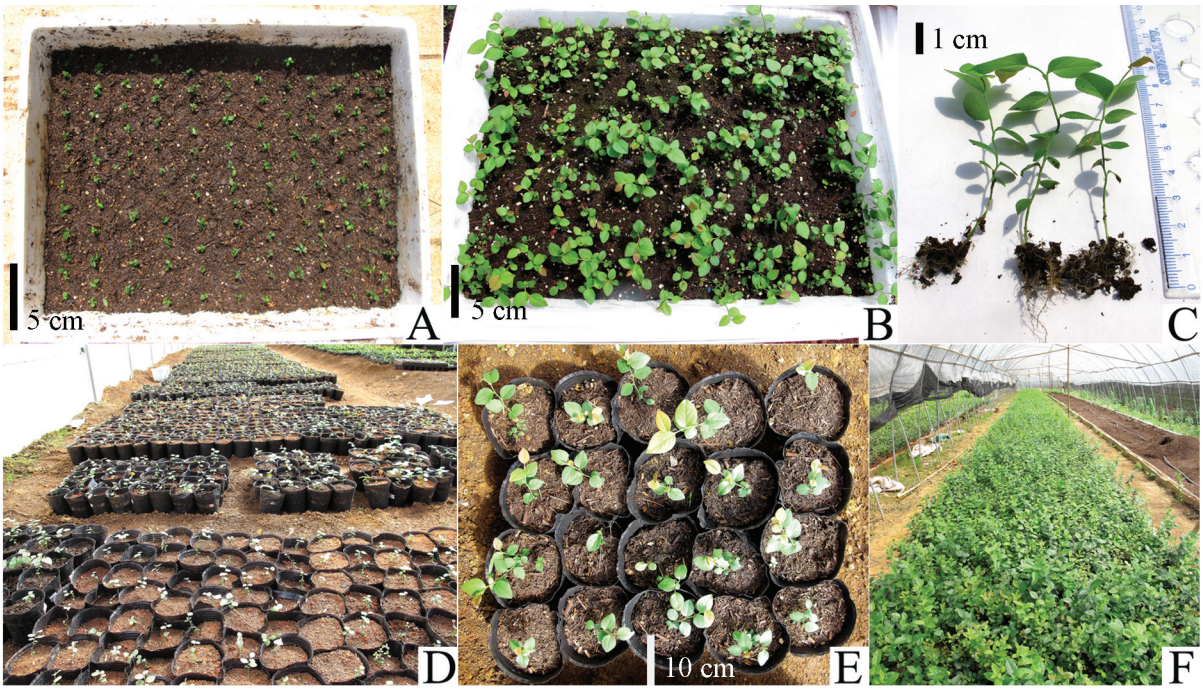

Figure 6. *Ex vitro* rooting and transplanting of Southern highbush blueberry cultivar 'O'Neal. A. Stems initially out of the bottle to *ex vitro* rooting maintained in a bubble chamber. B-C. *Ex vitro* rooted shoots maintained in bubble chamber for 90 days. D-E. Shoots transplanted in the nutrition bags. F. *Ex vitro* rooted shoots after transplanted it in the field for 180 days.

initiation and the growth of shoots (Smith *et al*. 2000). The number of shoots from original explants was correlated to the content of phosphorus in medium (Sharma & Thorpe 1989). In the present study, regardless of shoot number and vigor, red shoots have occurred on WPM medium, and eventually, poor growth was observed (Figure 1). Red shoots were also reported by Testsumura *et al*. (2008), which was believed to indicate a nitrogen deficiency. In the present study, nitrogen-rich OM, MS and IM WPM media were superior to nitrogen-poor WPN medium in terms of the growth of explants (Rowland & Ogden 1993) (Table 3, 4 and 5). Therefore, it was speculated that red shoots of highbush blueberry were caused by a deficiency in nitrogen.

Many researchers have reported that plant regeneration of highbush blueberry. Meiners *et al*. (2007) investigated adventitious shoots induction on sterile leaves of blueberry *cv*. 'Ozarkblue'. Ostroluckà *et al*. (2004) conducted plant regeneration from leaves of highbush blueberry (*Vaccinium corymbosum*). Moreover, micropropagation of *cv*. 'O`Neal' has already been reported from nodal segments (Tetsumura *et al*. 2008). In the present study, stems explants incubated on C07 medium (OM coupled with 2.0 mg·L^{-1} ZT, 2.0 mg·L^{-1} NAA and 0.05 mg·L^{-1} KT) showed the highest frequency of adventitious shoots induction (Table 4). Stem tips and leaves were also examined for their regenerative ability on C07 medium, in which an extremely low induction of adventitious buds in a low-quality and necrosis were observed (Figure 3G, H and I). It was speculated that the higher density of vascular tissue and thus the high level of PGR, which converged towards the proximal end and the transport of nutrients and PGRs (Blakeslee *et al*. 2005). Furthermore, the morphogenic superiority of stem to stem tip and leaf may be the result of a complex phenomenon including both transport of PGRs and tissue maturity (Welander 1988). The insensitive of the stem tip and leaf to various PGR treatments may be caused by factors such as explants' age, explants' origin, collecting time of explant, nature and balance of endogenous PGR in the explants, the lack of proper balance of the exogenous PGR and plant genotype (Van Eck & Kitto 1992, Martin *et al*. 2005, Steiner *et al*. 2007, Bhattacharjee *et al*. 2010). Among the three tissue types (stem with bud segment, stem tip and leaf) tested which incised from aseptic seedling, proved to be primary importance to induce adventitious shoots, competence for shoot organogenesis was restricted to the stem with bud segment. Despite numerous attempts using various PGR treatments in stem tip and leaf in this study, they were inferior in terms of the adventitious shoot formation because of their poor regenerative ability (Figure 3G, H and I). In this study, factors affecting shoot regeneration were tissue type, plant growth regulator, plant genotype and medium.

