In vitro seed germination and protocorm development of Eulophia graminea Lindl. (Orchidaceae).

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1 ***In vitro* seed germination and protocorm development of *Eulophia graminea* Lindl. (Orchidaceae).**

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1)

Abstract

Effects of various factors on seed germination and subsequent protocorm growth of *Eulophia graminea*, an endangered terrestrial orchid species native to Japan, were examined using New Dogashima medium without applying plant growth regulators and natural ingredients. For the culture, aseptic mature seeds collected from undehiscent pods were used throughout of the study, and New Dogashima medium supplemented with 29.2 mM sucrose and 8 g/L agar was used as standard medium. For examining the effect of calcium hypochlorite on seed germination, the seeds were pre-treated for 0-60 min with the solution containing 1% available chlorine and cultured on standard medium. The effects of other factors were examined using the seeds without calcium hypochlorite pretreatment. The effects of culture temperature on seed germination and subsequent growth were examined at the temperature range of 15–35°C. Optimum sucrose concentration and suitable kind of carbohydrate were examined using 0-175.3 mM of sucrose and 29.2 mM of sucrose, maltose, trehalose and glucose, respectively. As the results, germination rate increased with increase in the treatment duration of calcium hypochlorite solution and the maximum germination rate was 85.8% at 60 min treatment. Optimum sucrose concentrations for seed germination were 29.2-58.4 mM, which gave low germination rates (7-8%). Among the four kinds of carbohydrates tested, trehalose gave the highest germination rate (26.8%). On the effect of temperatures, both the highest germination rate and subsequent growth with shoot development were obtained at 30°C. Direct shoot development from protocorm without forming rhizome was shown at this high temperature.

Keywords Seed germination, Temperature, Calcium hypochlorite, Carbohydrates, Shoot induction, *Eulophia graminea*

Introduction

Orchids are wide spread all over the world through tropical to cold latitude. Since they mostly have beautiful flowers with variable characteristics, they attract many people around the world. Natural population of orchids has been decreasing mainly by the human activities on natural habitats such as exploitation and illegal collection. Many species are now endangered due to quite low natural reproduction efficiencies with the non-endospermic tiny seeds which require specific symbiotic fungi for seed germination (Hadley 1982). For the conservation of endangered orchid species, it is necessary to establish *in vitro* asymbiotic seed germination methods. Although many species have successfully been propagated by seeds with asymbiotic seed germination method established by Knudson (1922), it is still difficult to germinate asymbiotically in various terrestrial orchids, such as *Calanthe*, *Paphiopedilum* and temperate *Cymbidium* species (Arditti et al. 1982; Miyoshi and Mii 1995; Chen et al. 2015; Tsutsumi et al. 2020; Perner et al. 2022).

The genus *Eulophia* is a member of Orchidaceae widely distributing in tropical and subtropical regions in the world consisting of about 280 species (Kew Sciences 2023a). Among them, *E. graminea* is a terrestrial species distributing in South China, Taiwan, Southeast Asia, India, Sri Lanka, Nepal, Pakistan

47 and Ryukyu Islands (Kew Science 2023b). This species grows in hot to cool climate regions, under grassy
48 places in open hill and mountain forest where the sun shines well. It has underground large psuedobulbs,
49 and bears several inflorescences having many brown flowers with 3 cm in diameter in spring (Fig. 1). In
50 Ryukyu Islands, this species is ranked as rank II by Natural Conservation Division of Okinawa Prefecture
51 Red Data due to decreasing natural population (Okinawa Prefectural Government 2018). Therefore, it is
52 necessary to establish a useful method for seed propagation as the means for *ex situ* conservation of this
53 species.

54 There are several reports of germination on *Eulophia* genus (McAlister and van Staden 1998; Johnson
55 et al. 2007; Chang et al. 2010). In these reports, plant growth regulators (PGRs) and natural organic
56 substances such as coconut water were used to enhance seed germination and shoot or root induction from
57 protocorms. In *Eulophia* species, protocorms usually elongate to develop into rhizomes without shoot
58 induction after germination. Consequently, it needs a long culture period to establish plantlets after sowing
59 seeds. Many of these reports did not provide any detailed data of germination and subsequent development
60 of protocorms. Although there is only one report on seed germination of *E. graminea* (Chang et al. 2010),
61 germination rate and detailed culture conditions were not described in this paper and natural organic
62 substances such as coconut water were used in culture media. Moreover, after germination, protocorm
63 developed into rhizome for long time periods and detailed conditions for shoot induction were not shown.
64 In the present study, therefore, we examined the influences of culture temperature, sucrose concentration,

carbohydrate sources and immersion time of seeds in $Ca(ClO)_2$ solution which has often been used as surface sterilizer of the seeds in orchids, on seed germination and shoot development from protocorm.

