Vitis **53** (4), 207–214 (2014)

In vitro conservation of native Chinese wild grape (*Vitis heyneana* Roem. & Schult) by slow growth culture

Xue Jun Pan[1), 2)], Wen E. Zhang[2)] and Xia Li[1), 2)]

[1)] Engineering Research Center for Fruit Crops of Guizhou Province, Guiyang, Guizhou, P. R. China
[2)] College of Agriculture, Guizhou University, Guiyang, Guizhou, P. R. China

Summary

The aim of the present research work was to develop a protocol to preserve Chinese wild grape by slow growth conservation. Spectacular success was achieved in preserving shoot apices of *Vitis heyneana* under slow growth conditions. The optimized nutrient formulation to maintain slow growth of cultures was Murashige and Skoog (MS) media contained 5 g·L^{-1} agar, 0.05 mg·L^{-1} indole-3-butyric acid (IBA) and 0.1 mg·L^{-1} indole acetic acid (IAA) and 0.5 mg·L^{-1} abscisic acid (ABA). The best osmotic adjustment of nutrient medium was achieved by employing 10 g·L^{-1} mannitol where 47.78 % cultures could be conserved up to 12 months without any subculture. Among different combination of air breathable film area (ABFA), light intensity and chlorocholine chloride (CCC) concentration, used for increasing the subculture period, 19.63 mm^2 ABFA with 5.0 g·L^{-1} CCC cultured under lower light intensity suited best for slow growth conservation with 48.00 % microplants were able to survive 10 months without subculture. Further tests showed that the CCC had a negative effect to grape conservation. Cultures responded better when incubated at 10 °C compared with the control (25 °C). Our study also found that the combination of factors were also more beneficial to grape conservation than that of a single factor. 100 % survived shoots by slow growth conservation could regenerate to normal plantlets and transplant successfully. Transplanting plantlets showed no obvious difference in morphology with the control and the maternal parent in the field.

Key words: Basal media, Culture conditions, Growth retardants, Osmotic agents, Slow growth conservation, *Vitis*.

Introduction

Among the Vitaceae family, only grapevine (*Vitis* spp.) is one of the most important fruit crops in the world. Species of European (*V. vinifera* L.) origins stand out for its high economic value in two markets: table grape and wine production (He 1999, Silva *et al.* 2012). However, global grape production has encountered problems, mainly related to the occurrence of diseases and pests, which are causing a gradual decline in the productive vitality of the plants (Skiada *et al.* 2009). Consequently, some species are being included in germplasm banks to maintain the genetic diversity necessary for plant breeding programs (Schuck *et al.* 2011). China is one of the major gene centers of *Vitis*-species origination. More than 38 *Vitis*-species have their origin in China (He 1999, Kong 2004). China is a vast country with complex geographical environments, greatly differing in climate, soil and topography (Wan *et al.* 2008b). Under these conditions, there are many plant species which are abundant in *Vitis*-germplasm resources (Wan *et al.* 2008a, Pan *et al.* 2010). Chinese wild grape species have enormous economic potential, such as desirable disease resistance, drought tolerance and cold hardiness genes (Li *et al.* 2008, Wan *et al.* 2008a, b and c). This genetic diversity can provide options to develop new and more productive grape cultivars through selection and breeding resistant to biological and environmental stresses (Liu *et al.* 2012).

The genetic diversity of perennial plants, including grapevines, is usually sampled and maintained as live plants in field gene banks (Leão and Motoike 2011, Santana *et al.* 2008). These *in situ* collections are constantly in danger of being lost by exposure to environmental adversity, pest attacks, propagation issues, and frequent budget constraints, not to mention their potential to spread pests and diseases, which are important barriers to germplasm exchange (Engelmann 2011). Biotechnological strategies, based on concepts of *in vitro* plant cell, tissue and organ culture have been developed as an alternative and additional value in response to the problems related to the conservation of plant germplasm in the field (Engelmann 2011, Vasanth and Vivier 2011). These techniques also have the potential to overcome some of the limitations inherent to conventional methods of conservation *ex situ*, and to facilitate the exchange of pest-free germplasm with other research institutions (Ray and Bhattacharya 2010). They have been used for storage of grape germplasm (*V. vinifera*) in many ways such as storage at low temperature or growth retardation with the help of osmotic compounds for conservation on short term and medium term basis (Silva *et al.* 2012), whereas cryopreservation has been in practice for long term storage of germplasm (Vasanth and Vivier 2011). But the preservation effects were different in different species and cultivars, the different genotypes had different requirement in storage conditions (Du *et al.* 2012). Right now, studies on Chinese wild grapes (*V. heyneana* Roem. & Schult) *in vitro* maintenance is virtually non-existent in the literature. In this paper, the optimum nutrient formulation, including MS content, the concentration of

Correspondence to: Dr. X. J. Pan, Engineering Research Center for Fruit Crops of Guizhou Province, Guiyang, Guizhou, 550025, P. R. China. E-mail: pxjun2050@aliyun.com

sucrose, mannitol, CCC and ABA were selected to conserve *V. heyneana*. The effects of culture conditions such as temperature, light intensity and air breathable film area (ABFA) on shoots survival rate were investigated.

Material and Methods

Establishment of *in vitro* regeneration system: One accession of *Vitis heyneana* 'Huaxi-9' was used for the experiment, which was collected from the southwest areas of China and established at the field gene bank in the Guizhou University. The annual shoots were collected from April to June, cut into small pieces about 5 cm in length and rinsed with tap water for 40 min, then surface-sterilized for 30 s in 70 % (v/v) alcohol, immersed in 0.1 % (v/v) $HgCl_2$ for 15 min, washed three times with sterile water in the clean bench. Single nodes of about 1 cm were inoculated on MS medium, supplemented with 30 g·L^{-1} sucrose, 5.0 g·L^{-1} agar, 3.0 mg·L^{-1} 6-benzylaminopurine (6-BA) and 0.01 mg·L^{-1} indole-3-butyric acid (IBA) in PC culture flask (ZP5-330), 57 mm × 36 mm × 34 mm. The pH value of the medium was about 5.8. The bottle was wrapped in a polyethylene film. The single nodes were cultured at 25 ± 1 °C with 12 h day light at an intensity of 40 μmol·m^{-2}·s^{-1}. After four weeks, regenerated cluster axillary buds were excised and given various treatments in PC culture flasks containing 50 mL medium for *in vitro* conservation.

