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ORIGINAL ARTICLE

Highly compatible Epa-01 strain promotes seed germination and protocorm development of *Papilionanthe teres* (Orchidaceae)

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Abstract Symbiotic seed germination serves as a preferred method for orchid multiplication related to conservation and reintroduction programs, which involves isolation, identification, and germination-enhancing testing of symbiotic fungi. This study uses seeds of *Papilionanthe teres*, a locally endangered and medicinally valuable epiphytic orchid, to attract germination-enhancing fungi on its four host plants. Only one common and highly compatible fungus (Epa-01 strain, *Epulorhiza* sp.), isolated from seed baiting near three host plants (*Averrhoa carambola*, *Lagerstroemia villosa*, *Callistemon rigidus*), enhanced seed germination by more than 80 % and promoted protocorm development to reach stage 5 (with two leaves). Seeds cocultured with the Epa-01 strain and oat meal agar medium significantly outperformed in germination and growth speed compared with those cocultured with asymbiotic germination medium only, indicating that symbiotic seed germination is an effective method for *P. teres* seedling production. Bark substrate types have profound effects on symbiotic seed germination and protocorm development possibly due to different abundance and growth vitality of the Epa-01 strain on the four host plants. A significant difference was found in the developmental speed of symbiotic seeds between *A. carambola* and the other three host plants under ex situ and ex vitro seed germination treatments (all $P < 0.05$). The results suggest that in situ seed baiting may be used to effectively capture germination-enhancing fungi in epiphytic orchids, and testing the effects of bark substrate types on seed germination and protocorm development contributes to selecting appropriate host plants for its reintroduction into natural habitats.

Keywords *Papilionanthe teres* · In situ seed baiting · Symbiotic seed germination · Germination-enhancing fungi

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Introduction

Orchids are produced from seeds using either asymbiotic or symbiotic methods (Rasmussen 1995; Yam and Arditti 2009). However, symbiotic seed germination is superior to asymbiotic ones for several orchid species, as symbiotic protocorms develop more rapidly (Johnson et al. 2007; Nontachaiyapoom et al. 2011a) and the resulting mycorrhizal plants are more resistant to stress and pathogens (Brundrett et al. 2003). Symbiotic seed germination is one of the popular tools for the study of orchid-fungus specificity as well as for the production of mycobiont-infected healthy seedlings, which are valuable for both horticultural and conservation purposes (Stewart et al. 2003; Nontachaiyapoom et al. 2011a). Therefore, symbiotic seed germination has become one of the research hotspots in orchid conservation.

Symbiotic orchid propagation involves isolation, identification, and culture of effective symbiotic fungi that promote seed germination and/or reintroduction of threatened orchid species. The aforementioned steps are essential and can determine the success of conservation and

reintroduction programs (Stewart and Kane 2006; Swarts and Dixon 2009b). Most studies use the roots of mature orchids to screen germination-enhancing fungi (Stewart and Zettler 2002; Massey and Zettler 2007; Chutima et al. 2011; Chen et al. 2012). The method may be difficult or time/resource consuming owing to high diversities of root fungal isolates. It also has limited success in facilitating seed germination due to low species specificity of root fungal isolates (Bidartondo and Read 2008; Swarts and Dixon 2009a). Seeds cocultured with species-specific mycorrhizal fungi could improve the success of seed-based conservation programs, in both in situ germplasm conservation and reintroduction efforts (Keel et al. 2011). The species-specific mycorrhizal fungi may be captured using the in situ seed baiting technique under natural conditions (Rasmussen and Whigham 1993). They are easily isolated from germinated seeds, protocorms, and seedlings (Zelmer et al. 1996). In the last decade, many studies on symbiotic seed germination mainly focused on terrestrial orchids (Wu et al. 2010; Chutima et al. 2011; Fracchia et al. 2013); only a few studies referred to epiphytic orchids (Wang et al. 2011; Zi et al. 2014; Cruz-Higareda et al. 2015), especially Asian species.