Materials and Methods

Seed materials

Three fully matured pods before dehiscence were collected on 6 August 2021 after sibling crossed on 25 June 2021 from the plants of *E. graminea* grown in our nursery, and used for the experiments to clarify the influences of the seed treatment with $Ca(ClO)_2$, culture temperature and sucrose concentration on seed germination. Moreover, three fully mature pods before dehiscence obtained by open pollination of *E. graminea* were also collected on 23 August 2022 and used for the experiments on influences of different carbohydrate types in culture medium on seed germination. These pods were sprayed with 70% ethyl alcohol before brief flaming of the surface. Then, they were surface sterilized with NaClO solution (1% available chlorine) for 20 min. After pods were washed three times with sterilized distributed water, they were cut longitudinally into two halves by surgical knife. Then the seeds were aseptically scooped out with micro spatula and put into a Petri-dish. These seeds were mixed well and used for experiments.

Preparation of media for seed germination

New Dogashima (ND) medium supplemented with 29.2 mM sucrose and 8 g/L agar was mainly used as the standard medium (Tokuhara and Mii 1993) except for the experiments on influence of sucrose concentration and different kinds of carbohydrates. All the media were adjusted to pH5.4 using 1N KOH before autoclaving for 20 min at 121°C. Ten ml of medium was poured into each cell of 6-well cell culture plate (AGC Techno Glass Co., LTD. Shizuoka Japan).

Influence of $Ca(ClO)_2$ treatments on seed germination

Aseptic seeds (more than 500) were put into 1.5 ml micro tube (Nolato Treff AG, Degersheim, Switzerland) containing 1.0 ml $Ca(ClO)_2$ solution (1% available chlorine) and agitated for 0, 5, 10, 15, 20, 30 and 60 min, respectively. After washing 3 times by sterilized distributed water, more than 100 seeds were inoculated onto the standard medium in each Petri-dish by forceps. In case of 0 min treatment, seeds were immersed and washed by sterilized distributed water as the same way. After sowing, the rest of seeds were subjected to TTC test by 1% 2, 3, 5-triphenyl tetrazolium chloride solution for 48 h to determine the viability of seeds by the percentage of red-stained embryos. Culture conditions were 24 ± 0.5°C and 8.7 μmol/m^2/s illumination by white fluorescent lamps for 16 h and dark for 8 h. All experiments were conducted with 6 replicates of cells.

Influence of culture temperature on seed germination

To examine the influences of culture temperature on seed germination, culture temperatures were set at 15, 20, 25, 30 and 35°C in 5-layer temperature gradient incubator (TG-300WLED-5LE, by Nippon Medical & Chemical Instruments Co., LTD. Osaka Japan). Aseptic seeds (100-200) were inoculated onto the medium in each cell of the plate using forceps without surface sterilization treatment, and 6 replicates of cells were made for each temperature treatment. All cultures were kept under a 16 h photoperiod provided by white LED lights with a light intensity of 8.7 μmol/m^2/s.

Influences of sucrose concentration and different carbohydrate types on seed germination

To examine the influences of sucrose concentration on seed germination, ND medium supplemented with different sucrose concentrations (0, 29.2, 58.4, 87.6 and 175.3 mM) were used. The influences of carbohydrate types on seed germination were also examined on ND medium supplemented with each of the carbohydrates (sucrose, maltose, trehalose and glucose) at 29.2 mM. In each medium, 100-200 seeds without surface sterilization treatment were inoculated onto the media in each cell of the plate using forceps. For each treatment, 6 cells were prepared. All cultures were kept at 24 ± 0.5°C under 16 h photoperiod provided by white fluorescent lights with a light intensity of 8.7 μmol/m^2/s.

Process of seed germination

Seed germination and subsequent protocorm growth were classified into 6 stages as follows:

Stage 0: No germination stage, where no growth of embryo occurs (Fig. 2-A)

Stage 1: Pre-germination stage, where embryo swells in seed coat (Fig. 2-A)

Stage 2: Germination stage, where embryo raptures seed coat, then develops into protocorm (Fig. 2-B)

Stage 3: Meristem stage, where meristem area appears at the top of protocorm (Fig. 2-C)

Stage 4: Shoot induction (Fig. 2-D)

Stage 5: Root induction (Fig. 2-E)

In the present study, seeds at Stage 2 or later were categorized as 'germinated' and those at Stage 0 – 1 were regarded as 'non-germinated'. Twelve weeks after sowing, seed germination stages were examined under a stereo microscope (x 20).