Experimental Design

Experiment 1 - Effects of basal media and osmotic agents on conservation of *V. heyneana*: Cluster axillary buds obtained from previous tests were used as explants for conservation. Axillary buds of 'Huaxi-9' whose length were about 1 cm were cultured on MS, ½ MS or ¼ MS medium with 5.0 g·L^{-1} agar, 0.05 mg·L^{-1} IBA and 0.1 mg·L^{-1} indole acetic acid (IAA). Four concentrations of sucrose (40, 50, 60 and 70 g·L^{-1}) or five concentrations of mannitol (10, 20, 30, 40 and 50 g·L^{-1}) were tested as the osmotic adjustment in the media. Control explants were propagated on ½ MS medium with 30 g·L^{-1} sucrose. Each PC culture flask containing 50 mL of medium was inoculated with three axillary buds. A total of 54 plantlets were used for each treatment with six replications. These were incubated under a 16 h photoperiod with cool white fluorescent lamps (approx. 40 μmol·m^{-2}·s^{-1} light intensity) at 25 ± 1 °C. The survival rate was recorded after cultured 4, 6, 8, 10 and 12 months of conservation, respectively.

Experiment 2 - Effects of combinations of light intensity, air breathable film area (ABFA) and chlorocholine chloride (CCC) concentration on conservation of *V. heyneana*: The same explants as in experiment 1 were cultured on ½ MS medium supplemented with 30 g·L^{-1} sucrose, 5.0 g·L^{-1} agar, 0.05 mg·L^{-1} IBA and 0.1 mg·L^{-1} IAA. The combination of air breathable film area (ABFA), light intensity and chlorocholine chloride (CCC) concentration was designed in an orthogonal experiment (L9 (33)) and showed in Tab. 1. In this experiment, the ABFA was regulated by the capsule and sealing film whose ABFA was 113.10 mm^2. The capsule consists of two layers, there are five holes in one layer, the diameter of each hole is 5 mm, the other layer can be rotated up to five hole fully open or open any of several. In the basis of orthogonal experiment, the single factor tests of ABFA (19.63, one hole opened; 58.90, three holes opened; 98.15, five holes opened and 113.10 mm^2 (no capsule, control)) and CCC (5, 10, 20 and 0 mg·L^{-1} (control)) were conducted to verify the effect of ABFA and CCC. The culture conditions were the same as experiment 1. 54 plantlets were used for each treatment with six replications. The survival rate was recorded after 4, 6, 8, 10 and 12 months of conservation, respectively.

Table 1

Orthogonal experimental design of light, ABFA and CCC

Code	ABFA (mm^2)	Light (μmol·m^{-2}·s^{-1})	CCC (g·L^{-1})
1	19.63 (1)	10 (1)	5 (1)
2	19.63 (1)	20 (2)	10 (2)
3	19.63 (1)	30 (3)	20 (3)
4	58.90 (2)	10 (1)	10 (2)
5	58.90 (2)	20 (2)	20 (3)
6	58.90 (2)	30 (3)	5 (1)
7	98.15 (3)	10 (1)	20 (3)
8	98.15 (3)	20 (2)	5 (1)
9	98.15 (3)	30 (3)	10 (2)

Experiment 3 - Effects of combinations of temperature and abscisic acid (ABA) on conservation of *V. heyneana*: In order to study the effect of combinations of temperature and abscisic acid (ABA), cluster axillary buds of 'Huaxi-9' whose length were about 1 cm were cultured on ½ MS medium with 30.0 g·L^{-1} sucrose, 5.0 g·L^{-1} agar, 0.05 mg·L^{-1} IBA, 0.1 mg·L^{-1} IAA and four different concentrations of ABA (0.5, 1.0, 2.0 and 3.0 mg·L^{-1}). The material was divided into two groups cultured on the same media. One group was placed at 25 °C (12 h/d, 40 μmol·m^{-2}·s^{-1}). The other group was placed at 10 °C (12 h/d, 40 μmol·m^{-2}·s^{-1}) for conservation. The treatments were listed in Tab. 5. 54 plantlets were used for each treatment with six replications. The survival rate was recorded after cultured 6, 8, 10 and 12 months of conservation.

Growth after storage: Initially MS medium with 2.0 mg·L^{-1} BA and 0.1 mg·L^{-1} IAA was used for recovery culture. The survived shoots were subcultured on ½ MS medium supplemented with 0.15 mg·L^{-1} IBA and 0.1 mg·L^{-1} IAA for rooting. The culture conditions were the same as those in the establishment of *in vitro* regeneration system. The rooting tube plantlets were transplanted into the field according to PAN *et al.* (2004) and used to test morphological stability.

Statistical analysis: Statistical analysis of the results was carried out according to Duncan's multiple range tests using SAS statistical software (edition). Data were analyzed using one-way ANOVA tests. Differences were considered significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$. The data presented in tables or figures are mean values of three replicates ± SD.

Results

The effects of basal media and sucrose concentrations on the preservation: The effect of various media with different concentrations of sucrose on the conservation of 'Huaxi-9' was presented in Tab. 2. After 4 months of conservation, microplant survival rate in various media ranged from 43.33 % to 100 %, and decreased to 0-46.67 % after 10 months of conservation. After 10 months of conservation, survived plantlets could be observed only at 40 g·L^{-1} sucrose (Tab. 2). The lowest survival rate at all the storage periods was recorded when microplants were cultured on ¼ MS medium containing 70 g·L^{-1} sucrose, and the highest was on MS medium with 40 g·L^{-1} sucrose. When plantlets were cultured on the same basic media (½ MS), the sucrose concentrations had significant influences on the grape plantlets survival, the highest survival rate was observed in the 40 g·L^{-1} sucrose. The decrease of mineral elements (½ MS and ¼ MS) and/or the addition of high sucrose concentrations (50 to 70 g·L^{-1}) had an obvious negative effect on the microplant survival. The analysis of variance showed that the basic media, and sucrose concentrations as well as the interaction of two factors had highly significant effect ($p < 0.001$) on survival percentage at all storage periods. Among the two factors and their interactions (media and sucrose concentrations), the sucrose concentrations had the highest effect on the microplant survival rate except storage after 4 months.

