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Conservation of Gene-resources in Epiphytic *Vanda pumila* (Orchidaceae) by Tissue-cultured Shoot Primordia

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Shoot apices of 60-day-old protocorms of *Vanda pumila* Hook. f., were transplanted and cultured in B5 liquid media supplemented with 2% sucrose and NAA and BAP at various concentrations at pH 5.8 by shaking at 2 cycles/minute on a rotary culture apparatus under 2,000-10,000 lux illumination for 24 hours daily. Tissue-cultured shoot primordia were primarily induced from some of those cultures and were subcultured in B5 liquid medium supplemented with 2% sucrose and 0.02 mg/*l* BAP. Those tissue-cultured shoot primordia showed a high ability to regenerate plantlets approximately 120 days after they were placed onto 1/2 B5 agar medium supplemented with 1% sucrose and no plant growth substance at pH 5.8. The subculture line has been retaining the diploid chromosome number of $2n=38$, typical of this species, for three years. This method may have promise and be applied to certain important clones of *Vanda* for micropropagation and conservation of genetic resources.

Introduction

Since the mass propagation method in orchidaceous plants by using the protocorm-like-body (PLB) was successfully discovered in *Cymbidium* by Morel[1], many orchid genera such as *Cattleya, Cymbidium, Dendrobium* and *Oncidium* have become extensively popular in the commercial world.

On the other hand, Tanaka and Ikeda[2] developed another clonal-micropropagation method called "tissue-cultured shoot primordium" (Patented in Canada, France, Germany, and Japan). This technique may be applied to long-term, clonal micropropagation of various plant-species without any chromosomal aberration, genetic unbalance or virus contamination according to our experiences[3-6]. The shoot primordium originally arose from an apical dome at the shoot apex and can be maintained and propagated only in liquid medium stirred in test tube at 2 cycles per minute by a rotary culture apparatus under 2,000-10,000 lux illumination for 24 hours daily[2]. By using this method a terrestrial orchid, *Spiranthes sinensis* (Pers) Ames has been successfully micropropagated and maintained in a chromosomally and genetically stable condition and furthermore stable colchi-tetraploid plants were induced for more than seven years[6]. However, no tissue-cultured shoot primordium in the epiphytic orchid species has been induced and maintained.

Vanda pumila Hook. f. is an epiphytic orchid-species which has a wide distribution in Southeast Asia but is threatened. It is found in only a few small areas as it has been collected and cultivated for ornamental purpose and its habitat has been destroyed by deforestation. Particularly, the local population of the species grown in altitude 530-1,800 m above sea-level in the southern part of Yunnan province, the People's Republic of China is endangered[7].

The tissue-cultured shoot primordium method holds promise for long-term conservation of the

gene resources of the Yunnan *Vanda pumila* and can be, furthermore, applied to stable, clonal mass-propagation of ornamentally important strains after artificial selection.

Materials and Methods

Vanda pumila Hook. f. was collected in the southern part of Yunnan Province, the People's Republic of China and was cultivated in Kunming Institute of Botany, Chinese Academy of Sciences, Kunming. A ripened fruit of the species was harvested and utilized for this study.

The ripened fruit was surface-sterilized with 1% (v/v) benzalkonium chloride solution for five minutes, 1-2% (v/v) sodium hypochlorite solution for five minutes, and finally rinsed four times with sterile, distilled water. The capsule was then dissected longitudinally and seeds were scooped for sowing on 0.3% (v/v) Hyponex, 0.2% (v/v) peptone and 1.5% (v/v) agar medium. After 30 days seeds were germinated and formed protocorms in a stationary culture at 22°C under 500 lux continuous illumination by fluorescent lamp.

