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Effect of Different Media and Mannitol Concentrations on Growth and Development of *Vandopsis lissochiloides* (Gaudich.) Pfitz. under Slow Growth Conditions

J. Linjikao, P. Inthima, A. Kongbangkerd

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Abstract*—In vitro* conservation of orchid germplasm provides an effective technique for *ex situ* conservation of orchid diversity. In this study, an efficient protocol for *in vitro* conservation of *Vandopsis lissochiloides* (Gaudich.) Pfitz. plantlet under slow growth conditions was investigated. Plantlets were cultured on different strength of Vacin and Went medium (½VW and ¼VW) supplemented with different concentrations of mannitol (0, 2, 4, 6 and 8%), sucrose (0 and 3%) and 50 g/L potato extract, 150 mL/L coconut water. The cultures were incubated at 25±2 °C and maintained under 20 µmol/m^{2}s light intensity for 24 weeks without subculture. At the end of preservation period, the plantlets were subcultured to fresh medium for growth recovery. The results found that the highest leaf number per plantlet could be observed on ¼VW medium without adding sucrose and mannitol while the highest root number per plantlet was found on ½VW added with 3% sucrose without adding mannitol after 24 weeks of *in vitro* storage. The results showed that the maximum number of leaves (5.8 leaves) and roots (5.0 roots) of preserved plantlets were produced on ¼VW medium without adding sucrose and mannitol. Therefore, ¼VW medium without adding sucrose and mannitol was the best minimum growth conditions for medium-term storage of *V. lissochiloides* plantlets.

***Keywords*—**Preservation, *Vandopsis*, germplasm, *in vitro.*

I. Introduction

VANDOPSIS lissochilodes (Gaudich.) Pfitz., an attractive terrestrial orchid, is in the Orchidaceae family. This orchid is also known as Khao Phra Wihan. In Thailand, this species was found in Sakon Nakhon, Ubon Ratchathani and Sisaket Province [1]. Nowadays, human activities are the main cause of orchid extinctions such as deforestation, over-collection and habitat destruction [2]. More than 170 species of Thai orchids are now listed as endangered species. Up to date, *V. lissochiloides* is classified as "rare" orchids by the Threatened Plants in Thailand. It is locally very rare and occurs in specific habitats [3]. Therefore, an efficient method for plant conservation needs to be studied. *In situ* conservation techniques are an effective way to conserve orchid germplasm. However, a problem with this method is the requirement for a large area, high cost operation and complicated management [4]. Thus, *in vitro* conservation has been developed as an alternative technique for the conservation of plant genetic resources. There are several advantages associated with *in vitro* conservation including reduced space requirements and labour costs for maintenance of germplasm collections [5]-[7]. Generally, this method can be performed by short-term *in vitro* culture, slow growth or minimal growth storage and cryopreservation. In the slow or minimal growth preservation, this approach can be a practical alternative for reducing the growth rate of the cells, tissues or plants by modifying the physical environments and/or the culture medium and increasing the intervals between subcultures. Furthermore, this preservation technique also facilitates convenient storage of the pathogen-free plant materials and reduction of the expenses in labor cost [4]. Factors affecting minimal growth on *in vitro* preservation are crucial issues to study and improve the efficiency of plant conservation. Media strength as well as osmotic agents such as mannitol, sorbitol and polyethylene glycol are the main factors affecting growth and development of *in vitro* storage and widely investigated and observed in many plant species [8]-[10]. Therefore, the objective of this research is to study the effect of different media and mannitol concentrations on growth and development *V. lissochiloides* seedlings under *in vitro* medium term storage.

J. Linjikao is MSc student of the Biotechnology Programs, Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand (e-mail: julaluck.l@hotmail.com).

P. Inthima is an instructor, Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand (e-mail: phithaki@nu.ac.th).

A. Kongbangkerd is Assistant Professor, Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand (corresponding author, e-mail: anupank@nu.ac.th).

II. Material and Methods

A. Plant Materials

V. lissochiloides plantlets regenerated from seed derived protocorm-like bodies (PLBs) were cultured on VW medium added with 150 mL/L coconut water, 50 g/L potato extract, 20 g/L sucrose and 2 g/L activated charcoal [11]. The pH of all media was adjusted to 5.2 before autoclaving at 121 °C for 20 min. The plantlets were cultured at 25 ± 2 °C under 20 µmol/m^{2}s light intensity with 16 h photoperiod and subcultured once every 3 months.