Effect of culture temperature on shoot induction from protocorm

Effect of temperature on shoot and root induction from protocorms was examined at 15, 20, 25, 30 and 35°C, respectively. Protocorms (Stage 2, Fig. 1-B) obtained 90 d after sowing on ND medium supplemented with 29.2 mM sucrose and 8 g/L agar were used as material. Twenty protocorms were put on to φ9 cm of Petri-dish containing 80 ml of the same fresh medium. For each treatment, 3 plates were prepared. They were kept under continuos illumination with white LED lamps at 6.9 μmol/m^2/s. After 150 d, numbers of protocorms with different developmental stages were counted using stereo microscope (x 20) and percentage of protocorms at each developmental stage was calculated.

136

137 **Statistical Analysis**

138 To investigate the influences of culture temperature, $Ca(ClO)_2$ treatment, sucrose concentration, different
139 carbohydrate types and sucrose concentrations on seed germination, the rate of the seeds in each
140 developmental stage and that showing necrosis were recorded. Data of each experiment were logit-
141 transformed before statistical analysis of ANOVA using IBM SPSS ver. 24.0. For multiple analysis of
142 comparison between mean values, Tukey's HSD test was used. To investigate the influence of temperature
143 for induction of shoots and roots, statistical analysis was done by the same way without logit-transformation.

144

145 ## Results

146 **Effect of $Ca(ClO)_2$ treatment**

147 The rate of TTC-stained seeds after treatment with $Ca(ClO)_2$ solution decreased with increase in the
148 immersion time (Table 1). Although TTC staining rate of embryo was 86.7% before the treatment (0 min),
149 it decreased to 42.1% by the treatment for 60 min. In contrast, the rate of germination increased with
150 increase in the immersion time with $Ca(ClO)_2$ solution. The highest germination rate (86.7%) was shown
151 at 60 min of immersion time and the lowest rate (9.8%) was shown at 0 min immersion time. Also, the rate
152 of Stage 3 protocorms increased with increase in immersion time especially at 30 to 60 min. In contrast, no
153 significant difference was observed in the rates of Stage 2 protocorms among all the treatment times tested

(Table 1). Although the rate of necrosis was not so much affected by the immersion time, slightly higher rates (ca. 2%) were observed at 10-15 min immersion time. Consequently, significant difference was observed at $p < 0.001$ or 0.01 in all the parameters examined except for the rate of stage 2 protocorms (Table 1).

Effect of temperature on germination

The rate of seed germination increased with increase in culture temperature (Table 2). Germination was not observed at all under 15°C culture temperature. The highest seed germination rate (53.9%) was shown under 35°C culture, where rates of protocorms at Stage 2 and Stage 3 were also the highest. Significant differences at $p < 0.001$ were found in the rates of germination, Stage 2 and Stage 3 protocorms except for the rate of necrosis after germination. The color of protocorms at the appearance of meristem area (Stage 3) was cream yellow or light brown yellow under 35°C culture temperature, whereas light green at 20°C to 30°C (Fig. 3).

Effect of sucrose concentration on seed germination

After 12 weeks of sowing, the highest germination rate (8.3%) was shown at 29.2 mM sucrose (Table 3) followed by 7.1% at 58.4 mM, whereas no seed germination was observed on medium at 0 mM. The results of ANOVA showed significant differences in the rates of germination, Stage 2 protocorms, Stage 3 ptotocorms and necrosis. Moreover, results of multiple comparison procedure by Tukey's HSD showed no

173 significant difference between 28.2 mM and 58.4 mM on the frequencies of germination, Stage 3
174 protocorms and necrosis.
175
176 **Effect of different carbohydrate types on seed germination**
177 Table 4 shows that the highest rates of germination (26.8%) and Stage 3 protocorms (23.1%) were obtained
178 on ND medium supplemented with 29.2 mM trehalose as carbon source. In other media supplemented with
179 sucrose, maltose or glucose at the same concentration, both germination rate and the rate of Stage 3
180 protocorms were almost the same (13-14% and 10-11%, respectively) and no significant differences were
181 found among these 3 carbohydrates according to the multiple comparison procedure by Tukey's method.
182 In other parameters (Stage 2, Stage 4 and Necrosis), no significant differences were found among the 4
183 carbon sources tested by ANOVA.
184
185 **Effect of culture temperature on shoot induction from protocorm**
186 Culture temperatures gave significant differences in the frequencies of protocorms at all stages except for
187 Stage 4 by ANOVA at $p < 0.001$ when examined 150 d after transfer the stage 2 protocorms onto fresh
188 medium (Table 5). Most of the protocorms cultured under 15°C did not develop into the successive stages,
189 i.e., Stage 3 to Stage 5 (Fig. 4). However, in the cultures under higher temperature conditions, the
190 developmental stage of protocorms progressed with increasing culture temperature. Namely, protocorms

