

Effects of Sterilization Time, Medium Composition, and Temperature on Germination of *Calypso bulbosa* (L.) Oakes var. *bulbosa* (Orchidaceae) *In vitro*

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Abstract

The effects of sterilization time, medium composition, and temperature on the germination of *Calypso bulbosa* were studied. Mature seeds treated with sodium hypochlorite solution (1% available chlorine) for four to eight min showed high germination percentages. Although there were no differences in germination percentage among the four media tested, protocorm growth on Tsutsui and Tomita medium was slightly better. Incubation temperature significantly affected both germination and protocorm development. Transplanting damage to seedlings was reduced when the cultures were maintained between 17.5 and 20°C in the dark. Bulb formations were observed on the protocorm masses when they were moved to continuous light condition (500 lux).

1. Introduction

Calypso bulbosa (L.) Oakes var. *bulbosa* (Orchidaceae), native to the north temperate region of North-East Asia and North America, is one of the more attractive terrestrial plant species. This species is becoming rare as its native habitats have been reduced through human interference [1]. Although it has great horticultural value, it is difficult to establish the cultivation system for this species because little is known about its germination requirement [2]. Arditti [2] reported that seeds from mature capsules of *Calypso bulbosa* germinated very poorly. He incubated its *in vitro* seedlings at $22 \pm 2^\circ\text{C}$ and reported that germinated seedlings were very sensitive to environmental conditions, and that they died when transplanted. There were some reports about the stimulatory effect of seed treatment with sodium hypochlorite on seed germination [3-6]. In some terrestrial orchids distributed in the north temperate region of Europe and Japan, it is known that both *in vitro* germination and subsequent seedling growth are highly affected by temperature [7-9]. This study was performed to obtain a basic information on the germination and development of *Calypso bulbosa* so as to establish an *in vitro* propagation system for this species with poor germination ability.

2. Materials and Methods

In 1994 and 1995, flowers of *Calypso bulbosa* cultivated in a greenhouse of Hirosaki University were hand-pollinated and bagged for two weeks to prevent from cross pollination in May. In June, seeds were harvested from fully ripened capsules (just prior to dehiscence) and used for experiments. Their seed coat was not removed for enhancing germination [6].

Experiment 1. Effects of sterilization time of seeds and medium composition

Seeds were surface-sterilized with sodium hypochlorite solution (1% available chlorine) containing 2-3 drops of "Tween 20" for 1, 2, 4, 8, 12, 16 and 32 min, then rinsed in sterile distilled water. After sterilization, some seeds were used for an evaluation of their viability by the tetrazolium test. The viability was determined by the percentage of red-stained embryos after soaking in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) solution for 24hr at 30°C in the dark [5]. Culture media used included modified Curtis medium by Arditti [2], half the concentration of major elements of Norstog medium [10], half the concentration of major elements of Murashige and Skoog [11], and Tsutsui and Tomita medium [12]. All media were supplemented with 10 g/l sucrose, adjusted to pH 5.5 and solidified with 3 g/l gellan gum. Each tube (25 × 150 mm in size) containing 20 ml of medium was sealed with aluminum foil. The media were auto-

claved at 121°C for 12 min and cooled in a slanted position.

Sterilized seeds were transferred onto culture media with an inoculation loop. Approximately 180-300 seeds containing embryos were inoculated in each tube, and at least four replicates (tubes) of each treatment were made. All cultures were incubated in the dark at 25°C. After 16 weeks of culture, germination (embryo emergence from the testa) rate was counted. Germination rate was expressed as a percentage of the total number of seeds inoculated [13]. The size of protocorms produced was measured under a stereoscopic microscope.

Experiment 2. Effect of temperature on germination

Seeds were inoculated on Tsutsui and Tomita medium after six min of sterilization. Other conditions were the same as in experiment 1. After incubation in the dark at 15, 17.5, 20, 22.5, 25, and 27.5°C for 16 weeks, germination percentages and protocorm sizes were recorded.

After observation, protocorms were transplanted onto 30 ml fresh medium in 100 ml Erlenmeyer flasks to stimulate plant development.

3. Results

Experiment 1. Effects of sterilization time of seeds and medium composition

Mature seeds did not change their color after one to two min of sterilization. When seeds were treated from four to eight min, embryo color changed from reddish brown to creamy brown, and testa color turned white. The seeds turned completely white after more than 12 min of sterilization. About 18-70 % of the embryos showed a red coloration by the tetrazolium test (Table 1).

