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Developmental Morphology of Seeds and Micropropagation of *Orchis aristata* Fischer (Orchidaceae) in Axenic Culture*

by

Katsuhiko KONDO**, Chisako TANAKA***, Takiko SHIMADA**** and Motoyasu OHTANI****

近藤勝彦**・田中知佐子***・島田多喜子****・大谷基泰****：ラン科ハクサンチドリの一者培養における種子発芽形態と増殖に関する研究*

Since the technique of axenic seed germination in orchids was discovered by Knudson (1946), it has performed and contributed to propagate numerous species especially of epiphytic orchids and to produce their cross-pollinated hybrid plants (*e.g.*, Arditti 1967). Then, a mass micropropagation method by using protocorm-like bodies (PLB's) found in *Cymbidium* by Morel (1960) has been extensively popular to propagate commercialized epiphytic genera such as *Cattleya, Cymbidium, Dendrobium* and *Oncidium*. Additionally, the shoot primordium method described by Tanaka and Ikeda (1983) has also promised micropropagations of various orchids (Na and Kondo 1995, Tanaka *et al.* 1997). However, these methods have been applied to only a few terrestrial wild orchids (*e.g.*, Nagashima 1993, 1994, Hoshi *et al.* 1994).

The majority of the wild terrestrial orchid species has recently become severely threatened by degradation of their habitats, change in land use including drainage of boggy areas and increased use of fertilizers and so on. One way of protecting wild terrestrial orchids is to learn how to grow them well in cultivation and to propagate them in order to supplement and extend the natural populations (Ramsay in the Sainsbury Orchid Conservation Project leaflet). Seeds of those threatened, wild, terrestrial orchids are used in propagation instead of tissue culture in order to maintain genetic diversity (Ramsay in the Sainsbury Orchid Conservation Project leaflet).

Orchis aristata is a terrestrial orchid distributed in the alpine zone from Korean Peninsula to Alaska (Ohwi 1965). Some local races of the species such as the Mt. Hakusan race are endangered

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Contribution Number 51 from Laboratory of Plant Chromosome and Gene Stock, Faculty of Science, Hiroshima University.

** Laboratory of Plant Chromosome and Gene Stock, Faculty of Science, Hiroshima University, 1-4-3 Kagamiyama, Higashi-Hiroshima, Hiroshima, 739. 広島大学理学部附属植物遺伝子保管実験施設. 〒739 広島県東広島市鏡山1-4-3.

*** Mukaishima Orchid Center, 3091 Mukaishima, Hiroshima, 724. 向島洋ランセンター. 〒724 広島県向島町3091.

**** Research Institute of Agricultural Resources, Ishikawa Agricultural College, 1-308 Suematsu, Nonoichi, Ishikawa-Gun, Ishikawa, 921. 石川県農業短期大学農業資源研究所. 〒921 石川県石川郡野々市町末松1-308.

while the others are threatened. *Ex situ* conservation of the species in laboratory has to be established by axenic seed-germination and micropropagation.

Materials and Methods

Fruits of *Orchis aristata* Fischer were harvested in cultivated condition at Laboratory of Plant Chromosome and Gene Stock, Faculty of Science, Hiroshima University and wild condition in Mt. Hakusan at intervals of ten days from the 30th day stage after the artificial hand pollination until the 66th to 75th day stage when the capsules were matured, dried and dehisced. The non-dehisced, immatured fruits were surface-sterilized with 70% ethanol for ten seconds, 1–2% (v/v) sodium hypochlorite solution for 20 minutes and rinsed three or four times with sterile, distilled water. The capsules were, then, dissected longitudinally and seeds were scooped for sowing. Mature seeds obtained from the dried, dehisced capsules were soaked in distilled water containing a few drops of Tween 20 or a commercialized wetting agent overnight, before they were surface-sterilized with 1–2% sodium hypochlorite solution for ten minutes and then, rinsed three times with sterile, distilled water. Many of those surface-sterilized seeds were sown and cultured on Hyponex medium (3 g/l Hyponex, 2 g/l peptone and 30 g/l sucrose) at pH 5.8 (Kano 1968) and Knudson C medium (Knudson 1946) both supplemented with 1.5% gelrite at 22±1℃ under ca. 500 lux for 16 hours photoperiod per day. On the other hand, some seeds at the 40th day stage after the hand pollination were sown and cultured on Hyponex medium supplemented with 1.5 % gelrite at 22±1℃ under ca. 500 lux under controls of daily photoperiods at hour rates of light/dark of 24/0, 16/8 and 0/24. Germination frequency of seeds were counted at the 150th day stage after sowing.