were mostly remained at Stage 2 under 20 °C culture condition, whereas they proceeded to Stage 3 at 25°C, Stage 3 and Stage 5 at 30°C and Stage 5 at 35°C, respectively. Under 20°C and 25°C of culture temperature, few protocorms developed into plantlets with root (Stage 5). Higher frequencies of plantlets were shown at 30°C (36.7 %) and 35°C (38.3 %), respectively, where no significant difference was found between both temperatures by the results of multiple comparison procedure (Table 5). Color of plantlets were pale yellow at 35°C and pale green at 30°C, respectively (Fig. 4). The highest frequency of necrosis of protocorms was shown under the culture at 35°C (20.0%), whereas lower temperatures gave negligible frequencies of necrosis, 0% at 25 and 30°C and 1.7% at 15 and 20°C, respectively.

Growth of plantlets and acclimatization

Plantlets obtained 150 d after culture of protocorms were subcultured on ND medium supplemented with 58.4 mM sucrose and 8 g/L agar adjusted to pH5.4. Four months after the subculture, i.e., 12 months after sowing, plantlets with 3-4 leaves and 5 – 10 cm height were obtained (Fig. 5 left). For acclimatization, they were put into 6 cm clay pots with sphagnum moss as compost. Then *E. graminea* plants were successfully obtained after one year of culture in green house (Fig. 5 right).

Discussion

Numerous studies have been reported on *in vitro* seed germination of orchids using mature or immature seeds (Arditti et al. 1982; Kauth et al. 2008; Jolman et al. 2022). In most of these studies, seeds or seed

210 pods were sterilized with sodium hypochlorite (1.0 – 2.5%) or calcium hypochlorite solutions (2 – 7%) for
211 5 to 20 min. However, there have been a few studies on the effect of sterilization time of seeds on the
212 germination. Miyoshi and Mii (1998) reported that *in vitro* seed germination rate increased with increase
213 in the duration of sterilization using sodium hypochlorite (7-60 min of 0.5% and 7-30 min of 1.0%) in
214 *Cypripedium macranthos*. Yeh et al. (2021) investigated the effects of treatment time (0-90 min) and
215 concentration (0.5-4%) of sodium hypochlorite on the *in vitro* seed germination of *Vanilla planifolia* and
216 showed that the germination rate increased with increase in the treatment time and the concentration.
217 Katsalirou et al. (2019) also showed that seed germination rates of *Anacamptis laxiflora* and
218 *Himanthoglossum robertianum* increased with increase in the treatment time (5 - 85 min) of 0.5% sodium
219 hypochlorite solution. In *Eulophia dabia*, Panwar et al. (2022) showed the depletion of seed coat by the
220 treatment with sodium hypochlorite through the observation with scanning electron microscopy. Therefore,
221 it can be considered that increased seed germination by the prolonged treatment with calcium hypochlorite
222 solution observed in the present study might be also due to seed coat breakdown, which made it easy to
223 penetrate the components of culture medium into the seed. On the other hand, the ratio of embryo staining
224 by TTC test decreased with increase in treatment time. These results suggest that TTC test is not a reliable
225 method for assessing the viability of the seeds after the treatment with hypochlorite solution in *E. graminea*.
226 Since instable stainability of TTC for assessing seed viability of both epiphytic and terrestrial orchids was
227 also reported by Pradhan et al. (2022). Reduced stainability of the viable embryo after hypochlorite solution

228 treatment observed in the present study might be caused by inhibition of the activity of dehydrogenase in
229 the embryo, which in turn gave negative effect on the formation of the red substance, triphenyl formazan
230 from TTC.

231 Most of the experiments on seed germination of orchids *in vitro* were cultured under the temperature
232 ranges of 20-25°C (Arditti et al. 1982). However, not so many papers have been reported on the relationship
233 between germination rate and culture temperature. In *Galeola septentrionalis*, seed germination was
234 observed only in an airtight vessel at 30°C (Nakamura et al. 1975). Godo et al. (2010) reported that the
235 highest seed germination rate of *Calanthe tricarinata* was 20°C among the temperature ranges of 5 - 25°C.
236 Moreover, Tsutsumi et al. (2011) investigated the seed germination of 6 species of *Liparis* under the 5 -
237 20°C and showed that the highest rate of germination was observed at 20°C. In the present study, we showed
238 that optimum temperature of seed germination in *E. graminea* was 30 – 35°C which was higher temperature
239 compared to that of other species.

