Asymbiotic Seed Germination, Induction of Calli and Protocorm-like Bodies, and In Vitro Seedling Development of the Rare and Endangered *Nothodoritis zhejiangensis* Chinese Orchid

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Abstract. *Nothodoritis zhejiangensis* Z. H. Tsi is a rare and endangered epiphytic orchid restricted to a narrow distribution in China. The species is threatened with extinction as a result of loss of suitable habitats. An efficient propagation system is part of this species' conservation plan. Seed germination reached 64.7% on Knudson's C (KC) medium containing 1.0 mg·L⁻¹ α-naphthaleneacetic acid (NAA), 10% coconut water, and 0.1% activated charcoal. After 50 days culture, most callus (71.3%) from seed-derived protocorms formed on KC medium with 1.0 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid and 10% coconut water. Callus could be subcultured 12 times during ≈2 years with more than a 3.0-fold increase from the third to the twelfth subculture. Furthermore, 84% of callus from the tenth sub-culture on KC media supplemented with 1.0 mg·L⁻¹ NAA, 5% coconut water, and 0.1% activated charcoal formed protocorm-like bodies (PLBs). Many (57%) protocorms on KC medium containing 1.0 mg·L⁻¹ 6-benzylaminopurine and 10% coconut water formed PLBs. Both callus and PLBs formed simultaneously from different protocorms on KC medium containing 0.01 to 1.0 mg·L⁻¹ thidiazuron and 10% coconut water. PLBs were incubated 12 times during ≈2 years with an approximate 2.4-fold increase per sub-culture. Both callus and PLBs maintained their competence to regenerate plantlets. Hyponex N026 medium supplemented with 1.0 mg·L⁻¹ NAA, 50 g·L⁻¹ banana homogenate, and 0.1% activated charcoal was suitable for plantlet formation and growth of 95.5% of plantlets that developed from PLBs. The roots of plantlets 2 cm in height or taller were wrapped in Chilean sphagnum moss and fixed to a fir bark block; 69.3% of plantlets survived after 180 days in a greenhouse. This protocol is an efficient means for the large-scale propagation of this endangered orchid.

The only member of the monospecific genus *Nothodoritis* Z. H. Tsi, which belongs to the Orchidaceae family, is *Nothodoritis*

zhejiangensis Z. H. Tsi. It has a narrow geographic distribution: the districts of Linan and Ningbo (Zhejiang Province), Kuangxian

(Guansu Province), and Zhengping (Shaanxi Province) in China (Shi et al., 2008; Tsi, 1989, 1999; Wu et al., 2009). The genus is locally known as “elephant nose orchid” because of its long, slender rostellum (Fu and Hong, 2002). *N. zhejiangensis* has a similar morphology to *Doritis* Lindl. and *Phalaenopsis lowii* Rich. f. (Christenson, 2001; Tsi, 1999). Its quite peculiar flowers are white with purple transverse bands on sepals and petals and with purple lip markings (Fig. 1A; Fu and Hong, 2002; Wu et al., 2009). It was first found on Xitianmu Mountain, Linan, Zhejiang Province, in 1970 and was named in 1989 (Tsi, 1989). *N. zhejiangensis* is a typical epiphytic orchid that grows on tree branches in forests or at forest margins at elevations of 300 to 900 m (Wu et al., 2009). It usually blooms in the summer and its florescence can last several weeks. This species has great horticultural value as an ornamental plant because of its flower color and shape.

N. zhejiangensis is threatened with extinction as a result of loss of suitable habitats (Zeng, 2009). To preserve and use this rare and endangered endemic orchid, it is necessary to establish an efficient propagation system for large-scale propagation to meet the demands of the horticultural trade and hobbyists without further damage to natural populations. Under natural conditions, the main means of propagation of *N. zhejiangensis* is sexual reproduction, although seeds germinate slowly and inefficiently as a result of the absence of an endosperm. In addition, germination usually requires symbiotic fungi (Zeng, 2009).

An in vitro seed germination protocol has been described for *Doritis* (Wu et al., 2005), whereas for *Phalaenopsis*, many protocols for in vitro propagation have been described that involve in vitro seed germination (Qiu et al., 2009), PLB formation from flower stalk buds (Tokuhara and Mii, 1993), callus induction, and subsequent somatic embryogenesis from leaves (Chen and Chang, 2006; Ishii et al., 1998). A detailed review on in vitro orchid seed germination was provided by Kauth et al. (2008). However, to date, no efforts have been made to develop a protocol for the in vitro propagation or conservation of *N. zhejiangensis*. The goal of this study was to establish an effective propagation system for the large-scale propagation of this endangered orchid.