Grown protocorms ca. 1.5 mm long were transplanted on Murashige and Skoog (MS; Murashige and Skoog 1962) and Gamborg's B5 (B5; Gamborg *et al.* 1968) gelrite media without any hormone and their 1/2 and 1/4 diluents at a stationary culture at 22±1℃ under ca. 500 lux for 16 hours photoperiod per day.

Shoot apices of young plants were cut-off, individually placed and cultured in MS or B5 liquid media supplemented with α-naphthalene acetic acid (NAA) and N6-benzyladenine (BA) both at concentrations of 0.00, 0.02, 0.20 and 2.00 mg/l in combination. The cultures were maintained by shaking at 2 rpm on the rotary culture apparatus at 24±1℃ under 2,000–10,000 lux continuous illumination by halogen lamp.

Results and Discussion

Axenic seeds of *Orchis aristata* began to germinate 150 days after they were sown. Hyponex medium showed better axenic seed-germination rates than Knudson C medium (Table 1). Among the seeds harvested at five different stages at intervals of ten days after hand pollination those at the 40th day stage exhibited the best germination rates (Table 1). Immature seeds are often thought to perform better germination rates than the mature seeds (Withner 1974). However, seed maturation of the species could not be correlated with seed germination rares in axenic culture (Table 1). In a case of *Dactylorhiza maculata* (L.) Soo S. L., the mature seeds contained 15 times more abscisic acid (ABA) than immature seeds, that could influence seed dormancy (Van der Kinderen 1987). Thus, high concentration of ABA in the mature seeds of *Orchis aristata* might lead difficulty of germination. In

Table 1. Differences in seed germination in *Orchis aristata* directly related to differences in seed maturation in days after hand pollination

Days after hand pollination	No. of seeds germinated/No. of seeds sown	
Medium	Hyponex	Knudson C
30	0/1,829	0/1,247
40	30/2,631	2/1,930
50	24/2,344	0/2,008
60	0/1,546	0/1,451
seeds from dehisced capsule	3/1,093	0/895

Table 2. Axenic seed germination in *Orchis aristata* on Hyponex gelrite medium with no hormone in different photoperiodicity

Photoperiod (L/D)	No. of seeds germinated/No. of seeds sown
24/0	0/5,755
16/8	9/4,585
0/24	122/9,173

L = light; D = dark

Table 3. Effects of concentrations and combinations of NAA and BA in MS basal medium on PLB formation in shoot tips of *Orchis aristata*

BA (mg/l)	No. of PLB's formed/No. of explants used			
NAA (mg/l)	0.00	0.02	0.20	2.00
0.00	0/5	0/5	1/5	0/5
0.02	0/5	0/5	0/5	1/5
0.20	0/5	1/5	1/5	0/5
2.00	0/5	0/5	1/5	0/5

Table 4. Effects of concentrations and combinations of NAA and BA in B5 basal medium on PLB formation in shoot tips of *Orchis aristata*

BA (mg/l)	No. of PLB's formed/No. of explants used			
NAA (mg/l)	0.00	0.02	0.20	2.00
0.00	0/5	1/5	1/5	2/5
0.02	1/5	2/5	2/5	3/5
0.20	2/5	3/5	3/5	1/5
2.00	0/5	1/5	0/5	0/5