240 In asymbiotic seed germination of orchids, sucrose at 59.4 or 87.6 mM has been predominantly used as
241 a carbon source for the culture medium (Arditti et al. 1982). Udomdee et al. (2014) examined the effect of
242 different concentrations of sucrose (0 -116.9 mM) on the germination rate of *Dendrobium nobile* hybrids
243 and found no significant differences in the rate among the concentrations tested. Whereas, Johnson et al.
244 (2011) reported that addition of sucrose to the culture medium (10, 50 mM) gave much higher germination
245 rate than the no carbon source control in *Bletia purpurea*. In *Vanda dearei*, Jualang et al. (2014) showed

246 that the highest germination rate was obtained at 29.2 mM sucrose, and the germination rate decreased with
247 further increase in sucrose concentration. In the present study, the highest germination rate was also
248 obtained at the lowest concentration, 29.2mM (Table 3). These results suggest that low concentration of
249 sucrose (10 - 29.2 mM) would be appropriate for seed germination of most orchid species. Stewart and
250 Kane (2010) also investigated seed germination on the effects of three kinds of carbohydrates (fructose,
251 glucose and sucrose) at the same concentration (50 mM) on *Habenaria macroceratitis*. They reported that
252 there was no significant difference in the rate of seed germination among the three carbohydrate types.
253 Johnson et al. (2011) investigated the effect of 6 carbohydrate types (fructose, glucose, trehalose, sucrose,
254 mannitol and sorbitol) on the seed germination of *Bletia purpurea*, in which there were no significant
255 differences at least among the 4 types, i.e. fructose, glucose, sucrose and trehalose, whereas mannitol and
256 sorbitol gave very low germination rates compared to other 4 types. In our previous study (Tokuhara et al.
257 2023), trehalose was suitable for seed germination and subsequent growth until shoot induction in
258 *Gastrochilus japonicus*. In the present study, trehalose was also shown to be suitable carbohydrate for seed
259 germination of *E. graminea* (Table 4). These results suggest that different species or genus requires different
260 carbohydrate type for the optimum seed germination, which might be related to the natural carbohydrate
261 sources provided by their symbiotic fungi species (Smith 1967; Li et al. 2022).

262 In the previous studies on seed germination of *Eulophia*, protocorms usually develop into rhizomes,
263 which were then transferred onto media supplemented with plant growth regulators (PGR) or coconut water

264 (CW) for shoot induction (McAlister and van Staden 1998; Jonson et al. 2007; Chang et al., 2010; Hossain,
265 2015). In these studies, temperatures were kept at 22-25°C through the whole process from seed
266 germination. In the present study, shoot induction from protocorm was directly achieved without rhizome
267 formation by culturing at high temperature (30 - 35°C) without using PGR and CW (Table 4, Fig. 5).
268 Although culture at 35°C gave higher rate of protocorms with shoot induction (stages 4 and 5) than that at
269 30°C, the former induced relatively high rate (20%) of protocorm necrosis. Therefore, 30°C might be better
270 for the practical production of plantlets by avoiding the loss of protocorms. By applying the optimum
271 conditions revealed by the present study, efficient seed propagation method of this species would be
272 established, which will be eventually utilized for *ex situ* conservation.
273

274 ## Conclusion

275 In the present study, we investigated the effects of several factors on seed germination and protocorm
276 growth of *E. graminea*. On the seed germination, seed treatment for 60 min with $Ca(ClO)_2$ solution (1%
277 available chlorine) before sowing, culture temperature under 30°C, use of 29.2 mM trehalose as
278 carbohydrate in culture medium were revealed to be appropriate conditions. For shoot induction from
279 protocorm, culture at 30°C gave the highest shoot induction frequency without forming rhizomes among
280 the temperature range from 15 to 35°C. By applying these optimum conditions, in vitro seedlings were
281 obtained efficiently and the method established would be utilized for *ex-situ* conservation of this species.

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Statements and Declarations

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and

analysis were performed by the authors. The first draft of the manuscript was written by Tokuhara K. and final manuscript was revised and edited by Mii.M. All authors read and approved the final manuscript.

Table 1. Effects of immersion time of seeds with $Ca(ClO)_2$ solution before sowing on the seed germination and protocorm development of *Eulophia graminea* after 12 weeks of culture.

Treatment (min)	TTC staining (%)	Germination (%)	Stage 2 (%)	Stage 3 (%)	Necrosis[1)] (%)
0	86.7 ± 2.8a	9.8 ± 0.8d	5.9 ± 0.8	3.4 ± 1.1f	0.5 ± 0.2ab
5	85.3 ± 4.0a	10.5 ± 1.6d	4.4 ± 0.8	6.0 ± 1.1e	0.1 ± 0.1b
10	82.5 ± 0.8a	17.5 ± 0.8c	7.2 ± 1.0	8.0 ± 0.7de	2.3 ± 0.4a
15	84.6 ± 1.7a	21.0 ± 1.6c	6.7 ± 0.4	12.8 ± 1.5cd	1.6 ± 0.2a
20	73.3 ± 4.4b	24.7 ± 1.2c	7.0 ± 1.1	16.9 ± 0.7c	0.7 ± 0.3b
30	53.9 ± 6.5c	70.1 ± 2.8b	6.9 ± 1.0	62.3 ± 3.4b	0.8 ± 0.3ab
60	42.1 ± 3.1d	86.7 ± 0.9a	5.4 ± 0.8	80.4 ± 0.9a	0.9 ± 0.3ab
	***[2)]	***	ns[2)]	***	**[2)]