The effects of basal media and mannitol concentrations on the preservation: As shown in Tab. 3, the influences of various media with different concentrations of mannitol on the conservation of 'Huaxi-9' were different. When the mannitol concentration increased from 10 g·L^{-1} to 50 g·L^{-1}, the survival rate reduced from 100 % to 94.44 %, 100 % to 75.50 %, 85.56 % to 22.41 %, 78.44 % to 0 %, 47.78 % to 0 % with MS media after storage of 4, 6, 8, 10 and 12 months, respectively. The mannitol concentrations in other basal media treatments also had similar effects. Compared to the MS media, reduction of mineral elements content (½ MS, ¼ MS) had also an obvious restraint effect on the survival rate of grape. This result was the same as the effect of sucrose (Tab. 2). MS medium with 10 g·L^{-1} mannitol was the optimal medium for 'Huaxi-9' conservation at each storage period. It was better than all other media including the control medium (½ MS + 30 g·L^{-1} sucrose) (Tab. 3). Microplant condition degraded dramatically after 8 months of storage. After 12 months of storage, more than 50 % of the plantlets had turned yellow and dead even on the best treatment. But the effect of mannitol was better than that of sucrose (Tab. 2) because cultures grown in mannitol could be stored longer than those maintained on sucrose. After the same storage period, the survival rate was higher on mannitol than those on sucrose. The analysis of variance showed highly significant differences ($p < 0.001$) among the basic media, mannitol concentrations and interaction of two factors for survival percentage at all storage periods. Among the two factors and their interactions (media and

Table 2

Microplant survival on various media with four sucrose concentrations after different periods of storage

Media with sucrose	Microplant survival (%) after			
	4 Months	6 Months	8 Months	10 Months
MS + 40 g·L^{-1} sucrose	100 ± 0 Aa	70.33 ± 1.79 Cc	66.67 ± 3.60 Aa	46.67 ± 1.25 Aa
MS + 50 g·L^{-1} sucrose	100 ± 0 Aa	61.67 ± 2.36 Dd	43.33 ± 4.08 Cc	0 Ee
MS + 60 g·L^{-1} sucrose	100 ± 0 Aa	31.00 ± 1.65 Fg	0 Ee	0 Ee
MS + 70 g·L^{-1} sucrose	100 ± 0 Aa	10.67 ± 0.94 Ij	0 Ee	0 Ee
½ MS + 40 g·L^{-1} sucrose	100 ± 0 Aa	80.33 ± 0.47 Bb	56.67 ± 2.36 Bb	33.33 ± 2.72 Bb
½ MS + 50 g·L^{-1} sucrose	100 ± 0Aa	34.33 ± 0.72 Ff	0 Ee	0 Ee
½ MS + 60 g·L^{-1} sucrose	100 ± 0 Aa	24.33 ± 0.94 Gh	0 Ee	0 Ee
½ MS + 70 g·L^{-1} sucrose	94.44 ± 3.140 Ab	21.11 ± 1.57 GHi	0 Ee	0 Ee
¼ MS + 40 g·L^{-1} sucrose	100 ± 0 Aa	86.33 ± 1.89 Aa	43.33 ± 4.08 Cc	21.11 ± 2.83 Cc
¼ MS + 50 g·L^{-1} sucrose	100 ± 0 Aa	40.67 ± 0.94 Ee	16.67 ± 2.36 Dd	0 Ee
¼ MS + 60 g·L^{-1} sucrose	66.67 ± 4.71 Cd	31.33 ± 1.43 Fg	0 Ee	0 Ee
¼ MS + 70 g·L^{-1} sucrose	43.33 ± 2.72 De	19.67 ± 0.47 Hi	0 Ee	0 Ee
½ MS + 30 g·L^{-1} sucrose (CK)	80.25 ± 3.78 Bc	20 ± 1.63 Hi	0 Ee	0 Ee
F_{Media}	386.66***	22.52***	96.85***	57.66***
$F_{sucrose}$	182.45***	2243.97***	878.30***	1202.65***
$F_{Media \times sucrose}$	143.95***	102.01***	56.27***	57.66***

The values are mean ± SD (n = 6); different lower-case and capital letters in the same column represent the significant difference at $p < 0.05$ and $p < 0.01$ respectively. *** represent the significant difference at $p < 0.001$.

Table 3

Microplant survival on various media with five mannitol concentrations after different periods of storage