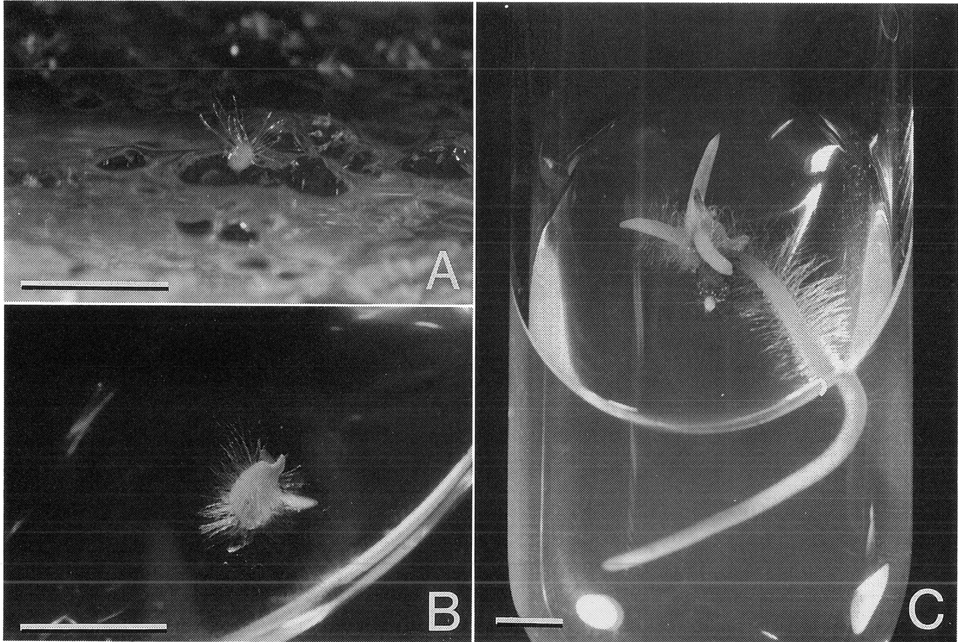

Fig. 1. Developmental morphology of seeds and protocorms of *Orchis aristata*. **A.** Newly occurred protocorm with numerous hairoids on Hyponex gelrite medium 150 days after sowing. **B.** Developed protocorm with numerous hairoids on 1/2 MS gelrite medium 60 days after transplantation. **C.** Young plant with a shoot and a few roots on 1/2 MS gelrite medium 120 days after transplantation. Bar = 10mm.

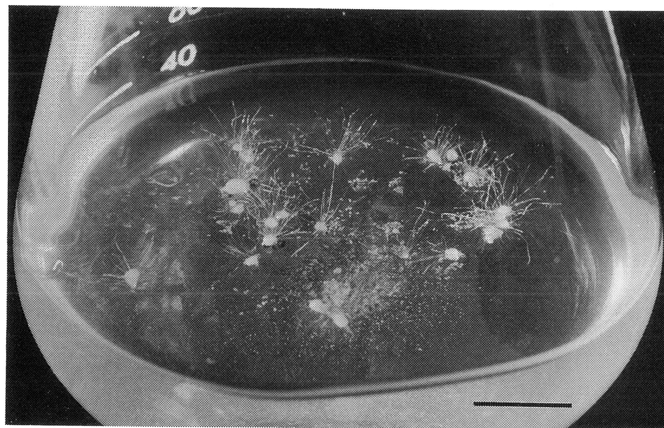

Fig. 2. Protocorm germination of 40-day-old immature seeds of *Orchis aristata* on Hyponex gelrite medium under all day dark. Bar = 10mm.

