

Culture Conditions suitable for *in vitro* Seed Germination and Development of Seedlings in *Paphiopedilum*

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Summary

Suitable culture medium and light condition for each step of *in vitro* seed germination, growth of protocorms and subsequent development of seedlings were determined in *Paphiopedilum* spp. The best seed germination and best growth of protocorms were obtained on Norstog medium in darkness. However, no differentiation of leaf nor root occurred in protocorms cultured on Norstog medium regardless of light condition. Although cultures on Burgeff EG-1 medium resulted in poor seed germination, germlings developed readily into seedlings with leaves and roots if cultured in light. Duration of the dark culture on Norstog medium to yield the maximal percentage of seed germination was found six weeks and longer. Healthier protocorms were produced in the culture with longer dark period. Protocorms grown to various developmental stages on Norstog medium in darkness were transplanted onto different media in different light conditions. The best development to seedlings was obtained from the protocorms with diameter 0.5 to 1.0 mm transplanted onto Burgeff EG-1 medium and cultured in light.

Introduction

Technique for seed propagation in orchids has been well improved, and now seeds of most orchid species can be easily germinated on several types of synthetic culture media (1,2,10). However, there are still some species of which seed propagation technique is not yet established. *Paphiopedilum* is among such orchids. The present work aimed to find out the most suitable culture medium and light condition for each step of seed germination, growth and development of protocorms and seedlings in *in vitro* seed propagation of *Paphiopedilum*.

Materials and Methods

1. Plant materials.

Paphiopedilum sukhakulii and its hybrids with other *Paphiopedilum* species listed in Table 1 were used as experimental materials.

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Capsules were harvested six months after artificial self- or cross-pollination. The capsules were surface sterilized by soaking them in 75% aqueous ethanol for 30 seconds followed by 15-min immersion in 0.5% sodium hypochloride solution containing Tween 20, and rinsed with sterilized water. The sterilized capsules were split to open and seeds were dispensed onto culture medium.

The cultures were classified into the following six groups according to their developmental stages ; dormant seed (Stage 0), with swollen embryo (Stage 1), with embryo burst out of seed coat (Stage 2), with diameter less than 0.5 mm (Stage 3), with diameter between 0.5 and 1.0 mm (Stage 4) and with diameter over 1.0 mm or with leaves and/or roots (Stage 5). In this study, swelling of embryo was regarded as germination of seed, the plants at developmental stages after germination and before initiation of leaf and/or root were called protocorms, and those after the initiation of leaf and/or root were seedlings.

2. Culture conditions

Norstog(6) and modified Burgeff EG-1(3) media were used. The media were supple-

mented with 2% sucrose, adjusted to pH 5.0 and solidified with 1% agar. Burgeff EG-1 medium was autoclaved at 120°C for 15 min, while Norstog medium was sterilized by passing nutrient solution through a milipore filter (pore size 0.22 μ m) to prevent a possible degradation by autoclaving of organic elements in it. Ten ml of medium was poured into a culture tube (20 mm in diameter and 150 mm in height). All cultures were incubated at 25°C in darkness or continuous white light of ca. 3,500 lx at plant level provided by fluorescent tubes.

Results

1. Suitable medium and light condition for seed germination.

Seed germinations scored six months after the inoculation in *P. sukhakulii* and its hybrids with other *Paphiopedilum* species on Norstog and Burgeff EG-1 media in darkness and light are shown in Table 1. Seeds produced by self-pollination germinated poorly or did not germinate at all, while germination percentages in hybrid seeds were generally high. The different seed sources showed similar trend in responses to the media and light condition although germination percentages differed among them. Namely, the highest germination percentage was obtained on Norstog medium in darkness. When incubated in light, however, germination percentage on Norstog medium was markedly reduced. On the other hand, no significant difference between dark and light cultures

was observed on Burgeff EG-1 medium.

