Seed Germination *In Vitro* and Plantlet Establishment of *Dendrobium victoria-reginae* Loher. var. *miyakei* (Schltr.) Liu & Su

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Summary

The seeds of *Dendrobium victoria-reginae* Loher var. *miyakei* was sown on six basal media such as, ¼ MS, ½ MS, ½ VW, VW, or Hyponex-1 *in vitro*. One quarter of MS supported the higher percentage of seed germination. ¼ MS Liquid medium gave the higher percentage for seed germinate tham that of seeds on solid medium. ½ MS medium supplemented with activated charcoal (0, 0.1, 0.5, 1, and 2 g L^{-1}) further more. 2 g L^{-1} enhanced plantlet growth, in response heigh of plantlets, number of leaves, and number of roots. The seedlings were successfully transplanted to pots in the green house and gave the better response of the stem growth and width leaves.

Introduction

Dendrobium is one of the largest genus in *Orchidaceae* (Zhu *et al.*, 2009). It was established by Olof Swartz in 1799 (Lavarack *et al*., 2000) distributed from Himalayas, Asia, Australia, Tasmania and the pacific island (Kamemoto *et al*., 1999). There are 900 to over 1400 species in *Dendrobium* (Stewart and Griffiths, 1995). Nowdays, over exploitation of *Dendrobium* due to

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various factors caused some species of this genus started to become scarce. Moreover, the low seed germination in nature makes the production of *Dendrobium* becomes less anyway.

One of those species is *Dendrobium victoriae-reginae* Loher. var. *miyakei* (Schltr)**.** It's native to Taiwan at the thickets of Island Lanyu (Botel Tobago), Taiwan (Su, 2000). It has small flowers with beautiful color. Since *Dendrobium victoriae-reginae* Loher. var. *miyakei* started to become scarce in Taiwan as a native species. Seed germination in *in vitro* is an appropriate solution for rapid multiplication.

The availability of the qualified planting materials *in vitro* is an important aspect to support the plantlet propagation of this species for breeding purposes. One of the important aspect is using activated charcoal in appropriate concentrations. Charcoal is able to create an environment within the planting medium *in vitro* getting darker. Pan and Staden (1998) reported that charcoal is any form of carbon characterized by a high adsorptive capacity for gases, vapors and colloidal solids prevented discoloration by adsorbing phenolics and rendered polyphenol oxidase and peroxidase inactive.

From tissue culture environment to the green house causes tissue stress and have slow growth. The seedling requires a phase of adjustment to new environmental stress after move out to the green house. These plantlets might easily be impaired by sudden change in environmental conditions (Pospisilova *et al.*, 1999). One of the factors that need attention during seedling growth is fertilization. Provision of appropriate frequency, required to get the optimum seedling growth. Spraying foliar fertilizer that too often can lead to poisoning of orchid plant so that the plant will be dry, but if it is not given then the orchid growth will slow (Dwiyani, 2012). Therefore, needed to do research to know the exact concentration of application Hyponex-2.

Materials and Methods

1. Seed germination

Materials

The *Dendrobium victoria-riginae* Loher. var. *miyakei* (Schltr) seed harvested (collected) from Lanyu Island (Taiwan) were used for seeds germination *in vitro* experiments. The capsules harvested on 2012 and July 24th, 2013, and were stored in a centrifuge tube containing silica gel and were used for germination test after 6 months. In laminar flow the seeds were sterilized in sodium hypochlorite (NaOCl, 0.5% w/v) solution with two drop Tween 20 and were shaked for 15 minutes, and then were washed with autoclaved distilled water three times by using a sieve with a filter paper inside to hold the seeds.

Different basal media for seed germination

Six basal media were used (Table 1) as a first treatment and each media with medium volume 8 ml in test tube (Pyrex no. 9820). The seeds were sown in test tubes for each medium and then were wrapped with aluminium foil as a cover.

Solid or liquid medium

In this treatments the PA3 medium were used for seed germination. In test tubes (Pyrex no. 9820) the seeds were sown on 8 ml solid medium or 5 ml liquid medium in a plastic petri dish (Alpha Plus Scientific Corp. 90 x 15 mm, Taoyuan, Taiwan). The test tube was covered with one layer alumium foil. The petri dishes were sealed with two layers of parafilm (American National Can Co., Menasha, WI).