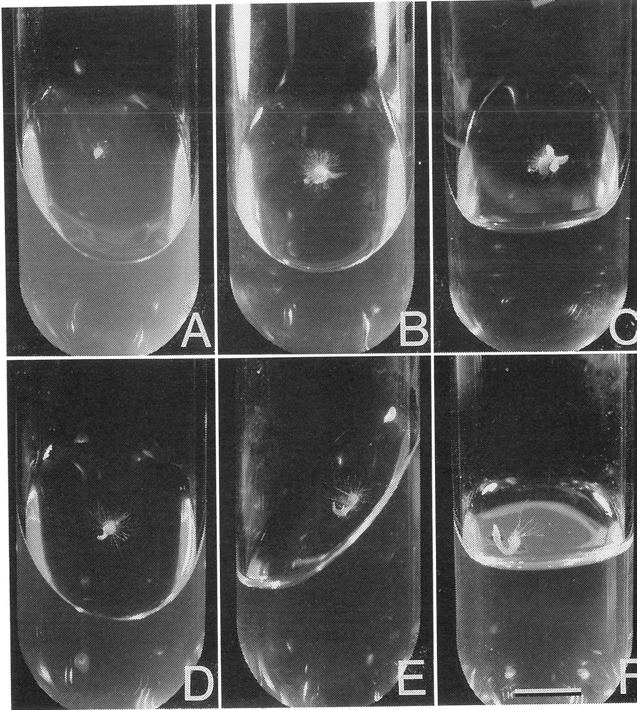

Fig. 3. Effects of basal gelrite media and 1/2 and 1/4 diluents of MS and B5 on protocorm growth in *Orchis aristata*. **A.** Planted on MS. **B.** Planted on 1/2 MS. **C.** Planted on 1/4 MS. Diluted MS exhibited shoot and root growth in protocorm. **D.** Planted on B5. **E.** Planted on 1/2 B5. **F.** Planted on 1/4 B5. Diluted B5 exhibited better growth of shoot but no root. Bar = 10 mm.

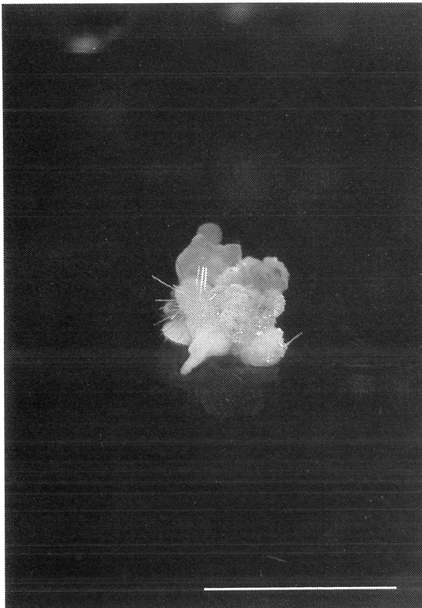

Fig. 4. A PLB of *Orchis aristata* occurred in B5 liquid medium supplemented with NAA and BA both at concentration of 0.2mg/l. Bar = 10 mm.

contrast, the 30-day-old seeds could be too young or immature to get germination. White-colored, globular-shaped protocorms of *Orchis aristata* produced numerous hairoids (Fig. 1A).

Concerning the photoperiodicity, any 40-day-old seed of *Orchis aristata* on Hyponex medium showed no germination under continuous light illumination (Table 2). Darkness for longer time-duration could be correlated with germination rates of seeds, and thus, continuous dark performed the best germination (Table 2).

As a conclusion, the best germination rate in *Orchis aristata* in axenic culture was shown in the conditions of the 40-day-old seeds, Hyponex gelrite medium without any hormone and continuous dark (Fig. 2).

Among the protocorms of *Orchis aristata* continuously subcultured on Hyponex gelrite medium, some showed differentiation of shoots and roots, but the others died. Then, protocorms were transplanted to the gelrite media of MS, B5 and their 1/2 and 1/4 diluents for differentiation and development of plantlets. Figure 3 shows the results 60 days after the protocorm transplantation: The protocorms transplanted on MS enlarged themselves but did not differentiate any shoot and root (Fig. 3A); those transplanted on 1/2 and 1/4 MS differentiated a shoot and a root (Figs. 1B, 3B, C); and those on B5, 1/2 B5 and 1/4 B5 differentiated in inverse proportion to shoot growth but, however, no root formation (Figs. 3D-F). Sixty days after transplantation those protocorms grew up large-enough young plants which was ready to be acclimatized (Fig. 1C).