Media with mannitol	Microplant survival (%) after				
	4 Months	6 Months	8 Months	10 Months	12 Months
½ MS + 30 g·L^{-1} sucrose (CK)	80.25 ± 3.78 Dd	20.00 ± 1.63 Hh	0 Ij	0 Hh	0 Ff
MS + 10 g·L^{-1} mannitol	100 ± 0 Aa	100 ± 0 Aa	85.56 ± 1.57 Aa	78.44 ± 1.37 Aa	47.78 ± 1.57 Aa
MS + 20 g·L^{-1} mannitol	100 ± 0 Aa	100 ± 0 Aa	74.76 ± 2.70 Bb	48.89 ± 0.79 Cc	20.33 ± 2.32 Cc
MS + 30 g·L^{-1} mannitol	100 ± 0 Aa	100 ± 0 Aa	37.96 ± 1.65 Eg	26.67 ± 1.87 Ff	0 Ff
MS + 40 g·L^{-1} mannitol	100 ± 0 Aa	93.33 ± 2.72 Bb	22.41 ± 1.75 FGHi	15.00 ± 1.36 Gg	0 Ff
MS + 50 g·L^{-1} mannitol	94.44 ± 1.57 Bb	75.50 ± 2.01 Dd	26.56 ± 1.75 FGh	0 Hh	0 Ff
½ MS + 10 g·L^{-1} mannitol	100 ± 0 Aa	100 ± 0 Aa	81.75 ± 1.37 Aa	66.67 ± 7.20 Bb	31.11 ± 1.57 Bb
½ MS + 20 g·L^{-1} mannitol	100 ± 0 Aa	100 ± 0 Aa	66.56 ± 0.81 Cc	33.33 ± 0 Ee	6.67 ± 0 Ee
½ MS + 30 g·L^{-1} mannitol	100 ± 0 Aa	100 ± 0 Aa	50.17 ± 1.62 Df	0 Hh	0 Ff
½ MS + 40 g·L^{-1} mannitol	100 ± 0 Aa	94.44 ± 1.57 Bb	27.43 ± 2.69 Fh	0 Hh	0 Ff
½ MS + 50 g·L^{-1} mannitol	100 ± 0 Aa	93.33 ± 0.38 Bb	21.37 ± 1.10 GHi	0 Hh	0 Ff
¼ MS + 10 g·L^{-1} mannitol	100 ± 0 Aa	91.11 ± 1.57 Bb	61.37 ± 1.42 Cd	41.67 ± 1.25 Dd	13.33 ± 2.36 Dd
¼ MS + 20 g·L^{-1} mannitol	100 ± 0 Aa	83.33 ± 2.97 Cc	55.19 ± 3.56 De	25.00 ± 1.36 Ff	0 Ff
¼ MS + 30 g·L^{-1} mannitol	100 ± 0 Aa	66.67 ± 2.72 Ee	34.11 ± 1.48 Eg	0 Hh	0 Ff
¼ MS + 40 g·L^{-1} mannitol	100 ± 0 Aa	43.33 ± 2.36 Ff	27.22 ± 2.98 Fh	0 Hh	0 Ff
¼ MS + 50 g·L^{-1} mannitol	89.86 ± 3.24 Cc	28.22 ± 2.51 Gg	20.63 ± 0.59 Hi	0 Hh	0 Ff
F_{Media}	11.91**	1239.83***	81.53***	505.54***	284.57***
$F_{mannitol}$	37.95***	344.83***	876.06***	657.82***	1006.27***
$F_{Media \times mannitol}$	11.91***	105.14***	25.23***	147.52***	119.76***

The values are mean ± SD (n = 6); different lower-case and capital letters in the same column represent the significant difference at $p < 0.05$ and $p < 0.01$, respectively. **and *** represent the significant difference at $p < 0.01$ and $p < 0.001$, respectively.

mannitol concentrations), the mannitol concentrations had the highest effect on the microplant survival rate except storage after 6 months.

The effects of combinations of light intensity, air breathable film area (ABFA) and chlorocholine chloride (CCC) concentration on conservation of *V. heyneana*: There were remarkable differences in *Vitis* preservation between different combination of air breathable film area (ABFA), light intensity and chlorocholine chloride (CCC) concentration (Tab. 4). The lowest ABFA (19.68 mm^2) had an apparent positive effect on preservation *in vitro*. Moreover, the lowest ABFA combined with 10 µmol·m^{-2}·s^{-1} light and 0.5 g·L^{-1} CCC was the best preservation method in all combinations after storage of 8 to 12 months. The survival rate was obviously higher compared to the other combinations ($p < 0.05$). The Duncan test showed significant differences among different ABFA-treatments ($p < 0.001$) and different CCC concentrations (except a storage of 6 months, $p < 0.001$ or $p < 0.01$) for the survival percentage at all storage periods. However, a negative concentration effect of CCC was observed on the survival rate of 'Huaxi-9' (Tab. 4, Figure, B). The effect of light on grape survival was different at different storage periods. The significant effect was presented only at the 8 months storage ($p < 0.001$) and 12 months ($p < 0.01$), different light treatments showed no obvious difference in survival rate after 6 months and 10 months of storage. The results of ABFA or CCC single factor test were shown in the Figure. Compared with the control (113.10 mm^2), decline of ABFA proved to be very effective in slow growth conservation strategy because it enhanced survival rate after storage (Figure, A). But addition of CCC decreased the survival rate with any concentrations, as the survival rate decreased obviously from 0 (control) to 20 g·L^{-1} CCC at all storage periods (Figure, B).

The effects of temperature and abscisic acid (ABA) concentrations on the preservation: Compared to control (25 °C + 0 mg·L^{-1} ABA), addition of ABA had an apparent effect on survival rate at both temperature treatments after 4 months to 10 months of storage (Tab. 5). After 4 months storage, there were no apparent changes in the survival rate among all treatments except for control, however, a remarkable difference was found among every treatment as the storage time was extended from 6 months to 10 months. The preservation effect was more significant on the medium with 0.5 mg·L^{-1} ABA and increasing concentration of ABA reduced the survival rate of 'Huaxi-9'. Compared with the conservation at 25 °C, the survival rate of microplants at 10 °C was significantly higher (Tab. 5). Variance analysis showed that the effects of different ABA concentration were significant at all storage periods. However, the effects of temperature treatments and the interactions of two factors were not similar to that of ABA after storage (Tab. 5). The effect of temperature was obvious only at 6 and 8 months of storage. When prolonging the storage time to 10 months, there were no significant differences between two temperature treatments. During storage period, the effect of interactions of ABA and temperature did not exist at 4 and 6 months ($p > 0.05$), then changed to be notable at 8 months ($p < 0.001$) and reduced again at 10 months of storage ($p < 0.05$). Growth after storage: As shown in Tab. 6, despite the conservation treatments had effects

Table 4

Microplant survival on various media with five mannitol concentrations after different periods of storage