Compatibility between fungus and orchid has a profound effect on successful symbiotic seed germination (Smith and Read 1997). It is mostly determined by the cocultivation of a fungus and orchid seeds on the oat meal agar (OMA) medium under laboratory conditions. Compared with other fungi, FCb4 strain (*Epulorhiza* sp.) and *Tulasnella* spp., which were isolated from ex situ or in situ seed baiting, showed higher promotion effects of seed germination and protocorm development in *Cymbidium mannii* and *Dendrobium aphyllum*, respectively (Sheng et al. 2012; Zi et al. 2014), suggesting that there was a high compatibility between germination-enhancing fungi isolated from developing protocorms and their host orchids. High compatibility contributes to beneficial reactions between fungi and orchids and promoting seed germination and protocorm development. But the beneficial reactions are easily affected by many ecological factors. Although clarifying the environmental effects on orchid seed germination and protocorm development under in situ conditions is difficult and time consuming due to the complexity of in situ conditions and variety of climate (Brundrett et al. 2003; Zettler et al. 2005; Diez 2007; Øien et al. 2007), the method of in vitro and ex vitro symbiotic seed germination has been found to be an effective way to elucidate the effects of testable environmental factors on germination (Kauth et al. 2008). Most studies focused on the impacts of temperature and photoperiod (Stewart and Kane 2006; Wang et al. 2011; Nikabadi et al. 2014; Zi et al. 2014) on symbiotic seed germination. For instance, our previous study confirmed that light significantly increased seed imbibition, protocorm formation, and two-leaved seed development of *Tulasnella* inoculated seeds (Zi et al. 2014). But few studies involved other ecological factors, such as humidity, pH, and species of substance. Analyses of effects of each testable ecological factor on seed germination and protocorm development contribute to selecting appropriate conditions of symbiotic seed germination and ensuring enough resources of healthy symbiotic seedling for orchid conservation.

Papilionanthe teres has medicinal value (Mazumder et al. 2010) and is often used as an ornamental plant in many countries. It is widely distributed in the tropical areas of Southeast Asia and usually grows on tree trunks in open forests or at forest margins (Chen et al. 2009). But in China, *P. teres* is only found in a very limited karst region of Xishuangbanna, Yunnan province, and is listed in the Chinese Red List as a Critically Endangered species due to its very narrow distribution and habitat degradation (Wang and Xie 2004). As part of a conservation research program, this investigation was conducted to restore and reestablish wild populations of *P. teres* and prevent the extinction of this species in this area.

In the present study, orchid seed packets were placed near the roots of adult *P. teres* plants to locate, isolate, and identify naturally occurring fungi capable of stimulating seed germination under natural conditions, and to test their effects on seed germination and protocorm development of *P. teres*. This study is the first to report the impacts of four epiphytic host substrates, *Averrhoa carambola*, *Lagerstroemia villosa*, *Callistemon rigidus*, and *Butea monosperma*, on symbiotic seed germination and protocorm development of *P. teres*. The data collected from this study will be used to propagate plants of *P. teres* and simultaneously provide references of epiphytic host selection during reintroduction, in situ restoration, and reestablishment of natural *P. teres* populations.

Materials and methods

Study sites and species

Papilionanthe teres (Fig. 1b) mainly occurs at the edge of forest or on the trunks of sparse woods with an altitude of about 600 m (Chen et al. 1999). At the study sites, the time of its anthesis is from May to June, and fruit (Fig. 1d) ripening occurs in March every year. Its flowers (Fig. 1c) are large and showy, and have high ornamental value.

This study was conducted in the Xishuangbanna Tropical Botanical Garden (XTBG; 21°45′N, 101°02′E; altitude, 580 m), which is contiguous to the Xishuangbannan National Nature Reserve, south of Yunnan Province, China. This area belongs to the tropical monsoon climate,