The tissue-cultured shoot primordium method was applied to the young protocorms. Each shoot apex 1-2 mm diameter of the 60-day-old protocorm was individually placed and cultured in test tubes of 30 mm diameter × 200 mm long containing 25 m*l* Gamborg's B5 liquid media (B5)[8] or Murashige and Skoog liquid media (MS)[9] supplemented with 0, 0.02 and 0.20 mg/*l* α-naphthalene acetic acid (NAA) and 0, 0.02, 0.20 and 2.00 mg/*l* 6-benzylaminopurine (BAP) in combination, pH 5.8. Sucrose concentrations were 2% for B5 media and 3% for MS media. The cultures were maintained by shaking at 2 cycles per minute on the rotary culture apparatus at 22°C under 2,000-10,000 lux continuous illumination by halogen lamp.

The tissue-cultured shoot primordia induced were subcultured in B5 liquid medium supplemented with 2% sucrose and 0.02 mg/*l* BAP every 21 days. Fresh weight of each mass of tissue-cultured shoot primordia 5 mm diameter was aseptically measured. Respective mass of tissue-cultured shoot primordia was cut into 2-3 segments each of which was cultured in the same subculture medium under the environmental condition described above. After 30 days, fresh weight of the grown segment of tissue-cultured shoot primordia was measured to calculate growth rate.

Regeneration of plantlets from the tissue-cultured shoot primordia was examined in B5 and 1/2 B5 agar media supplemented with no growth substance, or with 0.02 mg/*l* BAP, or with 0.02 mg/*l* NAA and 0.02 mg/*l* BAP in combination, and 1 or 2% (v/v) sucrose at 22°C with the day-length of 16 hours.

For the histological observation, the tissue-cultured shoot primordia were fixed in 4% (v/v) formaldehyde for 24-36 hours at 0°C, dehydrated in a series of 2-methoxyethanol, 100% ethanol, *n*-propanol and *n*-buthanol for 12-16 hours respectively at 0°C, and then infiltrated in monomer mixture solution of 94.5% purified glycol methacrylate, 0.5% (v/v) 2′2-azobis and 0.5% (v/v) polyethylene glycol 400 for 12 hours at 0°C three times. Then, they were transferred to polyethylene capsules, embedded in monomer mixture at 30°C for one day, 40°C for one day, and then 60°C for one day in an incubator, to prepare hard blocks. The embedded samples were sliced to 8-10 μm thick by a sledge microtome. The sections were affixed on glass slides and were stained with Toluidine blue O.

Chromosome number was counted in subcultured shoot primordia to judge whether or not those shoot primordia had chromosomal stability for a long-term subculture. After pieces of the 7-day-subcultured shoot primordia were pretreated in 0.05% colchicine for three hours at 18°C, they were fixed in modified Carnoy's solution (ethanol : chloroform : glacial acetic acid = 2 : 1 : 1) at 4°C for 24 hours. They were dehydrated by an ethanol series from 70%, 50%, 30%, to 15% for every five

minutes, macerated in 1N HCl at 60°C for eight minutes, and then, double-stained with Schiff's reagent (Feulgen method) and 2% aceto-orcein. Chromosome numbers were counted in 30 cells in each sample.

Results and Discussion

Most of the shoot apices of the protocorms of *Vanda pumila* placed in B5 liquid media supplemented with NAA and BAP at various concentrations in combination survived for the primary culture for a month, while those in MS liquid media died. This result was similar to that in the *Spiranthes sinensis* culture[6].

After three months of primary culture, the shoot apices differentiated into three culture types; (1) tissue-cultured shoot primordia, (2) protocorm-like-bodies (PLB's) and (3) calli (**Table 1**). *Confetti*-shaped masses of tissue-cultured shoot primordia were induced only in B5 liquid media supplemented with 0.02 or 0.20 mg/*l* BAP alone, or with 0.02 mg/*l* BAP and 0.02 or 0.20 mg/*l* NAA in combination. Each mass of tissue-cultured shoot primordia was, then, isolated and subcultured for propagation for three weeks in B5 liquid medium supplemented with 2% sucrose and 0.02 mg/*l* BAP to fill out 25 m*l* volume inside the culture tube (**Fig. 1**). Each mass of tissue-cultured shoot primordia obtained after the subculture consisted of bright green-colored nodules (**Fig. 1-B**) and each nodule consisted of a single cell layer and bigger, irregular-shaped inner cells (**Fig. 2-A**).