B. In vitro Slow Growth Storage

In vitro V. lissochiloides plantlets (approximately 0.5-1.0 cm in length with two leaves and 1-2 roots) were cultured on different strengths of VW medium (½VW and ¼VW)

augmented with different concentrations of mannitol (0, 2, 4, 6 and 8 %), 50 g/L potato extract, 150 mL/L coconut water and sucrose (0 and 3%) for a total of 13 treatments (Table I). The pH of all media was adjusted to 5.2 before autoclaving at 121 °C for 20 min. The cultures were kept at 25±2 °C under 20 µmol/m^{2}s light intensity with 16 h photoperiod for 24 weeks without subculture. The percentage of survival, leaf formation and rooting as well as leaves' and roots' induction numbers were measured and recorded.

TABLE I
THE MEDIA COMPOSITION FOR *IN VITRO* SLOW GROWTH STORAGE OF *V. LISSOCHILOIDES* PLANTLETS

Basal media [a]	Sucrose (%)	Mannitol (%)
VW	3	0
½VW	0	0
	3	0
	3	2
	3	4
	3	6
	3	8
¼VW	0	0
	3	0
	3	2
	3	4
	3	6
	3	8

[a] Basal media = modified Vacin and Went medium.

C. Recovery Growth of Plantlets after Slow Growth Storage

After the 24 weeks of *in vitro* storage, plantlets were removed from different preservation medium and then transferred to VW basal medium supplemented with 50 g/L potato extract, 50 g/L banana homogenate, 150 mL/L coconut water, 20 g/L sucrose and 2 g/L activated charcoal for growth recovery. The pH of all media was adjusted to 5.2 before autoclaving at 121°C for 20 min. The plantlets were cultured under normal growth conditions at 25±2 °C and grew under 20 µmol/m^{2}s light intensity with 16 h photoperiod. The survival percentage of preserved plantlets was determined after 10 weeks on recovery medium.

D. Experimental Design and Data Analysis

The experiment was performed in a completely randomized design (CRD) with three replications. Each treatment consisted of 20 plantlets per replicates. The experiment data were analyzed using ANOVA. Mean value were compared using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

III. RESULTS AND DISCUSSION

A. Effects of Different Media and Mannitol Concentrations on Survival Rate of V. lissochiloides

This study was conducted to investigate the effects of different media (VW, ½VW and ¼VW) and mannitol concentrations (0, 2, 4, 6 and 8%) on survival rate of *V. lissochiloides* plantlets. All plantlets were cultured on slow growth treatments for 8, 16 and 24 weeks without any subculturing. The results showed that, there were significant differences among different treatments for survival rate of all plantlets. After 8 and 16 weeks of storage, the survival rate of plantlets in various slow growth treatments ranged from 76.0 to 100% (Fig. 1). However, the survival rates decreased during the 24 weeks of preservation period. The lowest survival rate (76.3%) could be obtained on ¼VW medium added with 3% sucrose and 8% mannitol while the highest survival rate (100%) was recorded when plantlets were cultured on ¼VW medium without adding mannitol (Fig. 1).

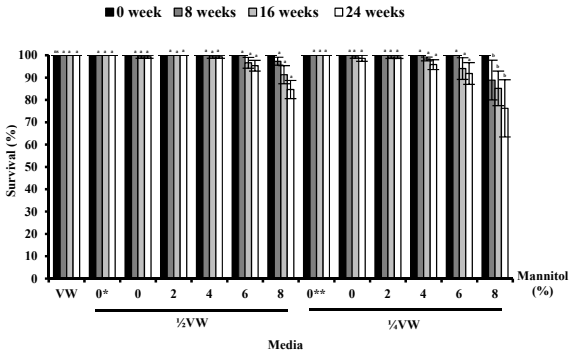


Fig. 1 The survival rate of *Vandopsis lissochiloides* on different media and mannitol concentrations after different periods of storage *0 = ½VW medium without added sucrose and mannitol **0 = ¼VW medium without added sucrose and mannitol