Materials and Methods

Seed source and sterilization. Thirty naturally pollinated mature seed capsules of *N. zhejiangensis* from 10 plants, which were yellow-green and ≈4 cm in length, were collected from Tianmu Mountain, Linan, Zhejiang Province, China, in Aug. 2006. The capsules were surface-sterilized by rubbing with 70% (v/v) ethanol for 3 to 5 s followed by agitation for 15 min in a solution containing 2% (v/v) sodium hypochlorite and 0.05% (v/v) Tween 20. The capsules were then rinsed five times with sterile distilled water.

Fig. 1. Asymbiotic seed germination and in vitro seedling development of *Nothodoritis zhejiangensis*. (A) Flowering specimen of *N. zhejiangensis* growing on the tree branches of *Cornus officinalis*. (B) Seed germination and protocorm development in vitro. (C) Yellowish callus mass developed from a protocorm. (D) PLBs developed from a callus mass. (E) Combination of shoot clusters, calli, and PLBs. (F) PLB-derived seedlings. (G) Transplanted seedlings on bark after growing in the greenhouse for 6 months. Bar is 2 cm (A) or 1 cm (B–G). PLBs = protocorm-like bodies.

Asymbiotic seed germination and culture conditions. Approximately 200 seeds from each capsule were subjected to a tetrazolium (TZ) viability test (Lakon, 1949). To determine the influence of basal medium on seed germination, the disinfected capsules were cut open vertically with a sterile scalpel, and the seeds were placed on five basal sowing media without plant growth regulators: Murashige and Skoog (MS) (Murashige and Skoog, 1962), half-strength MS (half-strength MS macro- and micronutrients), KC (Knudson, 1946), Vacin and Went (VW; Vacin and Went,

1949), and orchid seed sowing medium, Hyponex N026. This latter medium consisted of 1 g·L⁻¹ Hyponex 1 (Taihe Horticultural Co. Ltd., Taiwan, China), 1 g·L⁻¹ Hyponex 2, 100 mg·L⁻¹ myo-inositol, 2 g·L⁻¹ peptone, 1 g·L⁻¹ niacin, 1 g·L⁻¹ pyridoxine HCl, and 1 g·L⁻¹ thiamine HCl. NAA, coconut water (CW), and activated charcoal (AC) were added to media to improve orchid seed germination (Harvais, 1973; Hong et al., 2008; Rubluo et al., 1989). After initial trials, KC medium was shown to be the most appropriate basal medium for seed germination of *N. zhejiangensis*. Thus, KC medium containing NAA (0.5, 1.0, or 2.0 mg·L⁻¹), CW (5%, 10%, or 20%), and AC (0.05%, 0.1%, or 0.2%) were tested for improving seed germination. NAA and all plant growth regulators were purchased from Sigma Chemical Co. All media were supplemented with 30 g·L⁻¹ sucrose and 6 g·L⁻¹ agar. The CW used in these experiments was obtained from 6- to 7-month-old green coconuts from Hainan Province, China. The water was filtered through a single sheet of filter paper. The pH value of all media was adjusted to 5.8 with 1 N KOH or 1 N HCl before autoclaving at

121 °C for 18 min. For every treatment, ≈100 seeds were cultured in a 250-mL flat-bottomed culture flask containing 90 mL of medium. All experiments contained three independent replicates with 10 culture flasks per replicate. All cultures were incubated in a growth chamber at 25 ± 2 °C under a 16-h photoperiod provided by cool-white fluorescent lamps with a light intensity of 30 to 40 μmol·m⁻²·s⁻¹. Seed germination was estimated for each treatment by three parameters: the time required for germination, germination percentage of seeds, and whether viable embryos from seeds were round or ovoid hyaline. The former two parameters were recorded after 60 d of culture. The seed germination percentage was calculated as the number of germinated seeds out of the total number of cultured seeds in a flask.

