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In Vitro Propagation of *Phalaenopsis* via Culture of Cytokinin-Induced Nodes

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Abstract. A new procedure for in vitro propagation of orchids belonging to the genus *Phalaenopsis* was developed. In contrast to commonly employed propagation methods that make use of leaf, root, or shoot tip tissues, we have used elongated stems of 6-benzyladenine-induced young seedlings as starting material for propagation. The elongated stem consisted of several nodes of which top nodes were used for cyclic propagation of new explants and the middle nodes for producing shoots or multiple adventitious buds. The whole procedure of proliferation could be completed within 7 months, and about 2,300 plantlets were produced from a single induced stem in a single year. This method may be used for propagation of seedlings in the case of lack of seeds in orchid breeding or for propagation of vegetative buds developed on flower stalks of rare orchid varieties when available flower stalks are limited. It may also have great potential for the propagation of wild threatened orchid species.

Key Words. Phalaenopsis—In vitro propagation—Cytokinin—Stem elongation

Phalaenopsis is one of the most important orchids in commercial markets. Vegetative propagation is generally used in many genera of orchids, but it is difficult to propagate *Phalaenopsis* vegetatively because it is a monopodial orchid that cannot be propagated by offshoot culture. Leaf tissues (Tanaka et al. 1975), root tip tissues and segments (Tanaka et al. 1976, Yoneda and Momose 1988), shoot tips of mature plants (Intuwong and Sagawa 1974), and flower stalks (Arditti et al. 1977, Homma and Asahira 1985, Ichihashi 1992, Pieper and Zimmer 1976, Zimmer and Pieper 1978) have been used as starting material for in vitro propagation of orchids. Although some of these procedures are also suitable for commercial application, several problems remain to be solved. For example, leaf culture requires a considerable time before protocorm-like bodies (PLBs) are formed, and culture transfers must be made frequently to avoid damage from phenolics released from tissues (Arditti and Robert 1993). In flower stalk culture, it takes a long time before a flower stalk emerges from a plantlet, and the propagation rate is low. Shoot tip culture damages the mother plant, and again, the propagation rate is low. To overcome these problems, we have attempted to develop a new procedure using stem nodes that elongated as a result of treatment with cytokinin as starting material for propagation.

Materials and Methods

Plants

One-year-old plants of a *Phalaenopsis* hybrid, Morning Moon M-28 × Gladys Read St. Louis grown in vitro, were used to examine their growth as affected by cytokinins. Plants, from which the roots had been removed, were transplanted to a Hyponex medium consisting of 3.5 g liter^{-1} of Hyponex (N:P:K = 6.5%:6.0%:19%) to which zeatin, 2iP, kinetin, or BA had been added at concentrations of 2 and 10 mg liter^{-1}. The lengths of stems and numbers of leaves, roots, and adventitious buds were recorded 60 days after initiation.

One-year-old seedlings of the *Phalaenopsis* hybrid (Wataboushi × Grand City) × Ensyushirakawa were used to find the optimum concentration of BA for stem elongation. BA was added to Hyponex medium at concentrations from 0 to 20 mg liter^{-1}, and the lengths of stems were recorded 45, 90, and 135 days after initiation of culture.

Four explants were transplanted to each flask. Sixteen explants per treatment were used in all experiments, and each experiment was repeated at least once.

Abbreviations: PLB(s), protocorm-like body(ies); zeatin, 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine; 2ip, 6-(γ,γ-dimethylallylamino)purine; kinetin, 6-furfurylaminopurine; BA, 6-benzyladenine.