Tabel 1. The composition of six basal media

Media	Composition
PA1	1/4 MS salt(1962), 170 mg L^{-1} NaH_2PO_4, 20 g L^{-1} sucrose, 150 ml L^{-1} coconut water, 100 mg L^{-1} myo-inositol, 1 g L^{-1} Activated Charcoal, 1 g L^{-1} peptone, 6 g L^{-1} potato powder, 8 g L^{-1} agar
PA2	1/2 MS salt, other additives as PA1 media
PA3	1/4 MS salt, other additives as PA1 media, and remove Activated Charcoal
VW	VW (1949), other additives as PA1 media
1/2 VW	1/2VW, other additives as PA1 media
Hyponex1	Hyponex 1 g L^{-1} + 4 g L^{-1} gerlite

Percent seed germination

After 2, 4, 6, 8 or 10 weeks of sown in test tubes and plastic petri disehes, it was observed under light microscope. Seed germination percentage was calculated employing following formula:

$$\frac{\text{No. of seed showing sweeling of the embryo x 100}}{\text{Total No. of seeds}}$$

2. Plantlet culture *in vitro*

Eleven-months-old *in vitro* grown donor plantlets were obtained from seeds germination. The plantlets were selected from mother flask and cultured on PA2 medium (Table 1) with different concentration of activated charcoal (0, 0.1, 0.5, 1, and 2 g L^{-1}). After 16 weeks the plantlet were moved to green house.

3. Culture conditions

The pH of all media was adjusted to 5.2 with 0.1 N NaOH or HCl before autoclaving at 121°C for 20 minutes. The condition in environmental culture room was 25±2°C, and 12-h/d photoperiod was supplied by a cool white fluorescent lamp (FL 40D/ 38 ; China Electric Mfg. Co., Taipei, Taiwan) at 400 lux for germination and 5000 lux for plantlets growth. Each treatments of organic nitrogen and organic additives have 5 flask whereas activated charcoal treatments has 10 flask. All of treatments have 20 plantlets/ flask and were repeated three times to observed. Observations were made every four weeks after cultured. The plants growth (height (from base to leaf top), leaves and roots number) were determined for each trial.

4. Seedling growth in green house

A donor seedlings derived from plantlets cultured of activated charcoal treatments after moving out at 16^{th} week to the greenhouse and were applied Hyponex-2 after two months later. In this treatments have 4 concentration of hyponex-2 (0, 0.5, 1, and 2 g L^{-1}) that were applied to seedlings. Those plants were planted in the pot with size 1.5 inch and sphagnum moss as a potted media. Each pot planted 3 plants and the total pots in each treatments where Hyponex-2 applied were 10 pots. This treatments were repeated two times. Watering once a week with a method of alternating between hyponex-2 and water (50 ml/ pot). After 10 weeks. The plants growth height (from base to leaf top), leaves number, length of leaves, width of leaves, diameter of stem (in the midle of stem), roots number, and length of roots) were determined for each trial.

5. Statistical analysis

The experiments were conducted in a complete randomized design. The average values were analyzed by Costat (CoHort Software, Minneapolis, MN), and the differences were compared by analysis of variance and Duncan's multiple range test at $P \geq 0.05$.

Results

1. Seed germination

Effects of basal media on seed germination

PA1 medium gave the higher seed germination percentage (45.42%) after 10 weeks but it was not significantly different to PA3 and VW (44.09% and 43.19%). And PA2 medium with the higher concentration of inorganic salts, ½ VW medium, and Hyponex medium, the seed germination percentage respectively was 37.44%, 33.59%, and 27.24%, all significantly lower than PA1 medium. The hyponex still got the lower seed germination percentage until 10 week (Table 2).