Induction of callus and protocorm-like bodies from protocorms. Callus and PLB induction, development, and subculture from seed-derived protocorms were studied as possible methods for the large-scale propagation of *N. zhejiangensis*. Seed-derived protocorms were cultured on KC medium for inducing callus or PLBs by supplementing the medium with 10% CW and thidiazuron (TDZ) at 0.01, 0.1, 0.5, or 1.0 mg·L⁻¹, 6-benzylaminopurine (BAP) at 0.1, 0.5, 1.0, or 2.0 mg·L⁻¹, or 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.1, 0.5, 1.0, or 2.0 mg·L⁻¹ singly and some in combinations. The percentage of plantlets that formed, the amount of callus or number of PLBs that were induced, and the number of unresponsive or dead protocorms were calculated after 50 d of culture. Twenty protocorms were cultured per flask, and each experiment was repeated three times with 10 culture flasks per treatment.

Subculture of callus and protocorm-like body formation from callus. Callus from protocorms were subcultured every 50 d on KC medium supplemented with 10% CW and 1.0 mg·L⁻¹ 2,4-D to proliferate callus. Twelve subcultures were performed within a period of ≈2 years. Callus proliferation frequency was calculated as the ratio of the fresh weight of newly formed callus compared with incubated callus; fresh callus was weighed on sterile filter paper in a laminar flow cabinet to maintain sterile conditions. PLBs were induced to form callus on KC medium supplemented with 0.1% AC, 5% CW, and NAA at 0.1, 0.5, 1.0, or 2.0 mg·L⁻¹. Twenty callus clumps (≈2.0 g each, fresh weight) were cultured per flask, and each experiment consisted of three independent replicates with 10 culture flasks per replicate.

Protocorm-like body proliferation. PLBs were proliferated on KC medium supplemented with 10% CW and 1.0 mg·L⁻¹ BAP at 50-d intervals for each subculture. Twelve subcultures were performed during ≈2 years. PLB proliferation efficiency was calculated as the ratio of the number of PLB numbers newly formed compared with incubated PLBs. Twenty PLBs (1.5 mm in diameter) were cultured in each flask, and each experiment consisted of three independent replicates with 10 culture flasks per replicate.

Shoot formation and plant regeneration. Shoot formation percentage from protocorms

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or PLBs and subsequent plantlet growth status was assessed on KC or Hyponex N026 media containing 0.1, 0.5, 1.0, or 2.0 mg·L⁻¹ NAA, 0.1% AC, and 50 or 100 g·L⁻¹ banana homogenate. All experiments consisted of three independent replicates with 10 culture flasks per replicate with 20 protocorms or PLBs in each flask.

Greenhouse acclimatization. The effects of two transplanting methods and two planting media on ex vitro plantlet acclimatization were studied. Plantlets with roots 2 cm or longer were wrapped in Chilean sphagnum moss and grown attached to fir bark blocks or transplanted into pots with Chilean sphagnum moss or 2:1:1 (v/v) Zhijing stone for orchids (Northridge Enterprise Co., Ltd., Taiwan, China):sieved peat:shattered fir in April. The transplanted plantlets were grown in a greenhouse under no more than 800 μmol·m⁻²·s⁻¹ natural light. Plantlets were watered at 1- to 2-d intervals. After 1 month of acclimatization, plantlets were fertilized weekly with 150 mg·L⁻¹ 20N-20P-20K fertilizer (Peters Professional 20-20-20; The Scotts Co., Marysville, OH). Average temperatures ranged from 15 to 30 °C and humidity levels ranged from 70% to 98%. The percentage plantlet survival was recorded at 30 and 180 d after transplanting. Each experiment consisted of three independent replicates with 100 plantlets per replicate.

Statistical analysis. All experiments were established in a completely randomized design. Percentage data were converted to relative proportions, arcsine transformed, and then analyzed for significant differences. The data were analyzed with SPSS 11.0 for Windows (Microsoft Corp., Redmond, WA) using one-way analysis of variance, and means were separated using Duncan's multiple range test at *P* = 0.05.

Results

Asymbiotic seed germination and in vitro protocorm growth. TZ testing indicated that seeds were 85.5% viable. After 4 weeks of culture, seeds germinated on all five tested basal media, although the period required for germination and the germination percentage differed (Table 1). The seed germination percentage on KC media and Orchid Seed Sowing Medium Hyponex N026 were significantly higher than on MS, VW, and half-strength MS media; seed germination percentage on VW and half-strength MS media was significantly higher than on MS. The seed germination period on Hyponex N026 was significantly shorter than on MS or half-strength MS media. Although the seed germination percentage on KC media was not significantly higher than on Hyponex N026, more protocorms from germinated seed died after subsequent subculture and growth on Hyponex N026; therefore, KC basal media was the most appropriate basal medium for seed germination of *N. zhejiangensis* among all media tested.