Reduction of mineral element concentrations in VW medium (1/2-1/4 strength) and supplemented with high concentrations of mannitol (2-8%) had a significant adverse effect on growth of *V. lissochiloides* plantlets and survival rates. On these treatments, plantlets seem to have stunted growth, small leaves and short stem when compared to the control treatment. Abnormal plantlet morphology tended to be caused by reducing strength of VW medium from full to quarter and increasing the mannitol concentration to 8% (Fig. 2). Medium salt strength is the most important factor to reduce the growth of various plant species. The reduction of the concentrations of mineral salts (half and quarter) causes to decrease nutrient absorption, translocation and utilization resulting in reduction of plant growth [12], [13]. In addition, osmotic agents (i.e. mannitol and sorbitol) are widely used to mimic osmotic stress in plant tissue culture and causing on low root relative water content [14]. Addition of osmotic agents (i.e. mannitol and sorbitol) to the culture medium causes osmotic stress to plant cells. Mannitol was also used as osmoticum to reduce the hydric potential and restrict the water availability to the conserved plants [15]. In the present study, quarter strength VW without adding mannitol seem to be suitable for preserving plantlets up to 24 weeks without subculture with the highest survival rate (100%). Under these conditions, the plantlets remained green, healthy and could survive. Similar results were reported [16], [17] and stated that addition of mannitol at low level was effective to extend the subculturing interval. In addition, a lower concentration of mineral salts (¼VW) with high levels of mannitol (6 or 8%) can cause abnormal growth of plantlets such as stunted growth

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and necrosis. These results are in agreement with the previous reports which also mentioned that jojoba shootlets necrosis was observed when medium was supplemented with 60 g/L mannitol [18]. Moreover, some reports revealed that reducing the mineral concentration in the culture media (¼MS) led to decrease the survival rate of shoot apices of *Vitis heyneana* Roem. ("Huaxi-9") [19].

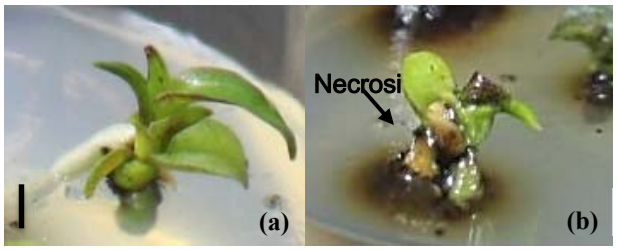


Fig. 2 Effects of different media and mannitol concentrations on necrosis in plantlets of *V. lissochiloides* (a) Healthy plantlet leaves with green color (control treatment) and (b) Plantlets grown in ¼VW added with 3% sucrose and 8% mannitol showing necrotic lesions at the leaf margin and stem after 24 weeks of storage. (bar = 0.25 cm)

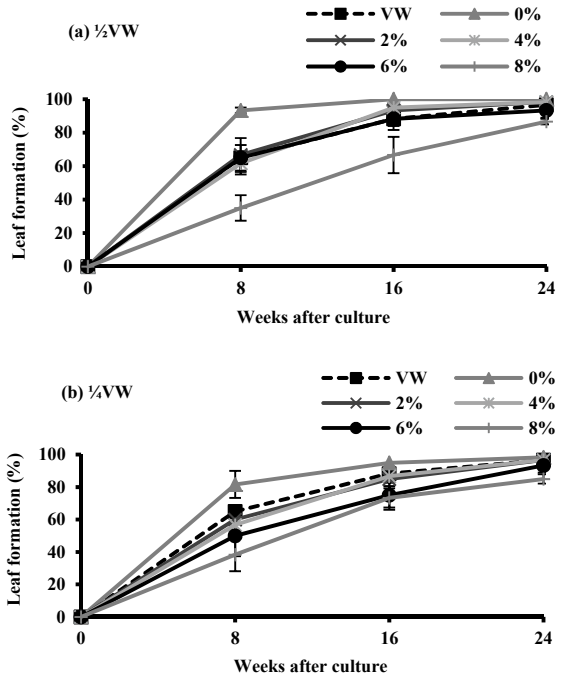


Fig. 3 Effects of different media and mannitol concentrations on leaf formation of *V. lissochiloides* on (a) ½VW and (b) ¼VW medium after 24 weeks of storage