Table 2. Effect different media on seed germination of *Dendrobium victoria-reginae* Loher var. *miyakei*

Culture period (Weeks)	Seed germination (%)[Z]					
	PA1	PA2	PA3	VW	1/2 VW	Hyponex 1
2	13.86 a[Y]	7.68 c	11.54 ab	11.91 ab	9.18 bc	8.53 bc
4	25.35 ab	28.64 a	24.82 ab	25.57 ab	19.17 bc	13.86 c
6	30.43 ab	31.90 ab	32.99 a	31.91 ab	26.29 b	18.67 c
8	38.92 a	34.74 ab	39.8 a	39.11 a	29.82 b	22.85 c
10	45.42 a	37.44 bc	44.09 ab	43.19 ab	33.59 cd	27.24 d

[Z] The values represent the mean (± SE) of 5 replicates

[Y]In row, mean values followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

Effect of solid or *liquid* medium

The seed germination in liquid medium had the higher percentage than solid medium. Table 3 showed that after 2 weeks the seed germination percentage was increase to 56.28% and significantly different to solid medium (35.18%) in 10 weeks. It also can be seen in Fig. 1b about different both of protocorms growth in liquid and solid medium.

Table 3. Effect of media phase on seed germination(%) *in vitro* of *Dendrobium victoria-reginae* Loher var. *miyakei*

	Culture period (weeks)				
Media phase	2	4	6	8	10
Liquid	8.42 a[Y]	27.27 a	35.5 a	46.02 a	56.28 a
Solid	8.38 a	18.16 b	23.27 b	29.83 b	35.18 b

[Z] The values represent of 5 replicates, basal medium was PA3

[Y]In column, mean values followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

2. Plantlet culture *in vitro*

The increasing of concentration activated charcoal made the affect to height of plantlets and roots number were increased. On the other hand, the reducing of concentration activated charcoal

has made the lower of height and number of roots. It can be seen in Table 4 where is the concentration activated charcoal at 2 g L^{-1} has the highest number of height and roots number also made significantly different compared to the other concentration (Fig. 1c). On the part of leaves, the effect of different concentrations activated charcoal more varied.

3. Effect of Hyponex-2 on potted plantlets

The concentration of 0.5 g L^{-1} Hyponex-2 give the better result for length of leave and diameter of stem, 1 g L^{-1} have a better response for width of leave and length of root, whereas 2 g L^{-1} can increase the plant height and roots number particularly seedlings growth in hight activated charcoal *in vitro* (Table 5 and Fig 1d).

Table 4. Effects of activated charcoal (AC) on the growth of *Dendrobium victoria-reginae* Loher var. *miyakei*

AC ($g.L^{-1}$)	4 w			8 w		
	Height (cm)	NO. of leaves	NO. of roots	Height (cm)	NO. of leaves	NO. of roots
0	1.0 e[Z]	4.0 bc	1.9 ab	1.1 e	4.3 a	2.6 b
0.1	1.1 d	4.4 a	2.5 a	1.4 d	5.4 a	3.0 b
0.5	1.4 c	3.8 c	1.7 b	1.6 c	4.7 a	2.8 b
1	1.7 b	3.8 c	2.0 ab	2.0 b	4.6 a	2.7 b
2	2.0 a	4.3 ab	2.5 a	2.3 a	5.0 a	3.5 a

AC ($g.L^{-1}$)	12 w			16 w		
	Height (cm)	NO. of leaves	NO. of roots	Height (cm)	NO. of leaves	NO. of roots
0	1.4 e	4.6 d	4.0 d	1.6 e	5.3 b	4.7 c
0.1	1.6 d	5.0 c	4.4 c	1.7 d	5.0 c	4.9 c
0.5	1.8 c	5.1 bc	4.6 b	2.0 c	5.4 b	5.6 b
1	2.1 b	5.2 b	4.6 b	2.2 b	5.8 a	5.4 b
2	2.7 a	5.4 a	5 a	3.1 a	5.9 a	6.1 a

The values represent the mean of 3 replicates (each replicates has 20 plants/flask).