KC medium supplemented with NAA (0.5, 1.0, or 2.0 mg·L⁻¹), with or without AC and CW, significantly shortened the germination period and increased germination percentage

compared with KC basal medium, except for KC medium supplemented with 2.0 mg·L⁻¹ NAA, which did not significantly increase germination percentage and KC medium supplemented with 1.0 mg·L⁻¹ NAA, 10% CW, and 0.2% AC, which did not significantly shorten the germination period (Table 2). KC medium supplemented with CW only (5%, 10%, or 20%) significantly increased germination percentage, but only KC medium supplemented with 10% CW significantly shortened the germination period compared with KC basal medium alone (Table 2). KC medium supplemented with AC only (0.05%, 0.1%, and 0.2%) did not significantly shorten the germination period and increased germination percentage compared with KC basal medium except for a high concentration of AC (0.2%), which significantly prolonged the germination period (Table 2; Fig. 1B). The KC media tested with combinations of NAA, CW, and AC significantly increased germination percentage and significantly shortened the germination period except for KC medium to which 1.0 mg·L⁻¹ NAA, 10% CW, and 0.2% AC were added, which did not significantly shorten the germination period compared with KC medium alone. KC medium containing 1.0 mg·L⁻¹ NAA, 10% CW, and 0.1% AC gave the highest percent germination, significantly higher than other media tested except for KC medium containing 0.5 mg·L⁻¹ NAA, 10% CW, and 0.1% AC.

Induction of calli and protocorm-like bodies from protocorms. In induction culture (Table 3), some protocorms formed plantlets with roots, some formed callus or PLBs, whereas others died or did not respond (protocorms formed were maintained indefinitely without further growth). PLBs and callus were easily differentiated, because PLBs were round, single, and easy to peel off from PLB clumps, whereas callus was lump-like and not easy to peel off, although for closely related *Cymbidium* hybrid orchids, callus is equivalent to miniaturized PLB clusters, which are identical to somatic embryos (Teixeira da Silva and Tanaka, 2006). No callus or PLBs formed, 91.7% protocorms formed seedlings, whereas only 5.7% of protocorms died on KC medium without plant growth regulators and with 10% CW. Callus could be induced on KC medium with 0.1 to 2.0 mg·L⁻¹ 2,4-D or a high concentration of BAP (2.0 mg·L⁻¹). The most suitable medium for callus induction was KC with 1.0 mg·L⁻¹ 2,4-D, which resulted in 71.3% callus induction. PLBs were induced on KC medium with 0.1 to 2.0 mg·L⁻¹ BAP. The most suitable medium for PLB induction was KC medium with 1.0 mg·L⁻¹ BAP, which resulted in 57.0% PLB induction. Callus and PLBs were induced synchronously on the same KC medium with 0.01 to 1.0 mg·L⁻¹ TDZ or with a high concentration (2.0 mg·L⁻¹) of BAP. The higher percentage of protocorm death occurred on KC medium with a high

Table 1. Effect of basal medium on the period and percentage of *Nothodoritis zhejiangensis* seed germination.^a

Basal medium	Germination period (days)	Germination (%)
KC	35.3 ± 3.2 ab	40.3 ± 2.7 a
VW	37.7 ± 1.5 ab	29.0 ± 2.3 b
Half-strength MS	38.7 ± 3.0 b	24.7 ± 3.5 b
MS	40.3 ± 3.0 b	15.7 ± 2.1 c
Hyponex N026	30.0 ± 1.2 a	46.0 ± 3.0 a

^aFor each treatment, ≈100 seeds were cultured in a 250-mL culture flask containing 90 mL of medium. All experiments consisted of three independent replicates with 10 culture flasks per replicate. Values followed by different letters within a column are significantly different at *P* < 0.05.

KC = Knudson's C; VW = Vacin and Went; MS = Murashige and Skoog.