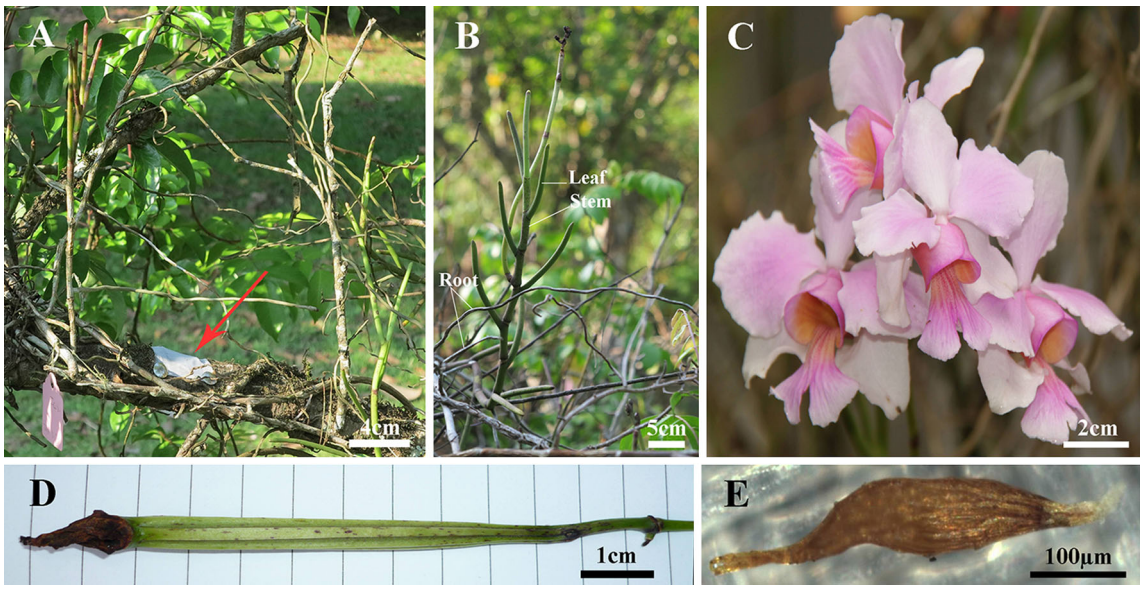

Fig. 1 Seed packet, plant, inflorescence, fruit, and seed of *P. teres* in XTBG. **a** Position of placing seed packets on plants (the *red arrow* shows seed packet). **b** Plant morphology of *P. teres*. **c** Inflorescence of *P. teres*. **d** Outcross-pollinated fruit of *P. teres* with 60-day developmental period. **e** Mature seed morphology of *P. teres*. (Color figure online)

and has an obvious alternation between rainy season (from May to October) and dry season (from November to March of next year). Its annual average precipitation and temperature are 1200–1500 mm and 18–22 °C, respectively (Ma et al. 1998), and the average relative humidity reaches about 86 % (Zhang and Cao 1995).

All six study sites located in XTBG were selected to conduct in situ seed baiting experiments of *P. teres* in 2014. The six study sites are at Tropical Rainforest (TR), Vine Garden, Fruit Garden (FG), Arboretum, Energy Plant Garden, and Ethnic Medicinal Garden. At each study site more than 10 plants of *P. teres* naturally occurs on the trunks of the host plants, *L. villosa*, *A. carambola*, *C. rigidus*, and *B. monosperma*. The kind of the host plant near which seed packets are placed and the number of host plants selected in each study site are summarized in Table 1. All laboratory experiments were conducted at the XTBG central laboratory, a research center for biodiversity conservation.

Seed collection and storage

Seeds of *P. teres* (Fig. 1e) were obtained from a wild population naturally grown on the trunks of the host plant *L. villosa* at TR. To avoid inbreeding depression and ensure seed availability, 15 flowers from 15 individuals were selected to conduct outcross-pollination experiments at TR on May 20, 2013. Pollinia used in artificial pollination were from another population from FG. In March 2014, the mature fruits, nearly dehisced capsules, were collected in sterilized Petri dishes and transported to the XTBG central laboratory for conducting laboratory experiments.

To harvest seeds, capsules were surface sterilized with 75 % ethanol for 10 min, rinsed three times with sterile double-distilled water, and opened with a scalpel under sterile conditions. The seeds were dried with anhydrous calcium chloride for 5 days at 22 °C and then stored at −20 °C for long-term preservation.

In situ symbiotic germination of *P. teres* seeds

An in situ seed packet technique (Rasmussen and Whigham 1993) was used to obtain fungi appropriate for germination of *P. teres* seeds. The seed packets were made of nylon with holes large enough to allow fungal spores to enter but not enough to allow the seeds to escape. Each seed packet contained 100 seeds. In April 2014, all seed packets were placed on the tree bark of different host plants and near the adult roots (Fig. 1a) or the seedling of *P. teres*. The seed packets were secured to the tree barks with four corners of the packets fixed with thumbtacks to avoid their losing (Fig. 1a). The number of seed packets placed near each plant at study sites is summarized in Table 1. In August 2014, the seed packets were recovered from the study sites and taken to the XTBG central laboratory for examination. Germinated seeds were collected for further experiments.

