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

Song-jun Zeng
Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China; and Graduate University of Chinese Academy of Sciences, Beijing 100039, China

Zhi-lin Chen
Horticulural Research Institute of Guizhou Province, Guiyang 550006, China

Kun-lin Wu and Jian-xia Zhang
Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China

Cheng-ke Bai
College of Life Science, Shaanxi Normal University, Xian 726200, China

Jaime A. Teixeira da Silva
Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

Jun Duan
Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China

Additional index words. coconut water, organic supplement, acclimatization

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 subculture 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 thiadiazuron 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. Hyponeks 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), Kuanxian (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 (Qu 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.
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 Hypoxen 1 (Taiyte Horticultural Co. Ltd., Taiwan, China), 1 g·L⁻¹ Hypoxen 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; Rublou 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...
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-day 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 _Nothodoritis 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).

### Table 1. Effect of basal medium on the period and percentage of _Nothodoritis zhejiangensis_ seed germination.*

| Basal medium | Germination period (days) | Germination (%) |
|--------------|---------------------------|-----------------|
| KC           | 35.3 ± 3.2 ab             | 40.3 ± 2.7 a    |
| VW           | 37.7 ± 1.5 a              | 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    |

*For each treatment, \(\approx 100\) 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 \(P < 0.05\).

### Table 2. Effect of NAA, coconut water, and activated charcoal on period and percentage of _Nothodoritis zhejiangensis_ seed germination.**

| 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 fgf  |
| 0                   | 5                 | 0                      | 36.0 ± 2.0 c            | 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 e            | 38.7 ± 2.4 hi   |
| 0                   | 0                 | 0.1                    | 32.0 ± 1.2 e            | 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 bde          | 50.0 ± 1.7 cde  |

**The basal medium was KC. For each treatment, \(\approx 100\) 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; VW = Vacin and Went; MS = Murashige and Skoog.

**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

---

*For each treatment, \(\approx 100\) 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.
concentration of TDZ (1.0 mg L\(^{-1}\)) and BAP (2.0 mg L\(^{-1}\)), or 2,4-D (2.0 mg 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 mg L\(^{-1}\) banana homogenate. Hyponex N026 medium supplemented with 50 mg L\(^{-1}\) AC and 1.0 mg L\(^{-1}\) Hyponex N026 medium supplemented with 1.0 mg L\(^{-1}\) TDZ = thidiazuron; BAP = 6-benzylaminopurine; 2,4-D = 2,4-dichlorophenoxyacetic acid; PLB = protocorm-like body; KC = Knudson’s C.

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

| Plant growth regulators (mg L\(^{-1}\)) | Seedling formation (%) | Callus induction (%) | PLB induction (%) | Protocorm with no response (%) | Protocorm death (%) |
|---------------------------------------|-----------------------|---------------------|------------------|-------------------------------|-------------------|
| TDZ BAP 2,4-D                         |                       |                     |                  |                               |                   |
| 0 0 0                                  | 91.7 ± 0.9 a          | 0 g                 | 0 f              | 2.7 ± 0.3 a                   | 5.7 ± 0.1 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 c         | 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       |
| 1.0 0 0                                | 31.3 ± 1.5 f          | 0 g                 | 57.0 ± 1.7 a     | 0 b                           | 14.0 ± 2.7 cd     |
| 2.0 0 0                                | 15.7 ± 3.0 g          | 53.9 ± 0.9 f        | 54.0 ± 1.5 b     | 0 b                           | 25.0 ± 1.2 a      |
| 0 0 1 0                                | 50.0 ± 1.4 d          | 30.3 ± 0.9 f        | 0 f              | 3.7 ± 0.7 a                   | 5.7 ± 1.2 f       |
| 0 0 0 0                                | 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              | 13.0 ± 1.5b                   |                   |
| 0 0 2 0                                | 4.7 ± 0.9 h           | 62.0 ± 1.2 b        | 0 f              | 33.3 ± 1.7 a                  |                   |

The general regeneration procedure of orchid regeneration through in vitro seed germination in 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 orchid conservation. As part of a conservation plan for this monoecious 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 DW media all contained a low salt concentration, although seed germination percentage was significantly higher on KC medium than on half-strength MS and DW media.