The dependence of plant tissue on exogenous growth regulators to induce adventitious shoots was well documented in blueberry (Sharma & Thorpe 1989, Gajdošová *et al*. 2006, Meiners *et al*. 2007, Liu *et al*. 2010). Many of them were reported that the combination of cytokinins and auxins to induce plant regeneration (Rowland & Ogden 1993). Cytokinins are commonly used in plant tissue culture to promote cell division, shoot proliferation, and shoot morphogenesis. Auxins

commonly promote cell elongation. In this study, we used ZT with KT and NAA to induce callus and adventitious shoots from leaf, stem tips and stem with bud segment explants (Table 4). IBA and BA were also investigated in the single factor experiments, and unlike many other studies, they were unsuitable for the propagation of *cv*. 'O`Neal' (Figure 2B and D), which was different from many other studies (Litwińczuk *et al*. 2005, Ružić *et al*. 2012). During the stage of callus induction and adventitious shoots formation, ZT, KT and NAA have turned out to be the main factors. Although ZT significantly influenced the formation of callus, it was not beneficial for adventitious shoots formation (Table 4 and 5). 2.0 $mg \cdot L^{-1}$ ZT was determined the most effective for callus induction and differentiation of callus to adventitious shoots. ZT concentration was not correlated with adventitious shoots formation. Similarly, NAA was the main factor in callus induction and insensitive to adventitious shoots formation. KT was effective for adventitious shoots formation, but not suitable for inducing of callus (Table 4 and 5).

Plant regeneration was a crucial aspect of tissue culture that facilitates the production of true-to-type plantlet. However, when the effects of the various compounds were combined, an increased shoot proliferation rate was antagonistic to the qualitative growth of shoot cultures of highbush blueberry in the present protocol. Seedlings in a high proliferation rate were commonly weak. Different concentration of IBA, NAA and CCC were supplemented with rejuvenation media to obtain the well-developed seedling (Table 6). Results showed that the well-developed degree of seedling was mainly influenced by CCC which promoted the seedlings rejuvenation in low concentrations (0.01 $mg \cdot L^{-1}$) (Table 7). As far as we know, the CCC has been used in this study for the first time in China to rejuvenate the highbush blueberry seedling. Moreover, rejuvenation facilitated rooting *ex vitro*.

Generally, *ex vitro* rooting of seedlings was propitious to reduce the cost of production, but the process was often slower than *in vitro*. However, the new protocol proposed from this study could reduce both the cost and time consumption. *In vitro* rooting could be induced in the rejuvenation and rooting medium, which was 1/2 OM supplemented with 2.0 $mg \cdot L^{-1}$ IBA along with 1.0 $mg \cdot L^{-1}$ NAA, 0.01 $mg \cdot L^{-1}$ CCC and 0.5 $g \cdot L^{-1}$ AC (Table 6 and 7). It was hard to survive for seedlings of *in vitro* rooting after transplanting. The current study developed a system of *ex vitro* rooting by micro-cutting with stems of 2 cm long, which was excised from plantlet *in vitro* (Figure 6). This protocol could enhance the surviving rate (> 95 %) and considerably reduce the cost per plantlets in tissue culture. In other words, it could eliminate the stage of rooting *in vitro*, which would be helpful for faster micropropagation of highbush blueberry and less cost. Therefore, the procedure described here is of considerable practical value for the large-scale production of *cv*. 'O`Neal'.

To conclude, adventitious shoot regeneration and *ex vitro* rooting protocol were achieved *cv*. 'O`Neal' and has been presented in this study. A high frequency of adventitious shoots formation was obtained by culturing stem with bud explants on induction medium: OM supplemented with 2.0 $mg \cdot L^{-1}$ ZT, 2.0 $mg \cdot L^{-1}$ NAA and 0.05 $mg \cdot L^{-1}$ KT. Subsequently, high multiplication coefficient and surviving rate acquired after incubating the weak proliferation plantlet on rejuvenation medium: 1/2 OM media supplemented with 2.0 $mg \cdot L^{-1}$ IBA, 1.0 $mg \cdot L^{-1}$NAA, 0.01 $mg \cdot L^{-1}$ CCC and 0.5 $g \cdot L^{-1}$ AC. Established effective recurrent shoots organogenesis provided a continuous supply of propagule free of important pathogens, insect pests and weeds over a prolonged period of about six months. The inclusion of cytokinins and auxins in the culture medium improved the plant regeneration, which displayed normal morphological and reproductive characteristics similar to those of stock plant. The utility of this new protocol has been particularly used for a blueberry plantation in Yunnan region. This effective regeneration system can be used for multiplication desirable genotypes and blueberry breeding of regionalization for Southwest of China *in vitro*, which is highly adapted to indigenous cultivation. Furthermore, as the essential approach, it can be used to improve blueberry cultivar via transferring the important gene from *Vaccinium* species native to Southwest of China.

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