Protocorms at different developmental stages were observed at the end of six-months culture because the seeds had germinated sporadically. Therefore, development of the protocorms was also scored. Frequency distribution of protocorms at respective developmental stages in *P. 'Clair de Lune' × P. sukhakulii* is shown in Fig 1. Trend was the same in other hybrids. On Norstog medium, almost 90% of the protocorms cultured in light remained at the Stage 1, while many protocorms cultured in darkness developed to the Stage 5 although most of them did not differentiate leaf nor root. On the other hand, protocorms cultured on Burgeff EG-1 medium differentiated leaves and roots when cultured in light.

2. Length of dark period required for seed germination.

Since the previous experiment showed that the best seed germination was obtained in dark culture on Norstog medium, it was then examined how long period of dark culture was required to yield the maximal seed germination percentage. Seeds of *P. sukhakulii × P. 'Alma Gevaert'* were incubated on Norstog medium in darkness for 0 to 12 weeks and then transferred to lighted condition, or incubated in darkness throughout the culture period of four months (Fig. 2).

The seeds incubated in continuous light (0 week dark) germinated poorly and the germination percentage increased with increasing length of dark period up to six

Table 1. Seed germination in *Paphiopedilum sukhakulii* and its hybrids with other *Paphiopedilum* species on different culture media in darkness and light.

Seed source	Germination percentage ^z			
	Norstog		Burgeff EG-1	
	Dark	Light	Dark	Light
<i>P. sukhakulii</i> (self-pollinated) ^y	46.0±0.5	8.7±0.5	4.0±0.1	3.0±0.2
<i>P. sukhakulii</i> (self-pollinated) ^x	0±0	0±0	0±0	0±0
<i>P. sukhakulii × P. neivium</i>	60.3±3.1	30.8±1.7	25.1±0.9	17.3±0.3
<i>P. sukhakulii × P. acmodontum</i>	97.2±1.8	79.4±0.3	91.5±1.0	83.0±1.8
<i>P. sukhakulii × P. bellatum</i>	46.9±0.4	11.5±1.0	19.5±3.2	7.5±0.2
<i>P. 'Clair de Lune' × P. sukhakulii</i> ^y	96.2±1.0	46.0±10	88.2±0.7	73.5±0.8
<i>P. 'Clair de Lune' × P. sukhakulii</i> ^x	82.7±1.8	27.3±1.9	50.9±3.9	44.6±9.9

^z Scored six months after the inoculation.

^{y, x} Seeds originated in different capsules.

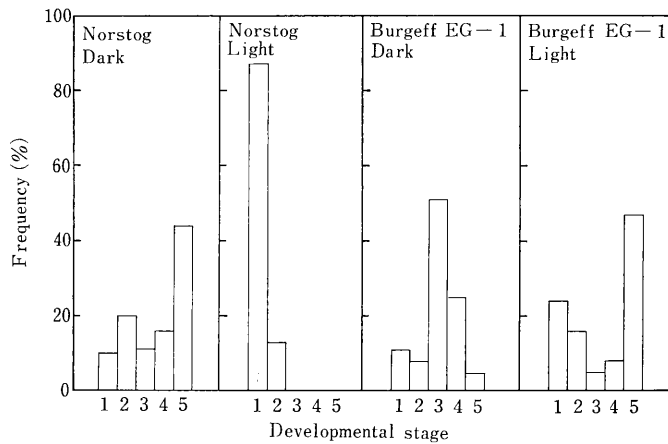


Fig. 1. Development of protocorms cultured on different culture media in darkness and light in *Paphiopedilum* 'Clair de Lune' \times *P. sukhakulii*. Frequency of the protocorms at each developmental stage (see Materials and Methods) was scored six months after the seed inoculation.