Values (mean ± standard error) within a column followed by the same letters are not significantly different at $p < 0.05$ according to Tukey HSD multiple comparison procedure. [1)] Protocorms showing necrosis after germination. [2)]: no significant (ns), significant at $p < 0.01$ (**) and $p < 0.001$ (***) by the results of ANOVA, respectively.

Table 2. Effect of culture temperature on the seed germination of *Eulophia graminea* 12 weeks after sowing.

Temperature (°C)	Germination (%)	Stage 2 (%)	Stage 3 (%)	Necrosis[1)] (%)
15	0.0 ± 0.0d	0.0 ±0.0b	0.0 ± 0.0c	0.0 ± 0.0
20	6.0 ± 1.3cd	2.5 ± 0.5b	3.5 ± 1.0b	0.0 ± 0.0
25	10.0 ± 1.0c	5.2 ± 1.1b	4.5± 1.2b	0.3 ± 0.2
30	33.0 ± 2.3b	6.7 ± 1.0ab	26.2 ± 2.8ab	0.1 ±0.1
35	53.9 ± 3.2a	14.3 ± 3.6a	39.6 ± 6.2a	0.0 ± 0.0
	***[2)]	***	***	ns[2)]

Values (mean ± standard error) within a column followed by the same letters are not significantly different at $p < 0.05$ according to Tukey HSD multiple comparison procedure. [1)] Necrosis of protocorms after germination. [2)]: no significant (ns), significant at $p < 0.001$ (***) by the results of ANOVA, respectively.

Table 3. Effects of sucrose concentration on the seed germination and protocorm development of *Eulophia graminea* 12 weeks after sowing.

Concentration (mM)	Germination (%)	Stage 2 (%)	Stage 3 (%)	Necrosis[1)] (%)
0	0.0 ± 0.0c	0.0 ±0.0b	0.0 ± 0.0c	0.0 ± 0.0c
29.2	8.3 ± 0.8a	1.2 ± 0.3a	5.5 ± 0.4a	1.6 ± 0.2a
58.4	7.1 ± 1.0a	0.1 ± 0.1bc	3.5± 0.5a	3.4 ± 0.6a
87.6	2.3 ± 0.8b	0.6 ± 0.3ab	0.3 ± 0.1b	1.4 ± 0.6ab
175.3	2.0 ± 0.6b	0.3 ± 0.2bc	0.6 ± 0.4bc	0.5 ± 0.1b
	***[2)]	***	***	***

Values (mean ± standard error) within a column followed by the same letters are not significantly different at $p < 0.05$ according to Tukey HSD multiple comparison procedure. [1)]: Necrosis of protocorms after germination. [2)]: Significant at $p < 0.001$ by the results of ANOVA.

Table 4. Effect of several kinds of carbohydrates on the seed germination and protocorm development in *Eulophia graminea* 20 weeks after sowing.

Carbohydrate (29.2 mM)	Germination (%)	Stage 2 (%)	Stage 3 (%)	Stage 4 (%)	Necrosis[1)] (%)
Sucrose	13.3 ± 1.4b	0.0 ±0.0	10.8 ± 0.9b	0.0 ± 0.0	2.5 ± 0.7
Maltose	13.4 ± 0.9b	0.2 ± 0.1	10.3 ± 0.8b	0.0 ± 0.0	2.9 ± 0.2
Trehalose	26.8 ± 3.0a	0.6 ± 0.2	23.1 ± 2.2a	1.0 ± 0.6	2.1 ± 0.6
Glucose	14.3 ± 0.7b	0.0 ± 0.0	11.4 ± 1.3b	0.0 ± 0.0	2.9 ± 1.2
	***[2)]	ns[2)]	***	ns	ns

Values (mean ± standard error) within a column followed by the same letters are not significantly different at $p < 0.05$ according to Tukey HSD multiple comparison procedure. [1)]: Necrosis of protocorms after germination. [2)]: No significant (ns), significant at $p < 0.001$ (***) by the results of ANOVA.

Table 5. Effect of culture temperature on shoot induction from Stage 2 protocorms of *Eulophia graminea* after 150 d of culture.