[Z]In column, mean values followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

AC = Activated Charcoal

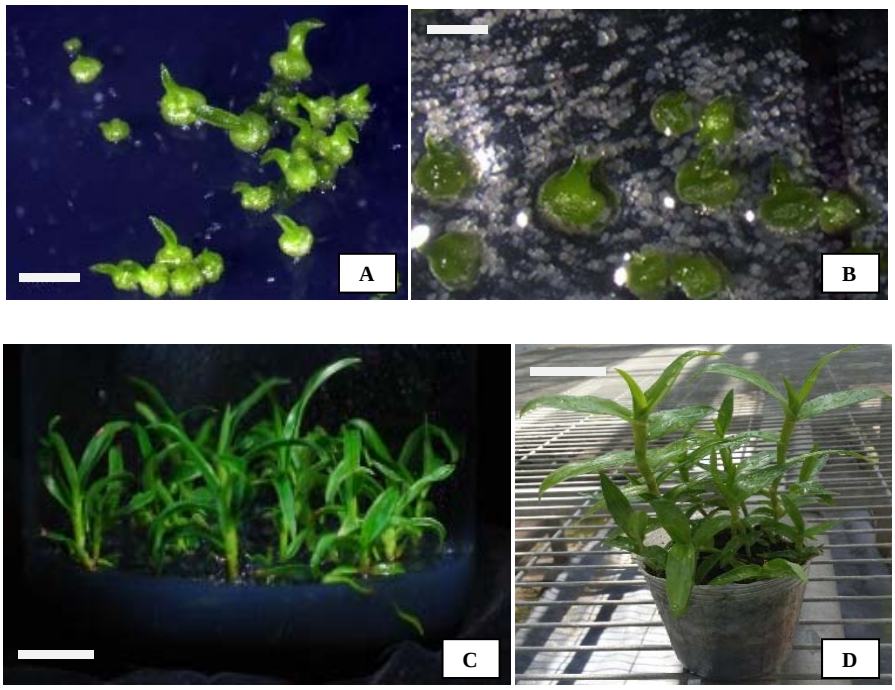

Fig. 1. The plant growth of *Den. victoria-reginae* Loher var. *miyakei.* Protocorms in PA1 solid medium and liquid (phase) medium (A and B) *in vitro* after 10 weeks, plantlet in PA2 medium at 2 g L^{-1} activated charcoal *in vitro* after 16 weeks (C), seedling growth in green house (D). (bar a and b = 0.8 cm, c and d = 1 cm)

Discussion

Fig 1a showed that the protocorms in PA1 medium had highest percentage. PA1 was used a quarter MS that had been reported by Tarek (2004) who explained that reducing the salt concentration of MS-medium to the a quarter strength may have accelerated the biochemical activity. A quarter MS-medium produced the longest shootlet of *Aspidistra elatior* (Tarek, 2004), enhanced shoots number of *Cassava* (Mushiyimana *et al.*, 2011).

In this treatments the liquid medium is the best medium because besides has the higher percentage in germination, it also germinated faster than solid medium. It was explained by Yoder *et al.* (2000) that the delay of germination in asymbiotic sowing was induced by the lack of rapid increase of water acquisition. It the reason of the solid media had lower percentage of seed germination. Tsai and Chu (2008) reported that the liquid culture can improve the growth of *Doritaenopsis.* They said that the seed of *Doritaenopsis* not only germinated but also grew

Table 5. Effect different concentration of Hyponex-2 on plant growth of *Dendrobium victoria-riginae* Loher var. *miyakei* in greenhouse after 10 weeks

