The Cytokinin Preference for Immature Embryo Culture of Some Terrestrial Orchids©

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Cytokinin preference and optimum levels were investigated for in vitro germination and protocorm growth of both *Cypripedium macranthos* and *Dactylorhiza aristata* on ½-strength Norstog medium. Cytokinins, BA, and zeatin, in low concentrations enhanced germination and protocorm growth in comparison to control without cytokinins. Kinetin did not enhance germination.

INTRODUCTION

The requirements for the in vitro culture of immature orchid seeds from many terrestrial orchid species have been determined (Arditti and Ernst, 1984). Harvais (1982) reported that cytokinins are the most important growth regulators affecting in vitro germination of native terrestrial orchids. However, little is known about the effects of cytokinins on immature seed culture of terrestrial orchids (De Pauw et al., 1995). In this experiment, the effects of cytokinins on immature seed culture of two terrestrial orchids were studied.

MATERIALS AND METHODS

Seed capsules were collected from cultivated plants of *Cypripedium macranthos* SW (*C. macranthos* var. *hotei-atsumorianum* Sadovski) and from wild plants of *Dactylorhiza aristata* (Fisch.) Soo. Seed capsules of *C. macranthos* were collected 8 weeks after pollination, and seed capsules of *D. aristata* were collected 25 days after pollination. Those are intermediate-stage materials (Tomita et al., unpublished data). Capsules were surface sterilized as previously described (Tomita and Tomita, 1997). Immature embryos were cultured on ½-strength Norstog medium (Norstog, 1973) supplemented with 30 g liter^{-1} sucrose and 4 g liter^{-1} Gellan gum. This basal medium was supplemented with benzyladenine (BA), kinetin, or zeatin at concentrations of 1.0, 3.14, and 10.0 μM, and the control had no cytokinin. All cultures were incubated in the dark at 20±2°C. After 16 weeks of culture, germination rate was counted and the protocorms were assessed on a scale I to III as follows: (I) single protocorm, bud (and root) beginning to differentiate (Fig. 1a); (II) protocorm like bodies (PLBs: multiple protocorm as described by De Pauw et al., 1995) (Fig. 1b); (III) abnormal protocorm (Fig. 1c). After the investigation, all protocorms were transferred onto fresh ½-strength Norstog medium without cytokinin. Thirty-two weeks after sowing, seedlings were thinned, transplanted into soil-based medium in pots, and grown for 12 weeks at 5°C (Tomita 1999).

RESULTS AND DISCUSSION

The pattern of germination over time in culture varied with cytokinin type. Kinetin did not enhance germination (data not shown).

Cypripedium macranthos. Results were summarized in Fig. 2. Germination had occurred on all tested media within 4 weeks after inoculation. After 16 weeks of culture, germination was significantly stimulated in medium supplemented with BAP or zeatin in comparison to control without cytokinin. The germination and subsequent development of protocorms were superior on the media with low concentrations of BA or zeatin (1.0 or 3.14 μM) among all tested media.

Dactylorhiza aristata. Results are sμmarized in Fig. 3. Germination had occurred on all tested media within 3 weeks after inoculation. The cytokinins, BA, and zeatin, in concentrations of up to 3.14 μM enhanced germination rate significantly in comparison to controls without cytokinin. Benzyladenine at a concentration of 10 μM caused abnormal growth (Type III) and decreased germination rate.