Fungal isolation

Mycorrhizal fungi were isolated from the developing protocorms collected from the seed baiting packets according to the methods described by Zettler and Piskin (2011) with

Table 1 Results of in situ seed baiting experiments of *P. teres* after 4 months

Study sites	Host plants of placing seed packets	Number of plants selected	Number of seed packet placed in each plant	Number of recovered seed packets/number of seed packets with protocorms or seedlings	Total number of protocorms and seedlings in each plant
TR	*Lagerstroemia villosa*	1	5	5/0	0
	Averrhoa carambola	2	10	8/5	13
VG	*Lagerstroemia villosa*	5	25	24/5	12
FG	*Averrhoa carambola*	4	20	19/9	27
AR	*Callistemon rigidus*	4	20	18/3	7
	Butea monosperma	2	10	7/0	0
EPG	*Butea monosperma*	4	20	19/0	0
EMG	*Callistemon rigidus*	2	10	10/0	0

TR Tropical Rainforest, *VG* Vine Garden, *FG* Fruit Garden, *AR* Arboretum, *EPG* Energy Plant Garden, *EMG* Ethnic Medicinal Garden

slight modifications as follows. The developing protocorms were washed with tap water and surface sterilized using sodium hypochlorite solution containing 1 % available chlorine for 3–5 min, and then rinsed three times with sterile double-distilled water. These protocorms were cut into small pieces using a sterile blade and placed in Petri dishes containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 26.0 ± 0.5 °C in the dark for 3–5 days. The fungal hyphae emerging at the edge of the broken protocorms were excised from the PDA medium and transferred to new Petri dishes containing a fresh PDA medium and incubated at 26.0 ± 0.5 °C in the dark for 3–5 days. Fungal colonies from actively growing isolates were subcultivated on new PDA dishes for three to four or more times until purified fungal strains were obtained (Wang et al. 2007).

Effects of fungal isolates on seed germination and seedling growth of *P. teres*

For each fungus isolated from developing protocorms, the following three treatments were conducted to examine its ability of promoting germination of *P. teres* seeds: OMA medium (oat 4 g/L, agar 8 g/L, pH = 5.8) without fungal inoculation, OMA medium with fungal inoculation ($OMA^{+Epa-01}$), and AGS medium (MS 0.9 L/L, natural mature coconut juice 0.1 L/L, carbon powder 1 g/L, sucrose 20 g/L, and agar 6 g/L). Each treatment included 10 replicates. Inoculation was done by placing a block of PDA containing purified active mycelia at the center of the OMA medium. The AGS medium was used as a nutrient-rich control medium according to the methods described by Chen et al. (2007) with slight modifications. The OMA medium without fungal inoculation was used as a nutrient-poor control medium.

Seeds of *P. teres* were removed from storage at −20 °C and moved to ambient temperature for 10 h. Then these seeds were surface sterilized using sodium hypochlorite solution containing 0.1 % available chlorine for 3 min with subsequent washing with ddH_2O for three times. Each sterilized circular nylon cloth with a diameter of 2.6 cm and spores of 45 μm was inoculated with 50–100 surface-sterilized seeds using a pipette. Then it was transferred separately to different cylindrical glass bottles (height 9 cm, diameter 6.5 cm) containing 35 mL of OMA medium without fungal inoculation, $OMA^{+Epa-01}$ such that its surface was completely covered with the fungal colony, and AGS medium.

All cylindrical glass bottles of the three treatments were incubated at 26.0 ± 0.5 °C and 14/10-h light/dark cycle for 60 days. Developmental stages of *P. teres* seeds were determined according to the methods described by Stewart and Kane (2006). *P. teres* seeds that reached developmental stage 2 or more were considered as germinated. Seed states, including developmental stage, germination rate, and fresh weight of germinated seeds, were monitored and recorded every 15 days after incubation. During incubation, cylindrical glass bottles contaminated with other fungi were discarded. The calculation of average developmental stage for each treatment referred to the methods described by Nontachaiyapoom et al. (2011b).

Molecular identification of fungi appropriate for germination of *P. teres* seeds

Fungi isolated from the developing protocorms appropriate for germination of *P. teres* seeds were incubated on a fresh PDA medium in the dark at 26 ± 0.5 °C for 7–10 days. A total of 0.2 g of the actively growing mycelia on the PDA medium were scraped off using a sterile blade and collected in a 1.5-mL sterile centrifuge tube. DNA was extracted using the E.Z.N.A. Fungal DNA Mini Kit (Omega Biotek, GA, USA) according to the manufacturer's instructions.