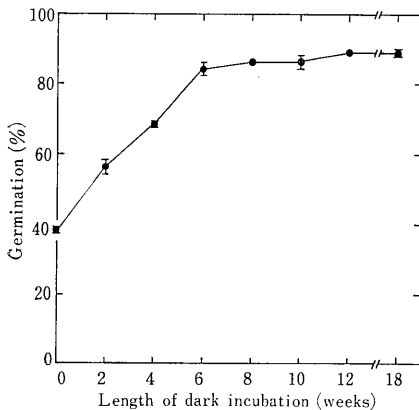


Fig. 2. Seed germination in *Paphiopedilum sukhakulii* \times *P.* 'Alma Gevaert' incubated on Norstog medium in darkness for different length of period and then transferred to lighted condition. Percent germination was scored four months after the seed inoculation, and shown as a mean with a S. E.

weeks. No significant difference was observed among the cultures incubated in darkness for six weeks and longer. Development of the resulted protocorms is shown in Fig. 3. Most of the protocorms in continuous light turned brown and died off at the early stage of development. The longer the period of dark culture was extended, the healthier protocorms were produced. However, no differentiation of leaf and root was observed in all cultures by the end of four-months

culture.

3. Suitable medium and light condition for development of seedlings.

Protocorms of *P.* 'Clair de Lune' \times *P. sukhakulii* were obtained from seeds cultured on Norstog medium in darkness. The protocorms at different stages of development (see Materials and Methods) were selected and transplanted onto different media and light conditions to determine the most favourable medium and light condition for development of protocorm to seedling and subsequent growth of the seedling, and the most suitable developmental stage of protocorm to be transferred to such conditions. Survival rate in the protocorms transplanted (Table 2) and growth and differentiation in the survived protocorms (Figs. 4 to 6) were scored four months after the transplantation.

Protocorms transplanted when they were at the Stage 3 and later showed the highest survival rate, and the best growth was obtained in those transplanted when at the Stage 4. Protocorms grown on Burgeff EG-1 medium, when cultured in light, yielded the greatest growth and the best development of leaves and roots. When cultured in darkness, however, seedlings etiolated and the growth was inferior. On Norstog medium, protocorms showed an abnormal development

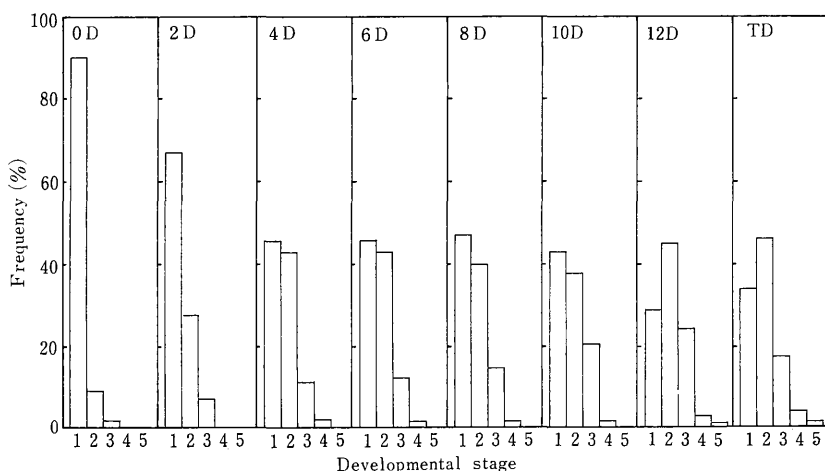


Fig. 3. Development of protocorms in *Paphiopedilum sukhakulii* × *P.* 'Alma Gevaert' cultured on Norstog medium in darkness for different length of period and then transferred to lighted condition. Frequency of the protocorms at each developmental stage (see Materials and Methods) was scored four months after the seed inoculation. *n*D and TD mean cultures incubated in darkness for *n* weeks and throughout the culture period, respectively.