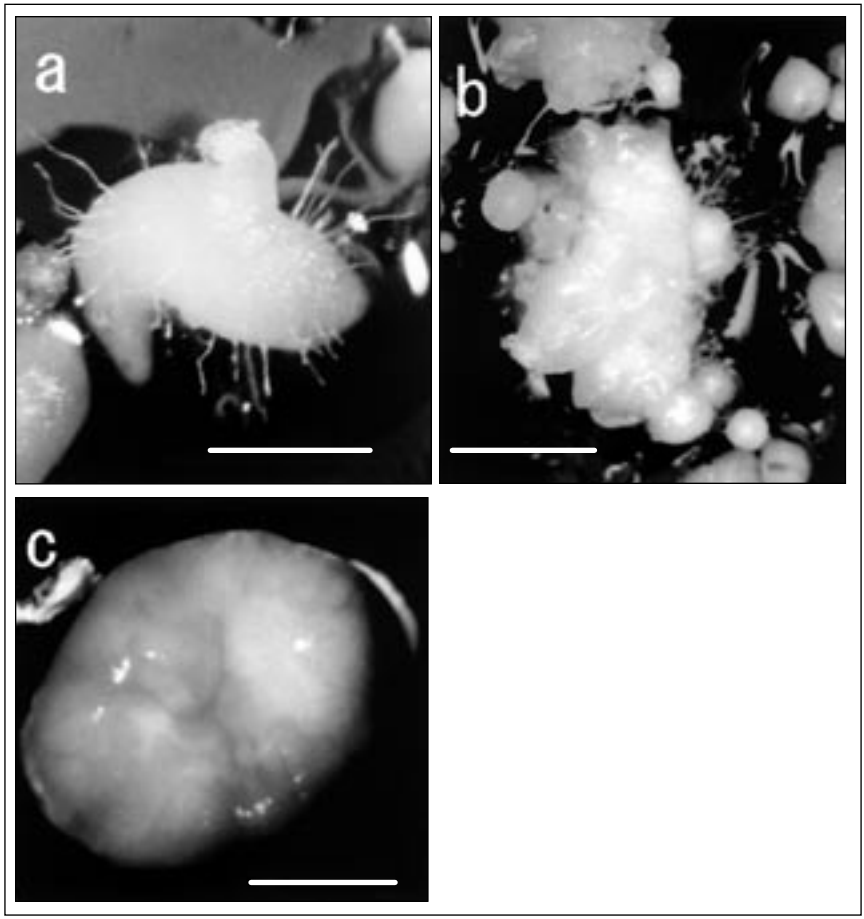

Figure 1. Morphological types of protocorm (*Cypripedium macranthos*).
a: Type I. Single protocorm.
b: Type II. Protocorm like bodies (PLB) (multiple protocorm; De Pauw et al. 1995)
c: Type III. Abnormal protocorm.
Bars=0.5 mm.

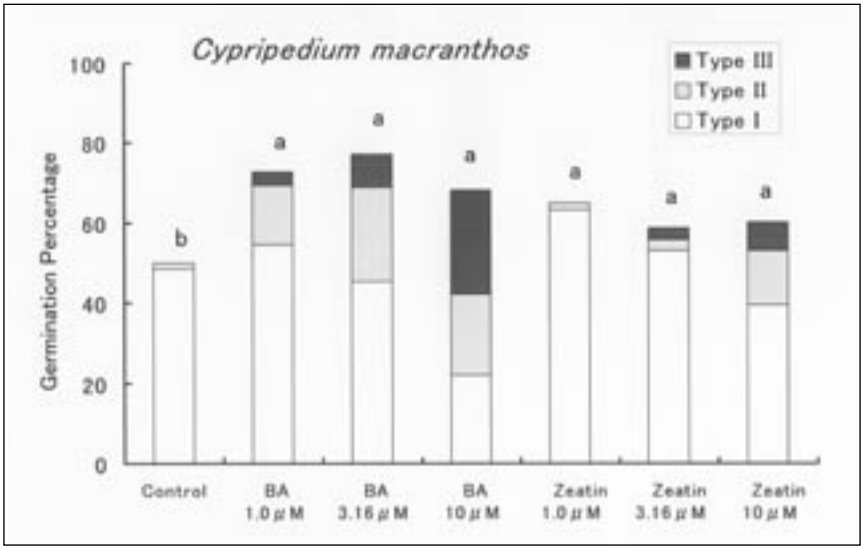

Figure 2. Effect of cytokinins on germination of *Cypripedium macranthos*. Germination percentages marked by the same letter on the top of bars are not significantly different when tested by Duncan's multiple range test at 5% level.