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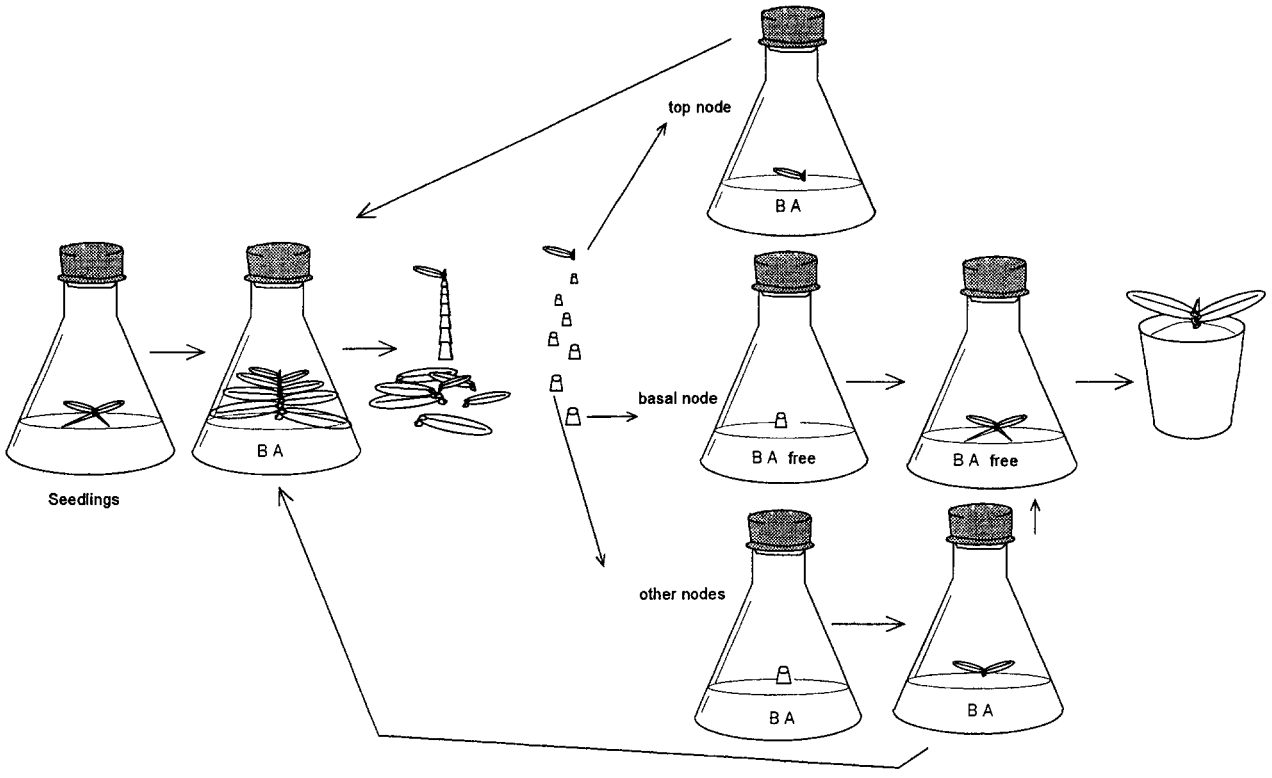

Fig. 1. Schematic outlines of the in vitro propagation of *Phalaenopsis* via culture of cytokinin-induced nodes.

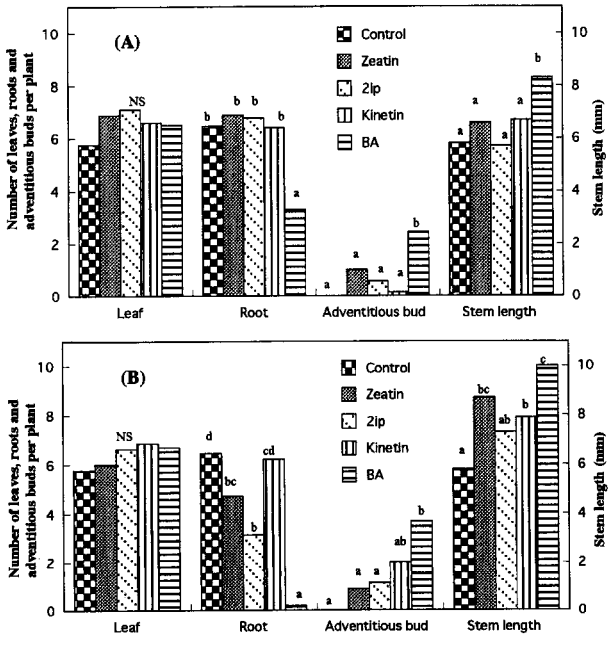

Fig. 2. Effects of zeatin, 2ip, kinetin, and BA in the medium on the growth and development of the *Phalaenopsis* hybrid, Morning Moon M-28 × Gladys Read St. Louis of seedlings, after 60 days in culture **(A)**, 2 mg liter^{-1}; **(B)**, 10 mg liter^{-1}. Different letters indicate significant differences at $p \leq 0.05$ (Duncan's multiple range test).