The internal transcribed spacer (ITS) region of rDNA was amplified using the polymerase chain reaction (PCR) with ITS4 and ITS5 primers (White et al. 1990) on a PCR Thermocycle Instrument (C1000 Tough Thermal Cycler, Bio-Rad, Hercules, California, USA). The PCR amplification reaction of 30-μL volume was as follows: 3 μL of 10× PCR buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM $MgCl_2$, pH = 8.3), 2 μL of 2.5 mM dNTP mixture, 2 μL each of 50 ng/μL primer (ITS4 and ITS5), 0.5 μL of TakaRa Taq DNA Polymerase (Dalian, China) (5 units/μL), 0.5 μL of DNA template (10–50 ng), and 20 μL of ddH_2O. The PCR thermal cycling program was 95 °C for 3 min, 40 cycles at 94 °C for 30 s, 55 °C for 40 s, 72 °C for 60 s, and a final extension step at 72 °C for 10 min. PCR products (5 μL) were electrophoresed in 1 % (w/v) agarose gel and stained with ethidium bromide. After visual inspection assisted by an ultraviolet light, the rest 25 μL of PCR products were purified and sequenced at Sangon Biotech Co., Ltd. (Shanghai, China).

The sequences obtained in this study were compared to those of fungal nucleotide sequences in the GenBank database in the National Center for Biotechnology Information (NCBI 2012; MD, USA), using the Basic Local Alignment Search Tool. They were considered as the same genus and species when the identity between the sequences found in this study and that of the database was greater than 99 %; only the genus was accepted when identity to the database matched to about 95 % (Sánchez et al. 2008).

Ex vitro symbiotic seed germination and determination of fungal growth rates on the bark substrates of the four host plants of *P. teres*

The dead and outermost barks of the four host plants of *P. teres* were collected and dried out at 37 °C for 5–10 days. Then they were broken to pieces with a small-size portable crusher (made in Guangzhou Xulang Machinery Co. Ltd, Guangdong, China).

Each cylindrical glass bottle was loaded with 20-mL ddH_2O and 10-g broken barks, and then they were mixed together to conduct the following three types of experiments: (1) ex situ symbiotic seed germination: 10-g broken barks were not sterilized; (2) ex vitro symbiotic seed germination: 10-g broken barks were sterilized and 20-mL ddH_2O was inoculated with newly grown active mycelia appropriate for germination of *P. teres* seeds; (3) determination of fungal growth rates: 10-g broken barks were sterilized. For the first two experiments, a circular nylon cloth inoculated with 50–100 seeds of *P. teres* was transferred to the cylindrical glass bottle and placed on the broken bark substrates. For the third experiment, a block of PDA medium containing purified active mycelia appropriate for germination of *P. teres* seeds was transferred to the cylindrical glass bottle and placed in the center of broken bark substrates. The size and type specification of circular nylon cloths and cylindrical glass bottles used were the same as specified in the section "Effects of fungal isolates on seed germination and seedling growth of *P. teres*." Each substrate underwent three treatments and each treatment included 30 replicates. The inoculation amount of newly grown active mycelia in 20-mL ddH_2O was not different in each treatment and replicate.

All cylindrical glass bottles were incubated at 26.0 ± 0.5 °C and 14/10-h light/dark cycle for 45 days in the first two treatments and for 14 days in the third treatment. The recording methods of seed states and calculation methods of average developmental stage for each treatment in the first two treatments were the same as described in the section "Effects of fungal isolates on seed germination and seedling growth of *P. teres*." Diameters of the fungal colonies in the third treatment were monitored and recorded every 2 days.

Statistical analysis

The statistical analysis was performed using the SPSS software 13.0. Fungal growth rates were calculated using the linear regression of the fungal colony diameter versus day. All comparisons of differences in experiments of in vitro symbiotic seed germination, and the differences of fresh weight of germinated seeds and developmental stages in experiments of ex vitro symbiotic seed germination between one treatment and another were determined using one-way analysis of variance followed by least significant difference or Games-Howell test. The other comparisons of differences were conducted with nonparametric tests followed by a test for two independent samples.