Treatment				Microplant survival (%) after			
Code	ABFA	Light	CCC	6 Months	8 Months	10 Months	12 Months
1	1	1	1	96.67 ± 3.06 ABa	77.78 ± 2.55 Aa	48.00 ± 4.58 Aa	16.67 ± 1.53 Aa
2	1	2	2	97.67 ± 2.08 Aa	75.22 ± 4.52 Aab	45.00 ± 4.00 ABa	12.44 ± 2.37 Bb
3	1	3	3	99.00 ± 1.73 Aa	70.00 ± 2.00 Ab	40.00 ± 3.00 BCb	6.67 ± 2.51 Cc
4	2	1	2	90.00 ± 3.00 BCb	41.22 ± 1.58 De	16.67 ± 2.08 De	0 Dd
5	2	2	3	88.00 ± 4.36 Cb	50.89 ± 2.59 Cd	19.89 ± 0.84 Dde	0 Dd
6	2	3	1	85.00 ± 3.46 Cb	59.78 ± 4.22 Bc	33.33 ± 2.88 Cc	0 Dd
7	3	1	3	70.00 ± 2.00 Dc	25.44 ± 2.37 Ef	20.00 ± 2.00 Dde	0 Dd
8	3	2	1	73.00 ± 2.00 Dc	42.22 ± 2.55 De	23.33 ± 2.89 Dd	0 Dd
9	3	3	2	75.00 ± 3.00 Dc	30.56 ± 7.19 Ef	6.67 ± 1.53 Ef	0 Dd
	F_{film}			147.72^{***}	288.56^{***}	81.93^{***}	108.10^{***}
	F_{light}			0.16^{ns}	10.89^{***}	0.74^{ns}	6.39^{**}
	Fccc			1.74^{ns}	26.93^{***}	15.03^{***}	6.39^{**}

The values are mean ± SD (n = 6); different lower-case and capital letters in the same column represent the significant difference at $p < 0.05$ and $p < 0.01$, respectively. Ns, **and *** represent no significance, significant difference at $p < 0.01$ and $p < 0.001$, respectively.

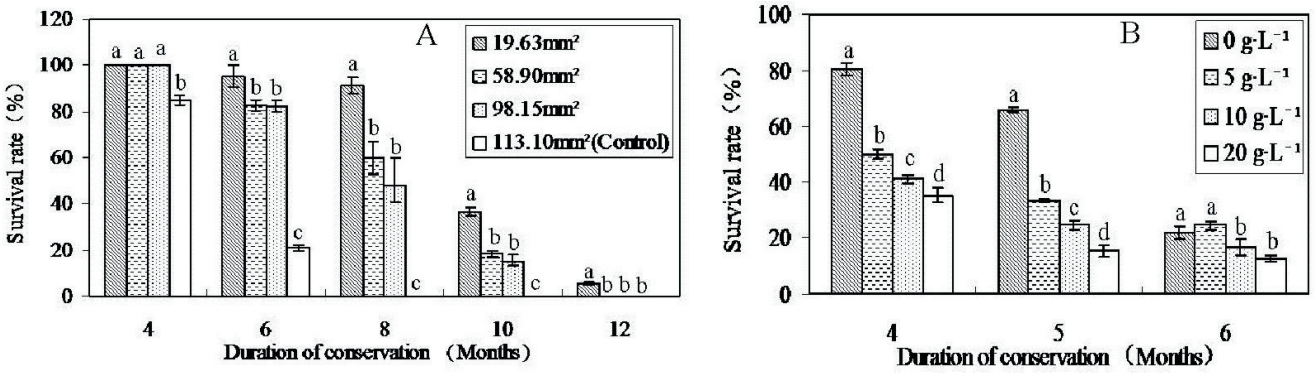

Figure: The effect of ABFA (**A**) and CCC concentrations (**B**) on the conservation of *V. heyneana.* Different letters (a, b, c and d) on top of error bars represent significant differences at p < 0.05 according to Duncan's multiple range tests. Vertical error bars represent SD (n = 6).

Table 5

Effect of different ABA concentrations on microplant survival after different periods conserved at 25 °C and 10 °C

Treatment	Microplant survival (%) after			
	4 Months	6 Months	8 Months	10 Months
10 °C + 0.50 mg·L^{-1} ABA	100 ± 0 Aa	89.12 ± 3.46 Aa	76.62 ± 1.29 Aa	26.33 ± 1.53 Aa
10 °C + 1.00 mg·L^{-1} ABA	100 ± 0 Aa	85.00 ± 4.58 ABab	65.00 ± 1.33 Bb	20.67 ± 1.15 Bbc
10 °C + 2.00 mg·L^{-1} ABA	100 ± 0 Aa	80.00 ± 4.41 BCbc	65.00 ± 1.15 Bb	16.67 ± 2.33 CDd
10 °C +3.00 mg·L^{-1} ABA	100 ± 0 Aa	76.67 ± 1.86 CDc	60.00 ± 1.33 Cc	16.67 ± 1.91 CDd
25 °C + 0.50 mg·L^{-1} ABA	100 ± 0 Aa	80.00 ± 2.88 BCbc	61.00 ± 1.73 Cc	23.00 ± 1.20 ABb
25 °C + 1.00 mg·L^{-1} ABA	100 ± 0 Aa	76.67 ± 1.63 CDc	59.33 ± 1.54 Cc	19.67 ± 1.96 BCc
25 °C + 2.00 mg·L^{-1} ABA	100 ± 0 Aa	70.00 ± 1.82 Dd	60.33 ± 1.34 Cc	20.33 ± 1.21 BCbc
25 °C +3.0 0mg·L^{-1} ABA	100 ± 0 Aa	70.00 ± 2.72 Dd	60.33 ± 1.53 Cc	15.67 ± 1.15 Dd
25 °C +0 mg·L^{-1} ABA (Control)	80.25 ± 3.78 Bb	20.00 ± 1.63 Ee	0 Dd	0 Ee
$F_{Temperature}$	—	45.02^{***}	123.28^{***}	0.40^{ns}
F_{ABA}	—	16.73^{***}	41.77^{***}	29.73^{***}
$F_{Temperature \times ABA}$	—	0.31^{ns}	33.43^{***}	4.97^{*}

The values are mean ± SD (n = 6); different lower-case and capital letters in the same column represent the significant difference at $p < 0.05$ and $p < 0.01$, respectively. Ns, * and *** represent no significance, significant difference at $p < 0.05$ and $p < 0.001$, respectively.

on the recovery growth, for example, the plant height on sucrose was shorter than the control, all surviving grape plantlets which had conserved in different media with different osmotic agents could turn to normal plantlets after regeneration. The regenerated plantlets showed no obvious difference in morphology compared with the control and maternal parent in the field (Tab. 6). The mean transplanted percentage after storage was 100 % for *V. heyneana*. Similar effects were obtained in survival plantlets after another treatment (data no shown).