Conc. of AC ($g.L^{-1}$)	Conc. of Hyponex 2 ($g.L^{-1}$)	Height	Leaf number	Length of leaf (cm)	Width of leaf (cm)	Diameter of stem (mm)	Root number	length of root (cm)
0	0	4.45abc[Z]	6.17a	3.08abc	0.75de	1.35bc	8.83bcd	4.35abc
	0.5	4.17bc	6.17a	3.03abc	0.78cde	0.85efgh	7.67cd	3.62bcd
	1	4.38bc	5.5a	2.9bc	0.75de	0.83efgh	8.83bcd	3.37cd
	2	3.13c	4b	2.45c	0.63e	0.61gh	6.67d	2.72d
0.1	0	5.4ab	6a	3.12abc	0.97abcd	1.38bc	7.5cd	4.23abc
	0.5	5.62ab	7a	3.85a	1.12a	1.65ab	10.17bcd	4.45abc
	1	5.62ab	6.17a	3.73ab	1abcd	0.92efg	8.67bcd	5.07a
	2	4.53abc	6a	3.37ab	0.95abcd	0.80efgh	8.33bcd	3.92abcd
0.5	0	5.27ab	6.83a	3.32ab	0.98abcd	1.04def	9.17bcd	4.78ab
	0.5	5.17ab	6.5a	3.1abc	0.88abcde	1.63ab	10.83bc	3.65bcd
	1	5.1ab	6.5a	3.1abc	0.98abcd	0.86efgh	11.83b	3.95abcd
	2	5.87a	6.5a	3.67ab	1.07abc	0.75fgh	11.67b	3.32cd
1	0	4.22bc	5.67a	2.97bc	0.8bcde	1.10cde	8.83bcd	4.05abc
	0.5	4.65ab	6a	3.22abc	0.93abcd	1.79a	9.33bcd	3.92abcd
	1	5.22ab	6.17a	3.47ab	1.08ab	0.91efg	10.5bc	3.97abcd
	2	4.53abc	6a	3.43ab	0.98abcd	0.78efgh	10.5bc	3.9abcd
2	0	5.38ab	6.5a	3.55ab	0.97abcd	0.95ef	8.83bcd	3.62bcd
	0.5	4.15bc	5.83a	3.07abc	0.88abcde	1.32cd	8.67bcd	4.18abc
	1	5.32ab	6.67a	3.73ab	1.1a	0.88efg	9.83bcd	3.9abcd
	2	4.97ab	6.67a	3.13abc	1.08ab	0.55h	15.67a	3.15cd
AC		***	**	**	***	***	***	*
Hyponex2		ns	ns	ns	ns	***	*	**
AC x Hyponex2		ns	ns	ns	ns	***	**	ns

The values represent the mean of 6 replicates

[Z] In column, mean values followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

faster in liquid media. It was supported the result in this study where is the higher percentage of seed germination is in liquid medium. The liquid medium provides better aeration and optimum conditions for nutrient uptake (Puchooa, 2004).

Based on this study concentration 2 g L^{-1} activated charcoal (AC) give the best result for plant growth of *Den. victoria-reginae* var. *miyakei* (Table 4 and Fig. 1b) that were observed. Pan and Staden (1998) concluded that the structure of activated charcoal is an important factor which intimately affects its adsorptive capasity. Martin *et al.* (2005) had been reported that the shoots of *Dendrobium* hybrids Sonia 17 and 18 were rooted well upon transfer to half-strength MS medium supplemented with 2 g L^{-1} AC. It's due to activated charcoal has a very fine pores with large inner surface area on which many substances can be adsorbed (Gnasekaran *et al.*, 2012).

Hyponex-2 with concentration 2 g L^{-1} give the better response to plant hight and root number. It supported by Dwiyani (2012) who reported that Hyponex fertilizer can increase the plant height and leave number of *Dendrobium* and Thepsithar *et al.* (2009) also reported that Hyponex with (20N-20P-20K) could enhanced number of seedlings. Table 5 showed that the highest on diameter of stem and length of leaves were used 0.5 g L^{-1} Hyponex-2. It supported by Pospisilova *et al* (1999) who said that after transfer of plantlets from *in vitro* cultures to the green house, substantial changes in leaf morphology, leaf thickness.

Therefore, this species for breeding practice, application of a quarter MS in liquid medium were enhanced percentage of seed germination to grown up being plantlets after moved to a half MS solid medium. Application of 0.5 g L^{-1} Hyponex to in green house for seedling growth gave the best response for stem growth and width of leaves.

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紅花石斛無菌播種與幼苗生長

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摘要：紅花石斛為產於臺灣蘭嶼的原生蘭科植物，本文建立其種子發芽的無菌培育條件，篩檢活性炭促進瓶內小苗發育的合適濃度，以及溫室育苗的施肥濃度。紅花石斛的種子在靜置液體培養基中發芽比固體培養基更快，在另一實驗中 1/4 MS 固體培養基得到了最高比例的種子發芽率。紅花石斛種子發芽後 1 cm 高幼苗，培養在含為 2 g L^{-1} 活性炭 16 週後可增加小苗的株高、葉數及根長。紅花石斛的瓶苗的移植到溫室栽培後，每二週於葉面噴施 50 ml 的 0.5 g L^{-1} 花寶 2 號液體肥料，有助於增加小苗的莖粗及葉片寬度。

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