Table 2. Effect of NAA, coconut water, and activated charcoal on period and percentage of *Nothodoritis zhejiangensis* seed germination.^a

NAA (mg·L ⁻¹)	Coconut water (%)	Activated charcoal (%)	Germination period (d)	Germination (%)
0	0	0	35.3 ± 3.18 e	40.3 ± 2.7 h
0.5	0	0	25.7 ± 1.8 a	48.3 ± 2.7 ef
1.0	0	0	28.0 ± 1.2 abc	46.0 ± 1.2 fg
2.0	0	0	28.7 ± 0.9 abc	43.0 ± 1.7 fgh
0	5	0	36.0 ± 2.0 e	49.3 ± 3.1 de
0	10	0	28.7 ± 1.5 abc	55.0 ± 1.2 bcd
0	20	0	35.0 ± 2.7 de	50.3 ± 1.5 cde
0	0	0.05	33.3 ± 1.8 cde	38.7 ± 2.4 hi
0	0	0.1	32.0 ± 1.2 cde	41.0 ± 1.5 ghi
0	0	0.2	36.7 ± 1.2 e	36.3 ± 1.8 i
0.5	10	0	27.3 ± 1.5 ab	58.0 ± 2.3 b
0.5	10	0.05	29.3 ± 0.7 abc	56.0 ± 2.1 bc
0.5	10	0.1	30.0 ± 2.0 abcd	60.3 ± 1.5 ab
1.0	10	0.1	27.3 ± 1.5 ab	64.7 ± 1.8 a
1.0	10	0.2	32.3 ± 1.2 bcde	50.0 ± 1.7 cde

^aThe basal medium was KC. For each treatment, ≈100 seeds were cultured in a 250-mL culture flask containing 90 mL of medium. All experiments consisted of three independent replicates with 10 culture flasks per replicate. Values followed by different letters within a column are significantly different at *P* < 0.05.

NAA = α-naphthaleneacetic acid; KC = Knudson's C.

concentration of TDZ ($1.0 \text{ mg}\cdot\text{L}^{-1}$), BAP ($2.0 \text{ mg}\cdot\text{L}^{-1}$), or 2,4-D ($2.0 \text{ mg}\cdot\text{L}^{-1}$).

Subculture of callus-induced callus and protocorm-like bodies. The callus proliferation efficiency was tested on KC medium supplemented with 10% CW and $1.0 \text{ mg}\cdot\text{L}^{-1}$ 2,4-D from the first to the twelfth subculture (Fig. 2). From the third to the twelfth subculture (Fig. 1C), callus proliferation efficiency was not significantly different but was significantly higher than the first and second subcultures, and the frequency in the second subculture was significantly higher than the first subculture.

Callus from the first to the twelfth subculture readily formed PLBs after transfer to KC medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA, 5% CW, and 0.1% AC. PLB formation percentage (82.7%, 83.3%, 85.0%, 84.7%, 83.3%, 87.7%, 86.3%, 85.0%, 82.0%, 84.0%, 82.7%, and 82.7% for the 12 subcultures, respectively) was not significantly different between subculture periods on the same media. Of the tested NAA concentrations, $1.0 \text{ mg}\cdot\text{L}^{-1}$ was most suitable for PLB formation with a significantly higher PLB formation percentage (84.0%) from the tenth subculture compared with other media tested (Table 4). PLBs from callus or protocorms were similar in shape to the protocorms of *N. zhejiangensis* formed after seed germination.

Protocorm-like body proliferation. The PLB proliferation efficiency was 2.2, 2.3, 2.4, 2.4, 2.3, 2.6, 2.5, 2.6, 2.4, 2.5, 2.3, and 2.5 for the 12 subcultures, respectively, but was not significantly different between the first and twelfth subcultures at ≈ 50 -d subculture intervals on KC medium supplemented with 10% CW and $1.0 \text{ mg}\cdot\text{L}^{-1}$ BAP (Fig. 1D).