Discussion

One advantage of slow growth conservation is that slow growth does not require frequent subculture. Another advantage of slow growth conservation is after prolonged period of conservation, the culture can easily be retrieved for production of new plants with full genetic integrity (AHMAD *et al.* 2011). Slow growth is generally achieved by adding osmotic agents in the form of varying concentrations of sucrose, sorbitol and mannitol (GONÇALVES and ROMANO 2007, LATA *et al.* 2010, SCHERWINSKI-PEREIRA *et al.* 2010). Due to the addition of osmotic agents in culture media, there is a significant increase in the storage period of *in vitro* tissues (SHARAF *et al.* 2012).To prolong the period of slow growth conservation, a set of experiments was planned by adjusting content of mineral elements and by adding osmotic agents in culture media. The objective was to improve plantlet survival rate and to prolong the subculture period. Number of plantlets increased first and decreased significantly with the increase of sucrose concentration, and a lower content of mineral elements also reduced the survival rate of 'Huaxi-9'. The maximum survival rates of 46.67 %, 33.33 % and 21.11 % were obtained after 10 months storage on MS, ½ MS and ¼ MS media, each supplemented with 40 g·L^{-1} sucrose (Tab. 2) and the plantlets after conservation could be retrieved (Tab. 6). A complete survival could be obtained only when microshoots of *V. heyneana* were cultured on the medium with 40 g·L^{-1} sucrose conserved for 4 months. It has already been reported that sucrose in high doses exhibited detrimental effect on general condition of plant due to osmotic stress (WITHERS 1991). Using mannitol as an osmotic agent, the storage time of 'Huaxi-9' could be significantly extended or the number of microshoots be raised at the same storage period, when compared with sucrose as osmotic agent (Tabs 2 and 3). The survival rate of plantlets was significantly reduced when the culture period was extended. The mortality of shoot tips was increased with an increase of mannitol concentration and with an increase of culture period up to 12 months. The maximum survival rate (47.78 %) after storage of 12 months was obtained on MS medium with 10 g·L^{-1} mannitol (Tab. 3). Higher concentration mannitol also increased deterioration of microshoots and complete necrosis. Similar results were also reported on plantlets of *Artemisia herba-alba* (SHARAF *et al.* 2012) and *Glycyrrhiza glabra* (ASTAVA *et al.* 2013) when conserved *via* slow growth culture. Some other reports also supported that osmotic agent reduced growth of *in vitro* grown cultures (BAJAJ 1995, MONTALVO-PENICHE 2007, DU *et al.* 2012).

For developing slow growth cultures, various growth regulators, such as abscisic acid (ABA), uniconazole and chlorocholine chloride (CCC), were used to inhibit the plants growth *in vitro* conservation (DU *et al*. 2012, SRIVASTAVA *et al.* 2013). It has been shown that ABA plays an important role in growth, cell division and cell elongation in plants (SWAMI and SMITH 1999). ABA also could alter the carbohydrate metabolism of plant cells (SHIBLI *et al.* 2006, SILVA and SCHERWINSKI-PEREIRA 2011) and significantly decrease growth of shoots in media when compared with controls (DU *et al.* 2012, KEATMETHA 2006, SRIVASTAVA *et al.* 2013). Some researchers reported that CCC was also responsible for inhibiting plant growth and could be used for slow growth conservation (GUNNING and LAGERSTEDT 1985, LI *et al.* 1992). In our study, compared with control (0 mg·L^{-1} ABA), addition of 0.5-3.0 mg·L^{-1} ABA in media could improve the grape survival rate, and the low concentration of ABA (0.5 mg·L^{-1}) proved to be the most beneficial, as 26.33 % (10 °C) and 23.00 % (25 °C) shoot tips remained green up to 10 months of conservation (Tab. 5). On the contrary, compared to the control, more shoots died when the CCC was supplied in media (Figure, B). So we could consider that the CCC promoted the plantlets growth and accelerated plants death in preservation progress. CHEN *et al.* (2006) had also reported that the CCC had no significant effect on Lily slow growth conservation.

The culture conditions, such as temperature, light intensity and oxygen concentration in the culture vessels, also played an important role in developing slow growth conservation (CHEN *et al.* 2006, DU *et al.* 2012, ENGELMANN

Table 6

Growth of the 'Huaxi-9' recovery culture for 40 d after 10 months of conservation in different media with different osmotic agent

Treatment	Plant height (cm)	Bud proliferation	Number of roots	Number of leaves	Leaf shape	Leaf color
MS + 40 g·L^{-1} sucrose	4.50 ± 0.50 b	6.33 ± 0.38 ab	2.33 ± 0.29 a	7.75 ± 0.46 a	Heat-shaped	Light green
½ MS + 40 g·L^{-1} sucrose	4.67 ± 0.34 b	5.96 ± 0.51 b	2.67 ± 0.58 a	7.47 ± 0.50 ab	Heat-shaped	Light green
MS + 10 g·L^{-1} mannitol	6.24 ± 0.55 a	6.77 ± 0.48 ab	2.64 ± 0.38 a	6.86 ± 0.55 abc	Heat-shaped	Green
½ MS + 10 g·L^{-1} mannitol	5.88 ± 0.84 a	7.56 ± 0.51 a	2.67 ± 0.58 a	6.67 ± 0.58 bc	Heat-shaped	Green
½ MS + 30 g·L^{-1} sucrose (CK)	5.22 ± 0.63 ab	7.33 ± 0.58 a	2.33 ± 0.58 a	6.33 ± 0 c	Heat-shaped	Green

The values are mean ± SD (n = 6); different lower-case letters in the same column represent the significant difference at $p < 0.05$.