Orchid seed germination and protocorm and/or PLB development is stimulated by organic amendments (Chu and Mu\*
...
Table 4. Effect of NAA concentration on PLB formation from callus of the tenth subculture of Nothodoritis zhejiangensis.*

| 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 c     |
| 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     |

*The 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.*

| 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          | 50                        | 20.0 ± 1.2 ab         | 73.7 ± 2.2 c           | 6.3 ± 1.2 b  | +            |
| 0.5          | 100                       | 17.7 ± 0.9 b          | 76.3 ± 0.7 c           | 6.0 ± 0.6 b  | ++           |
| 1.0          | 100                       | 4.7 ± 0.9 d           | 95.3 ± 0.9 a           | 0 c          | ++           |
| 1.0          | 100                       | 3.0 ± 1.5 d           | 87.5 ± 2.3 b           | 11.3 ± 0.9 a | +            |

*Values followed by different lowercase letters within a column or by different capital letters within a row 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.*

| 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 a A                               | 69.3 ± 2.2 a B                                |
| Chilean sphagnum moss   | 92.3 ± 1.5 a A                               | 56.0 ± 2.1 B                                  |
| Mixture media*           | 85.7 ± 4.0 a A                               | 45.3 ± 3.2 B                                  |

*Values followed by different lowercase 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.

Literature Cited

Arndt, J. 1967. Factors affecting the germination of orchid seeds. Bot. Rev. 33:1–97.

Arndt, J. 1982. Orchid biology: Reviews and perspective II, p. 243–370. Cornell Univ. Press, Ithaca, NY.

Arndt, J. 2008. Micropropagation of orchids. Vols. I–II. 2nd Ed. Blackwell Publishing Ltd., New York, NY.

Arndt, J. and R. Ernst. 1984. Physiology of germinating orchid seeds, p. 177–222. In: Arndt, J. (ed.). Orchid biology: Reviews and perspectives III. Cornell Univ. Press, Ithaca, New York.

Boase, M.R., S. Wright, and P.L. McLeay. 1993. Coconut water enhancement of axillary shoots growth of in vitro kiwifruit. N. Z. J. Crop Hort. Sci. 21:171–176.

Chu, J.T. and W.C. Chang. 2006. Direct somatic embryogenesis and plant regeneration from leaf explants of Phalaenopsis amabilis. Biol. Plant. 6:169–173.

Christensen, E.A. 2001. Phalaenopsis—A monograph. Timber Press, Portland, OR, pp. 44–45.

Chu, C.C. and K.W. Mudge. 1994. Effects of prechilling and liquid suspension culture on seed germination of the Yellow Lady’s slipper orchid, Cypripedium calceolus var. pubescens. Lindleyana 9:153–159.

DeMeir, E.M., Weimer, and P. Mudge 1991. In vitro germination and development of Showy Lady’ Slipper orchid (Cypripedium reginae Walt.) seeds. HorticScience 26:272.

Dutra, D., T.R. Johnso, P.J. Kauth, S.L. Stewart, M.E. Kane, and L. Richardson. 2008. Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid Bletia purpurea. Plant Cell Tissue Organ Cult. 94:31–121.

Fast, G. 1982. European terrestrial orchids. Symbiotic and asymbiotic methods, p. 203–293. In: Arndt, J. (ed.). Orchid biology: Reviews and perspectives, I. Cornell Univ. Press, Ithaca, NY.

Fu, L.K. and T. Hong. 2002. Higher plant of China. Springer, Berlin, New York.

Harvais, G. 1973. Growth requirements and development of Phalaenopsis coccinea in axenic culture. Can. J. Bot. 51:327–332.

Hong, P.L., J.T. Chen, and W.C. Chang. 2008. Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of a maudiae type slipper orchid. Acta Physiol. Plant. 30:755–759.

Ishii, Y., T. Kamaka, and M. Goi. 1998. Callus induction and somatic embryogenesis of Phalaenopsis. Plant Cell Rep. 17:446–450.