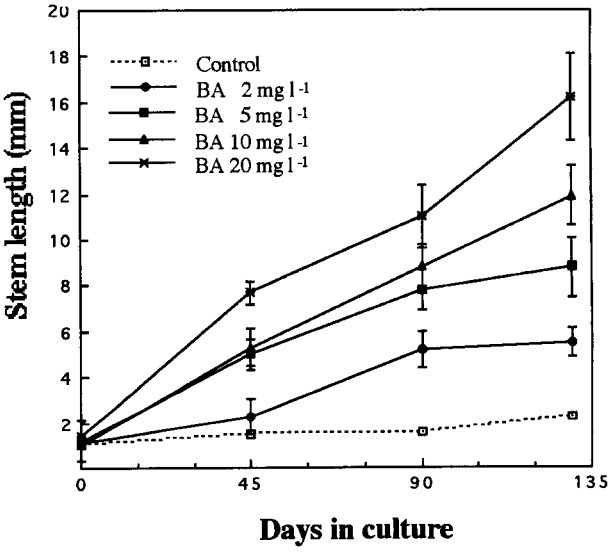

Fig. 3. Effects of BA at various concentrations on stem length in the *Phalaenopsis* hybrid (Wataboushi × Grand City) × Ensyushirakawa. *Vertical bars* represent standard errors.

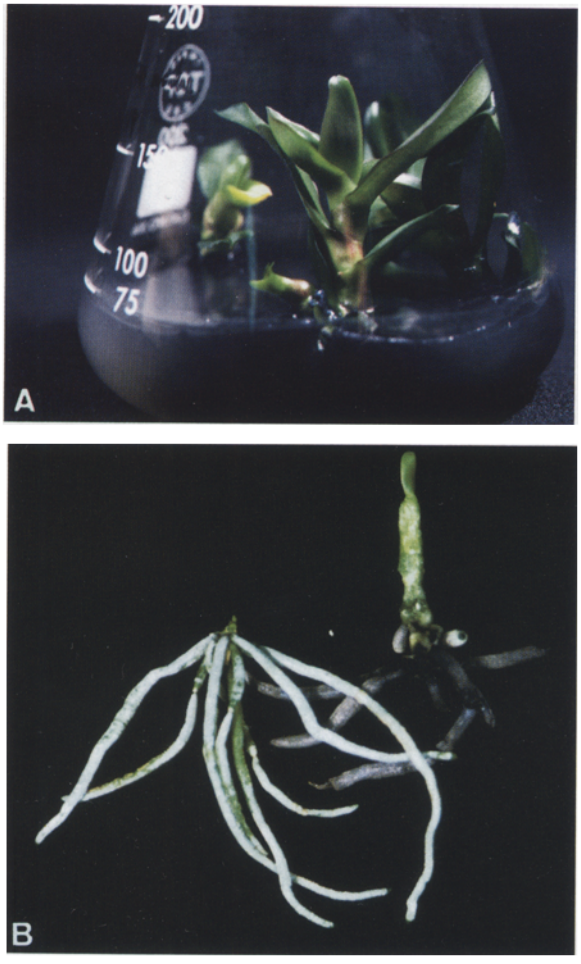

Fig. 4. (A), a seedling with a elongated stem cultured in Hyponex medium with 5 mg liter^{-1} BA for 90 days. **(B),** defoliated seedlings after 90 days of culture on cytokinin-free medium (*left*) or medium supplemented with 5 mg liter^{-1} BA (*right*). Note stem elongation and shorter roots in the seedlings exposed to BA.

Regeneration Conditions

Elongated stems (about 1 cm in length) from the *Phalaenopsis* hybrid (Wataboushi × Grand City) × Ensyushirakawa were obtained after culture of explants for 2 months in Hyponex medium supplemented with 5 mg liter^{-1} BA. The elongated stems, from which leaves had been removed, were cut into four node sections (the top node, including the shoot apex; and second, third, and basal node sections). The sections were placed separately on Hyponex medium containing 2 g liter^{-1} peptone (Difco Laboratories) with BA at various concentrations. Ten sections were tested per treatment, and the growth of sections was observed 2 months after the start of the experiment. When shoots had developed from the sections, they were transferred to fresh Hyponex medium without growth regulators to allow the formation of roots. Fig. 1 shows the whole procedure of proliferation developed in this study.