After the first subculture, different types of cultures including mass of tissue-cultured shoot primordia, PLB's and complex conglomeration mixed with tissue-cultured shoot primordia and

Table 1. Induction of tissue-cultured shoot primordia from shoot apices in 60-day-old protocorms of *Vanda pumila* in B5 liquid media supplemented with NAA and BAP for primary culture.

NAA (mg/*l*) \ BAP (mg/*l*)	Number of shoot apices developed / Number of shoot apices planted			
	0	0.02	0.20	2.00
0	PLB 4/4	SP 2/4	C 1/4	C 1/4
			C, SP 2/4	
		D 2/4	D 1/4	D 3/4
0.02	PLB 3/4	SP 1/4	C 1/4	
		SP, PLB 2/4		
	D 1/4	D 1/4	D 3/4	D 4/4
0.20	PLB 1/4	SP, PLB 2/4	C 1/4	
	D 3/4	D 2/4	D 3/4	D 4/4

Induction of: PLB=protocorm-like bodies; SP=shoot primordia; C=calli and D=dead.

Table 2. Comparison of growth rates among mass of tissue-cultured shoot primordia (SP), protocorm-like bodies (PLB), and complex conglomeration of SP and PLB (CC) in *Vanda pumila*.

Culture type	Fresh weight (g)		Growth rate
	Initial	30 days	
SP	4.01	19.35	4.83
PLB	4.08	8.76	2.14
CC	4.00	14.12	3.53

Growth rate: fresh weight 30 days after subculture / initial fresh weight.