Shoot formation and plant regeneration. Hyponex N026 medium supplemented with 0.1% AC and $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA with or without $50 \text{ mg}\cdot\text{L}^{-1}$ banana homogenate was most suitable for plantlet formation among the tested media, and 89.3% or 95.3% of PLBs from the tenth subculture formed plantlets in these media (Table 5). Plantlet formation efficiency was 88.3%, 89.7%, 90.0%, 93.7%, 92.0%, 94.7%, 89.7%, 88.7%, 92.7%, 95.3%, 96.0%, and 95.0% for the 12 subcultures, respectively, but was not significantly different from the PLBs of the first to twelfth subcultures. Some PLBs (6.0%, 11.3%, 6.3%, respectively) died on Hyponex N026 media supplemented with 0.5 or $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA and $100 \text{ g}\cdot\text{L}^{-1}$ banana homogenate or with $2.0 \text{ mg}\cdot\text{L}^{-1}$ NAA without banana homogenate. Hyponex N026 medium supplemented with 0.5 or $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA and $50 \text{ g}\cdot\text{L}^{-1}$ banana homogenate favored plantlet growth most (Table 5).

Greenhouse acclimatization. Plantlets grew vigorously after 30 d transplanting with high plantlet survival percentages that were not significantly different between media. After growth in a greenhouse for 180 d, plantlet survival rate on fir bark blocks was significantly higher than on Chilean sphagnum moss or on the sand/peat/fir bark mixture (Table 6). On all three media, plantlet survival percentage was significantly higher at 30 d transplanting compared with 180 d. Approximately 5000

Table 3. Effect of TDZ, BAP, and 2,4-D on induction of callus and PLB from *Nothodoritis zhejiangensis* protocorms.^z

Plant growth regulators (mg·L ⁻¹) TDZ BAP 2,4-D	Seedling formation (%)	Callus induction (%)	PLB induction (%)	Protocorm with no response (%)	Protocorm death (%)
0 0 0	91.7 ± 0.9 a	0 g	0 f	2.7 ± 0.3 a	5.7 ± 0.7 f
0.01 0 0	82.3 ± 1.5 b	1.0 ± 0.6 g	4.7 ± 0.3 e	4.7 ± 0.7 a	7.3 ± 0.6 ef
0.1 0 0	57.7 ± 2.0 d	7.0 ± 0.6 f	20.3 ± 0.9 d	0 b	15.0 ± 1.5 c
0.5 0 0	33.0 ± 1.5 f	9.7 ± 0.3 e	36.3 ± 0.9 c	0 b	21.0 ± 1.5 b
1.0 0 0	20.3 ± 2.0 g	9.7 ± 0.9 e	38.0 ± 0.6 c	0 b	32.0 ± 1.2 a
0 0.1 0	86.3 ± 2.3 ab	0 g	5.0 ± 0.6 e	4.7 ± 0.6 a	4.0 ± 1.0 f
0 0.5 0	75.3 ± 2.6 c	0 g	19.7 ± 1.5 d	0 b	5.0 ± 1.2 f
0 1.0 0	31.3 ± 1.5 f	0 g	57.0 ± 1.7 a	0 b	14.0 ± 2.7 cd
0 2.0 0	15.7 ± 3.0 g	5.3 ± 0.9 f	54.0 ± 1.5 b	0 b	25.0 ± 1.2 a
0 0 0.1	60.7 ± 2.4 d	30.3 ± 0.9 d	0 f	3.7 ± 0.7 a	5.7 ± 1.2 f
0 0 0.5	39.3 ± 1.8 e	50.0 ± 0.6 c	0 f	0 b	10.7 ± 1.2 de
0 0 1.0	15.7 ± 2.3 g	71.3 ± 1.2 a	0 f	0 b	13.0 ± 1.5b
0 0 2.0	4.7 ± 0.9 h	62.0 ± 1.2 b	0 f	0 b	33.3 ± 1.7 a

^zThe basal medium was KC with 10% coconut water. All experiments consisted of three independent replicates with 10 culture flasks per replicate and 20 protocorms per flask. Values followed by different letters within a column are significantly different at $P < 0.05$.

TDZ = thidiazuron; BAP = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; PLB = protocorm-like body; KC = Knudson's C.

plantlets from seed germination and plant regeneration of a mature seed capsule were produced and planted successfully within 2 years. Seedlings or plantlets were successfully acclimatized to greenhouse conditions and can be used for ornamental, reintroduction, and conservation purposes.

Discussion

The general regeneration procedure of orchid regeneration through in vitro seed germination is protocorm formation from seed, seedling development in vitro, and acclimatization of seedlings (Kauth et al., 2006, 2008; Shiau et al., 2005), which is effective for in vitro conservation of orchids (Dutra et al., 2008). Although in vitro propagation has long been associated with orchid propagation (Arditti, 1967, 1982, 2008), asymbiotic seed germination and tissue culture of *Nothodoritis zhejiangensis* have never been reported. As part of a conservation plan for this monophyletic species, an efficient propagation system is necessary. In this study, an effective micropropagation system was established through the induction of callus and PLBs and their successful subculture and proliferation from protocorms. This resulted in an efficient large-scale propagation of this endangered orchid with 5000 plantlets being propagated within 2 years.