Two kinds of containers were used: 200-mL flasks filled with 70 mL of medium, for stem elongation culture; and test tubes (25 × 150 mm) filled with 20 mL of medium for stem section culture. All media were solidified with 10 g liter^{-1} agar (Wako Pure Chemical Industries Ltd., Osaka, Japan) and supplemented with 25 g liter^{-1} sucrose. The pH of each medium was adjusted to 5.6 prior to autoclaving at 121°C at a pressure of 1.3 kg cm^{-2} for 15 min. All cultures were maintained in an incubator with a 16-h photoperiod and light at 2,500 lux, provided by fluorescent lamps, at a temperature of 25 ± 2°C.

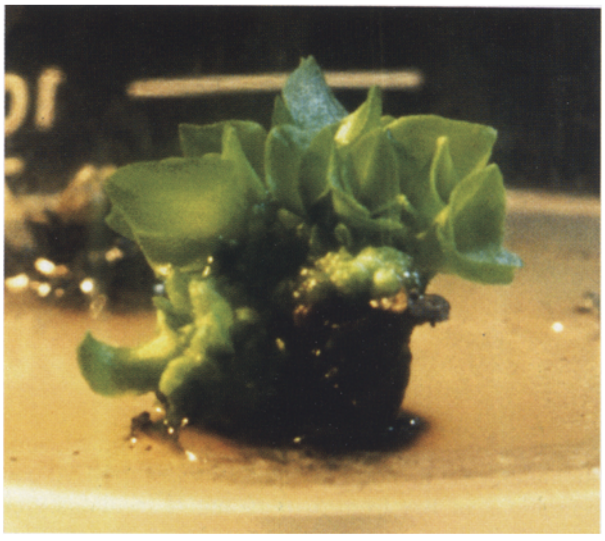

Fig. 5. Formation of shoots and multiple adventitious buds from the second node section of stem on Hyponex medium with 5 mg liter^{-1} BA after 70 days in culture.

Results

Effects of Different Cytokinins on Growth of Seedling

At low concentration (2 mg liter^{-1}), zeatin, 2ip, and kinetin, had little effect on the growth of seedling of the *Phalaenopsis* hybrid Morning Moon M-28 × Gladys Read St. Louis. BA inhibited the development of roots and increased the number of adventitious buds and stem length (Fig. 2A). The cytokinins were also tested at a higher concentration (10 mg liter^{-1}). As shown in Fig. 2B, BA had a dramatic effect on the seedlings, whereas zeatin, 2ip, and kinetin appeared to have similar but weaker effects. The number of adventitious buds formed during treatment with BA increased to 3.7 per explant, but no buds formed on the controls (no cytokinins added). BA promoted stem elongation by 37% compared with the controls. Even at the higher concentration, none of the cytokinins had a significant effect on leaf development (Fig. 2).

Optimum Concentration of BA for Induction of Stem Elongation

As shown in Fig. 3, the lengths of stems increased with BA concentration. The effect of BA became more obvious with increasing duration of culture. However, if the

Table 1. Effects of concentrations of BA on different node segments from elongated stem of *Phalaenopsis* hybrid (Wataboushi × Grand City) × Ensyushirakawa in Hyponex medium in vitro.

Concentration of BA (mg liter^{-1})	No. of shoots[a]				
	Basal node	Second node	Third node	Top node[b]	Total
0	3.6 ± 0.4	2.5 ± 0.4	1.5 ± 0.5	1.0 ± 0.0	8.6 ± 0.3
5	0.1 ± 0.1	4.4 ± 0.9	5.2 ± 1.2	1.0 ± 0.0	10.7 ± 0.6
10	0.1 ± 0.1	6.9 ± 0.9	4.5 ± 1.2	1.0 ± 0.0	12.5 ± 0.6
20	0.6 ± 0.4	3.3 ± 0.9	2.4 ± 0.4	1.0 ± 0.0	7.3 ± 0.4

[a] After 70 days of culturing, each result is presented as the mean ± S.E. (n = 10).
[b] Including stem tip.

concentration of BA was higher than 10 mg liter^{-1}, some slight deformation of leaves and plants occurred. At concentrations of BA from 5 to 10 mg^{-1}, seedlings grew normally without vitrification, and each developed a long stem compared with controls (Fig. 4). These results suggested that the optimum concentration of BA for stem elongation on Hyponex medium was 5–10 mg liter^{-1}.