The effects of sterilization time of seeds and medium composition on germination are summarized

in Fig. 1. Seeds treated with sodium hypochlorite solution from four to eight min showed significantly higher germination percentages. There were no significant differences in germination percentages among the four media tested. Protocorms of different sizes were obtained after 16 weeks of culture because the seeds had germinated sporadically. When seeds were treated with sodium hypochlorite from four to eight min, about 50% of the protocorms on Tsutsui and Tomita medium grew over 0.5 mm in size, while most of protocorms produced on the other three media were under 0.5 mm.

With all media, most protocorms formed were white in color, but some were brown and had died by the end of the investigation. After the investigation, all white protocorms were transplanted onto the same media as used for primary culture, but they turned brown and died within 20 weeks after sowing.

Experiment 2. Effect of temperature on germination

About 70% of the embryos showed a red coloration after six min of sterilization by the tetrazolium test. As shown in Fig. 2, germination was observed with all media tested. Significantly higher germination percentages (over 50%) were obtained at 17.5 and 20°C. Under 20°C, all protocorms were white in color. At 15°C, protocorm size was small (under 0.5 mm) compared to those grown at 17.5 and 20°C, at which temperature most protocorms grew 1-1.5 mm in size. At 22.5 and 25°C, some protocorms turned brown immediately after germination and had died by the end of the investigation. At 27.5°C, although germination occurred, all protocorms died within 10 weeks after sowing. All white protocorms (1.0-1.5 mm in size, cultured at 17.5 to 25°C) were transplanted under the same conditions as in initial culture. All protocorms cultured at 22.5 and 25°C died within four to eight weeks after transplanting, while those incubated at 17.5 and 20°C survived.

Table 1. The effect of the time of sterilization treatment on both seed color and stainability by tetrazolium test.

Sterilization time (min)	Seed color		Percentage of red stained embryos*
	embryo	testa	
1	reddish brown	reddish brown	20.6
2	"	"	27.8
4	creamy brown	white	58.5
8	"	"	69.7
12	white	"	34.1
16	"	"	22.3
32	"	"	17.9

* The seeds were soaked in 1% TTC solution for 24 hr at 35°C in the dark. At least 300 seeds were used in each treatment.

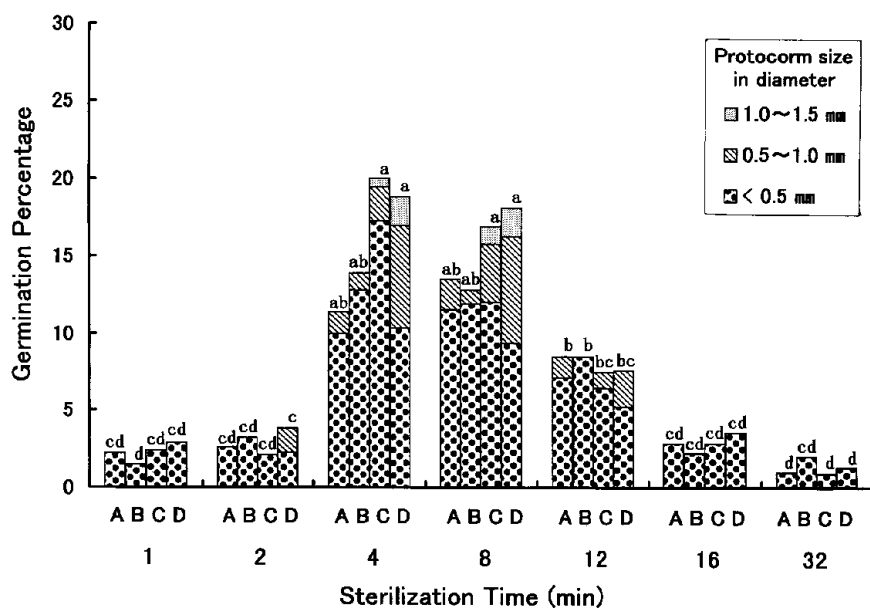


Fig. 1 The effects of sterilization time and medium composition on both seed germination and protocorm development of *Calypso bulbosa*.

A: Modified Curtis medium.

B: Half the concentration of major elements of MS.

C: Half the concentration of major elements of Norstog.

D: Tsutsui and Tomita medium.

Germination percentages marked by the same letter above the bars are not significantly different when tested by Duncan's multiple range test at 5% level.