Results

In situ symbiotic germination of *P. teres* seeds

The viability of all seeds was more than 90 %. The four host plants, *A. carambola*, *L. villosa*, *C. rigidus*, and *B. monosperma*, of *P. teres* were extracted, and in situ symbiotic germination of *P. teres* seeds at the six study sites was conducted. A total of 30 seed packets were placed on the trunks of each host plant (Table 1). After 4 months (August 15), 110 packets out of 120 were recovered, and only 22 seed packets contained germinated seeds (Table 1). Of all seeds in the 22 packets, 7 seeds were at developmental stage 1 (Fig. 2a), 36 seeds became protocorms (stage 2–3, Fig. 2a–c), 23 seeds developed into seedlings with 1 leaf (stage 4, Fig. 2d), and the rest were at developmental stage 0. The number of protocorms/seedlings occurring in the packets placed on the trunks of host plants

A. carambola, *L. villosa*, and *C. rigidus* was 27/13, 4/8, and 5/2, respectively. The seed germination rate in the packets placed on the trunks of *B. monosperma* was zero (Table 1). During the recovery of seed packets, relatively high seed germination rates occurred in the packets placed near the area with moss or much humus.

Effects of fungal isolates on seed germination and seedling growth of *P. teres*

Eight types of fungal isolates were obtained from protocorms of *P. teres*. Four strains of endophytic fungi were isolated from *A. carambola*, three from *L. villosa*, and three from *C. rigidus*. Only one common fungal strain was found in protocorms from the three host plants of *P. teres*. Each fungal isolate was cocultured with seeds and the OMA medium for some time. It was found that only the Epa-01 strain promoted seed germination and seedling growth of *P. teres*.

With the passage of time, seed developmental stages stopped at stage 0 or stage 1 when they were cocultured with the OMA medium only (Figs. 3a–d, 4C; Table 2), and the seed germination rate and fresh weight of germinated seeds were zero (Fig. 4A, B). During 60 days of incubation, both the $OMA^{+Epa-01}$ and the AGS medium made *P. teres* seeds reach developmental stage 5 (Table 2; Fig. 4D). At 60 days of incubation, the ratio of germinated seeds with developmental stage 5 to total seed number on $OMA^{+Epa-01}$ was significantly higher than the corresponding ratio using the AGS medium ($F = 10.556$, $P < 0.05$; Table 2; Fig. 4d). Differences in external appearances of protocorms and seedlings were noticed between the two treatment groups, $OMA^{+Epa-01}$ and AGS medium, with regard to size of protocorms (Fig. 3e, i, f, j), leaf length of seedlings (Fig. 3g, k), and average leaf number per seedling (Fig. 3h, l). The seed germination rate and average developmental stage on $OMA^{+Epa-01}$ were significantly higher than those on the AGS medium under the same incubation time (Fig. 4A, C) ($F^{15/30/45/60\ days}_{seed\ germination} = 55.639/486.542/120.230/62.131$, all $P < 0.01$; $F^{15/30/45/60\ days}_{developmental\ stage} = 242.573/53.133/622.246/27.99$, all $P < 0.01$). Although no difference was noted in the fresh weight of protocorms or seedling at 60 days of incubation ($F = 0.053$, $P > 0.05$), their fresh weight on $OMA^{+Epa-01}$ was significantly higher than that on the AGS medium at the early stages of incubation (Fig. 4B) ($F_{15/30/45\ days} = 42.297/18.235/14.22$, all $P < 0.01$).

Morphological characterization and molecular identification of fungus appropriate for germination of *P. teres* seeds

Coculture experiments confirmed that only the Epa-01 strain promoted seed germination and seedling growth. However, it did not form reproductive structure on the PDA medium, such as sporogonium. Its mycelia were absent from monilioid cells (Fig. 5b) and growth speed was 0.20–0.25 mm/hour on the PDA medium. White and cottony mycelium occurred on the outside area while white, starchy, and thickened liquid on the inside area of the Epa-01 colony (Fig. 5a).

The ITS and 5.8S rDNA sequence length of the Epa-01 strain was found to be 583 bp. The sequence was submitted to NCBI's GenBank, and its accession number was KU296050. Based on the BLAST research, a difference of only 7 bp was found in the ITS and 5.8S rDNA sequence between the Epa-01 strain and HK-03 strain (*Epulorhiza* sp.). The HK-03 strain was isolated from the roots of *Holcoglossum* plants (Tan et al. 2012). The sequence identity of the two fungal strains reached 99 %, suggesting that the Epa-01 strain belongs to a species of *Epulorhiza*.