Shoot tips of young plants of *Orchis aristata* were cut-off and placed in MS liquid media supplemented with NAA and BA at various concentrations in combination but all died within 40 days after the beginning of the primary culture although some formed PLB's (Table 3), while those in B5 liquid media supplemented with NAA and BA at various concentrations in combination mostly produced PLB's. Especially, shoot tips in B5 liquid media supplemented with BA at concentrations of 0.02 and 0.2 mg/l and NAA regardless of concentrations produced more white-colored PLB's (Fig. 4, Table 4); those in B5 supplemented with 0.20 mg/l BA and less than 0.20 mg/l NAA produced largest number of PLB's and furthermore average 5.5 PLB's 60 days after the beginning of the primary culture.

The individual plants germinated and grown from seeds of *Orchis aristata* of Mt. Hakusan origin in axenic culture and acclimatized may be reintroduced and restocked natural sites since many conservation biologists suggest that only plants of native origin should establish natural population. Clonal plants of the species micropropagated, regenerated and acclimatized may be supplied for ornamental and commercial purposes. These two artificial propagation systems may perform *in situ* and *ex situ* conservation of the threatened and endangered orchid species.

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Summary

Seeds of *Orchis aristata* at various maturation stages were harvested, surface-sterilized and immediately sown on Hyponex gelrite medium and Knudson C gelrite medium: The immature seeds at the 40-day-old stage after hand-pollination sown on Hyponex gelrite medium showed the highest

germination rate, while those passed gradually more maturation showed a gradual decrease of germination rates. On the basis of photoperiod, longer dark cultures performed highest germination rates; the 24-hour-dark culture did perform the highest germination rate. Satisfied protocorm development with root formation was exhibited on 1/2 and 1/4 MS gelrite media without any hormone at pH 5.8 at 22± 1 ℃ under ca. 500 lux under the control of daily photoperiod at hour rate of light/dark of 16/8, while that with shoot formation and growth was exhibited on 1/2 and 1/4 B5 gelrite media. The best induction and micropropagation of PLB's were displayed in B5 liquid media supplemented with less than 0.20 mg/1 NAA and 0.20 mg/1 BA by shaking at 2 rpm on the rotary culture apparatus at 24±1℃ under 2,000-10,000 lux continuous illumination.

摘　　要

ハクサンチドリにおいて，種子の熟度がハイポネックス・ゲルライト培地とノドソンＣ・ゲルライト培地での無菌発芽に及ぼす影響を調べたところ，受粉後40日目の未熟種子をハイポネックス・ゲルライト培地に蒔いたものが最も高い発芽率を示した。さらに，その受粉後40日の未熟種子をハイポネックス・ゲルライト培地に蒔き，日長条件が無菌発芽に及ぼす影響を調べたところ，明期が短くなるほど発芽率が上昇し，24時間暗期試験区で最も良い結果を得た。無菌播種により得たプロトコームを，0.3%ゲルライト添加，ホルモン無添加，pH5.8の MS ならびに B5基本培地および１/２，１/４，希釈培地に移植して，22± 1 ℃，16時間明期条件で，幼植物体分化に及ぼす影響を調べたところ，１/２，１/４ MS 培地で発根が促され，１/２，１/４B5培地でシュートの成長が良好であった。プロトコーム様体の形成と大量増殖は，0.2 mg/1以下 NAA と0.2 mg/1 BA 添加の B5液体培地，24± 1 ℃，2 rpm 回転培養，2,000-10,000 lux 24時間照明で最も良い結果を得た。

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