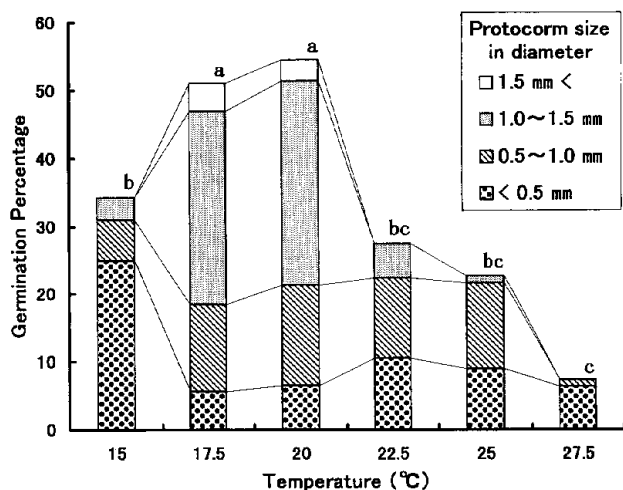


Fig. 2 The effects of incubating temperature on both seed germination and protocorm development of *Calypso bulbosa*.

Germination percentages marked by the same letter above the bars are not significantly different when tested by Duncan's multiple range test at 5% level.

Surviving protocorms were subcultured at 12-week intervals. Although their growth was very slow, about half of them developed to protocorm masses about 60 weeks after sowing (Fig. 3). Shoot-

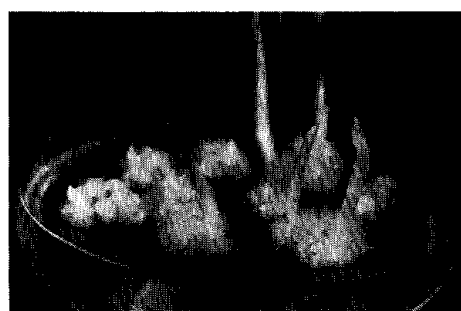


Fig. 3 Protocorm masses developing from seeds cultured on Tsutsui and Tomita medium (60 weeks after sowing).

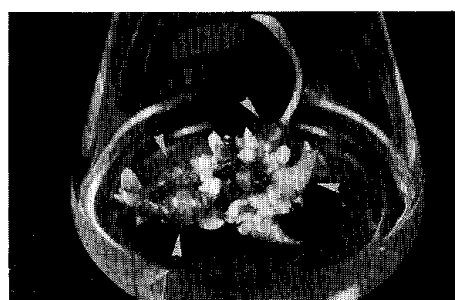


Fig. 4 Bulb formation (arrows) from a protocorm mass (76 weeks after sowing).

proliferating protocorm masses were transferred to a continuous light condition (500 lux) 64 weeks after sowing. Bulb formation was observed about 70 to 76 weeks after sowing (Fig. 4).

4. Discussion

Harvais and Hadley [3] improved the germination rate of *Dactylophiza purpurella* by sterilizing the seeds with calcium hypochlorite solution until they lost their dark color. The mechanism by which hypochlorite solution stimulates seed germination or breaks dormancy is thought to be its action in bringing about a partial degradation of the seed coat and/or a solubilization and oxidation of inhibitors [4-6, 8]. In experiment 1, differences in coloration of the seeds were observed according to the sterilization time showing that, in *Calypso bulbosa*, optimal treatment time with sodium hypochlorite should be between four to eight min. By the tetrazolium test, the viability of the seeds was found to be about 70% after those suitable treatments. Any shorter period of time of treatment would not be sufficient to break the suberin and to stain embryo with TTC solution, while with any longer time of treatment, the seeds would be damaged and the germination rate would decrease.

The results of experiment 2 suggest that the optimal temperature for both germination and seedling growth of *Calypso bulbosa*, which distributed in boreal zone of north temperate region, would lie between 17.5 and 20°C. For most orchid species, the temperature that yields the highest germination percentage lies between 22 and 25°C [8], but some species, which distributed in boreal zone such as *Aorchis* [9] and *Cypripedium* [14] germinate best below 20°C. Over-optimal temperatures have a pronounced negative effect on germination and seedling growth [4, 7, 8]. Rasmussen *et al.* [7] suggest that the highest part of temperatures' effect dealt with in such cases is probably of ecological relevance to the soil temperatures in the habitats of the north temperate region where these species occur. Further studies will be needed for the relationship between germination of orchid

species distributed in north temperate region, especially in boreal zone, and the environments of their native habitats.

Although further investigations are needed in the nutrient requirement of protocorms and also in the conditions under which aymbiotically produced seedlings would grow, these studies show that four to eight min of sterilization improves the germination rate of mature seeds, and relatively low temperatures (17.5 to 20°C) are suitable for the germination and initial protocorm growth of *Calypso bulbosa*, which is an endangered plant species.

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