Propagation of Elongated Stems

Each node section (top, second, third, and basal node sections) was placed on Hyponex medium supplemented with various concentrations of BA. After 70 days, shoots and multiple adventitious buds had been produced from inoculated nodes, and two or three leaves had formed. Most of the shoots were formed by axillary buds, and some of them developed from multiple adventitious buds. The highest number of shoots was obtained from the third nodes (5.2 shoots with 5 mg liter^{-1} BA) and the second nodes (6.9 shoots with 10 mg liter^{-1} BA) (Table 1). The basal node sections produced almost no shoots except in BA-free medium. Each inoculated top node only produced one shoot in all cases, regardless of the concentration of BA in the medium (Table 1). Therefore, the top nodes could be used repeatedly as a source for the next cyclic propagation, while the other nodes (middle and basal nodes) could also be cultured into plantlets (Fig. 1).

Although most of the nodes produced shoots within 60 days of culture, multiple adventitious buds were induced on 10% of the node sections (Fig. 5). The protrusions with multiple adventitious buds were divided into several parts and placed on Hyponex medium with BA. After 60–90 days in culture, about half of them had produced multiple shoots; the others were brown and subsequently died.

All shoots produced from inoculated nodes were transferred to BA-free Hyponex medium supplemented with 2 g liter^{-1} peptone for root formation. These shoots were well rooted and developed into plantlets after 60–70 days of culture (Fig. 6). The plantlets were transplanted from the flasks to clay pots filled with sphagnum moss in a greenhouse and were cultured in the same way as standard seedlings.

Fig. 6. Roots of explants from stem node sections on BA-free Hyponex medium after 60 days in culture.

Discussion

Our study showed that BA promoted stem elongation in *Phalaenopsis* seedlings and that node sections from elongated stems could be used as starting material for regeneration of multiple plantlets. This method allowed us to produce more than 2,300 plantlets from a single seedling with an induced stem in a single year. Many clonal propagation methods of *Phalaenopsis* are based on PLB production from the leaf, root, or flower stalk (Arditti and Robert 1993). However, the formation of PLBs from leaves or other tissues is not easily reproducible, and the culture conditions are stringent. For example, the production of PLBs decreases with increasing age of plantlets (Tanaka et al. 1975). The PLBs obtained from floral stalks do not proliferate readily, and their viability is low

(Tokuhara and Mii 1993). The formation of PLBs is not achieved easily. Some root tips of *Phalaenopsis* do not produce PLBs, and the cultures require lengthy incubation (Tanaka et al. 1976). In our previous study PLBs were obtained from cultures of leaf segments of plantlets of a *Phalaenopsis* hybrid, (Wataboushi × Grand City) × Ensyushirakawa but not in similar cultures of another *Phalaenopsis* hybrid, Pink Leopard Petra. In the procedure we succeeded in inducing 100% of seedlings or explants to elongate their stems, and almost 100% of the nodes from elongated stems developed into plantlets. We tested our method using another *Phalaenopsis* hybrid, White Dream × Dome Gules, and similar results were obtained. Interestingly, in the same way we also succeeded in inducing stem elongation of shoots from floral stalks of another orchid, × *Doriella* Tiny, and a propagation rate of about 6,000 times in one year was obtained by using the nodes of elongated stems (unpublished data). Our results suggested that BA-elongated stems are good starting material for the clonal propagation of orchids. Although it is easy to grow a large number of plants directly from seeds, this method may be used for propagation of seedlings or vegetative buds developed on flow stalks of rare *Phalaenopsis* varieties in the case of lack of seeds, especially in the *Phalaenopsis* breeding procedure. This method may have also great potential for the propagation of wild threatened orchid species.

The effects of BA on initiation of shoots or shoot elongation were clearly related to the positions of nodes on stems. The reason for this phenomenon is unknown. Cytokinins antagonize apical dominance and stimulate lateral bud development in many plants (Imre 1987). However, in the present study, the top node, including the main apical meristem, always formed only one shoot whether or not the medium contained BA (Table 1), and the shoot stem elongated in medium with added BA. It seems that the main apical meristem always maintained apical dominance and did not allow development of axillary shoots even in medium with BA. It is not known why the basal node sections almost all failed to form shoots in the medium with BA but did do so in the absence of BA.

A prominent feature of our method is its simplicity. Only three to four transfers need to be conducted during the entire propagation procedure, which reduces the possibility of contamination and the cost of cultures. The whole procedure can be completed within 7 months: 2 months for stem elongation, 3 months for development of multiple shoots from the node sections, and another 2 months for setting up plantlets and transplantation to clay pots.

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