2011). Previously, Moriguchi *et al.* (1988) has reported that mineral oil could be used for grape callus storage and plant regeneration by preventing the callus cultures from coming directly in contact with oxygen. In our test, the oxygen concentration in the culture vessel was regulated by changing breathable area of sealing film. The results showed that lower breathable area obviously increased the grape survival rate (Tab. 4 and Figure, A). 91 % 'Huaxi-9' could be conserved for more than 8 months only by regulating the permeable area to19.63 mm^2, though some plantlets died during storage. These results suggested that this method was an excellent method of regulating oxygen pressure for plant conservation by slow growth.

Decline of incubation temperature also proved to be very effective in slow growth conservation strategy because it enhanced subculture period (Divakaran *et al.* 2006, Engelmann 2011). The culture temperature was optimized by keeping the cultures at two different temperatures (10 °C and 25 °C), where cultures survived and responded well at 10 °C (Tab. 5). These observations were made on the basis of visual morphological changes and microplant survival. Although, the shoots incubated at such a low temperature of 10 °C turned yellowish in color, leaf size became very small but the shoot tips were quite fresh and green, whereas the shoots incubated at higher temperatures had poor survival and needed frequent subculture due to depletion of nutrients (Srivastava *et al.* 2013). The analysis of variance showed highly significant differences ($p < 0.001$) between the temperatures for survival percentage after 6 to 8 months storage at 10 °C (Tab. 5). It was also possible to limit growth by changing light intensity, mainly by lowering the light intensity (Niu *et al.* 2005). Our study also demonstrated this conclusion. However, the effect of light intensity was lower than those of CCC and ABFA (Tab. 4) and the combination of factors were also more beneficial to grape conservation than that of single factor (Tab. 2 to Tab. 5). The same conclusions were also reported by Srivastava *et al.* (2013) in *Glycyrrhiza glabra* and by Du *et al.* (2012) in Lily.

Conclusion

In the present work, a spectacular success was achieved in conserving shoot apices of *V. heyneana* 'Huaxi-9' under slow growth conditions. It may be concluded that the promising conservation medium was the MS with 0.05 $mg \cdot L^{-1}$ IBA, 0.1 $mg \cdot L^{-1}$ IAA, 0.5 $mg \cdot L^{-1}$ ABA, 5 $g \cdot L^{-1}$ agar, 10 $g \cdot L^{-1}$ mannitol and sealing film with 19.63 mm^2 breathable area. The optimum condition was at 10 °C and 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity for 12 h photoperiod a day which was suitable for medium-term preservation of *V. heyneana* germplasm resources.

Acknowledgements

This research was supported by the project of outstanding young scientists of Guizhou Province (2009-02).

References

Ahmed, M.; Anjum, M. A.; Ahmed, M. J.; Sajid, G. M.; Yoqub, A.; Shafqat, M.; 2011: Role of plant growth regulators in preservation of *Pyrus* germplasm *in vitro*. Afr. J. Biotechnol. **10**, 14029-14037.

Bajaj, Y. P. S.; 1995: Cryopreservation of plant cell, tissue and organ culture for the conservation of germplasm and biodiversity. In: Y. Bajaj (Ed.): Biotechnology in agriculture and forestry, 3-28. Springer-Verlag Press, New York, USA.

Chen, H.; Chen, X. L.; Chen, L. Q.; Lu, X. X.; 2006: Studies on germplasm conservation of Lily (*Lilium* L.) by restricting growth method. Acta Hortic. Sin. **33**, 779-789.

Divakaran, M.; Babu, K. N.; Peter, K. V.; 2006: Conservation of Vanilla species *in vitro*. Sci. Horticult. **10**, 175-180.

Du, Y. P.; Li, W. Y.; Zhang, M. F.; He, H. B.; Jia, G. X.; 2012: The establishment of a slow-growth conservation system *in vitro* for two wild Lily species. Afr. J. Biotechnol. **11**, 1981-1990.

Engelmann, F.; 2011: Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell Dev. Biol. Plant. **47**, 5-16.

Gonçalves, S.; Romano, A.; 2007: *In vitro* minimum growth for conservation of *Drosophyllum lusitanicum*. Biol. Plant. **51**, 795-798.

Gunning, J.; Lagerstedt, H. B.; 1985: Long term storage techniques for *in vitro* plant germplasm. Proc. Int. Plant Prop. Soc. **35**, 199-205.

He, P. C.; 1999: Ampeliology, 562-564. China Agric. Sci. Technol. Press, Beijing, China.

Keatmetha, W.; Suksa-Ard, P.; Mekanawakul, M.; Te-Chato, S.; 2006: *In vitro* germplasm conservation of *Garcinia mangostana* L. and *Lansium domesticum* Corr. Walailak J. Sci. Tech. **3**, 33-50.

Kong, Q. S.; 2004: Annals of Chinese grape, 16-25. China Agric. Sci. Technol. Press, Beijing, China.

Lata, H.; Moraes, R. M.; Bertoni, B.; Pereira, A. M. S.; 2010: *In vitro* germplasm conservation of *Podophyllum peltatum* L. under slow growth conditions. In Vitro Cell Dev. Biol. Plant **46**, 22-27.

Leão, P. C. S.; Motoike, S. Y.; 2011; Genetic diversity in table grapes based on RAPD and micro-satellite markers. Pesq. Agropec. Bras. **46**, 1035-1044.

Li, D.; Wan, Y. Z.; Wang, Y. J.; He, P. H.; 2008: Relatedness of resistance to anthracnose and to white rot in Chinese wild grapes. Vitis **47**, 213-215.