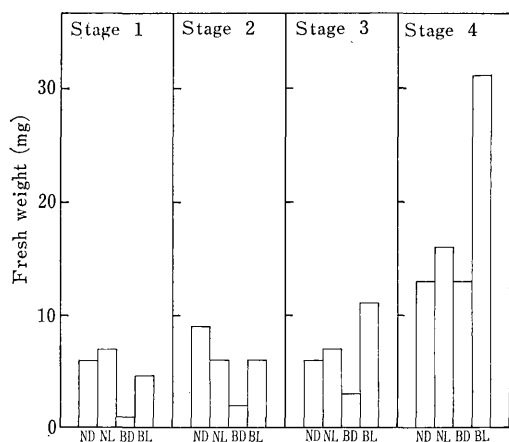


Fig. 4. Growth of protocorms in *Paphiopedilum* 'Clair de Lune' × *P. sukhakulii* which were obtained on Norstog medium in darkness and transplanted onto different culture media in darkness and light when they were at different developmental stages (see Materials and Methods). Fresh weight was measured four months after the transplanted. ND, NL, BD and BL mean cultures on Norstog medium in darkness and light and on Burgeff EG-1 medium in darkness and light, respectively.

with remarkable increase in size and the morphogenesis was delayed. Mortality of protocorm was high and those survived did not grow well especially in light.

Discussion

Burgeff EG-1 medium has been recommended for seed germination of *Paphiopedilum* (7) and Norstog medium was recently proved to be useful for it (9). The present work indicated that Norstog medium was more suitable than Burgeff EG-1 medium for seed germination in *Paphiopedilum*. This medium, however, was apparently not suited for further development of protocorms. Instead, Burgeff EG-1 medium was more favourable to differentiation of leaf and root and further growth of seedlings. Thus, seeds should be incubated on Norstog medium in darkness for six weeks or longer to allow the resulted protocorms to grow to the Stage 4, and then the protocorms should be transplanted onto Burgeff EG-1 medium in light to yield the best result in seed propagation of *Paphiopedilum*. The present study revealed that requirements for seed germination and growth of protocorm were different from those for differentiation of leaf and root. Therefore, it is apparent that different stages in the development should be studied separately. Most of the previous works on seed propagation in orchids have considered these developmental stages as a whole. This may be one of the reasons why the results

Table 2. Survival rate in protocorms of *Paphiopedilum* 'Clair de Lune' × *P. sukhakulii* which were obtained on Norstog medium in darkness and transplanted onto different culture media in darkness and light when they were at different developmental stages (see Materials and Methods).

Culture medium	Light condition	Survival rate (%) ^z			
		Developmental stage when transplanted			
		1	2	3	4
Burgeff EG-1	Dark	26.7 ± 14.5	93.3 ± 6.9	100 ± 0	100 ± 0
	Light	63.3 ± 6.8	80.0 ± 0	100 ± 0	100 ± 0
Norstog	Dark	53.3 ± 3.3	76.7 ± 6.5	93.3 ± 6.9	90.0 ± 5.8
	Light	6.7 ± 6.6	30.0 ± 5.8	50.0 ± 5.8	61.9 ± 9.1

^z Scored four months after the transplantation.

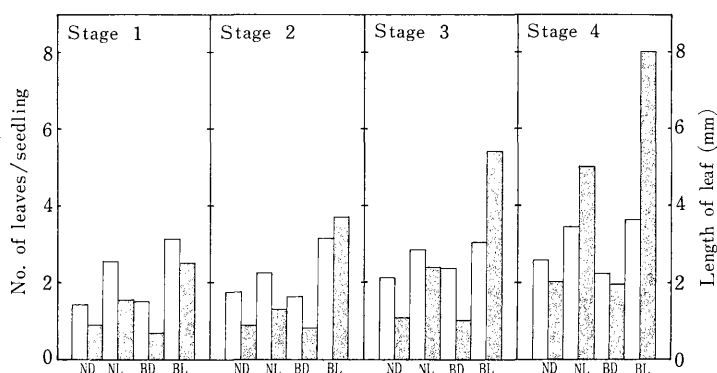


Fig. 5. Differentiation of leaves in protocorms of *Paphiopedilum* 'Clair de Lune' × *P. sukhakulii* which were obtained on Norstog medium in darkness and transplanted onto different culture media in darkness and light when they were at different developmental stages (see Materials and Methods). Number of leaves per seedling (open column) and length of the longest leaf (closed column) were scored four months after the transplantation. ND, NL, BD and BL mean cultures on Norstog medium in darkness and light and on Burgeff EG-1 medium in darkness and light, respectively.