Kauth, P.J., D. Dutra, T.R. Johnson, S.L. Stewart, M.E. Kane, and W. Vendrame. 2008. Techniques
and applications of in vitro orchid seed germination, p. 375–391. In: Teixeira da Silva, J.A. (ed.). Floriculture, ornamental and plant biotechnology: Advances and topical issues. 1st Ed., Vol V. Global Science Books, Isleworth, UK.

Kauth, P.J., W.A. Vendrame, and M.E. Kane. 2006. In vitro seed culture and seedling development of Calopogon tuberosus. Plant Cell Tissue Organ Cult. 85(1):91–102.

Kerbuay, G.B. and W. Handro. 1981. Cultures of orchid embryo in liquid medium. Orchid Rev. 89:316–318.

Knudson, L. 1946. A new nutrient solution for the germination of orchid seeds. Amer. Orchid Soc. Bull. 15:214–217.

Lakon, G. 1949. The topographical tetrazolium method for determining the germination capacity of seeds. Plant Physiol. 24:389–394.

Laurain, D., J.C. Chenieux, and J. Tremouillaux-Guiller. 1993. Direct embryogenesis from female haploid protoplasts of Ginkgo balboa L., a medicinal woody species. Plant Cell Rep. 12:656–660.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Qiu, L.W., J. Wang, L.H. Xiao, and H.D. Zhen. 2009. A highly effective propagation technology for Phalaenopsis. Guangxi Agr. Sci. 40(12):1523–1525.

Raghavan, V. 1977. Diets and culture media for plant embryos, p. 361–413. In: Rechcigl, M.J. (ed.). CRC handbook series in nutrition and food. Taylor & Francis Publisher, London, UK.

Rublue, A., V. Chavez, and A. Martinez. 1989. In vitro seed germination and reintroduction of Bletia urbana (Orchidaceae) in its natural habitats. Lindleyana 4(2):68–73.

Seei, S. and P.G. Latha. 1992. Foliar regeneration of the endangered Red vanda, Renanthera imbricata Rolfe. Plant Cell Tissue Organ Cult. 29:167–172.

Shi, C.K., X.J. Liu, and X.G. Sunday. 2008. Four taxa of Orchidaceae newly recorded from Gansu Province. Journal of Gansu Agri. Univ. 43:130–132.

Shiau, Y.L., S.M. Nalawade, C.N. Hsai, and H.S. Tsay. 2005. Propagation of Haemaria discolor via in vitro seed germination. Biol. Plant. 49(3):341–346.

Teixeira da Silva, J.A., M.T. Chan, Sanjaya, M.L. Chai, and M. Tanaka. 2006. Priming abiotic factors for optimal hybrid Cymbidium (Orchidaceae) PLB and callus induction, plantlet formation, and their subsequent cytogenetic stability analysis. Sci. Hort. 109(4):368–378.

Teixeira da Silva, J.A. and M. Tanaka. 2006. Embryogenic callus, PLB and TCL paths to regeneration in hybrid Cymbidium (Orchidaceae). J. Plant Growth Regul. 25(3):203–210.

Teixeira da Silva, J.A., T. Yam, S. Fukai, N. Nayak, and M. Tanaka. 2005. Establishment of optimum nutrient media for in vitro propagation of Cymbidium Sw. (Orchidaceae) using protocorm-like body segments. Propagation Ornamental Plants 5(3):129–136.

Tokuhara, K. and M. Miib. 1993. Micropropagation of Phalaenopsis and Doritaenopsis by culturing shoot tips of flower stalk buds. Plant Cell Rep. 13:7–11.

Tsi, Z.H. 1989. Nothodoritis Tsi—A new genus of Orchidaceae from China. Acta Phytotax. Sin. 27(1):58–61.

Tsi, Z.H. 1999. Flora Reipublicae popularis sinicae, Science Press, Beijing. Tomus 19:278–279.

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

Wu, C.H., X.L. Ye, and C.Y. Liang. 2005. In vitro seed germination in Doritis pulcherrima. Guihaia 25(2):149–151.

Zeng, S.J. 2009. Nothodoritis zhejiangensis Z. H. Tsi—A rare and endangered epiphytic orchid in China. Flower 8:29.