Seed germination and seedling development were prominently affected by the choice of medium. Some orchid species prefer a low salt and nitrogen medium for seed germination (Arditti and Ernst, 1984; Fast, 1982; Van Waes and Debergh, 1986) and PLB formation (Teixeira da Silva et al., 2005). *N. zhejiangensis* showed significantly lower seed germination on MS than on half-strength MS medium possibly because of the high salt concentration of MS. However, low salt content was not the only factor affecting seed germination of *N. zhejiangensis*, because KC, half-strength MS, and VW media all contained a low salt concentration, although seed germination

Fig. 2. Effect of subculture period of *Nothodoritis zhejiangensis* on callus proliferation on Knudson's C medium supplemented with 10% coconut water and $1.0 \text{ mg}\cdot\text{L}^{-1}$ 2,4-dichlorophenoxyacetic acid shown at 50-d intervals. Different letters within a column are significantly different at $P < 0.05$.

percentage was significantly higher on KC medium than on half-strength MS and VW media.

Orchid seed germination and protocorm and/or PLB development is stimulated by organic amendments (Chu and Mudge, 1994; DeMarie et al., 1991; Harvais, 1973; Teixeira da Silva et al., 2006). CW was supplemented in the germination medium because of its beneficial effect on seed germination, which had been reported for orchids like *Bletia urbana* (Rubluo et al., 1989), *Cattleya* (Kerbuay and Handro, 1981), and *Cymbidium* (Teixeira da Silva et al., 2005, 2006). CW contains amino acids, vitamins, sugar, and plant growth regulators such as cytokinin (Laurain et al., 1993) as well as various inorganic ions such as phosphorus, magnesium, potassium, and sodium (Raghavan, 1977), which are beneficial for orchid seed germination. Hence, the responses of in vitro asymbiotic germination of *N. zhejiangensis* to CW were investigated and the result was consistent. In addition, *Phalaenopsis* callus induction and somatic embryogenesis was stimulated by 20% CW (Ishii et al., 1998). CW was also essential for direct embryogenesis (Laurain et al., 1993) and enhancement of axillary shoot growth

Table 4. Effect of NAA concentration on PLB formation from callus of the tenth subculture of *Nothodoritis zhejiangensis*.^z

NAA (mg·L ⁻¹)	Callus proliferation (%)	Callus and PLB proliferation (%)	PLB formation (%)
0	30.7 ± 1.7 a	49.3 ± 2.3 a	20.0 ± 0.6 e
0.1	26.3 ± 1.5 a	44.3 ± 2.3 a	29.3 ± 2.3 d
0.5	15.0 ± 1.7 b	30.0 ± 2.5 b	55.0 ± 1.2 c
1.0	6.0 ± 1.0 c	10.0 ± 1.5 c	84.0 ± 2.1 a
2.0	11.3 ± 0.9 b	15.0 ± 1.5 c	73.7 ± 1.5 b

^zThe basal medium was KC with 5% coconut water and 0.1% activated charcoal. All experiments consisted of three independent replicates with 10 culture flasks per replicate and with 20 callus clumps per flask. Values followed by different letters within a column are significantly different at $P < 0.05$.

NAA = α -naphthaleneacetic acid; PLB = protocorm-like body; KC = Knudson's C.

Table 5. Effect of NAA and banana homogenate concentration on plantlet formation from PLBs derived from the tenth subculture of *Nothodoritis zhejiangensis*.^z

NAA (mg·L ⁻¹)	Banana homogenate (g·L ⁻¹)	PLB proliferation (%)	Plantlet formation (%)	Necrosis (%)	Growth status
0	0	25.7 ± 3.5 a	74.3 ± 3.5 c	0 c	++
0.5	0	20.7 ± 2.3 ab	79.3 ± 2.3 c	0 c	++
1.0	0	10.7 ± 1.2 c	89.3 ± 2.9 ab	0 c	++
2.0	0	20.0 ± 1.2 ab	73.7 ± 2.2 c	6.3 ± 1.2 b	+
0.5	50	22.0 ± 1.2 ab	78.0 ± 1.7 c	0 c	+++
0.5	100	17.7 ± 0.9 b	76.3 ± 0.7 c	6.0 ± 0.6 b	++
1.0	50	4.7 ± 0.9 d	95.3 ± 0.9 a	0 c	+++
1.0	100	3.0 ± 1.5 d	85.7 ± 2.3 b	11.3 ± 0.9 a	+