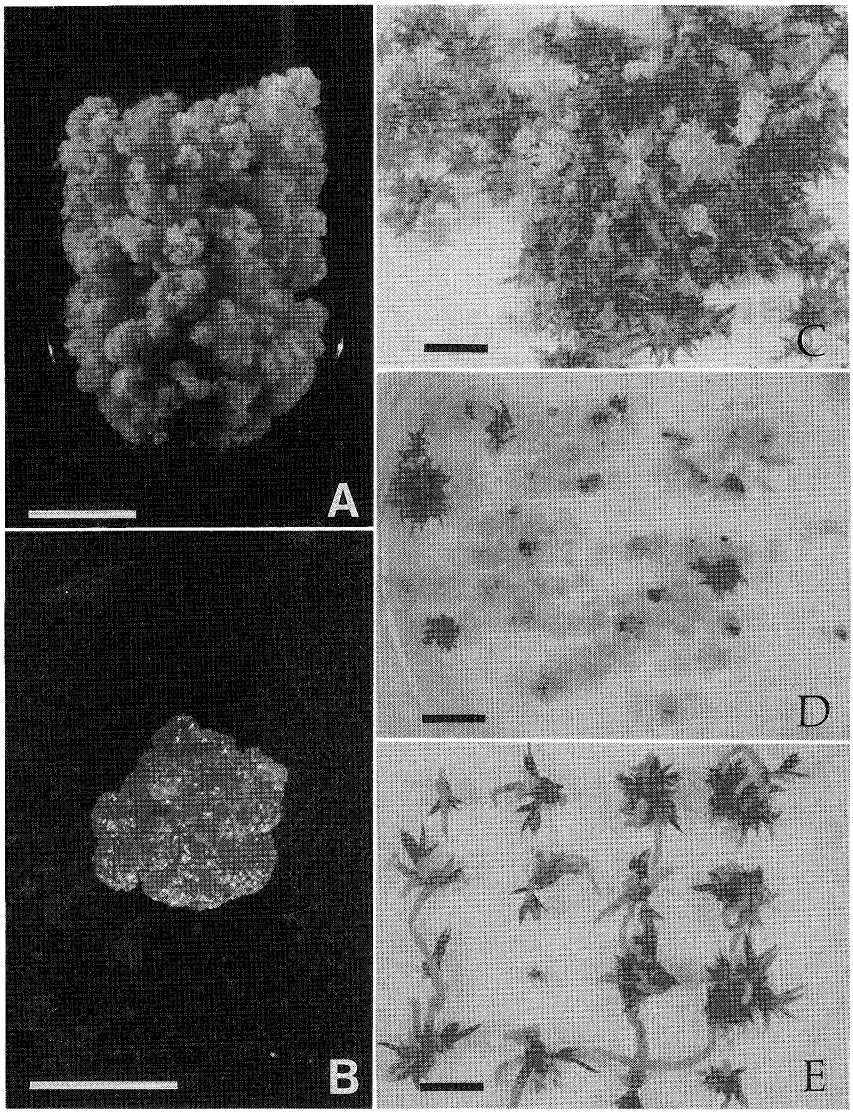

Fig. 1 Masses of tissue-cultured shoot primordia and their regeneration of plantlets in *Vanda pumila*.

A. Three weeks subculture of masses of shoot primordia in a test tube 30 mm diameter × 200 mm long contained 25 m*l* B5 liquid medium supplemented with 0. 02 mg/*l* BAP. B. A large-enough, individual mass of tissue-cultured shoot primordia. C. Highly efficient shoot regeneration from subcultured masses of shoot primordia after 80 days culture on 1/2 B5 agar medium with no growth substance. D. Poor shoot regeneration from subcultured masses of shoot primordia after 80 days culture on B5 agar medium with 0. 02 mg/*l* NAA and 0. 02 mg/*l* BAP. E. Regeneration of plantlets after 120 days culture on 1/2 B5 agar medium with no growth substance. Some plantlets formed multiple shoots. Bar in A and C-E = 10 cm; Bar in B = 5 mm.

PLB's occurred were cultured separately and fresh weighs of these cultures were measured at the initial stage and one month after the subcultures for determination of their growth rates (**Table 2**). Then, the tissue-cultured shoot primordia showed the highest growth rate. This rapid-growth behavior of the tissue-cultured shoot primordia in *Vanda pumila* was the same as that in *Haplopappus gracilis*[2] and *Spiranthes sinensis*[6].

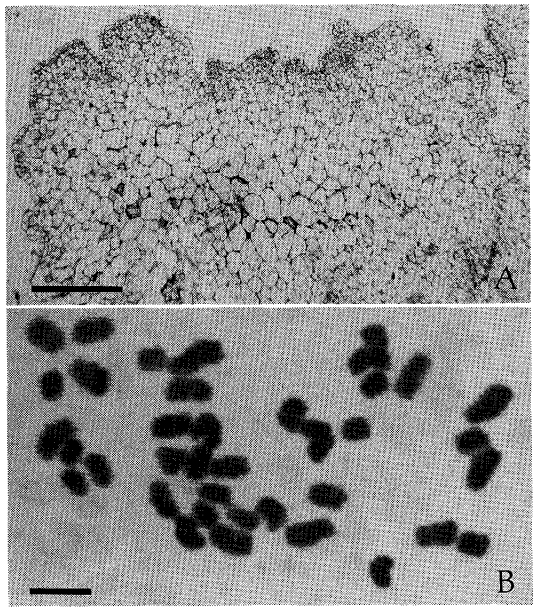

Fig. 2 Cross-section of a mass of tissue-cultured shoot primordia at ten-day-stage after the beginning of subculture and its mitotic chromosome complement in *Vanda pumila*. A. The presence of a single outer cell-layer and numerous different sized and shaped inner-cells is characteristic of a mass of tissue-cultured shoot primordia. B. The normal diploid chromosome number of $2n=38$ at mitotic metaphase in a somatic cell. Bar in A=30 μm; Bar in B=1 μm.