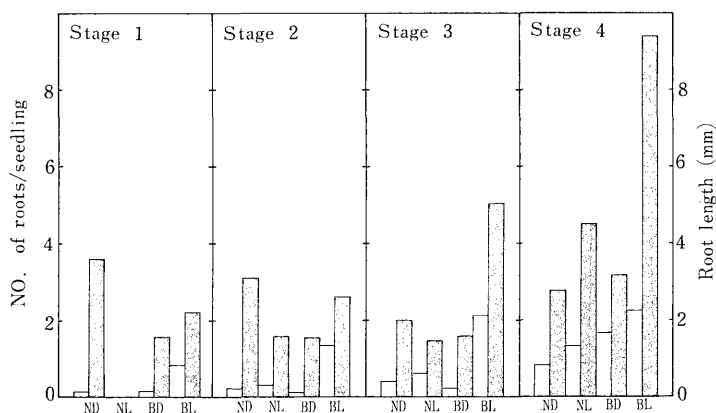


Fig. 6. Differentiation of roots in protocorms of *Paphiopedilum* 'Clair de Lune' × *P. sukhakulii* which were obtained on Norstog medium in darkness and transplanted onto different culture media in darkness and light when they were at different developmental stages (see Materials and Methods). Number of roots per seedling (open column) and length of the longest root (closed column) were scored four months after the transplantation. ND, NL, BD and BL mean cultures on Norstog medium in darkness and light and on Burgeff EG-1 medium in darkness and light, respectively.

obtained were not always consistent. Major difference in composition between Burgeff EG-1 and Norstog media is of nitrogen source, that is, ammonium salt is used in Burgeff EG-1 medium while amino acids are used in Norstog medium. Amino acids supported seed germination and protocorm growth but were inhibitory against shoot formation in *Dactylorhiza purpurella* and *Cypripedium reginae*(4, 5). It was reported in some orchids that most amino acids either inhibited growth or had no effect on it(1, 8). These facts and the present results indicate that amino acids play different roles before and after the Stage 4, or they play some important roles in transition from the Stages 4 to 5, namely, differentiation of root and/or leaf. The difference in media used influenced also the effects of light, that is, the photoinhibition of seed germination and early growth of protocorms was more evident on Norstog medium than on Burgeff EG-1 medium. This photoinhibition on Norstog medium might be through influence on the utilization of amino acids because the utilization of some amino acids was affected by light(1, 8).

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パフィオペディルム属における種子発芽と幼苗分化の
ための最適 *in vitro* 培養条件

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摘 要

パフィオペディルム属の幾つかの種及びそれらの間の交配種を材料として、種子発芽、プロトコームの生長及び幼苗への分化のそれぞれの過程における最適な培地及び光条件を検討した。種子発芽及びプロトコームの生長は Norstog 培地、暗条件下で最も優れていたが、この条件下ではすべてがプロトコームの段階にとどまり、幼苗への分化は起こらなかった。Burgeff EG-1 培地上では種子発芽率は低かったが、発芽したものは明下で培養されれば多くが幼苗に分化した。Norstog 培地上での暗培養期間が6週間以下の場合、暗培養期間が短くなるほど発芽率は低く、発芽後のプロトコームの生長も劣

った。暗培養期間が6週間より長い場合は、発芽率に顕著な差はなかったが、プロトコームの生長は暗培養期間が長くなるほど良好であった。しかし、Norstog 培地上での培養が続く限り幼苗への分化は起こらなかった。次に、Norstog 培地、暗条件下で培養して得た、発生段階の異なるプロトコームを材料とし、プロトコームの幼苗への分化に最適な培養条件を検討した。プロトコームを横径が0.5~1.0 mm の段階まで生長させてから Burgeff EG-1 培地に移植し、明条件下で培養したときに最も高い率で幼苗が得られた。