Ex vitro symbiotic seed germination and determination of fungal growth rates on the bark substrates of the four host plants of *P. teres*

No significant difference was found in the colony growth rate of Epa-01 strain on the bark substrates between *A. carambola* and *L. villosa* ($U = 64$, $W = 142$, $P > 0.05$), and between *L. villosa* and *C. rigidus* ($U = 44.5$, $W = 122.5$, $P > 0.05$) (Fig. 6F). But the colony growth rate of Epa-01 strain on the bark substrate of *A. carambola* was significantly higher compared with that of *C. rigidus* ($U = 14$, $W = 92$, $P < 0.01$) and *B. monosperma* ($U = 2$, $W = 80$, $P < 0.01$). The colony growth rate of Epa-01 strain on the bark substrate of *B. monosperma* was significantly lower compared with that of the other three host plants (all $P < 0.01$) (Fig. 6F).

Although *P.* seeds were only cocultured with four bark substrates for 45 days, protocorms occurred only on the bark substrates of *A. terescarambola* and *L. villosa* (Fig. 2i–l), and all seeds on the other two bark substrates stopped at developmental stage 0 (Fig. 6D; Table 3). The seed germination rate ($U = 12$, $W = 67$, $P < 0.01$), average fresh weight of a single germinated seed ($U = 16$, $W = 71$, $P < 0.05$), average developmental stages ($U = 13$, $W = 68$, $P < 0.01$), and percent of seed number with developmental stage 2 ($U = 11.5$, $W = 66.5$, $P < 0.01$) on the bark substrates of *A. carambola* were significantly higher than those of *L. villosa* (Fig. 6A–D).

Four bark substrates inoculated with the same quantity of mycelia of the Epa-01 strain were cocultured with seeds of *P. teres* for 45 days, and protocorms or seedlings occurred on the bark substrates of *A. carambola*, *L. villosa*, and *C. rigidus*. Their external appearance including size and number of protocorms with one leaf were different