Shoot regeneration from tissue-cultured shoot primordia was affected by the basal media used and the concentrations of NAA and BAP added (**Table 3; Fig. 1-C** and **D**). The higest shoot-regeneration rate from tissue-cultured shoot primordia was shown in 1/2 B5 agar medium supplemented with 1% sucrose and no growth substance (**Table 3**). After 56 days shoots began to be regenerated from tissue-cultured shoot primordia and after 120 days the regenerated shoots formed roots to become plantlets (**Fig. 1-E**). The inhibitory effect of growth substances on the proliferation of tissue-cultured shoot primordia in *V. pumila* was similar to that observed in the regeneration rate of somatic embryos in orchard grass calli[10].

During three years of subculture, the shoot primordium culture line of *Vanda pumila* has continuously shown the stable chromosome number of $2n=38$ (diploid) (**Fig. 2-B**), except that less than 5% cells have shown $2n=36$ (aneuploid) and $2n=76$ (tetraploid) (**Table 4**). Since the wild *V.*

Table 3. Effect of basal medium and growth substances on shoot regeneration from subcultured shoot primordia of *Vanda pumila* after 80 days culture.

Basal medium	Fresh weight of mass of shoot primordia planted (g)	Concentration of NAA (mg/*l*)	Concentration of BAP (mg/*l*)	Number of shoots regenerated
B5	0.98	0.00	0.00	116
B5	0.93	0.00	0.02	48
B5	1.01	0.02	0.02	30
1/2 B5	0.95	0.00	0.00	327
1/2 B5	1.04	0.00	0.02	249
1/2 B5	1.03	0.02	0.02	193

Each basal medium contained 1% sucrose.

Table 4. Stability of chromosome number in tissue-cultured shoot primordia of *Vanda pumila* during three-year-course of subculture.

Months in culture	No. of cells studied	No. of cells which have chromosome number of:		
		$2n=36$(%)	$2n=38$(%)	$2n=76$(%)
11	110	1(0.9)	107(97.3)	2(1.8)
17	113	2(1.8)	108(95.5)	3(2.7)
22	109		109(100.0)	
32	109	3(2.7)	104(95.5)	2(1.8)

Each cell studied was randomly taken and studied from a different mass of tissue-cultured shoot primordia.

pumila has been reported to have the chromosome numbers of $2n=38$ and 76[11], it could be mixoploid in nature.

Thus, the tissue-cultured shoot primordium of *Vanda pumila* could maintain a high ability for rapid propagation and regeneration of plantlets and high chromosome stability for a long time. The tissue-cultured shoot primordium method of Tanaka and Ikeda[2] promises to help develop and improve a system of gene storage for important species and genotypes of orchids including the genetic diversity of *Vanda pumila*. The method may also be useful for the clonal mass propagation of ornamental varieties of other *Vanda* species.

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《和文要約》

Vanda pumila の苗条原基法による保全とクローン大量増殖

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絶滅危惧種 *Vanda pumila* の無菌発芽後60日目のプロトコームの茎頂ドームを0.02 mg/*l* BAPと2%ショ糖を含むB5液体培地(pH 5.8)中で回転培養して，着生ランとして初めて組織培養苗条原基を作出し，継代培養して大量増殖系を確立した．小植物体の再分化は，苗条原基集塊を，1%ショ糖を含むホルモン無添加1/2 B5寒天培地に移植し，120日以上培養して最大値に達した．この小植物体再分化能力は継代を開始して3年後の現在も維持されている．そして，継代培養を続ける苗条原基で，常に安定した染色体数$2n=38$が得られている．以上の結果から，*Vanda pumila* の保全に苗条原基法が有効であることが証明された．またこの手法は，*Vanda* 優良個体の品種化を目的としたクローン大量増殖にも有効であると考えられた．