B. Effects of Different Media and Mannitol Concentrations on Leaf and Root Formation of V. lissochiloides Plantlet

Concerning the effect of different media and mannitol concentrations on leaf and root formation of *V. lissochiloides* plantlets after 8, 16 and 24 weeks of storage, the rate of leaf formation of preserved plantlets increased during the followed storage periods. However, using high concentration of mannitol together with low concentrations of mineral salts of culture media could suppress new leaf formation of plantlets. After 24 weeks of storage, results in Figs. 3 (a) and (b) indicated that the highest leaf formation percentage (100%) was noticed on ½VW medium without adding sucrose and mannitol when compared with the control treatment while the lowest leaf formation percentage (85.0%) was found on ¼VW medium added with 3% sucrose and 8% mannitol.

In case of root formation, the highest root formation percentage (81.7%) was detected on ½VW medium without adding sucrose and mannitol (Fig. 4 (a)). On the contrary, *V. lissochiloides* plantlets cultured on ¼VW medium added with 3% sucrose and 8% mannitol gave the lowest root formation percentage (8.3%) after 24 weeks of storage (Fig. 4 (b)). It was assumed that, high concentration of mannitol (8%) might have an adverse effect on root growth and development. A similar result was reported by [20] that root formation of wild pear micro shoots was not observed in conservation media added with mannitol. Furthermore, the absence of rooting hormones (i.e. IBA, IAA or NAA) combined with effects of mannitol in the conservation medium causing poor root formation of plants were also previously reported [21].

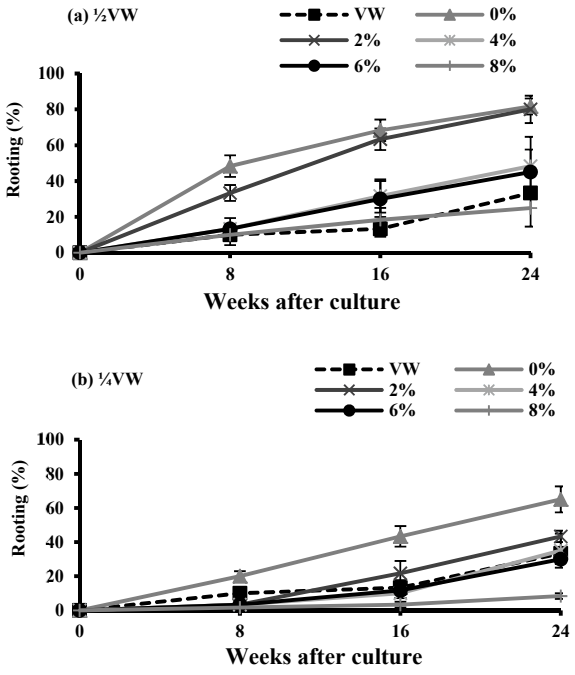


Fig. 4 Effects of different media and mannitol concentrations on rooting of *V. lissochiloides* on (a) ½VW and (b) ¼VW medium after 24 weeks of storage.

C. Effects of Different Media and Mannitol Concentrations on Leaf and Root Induction Number of V. lissochiloides Plantlets

After 24 weeks of storage, the results showed no significant differences among different treatments for leaf and root induction number. The highest number of leaves (4.6 leaves/ plantlet) was produced on ¼VW medium with the absence of sucrose and mannitol. In contrast, the lowest number of leaves (2.4 leaves/plantlet) was obtained when plantlets were cultured on ¼VW medium added with 3% sucrose and 8% mannitol (Table II). With regarding to the root induction number, as shown in Table II, the highest root number (2.5 roots/plantlet) was produced on ½VW medium added with 3%

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sucrose without adding mannitol and ¼VW medium without adding sucrose and mannitol while the lowest number of roots (1.4 roots/plantlet) was obtained when plantlets were cultured on ¼VW medium supplemented with 3% sucrose and 8% mannitol. Therefore, decreasing the mineral concentration in the culture media led to decrease leaf and root induction number. The 1/4 strength is the lowest concentration of conservation medium that was insufficient nutrients for using during preservation period under *in vitro* slow growth storage. Thus, the maintenance of explants over longer periods of storage could result in nutrient depletion [22]. Addition of mannitol to culture medium increases the osmotic potential and reduces nutrient uptake that leads to inhibit plant growth and photosynthesis [23], [24]. Some reports also mentioned that the addition of mannitol to the culture media reduced the growth of plant in terms of roots and leaves number [17], [25].