Temperature (℃)	Stage 2 (%)	Stage 3 (%)	Stage 4 (%)	Stage 5 (%)	Necrosis[1)] (%)
15	98.3 ± 1.7a	0.0 ± 0.0c	0.0 ± 0.0	0.0 ± 0.0b	1.7 ± 1.7b
20	60.0 ± 10.4b	28.3 ± 6.7b	8.3 ± 8.3	1.7 ± 1.7b	1.7 ± 1.7b
25	26.7 ± 7.3c	58.3 ± 4.4a	13.3 ± 7.3	1.7 ± 1.7b	0.0 ± 0.0b
30	20.0 ± 10.0c	38.3 ± 4.4ab	5.0 ± 2.9	36.7 ± 7.3a	0.0 ± 0.0b
35	8.3 ± 7.9c	21.7 ± 6.0bc	11.7 ± 1.7	38.3 ± 13.0a	20.0 ± 7.6a
	***[2)]	***	ns[2)]	***	**[2)]

Values (mean ± standard error) within each column followed by the same letters are not significantly different at $p < 0.05$ according to Tukey HSD multiple comparison procedure. [1)]: Necrosis of protocorms after germination. [2)]: No significant (ns), significant at $p < 0.01$ (**) and $p < 0.001$ (***) by the results of ANOVA.

Fig. 1 Flowering plants and flowers of *Eulophia graminea* Lindl., in the natural habitat in Okinawa, Japan.

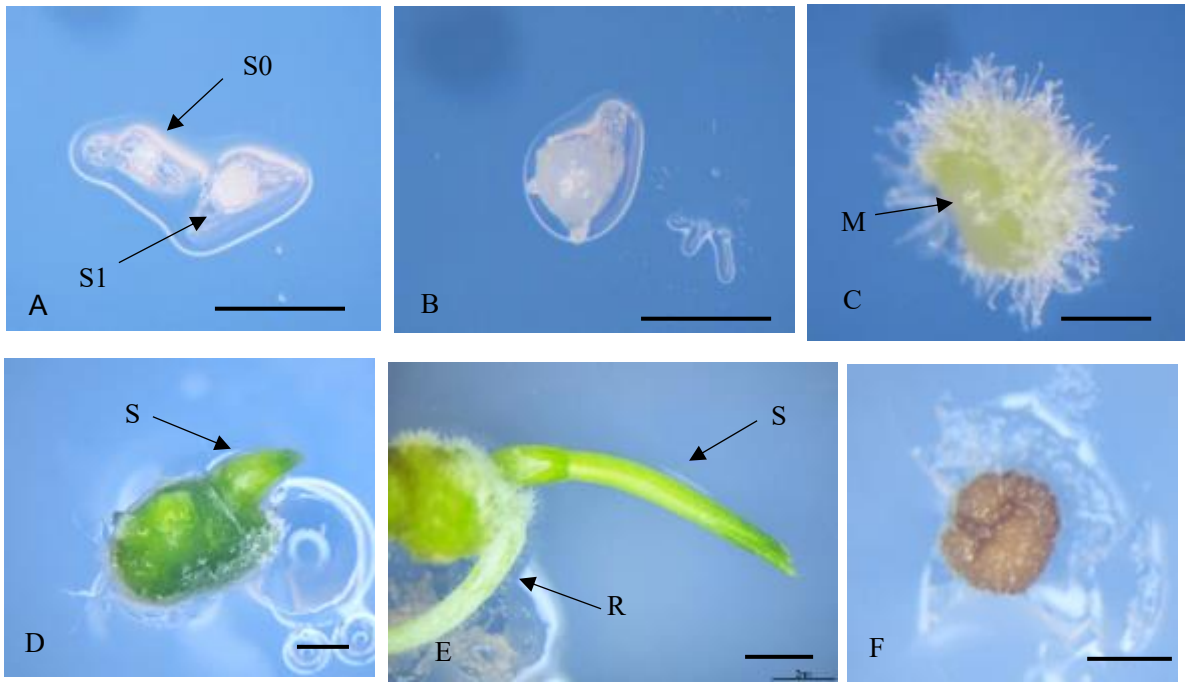

Fig. 2 Process of seed germination and plantlet induction. A: Seeds with no embryo growth at Stage 0 (S0) and swollen embryo at Stage 1 (S1) 21 d after sowing. B: Protocorm formation (Stage 2) with ruptured seed coat 40 d after sowing. C: Protocorm with appearing meristem area (M) (Stage 3) 80 d after sowing. D: Protocorm with shoot induction (arrow) (Stage 4) 120 d after sowing. E: Root induction (R) at the base of shoot (Stage 5) 150 d after sowing, F: Protocorm showing necrosis after germination. Bar = 0.5 mm (A and B), Bar = 1 mm (C, D, E and F).