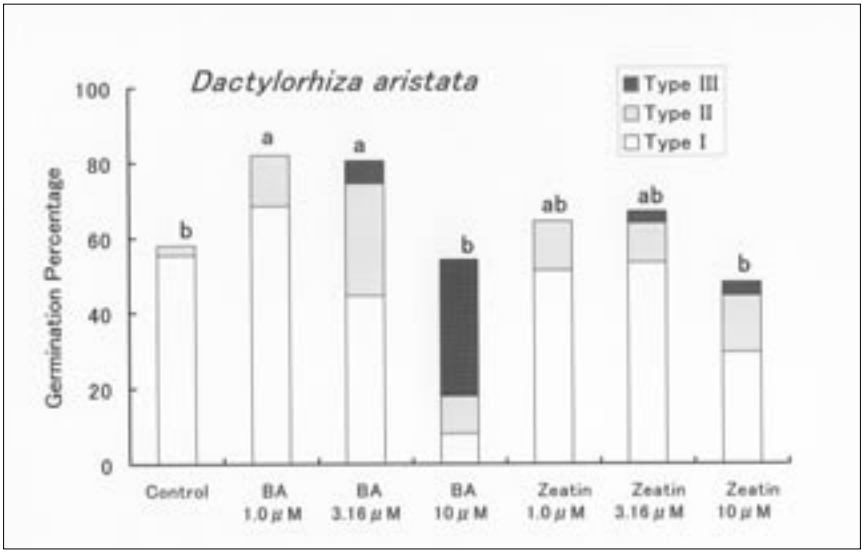

Figure 3. Effect of cytokinins on germination of *Dactylorhiza aristata*. Germination percentages marked by the same letter on the top of bars are not significantly different when tested by Duncan's multiple range test at 5% level.

On both species, protocorms developed faster and the PLBs (Type II) increased with BA or zeatin in comparison to the control. Benzyladenine at low levels (1.0 or 3.14 μM) were most effective at producing PLBs. Zeatin was less effective than BA for production of PLBs but results in more single (normal) protocorms (Type I).

After the investigation, protocorms were transferred onto fresh $^1/_2$-strength Norstog medium without cytokinin. Abnormal protocorms (Type III) died within 4 weeks after transplanting. Thirty-two weeks after sowing, seedlings were thinned out, transplanted into soil-based substrate in pots, and grown for 12 weeks at 5°C. Plantlets were well established.

Cytokinin requirement has been demonstrated for *C. reginae* (Harvais, 1982), *C. calceolus* (Van Waes and Debergh, 1986), and *C. candidum* (De Pauw et al., 1995). In this study, germination of both *C. macranthos* and *D. aristata* occurred without the addition of cytokinins to the medium but in the presence of two of three cytokinins tested, germination significantly improved. De Pauw et al. (1995) suggested that the requirement for cytokinins in the germination medium might be related to utilization of storage lipid mobilization, and they proposed a new model to establish an effective propagation system using cytokinins for immature seed culture of *C. candidum*. Their model could be applicable to *C. macranthos* and *D. aristata* for the purpose of developing the efficient micropropagation method for these species.

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LITERATURE CITED

Arditti, J. and **R. Ernst.** 1984. Physiology of germinating orchid seeds. pp. 176-222. In: J. Arditti (ed.) Orchid biology: Reviews and perspectives III. Cornell University Press. Ithaca, New York.

De Pauw, M. A., W. R. Remphrey, and **C. E. Palmer.** 1995. The cytokinin preference for in vitro germination and protocorm growth of *Cypripedium candidum*. Ann. Bot. 75: 267-275.

Harvais, G. 1982. An improved culture medium for growing the orchid Cypripedium reginae axenically. Can. J. Bot. 60:2547-2556.

Norstog, K. 1973. New synthetic medium for the culture of barley embryos. In Vitro, 8: 307-308.

Tomita, M. 1999. Effects of cold pretreatment and potting materials on growth of acclimated plantlets of Cypripedium macranthos var. speciosum. Comb. Proc. Intl. Plant Prop. Soc. 49:669-673.

Tomita, M. and **M. Tomita.** 1997. Effects of culture media and cold treatment on germination in asymbiotic culture of *Cypripedium macranthos* and *Cypripedium japonicum*. Lindleyana 12:208-213.

Van Waes, J. M. and **P. C. Debergh.** 1986. In vitro germination of some western European orchids. Physiol. Plantarum 67:253-261.