TABLE II
EFFECTS OF DIFFERENT MEDIA AND MANNITOL CONCENTRATIONS ON GROWTH AND DEVELOPMENT OF *V. LISSOCHILOIDES* PLANTLET AFTER 24 WEEKS OF STORAGE

Basal media [a]	Sucrose (%)	Mannitol (%)	Number per explant	
			Leaves	Roots
VW	3	0	4.1 ± 0.2 ab	1.8 ± 0.1 def
½VW	0	0	4.5 ± 0.2 a	2.0 ± 0.1 bcde
	3	0	4.4 ± 0.2 a	2.5 ± 0.1 a
	3	2	4.2 ± 0.3 ab	2.4 ± 0.1 ab
	3	4	4.0 ± 0.2 ab	2.1 ± 0.2 abcd
	3	6	3.2 ± 0.1 cd	1.8 ± 0.1 def
	3	8	2.7 ± 0.1 de	1.6 ± 0.1 ef
¼VW	0	0	4.6 ± 0.3 a	2.5 ± 0.1 a
	3	0	4.5 ± 0.1 a	2.3 ± 0.1 abc
	3	2	3.6 ± 0.1 bc	2.1 ± 0.2 abcd
	3	4	3.6 ± 0.1 bc	1.9 ± 0.2 cde
	3	6	3.2 ± 0.1 cd	1.7 ± 0.1 def
	3	8	2.4 ± 0.1 e	1.4 ± 0.0 f

Values represent the mean ± SE of three replicates (20 plantlets per replicates). Means within a column followed different letters are statistically significant at $p \leq 0.05$ according to DMRT.
[a] Basal media = modified Vacin and Went medium

D. Growth Recovery of Preserved Plantlets

After 24 weeks of *in vitro* slow growth storage, the plantlets of *V. lissochiloides* were transferred to recovery medium under normal growth conditions for 10 weeks to determine the survival rate. During recovery growth, plantlets were able to regenerate new leaves and roots. However, the preserved plantlets from conservation media added with mannitol could regenerate leaves and roots, but shoots regeneration did not occur after subculture to recovery medium. The results indicated that the survival rate of preserved plantlets in different preservation media varied from 84.4 to 100% and showed no statistically significant differences. Preservation media supplemented with lower concentrations of mannitol resulted in higher survival rates. However, the addition of 8% mannitol to the ¼VW preservation medium decreased the survival rate of *V. lissochiloides* plantlets. The results found that, the lowest survival percentage (84.4%) of preserved plantlets was observed in ¼VW medium added with 8% mannitol (Fig. 5). The maximum number of leaves (5.8 leaves/plantlets) and root number (5.0 roots/plantlets) were produced on ¼VW medium without adding sucrose and mannitol (Table III), (Fig. 6).

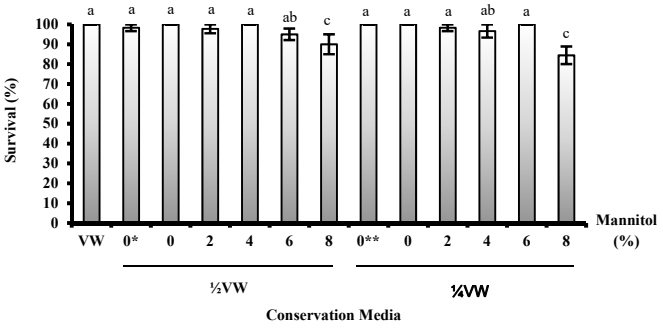


Fig. 5 Survival rate after 10 weeks of recovery growth of *V. lissochiloides* plantlets in recovery medium. *0 = ½VW medium without added sucrose and mannitol, **0 = ¼VW medium without added sucrose and mannitol