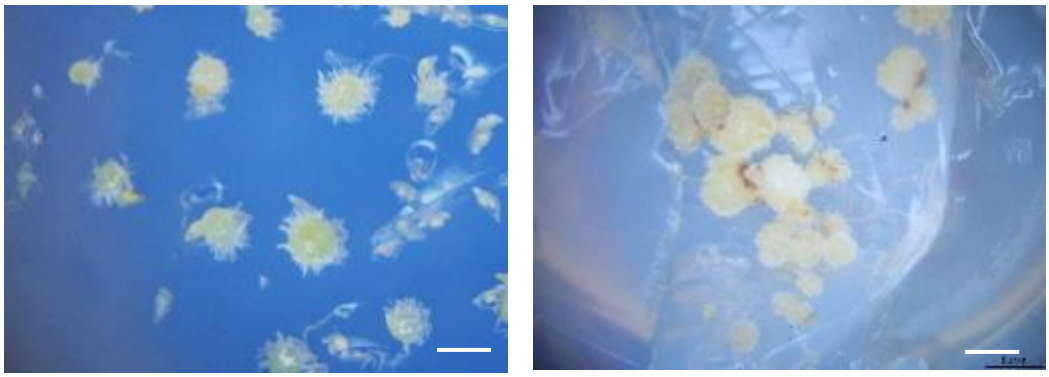

Fig. 3 Effect of temperature on color of protocorms with meristem area (Stage 3). Light green under 30°C (left) and cream yellow under 35°C (right) culture.

Bar = 0.5 mm.

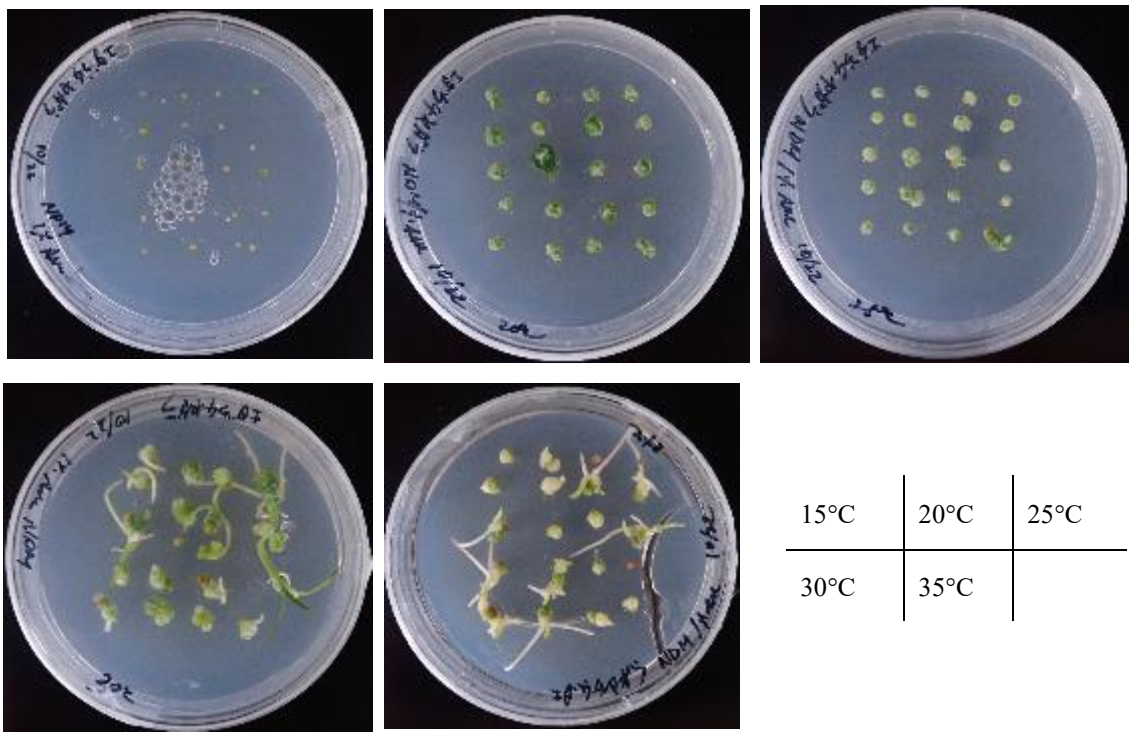

Fig. 4 Effect of temperature on shoot induction from protocorms of *Eulophia graminea* 150 d after transfer the protocorms to fresh medium. Shoots were directly formed from protocorms at high temperature conditions (30-35°C).

Fig. 5 *In vitro* plantlets obtained one year after seed sowing just before acclimatization (left) and plant established in 6 cm clay pot 1 year after acclimatization (right). Bar = 1 cm