Li, W.; Cao, Z. Y.; Zhang, L. P.; 1992: Effects of some plant inhibitors on the growth of grape plantlets in test-tube. Acta Hortic. Sin. **19**, 215-220.

Liu, C. H.; Fan, X. C.; Jiang, J. F.; Guo, D. L.; Sun, H. S.; Zhang, Y.; Feng, J. C.; 2012: Genetic diversity of Chinese wild grape species by SSR and SRAP markers. Biotechnol. Biotechnolog. Equip. **26**, 2899-2903.

Montalvo-Peniche, M. C.; 2007: *In vitro* germplasm conservation of habanero pepper (*Capsicum chinense* Jacq.). Hort. Sci. **42**, 1247-1252.

Moriguchi, T.; Kozaki, I.; Matsuta, N.; Yamaki, S.; 1988: Plant regeneration from grape callus stored under a combination of low temperature and silicone treatment. Plant Cell Tiss. Org. Cult. **15**, 67-71.

Niu, A. G.; Zhang, K. C.; Zhang, X. M.; Yan, G. H.; Li, W. S.; Jiang, L. J.; 2005: Preservation of *in vitro* growth shoot of cherry germplasm. Acta Hortic. Sin. **32**, 298-300.

Pan, X. J.; Wang, Y. J.; Zhang, J. X.; Jiang, S. P.; 2004: Study on transplanting techniques of grape seedlings from embryo rescue. Acta Bot. Boreal.-Occident. Sin. **24**, 1077-1082.

Pan, X. J.; Li, D.Y.; Zhang, W. E.; Fan, W. G.; Li, S. Y.; 2010: Investigation and analysis of wild *Vitis* resources in Guizhou province. J. Fruit Sci. **27**, 898-901.

Ray, A.; Bhattacharya, S.; 2010: Storage and conversion of *Eclipta alba* synseeds and RAPD analysis of converted plantlets. Biol. Plant. **54**, 547-550.

Santana, J. C.; Hidalgo, E.; Lucas, A. I.; Recio, P.; Ortiz, J. M.; Martín, J. P.; Yuste, J.; Arranz, C.; Rubio, J. A.; 2008: Identification and relationships of accessions grown in the grapevine (*Vitis vinifera* L.) germplasm bank of Castillay Léon (Spain) and the varieties authorized in the VQPRD areas of the region by SSR-marker analysis. Genet. Res. Crop Evol. **55**, 573- 583.

SCHERWINSKI-PEREIRA, J. E.; COSTA, F. H. S.; CAMILLO, J.; SILVA, D. B.; ALVES, R. B. N.; VIEIRA, R. F.; 2010: Tissue culture storage of Brazilian medicinal plants germplasm. Acta Hortic. **860**, 211-214.

SCHUCK, M. R.; BIASI, L. A.; MARIANO, A. M.; LIPSKI, B.; RIAZ, S.; WALKER, M. A.; 2011: Obtaining interspecific hybrids, and molecular analysis by microsatellite markers in grapevine. Pesq. Agropec. Bras. **46**, 1480-1488.

SHARAF, S. A.; SHIBLI, R. A.; KASRAWI, M. A.; BAGHDADI, S. H.; 2012: Slow-growth preservation of wild shih (*Artemisia herba-alba* Asso.) microshoots from Jordan. J. Food Agric. Environ. **10**, 1359-1364.

SHIBLI, R. A.; SHATNAWI, M. A.; SUBAIH, W.; AJLOUNI, M. M.; 2006: *In vitro* conservation and cryopreservation of plant genetic resources: A review. World J. Agric. Sci. **2**, 372-382.

SILVA, R. C.; LUIS, Z. G.; SCHERWINSKI-PEREIRA, J. E.; 2012: Short term storage *in vitro* and large scale propagation of grapevine genotypes. Pesq. Agropec. Bras. **47**, 344-350.

SILVA, T. L.; SCHERWINSKI-PEREIRA, J. E.; 2011: *In vitro* conservation of *Piper aduncum* and *Piper hispidinervum* under slow-growth conditions. Pesq. Agropec. Bras. **46**, 384-389.

SKIADA, F. G.; GRIGORIADOU, K.; MALIOGKA, V. I.; KATIS, N. I.; ELEFTHERIOU, E. P.; 2009: Elimination of grapevine leafroll-associated virus 1 and grapevine *rupestris* stem pitting-associated virus from grapevine cv. Agiorgitiko, and a micropropagation protocol for mass production of virus free plantlets. J. Plant. Pathol. **91**, 177-184.

SRIVASTAVA, M. S.; PURSHOTTAM, D. K.; SRIVASTAVA, A. K.; MISRA, P.; 2013: *In vitro* conservation of *Glycyrrhiza glabra* by slow growth culture. Int. J. Biotechnol. Res. **3**, 49-58.

SWAMY, P. M.; SMITH, B. N.; 1999: Role of abscisic acid in plant stress tolerance. Curr. Sci. **76**, 1220-1227.

VASANTH, K.; VIVIER, M. A.; 2011: Improved cryopreservation procedure for long term storage of synchronized culture of grapevine. Biol. Plant. **55**, 365-369.

WAN, Y. Z.; LI, D.; WANG, Y. J.; HE, P. H.; 2008a: Evaluation of agronomic traits in Chinese wild grapes and screening superior accessions for use in a breeding program. Vitis **47**, 153-158.

WAN, Y. Z.; SCHWANINGER, H.; LI, D.; SIMOB, C. J.; WANG, Y. J.; HE, P. H., 2008b: The eco-geographic distribution of wild grape germplasm in China. Vitis **47**, 77-80.

WAN, Y. Z.; SCHWANINGER, H.; LI, D.; SIMOB, C. J.; WANG, Y. J.; ZHANG, C. H.; 2008c: A Review of taxonomic research on Chinese wild grapes. Vitis **47**, 81-88.

WITHERS, L. A.; 1991: *In vitro* conservation. Biol. J. Linn. Soc. **43**, 31-42.

Received September 9, 2013