TABLE III
GROWTH RECOVERY OF *V. LISSOCHILOIDES* PLANTLETS AFTER RECULTURED IN RECOVERY MEDIUM FOR 10 WEEKS

Preservation media [a]	Sucrose (%)	Mannitol (%)	Number per explant	
			Leaves	Roots
VW	3	0	5.2 ± 0.7 ab	3.2 ± 0.3 bcde
½VW	0	0	4.5 ± 0.2 bc	2.8 ± 0.2 cdef
	3	0	4.1 ± 0.7 cd	2.4 ± 0.5 efg
	3	2	4.1 ± 0.2 cd	3.3 ± 0.1 bc
	3	4	4.2 ± 0.2 cd	2.7 ± 0.2 cdef
	3	6	3.9 ± 0.2 cd	2.5 ± 0.2 defg
	3	8	2.9 ± 0.3 e	1.8 ± 0.2 g
¼VW	0	0	5.8 ± 0.2 a	5.0 ± 0.1 a
	3	0	5.6 ± 0.1 a	3.9 ± 0.1 b
	3	2	4.0 ± 0.1 cd	2.4 ± 0.2 fg
	3	4	4.9 ± 0.1 bc	3.3 ± 0.1 bc
	3	6	3.2 ± 0.3 de	1.7 ± 0.3 g
	3	8	4.1 ± 0.0 cd	3.2 ± 0.1 bcd

Values represent the mean ± SE of three replicates (20 plantlets per replicates). Means within a column followed different letters are statistically significant at $p \leq 0.05$ according to DMRT.
[a] Preservation media = modified Vacin and Went medium

From the results, the preservation medium without adding mannitol could preserve *V. lissochiloides* plantlets for 24 weeks without any subculturing and plantlets showed no abnormality. However, the higher concentrations of mannitol cause water deficit symptom and lead to retardation of plant growth then plantlets finally died [24], [26]. Supplementing the culture medium with mannitol at higher concentration may be toxic and harmful to plants [27], [28]. These results were in line with the former report on *in vitro* preservation study of *Teucrium polium* L. [29]. Related result was also reported that the regrowth capacity of date palm shoot-tip decreases when preservation media was supplemented with mannitol [30]. Furthermore, some previous studies indicated that the higher concentration of mannitol in MS medium adversely affected survival rate and re-growth of the *Spilanthes acmella* plantlets [31].

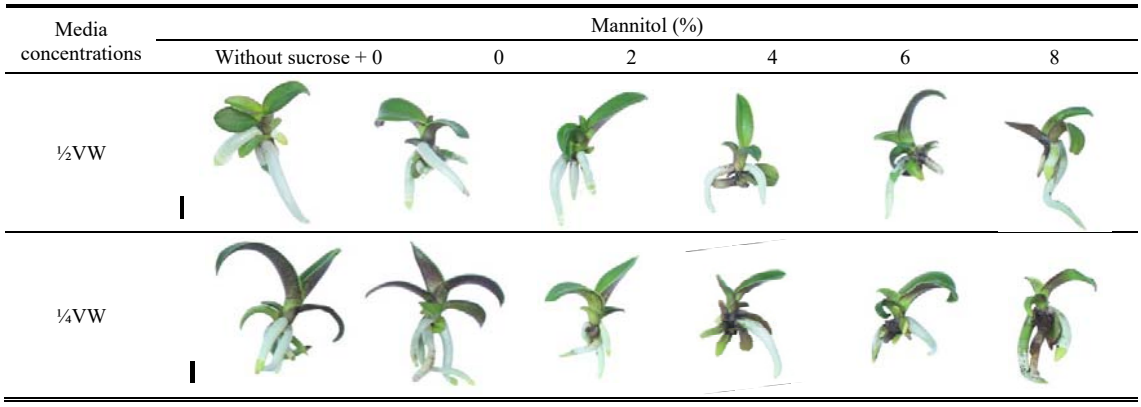


Fig. 6 Growth and development of *V. lissochiloides* plantlets after recultured in recovery medium for 10 weeks (bar = 0.5 cm)

IV. Conclusion

In conclusions, an optimal conservation to preserve *V. lissochiloides* plantlets under medium term storage was performed on ¼VW medium without adding sucrose and mannitol. Plantlets could be preserved for up to 24 weeks with high regrowth capacity and good recovery. On the contrary, addition of 8% mannitol to preservation medium resulted in growth suppression; promoting abnormal characteristics and low regrowth capacity after recultured in recovery medium.

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