^z+, ++, +++ = poor, normal, and good growth, respectively. All experiments consisted of three independent replicates with 10 culture flasks per replicate and 20 PLBs per flask. Values followed by different letters within a column are significantly different at $P < 0.05$. The basal medium was Hyponex N026 containing 0.1% activated charcoal.

NAA = α -naphthaleneacetic acid; PLBs = protocorm-like bodies.

Table 6. Survival rate of *Nothodoritis zhejiangensis* seedlings grown on three different supporting mixtures after 30 and 180 d.^z

Transplanting conditions	Survival rate after 30 d of transplanting (%)	Survival rate after 180 d of transplanting (%)
Fixed on fir bark blocks	90.0 ± 2.9 aA	69.3 ± 2.2 aB
Chilean sphagnum moss	92.3 ± 1.5 aA	56.0 ± 2.1 bB
Mixture media ^y	85.7 ± 4.0 aA	45.3 ± 3.2 cB

^zValues followed by different lower-case letters within a column or by different capital letters within a row are significantly different at $P < 0.05$. Each experiment consisted of three independent replicates with 100 plantlets per replicate.

^yContains 2:1:1 (v/v) Zhijiang stone for orchids:sieved peat:shattered fir bark.

(Boase et al., 1993). Therefore, CW was also used to induce and proliferate *N. zhejiangensis* callus and PLBs.

In the present report, callus and PLBs were induced from seed-derived protocorms, which could be subcultured and maintained on KC medium containing 2,4-D or BAP. Callus was totipotent by being able to regenerate plantlets through PLBs as an intermediate step. Callus and PLBs were subcultured ≈ 12 times over a 2-year period without losing their ability to regenerate plantlets. This protocol can thus be used for large-scale, continual propagation of this endangered orchid. In our study, 2,4-D, BAP, or TDZ played different roles in callus and PLB induction. 2,4-D alone was suitable for callus induction, BAP alone was more beneficial for PLB formation, whereas TDZ was not suitable for callus or PLB induction when used alone.

PLB formation from callus was significantly affected by NAA concentrations in our study; similar results have also been reported for a *Maudia*-type slipper orchid (Hong et al., 2008). Banana homogenate was used in medium to facilitate plantlet in vitro growth of

Renanthera imschootiana (Seeni and Latha, 1992); in our study, Hyponex N026 media supplemented with 50 g·L⁻¹ banana homogenate favored plantlet in vitro growth more than with or without 100 g·L⁻¹ banana homogenate.

N. zhejiangensis is only found growing as an epiphyte on the trunks of trees, and to our knowledge, there have been no reports of artificial planting of this endangered Chinese orchid. Plantlets exhibited vigorous growth with high survival rates from April to July; some plantlets died from August to September possibly as a result of inclement weather in greenhouse conditions in South China. However, when plantlets were planted on fir bark blocks, the survival rate was significantly higher than in pots with Chilean sphagnum moss or a sand/peat/fir bark mixture after 180 d transplanting (Table 6). A similar environment as that found in nature is likely to be suitable for growth of *N. zhejiangensis* plantlets, even in greenhouse conditions. How to increase survival rates of *N. zhejiangensis* plantlets needs further research.

In conclusion, this study reports a procedure for asymbiotic germination, in vitro seedling

culture, and regeneration system through PLBs and callus as well as greenhouse acclimatization of *N. zhejiangensis* that can be used for conservation and commercial production. Conservation of endangered or threatened orchid species can benefit from germination and acclimatization protocols that focus on propagating orchid seedlings for reintroduction or maintaining protocorms and seedlings in vitro. Callus and PLBs were incubated 12 times over 2 years and did not lose their competence to regenerate plantlets, enhancing the commerce importance for the mass production of *N. zhejiangensis*. Whether that callus or PLBs can be used for short-term or long-term germplasm conservation depends on further studies of genetic stability.

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