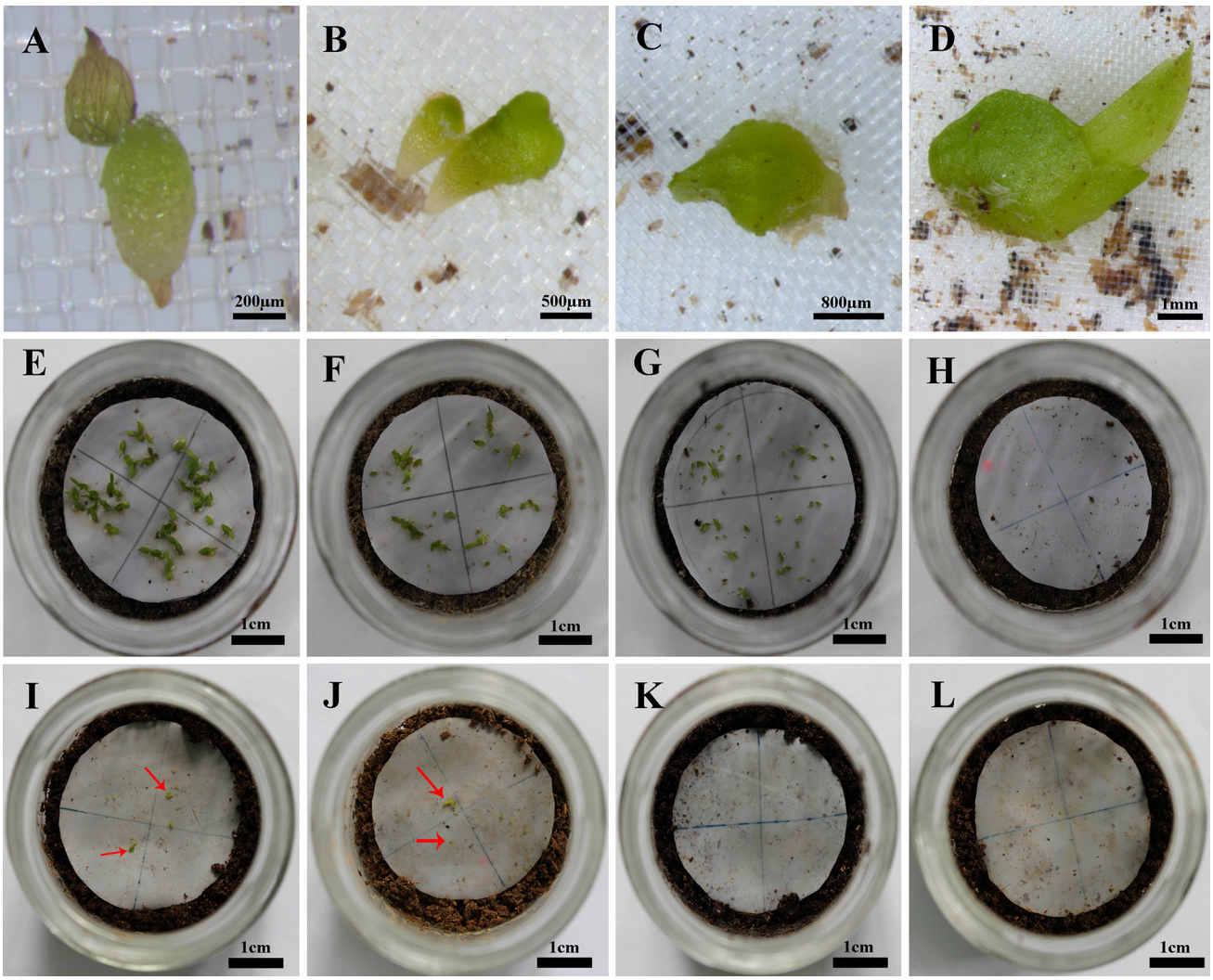

Fig. 2 Protocorms and seedlings of *P. teres* in/on seed packets and four bark substrates. **a–d** Protocorms and seedlings in seed packets recovered from in situ baiting experiments of *P. teres* showing different developmental stages. **a** Two protocorms showing developmental stage 1 and stage 2, respectively. **b** Two protocorms showing developmental stage 2 and stage 3, respectively. **c** One protocorm showing developmental stage 3. **d** One seedling showing developmental stage 4 with a leaf. **e–h** Seeds of *P. teres* sown on bark substrates inoculated with the Epa-01 strain for 45 days. **i–l** Seeds of *P. teres* sown on bark substrates without fungal inoculation for 45 days. **e**, **i** *A. carambola*, **f**, **g** *L. villosa*, **g**, **k** *C. rigidus*, and **h**, **l** *B. monosperma*. *Red arrows* in (**i**) and (**j**) show protocorms. No protocorm and seedling in parts (**h**), (**k**), and (**l**) indicate that all seeds were at developmental stage 0 or stage 1. (Color figure online)

(Fig. 2e–h). All seeds on bark substrates of *B. monosperma* stopped at developmental stage 0 (Fig. 6E; Table 3). Among the three bark substrates (A, *A. carambola*; L, *L. villosa*; C, *C. rigidus*), no significant difference was found in the seed germination rates ($U_{A–L/A–C/L–C} = 104/140.5/1.02$, $W_{A–L/A–C/L–C} = 209/293.5/207$, all $P > 0.05$) (Fig. 6A), while significant differences were found in the average fresh weight of a single germinated seed (all $P < 0.01$) (Fig. 6B), average developmental stages ($P_{A–L/A–C/L–C} = 0.039/0.001/0.320$) (except between *L. villosa* and *C. rigidus*) (Fig. 6C), and percent of seed number with developmental stage 4 ($U_{A–L/A–C/L–C} = 59.50/6.00/25.00$, $W_{A–L/A–C/L–C} = 164.50/216.00/235.00$, $P_{A–L} < 0.05$, $P_{A–C/L–C} < 0.01$) (Fig. 6E).

Discussion

The orchid in situ seed baiting technique (Rasmussen and Whigham 1993) has a wide applicability for investigating orchid mycorrhizal fungal inoculum levels and specificity (Brundrett et al. 2003). It has been successfully used in terrestrial orchids (Batty et al. 2001, 2002). As the seed packets were placed on the trunks or branches of host plants and exposed to air, the interaction processes between seeds of epiphytic orchids and fungi were deeply affected by many ecological factors. Disadvantageous ecological factors easily result in the failure of in situ symbiotic germination. So far, only limited successful case studies on the in situ seed baiting technique in epiphytic orchids are

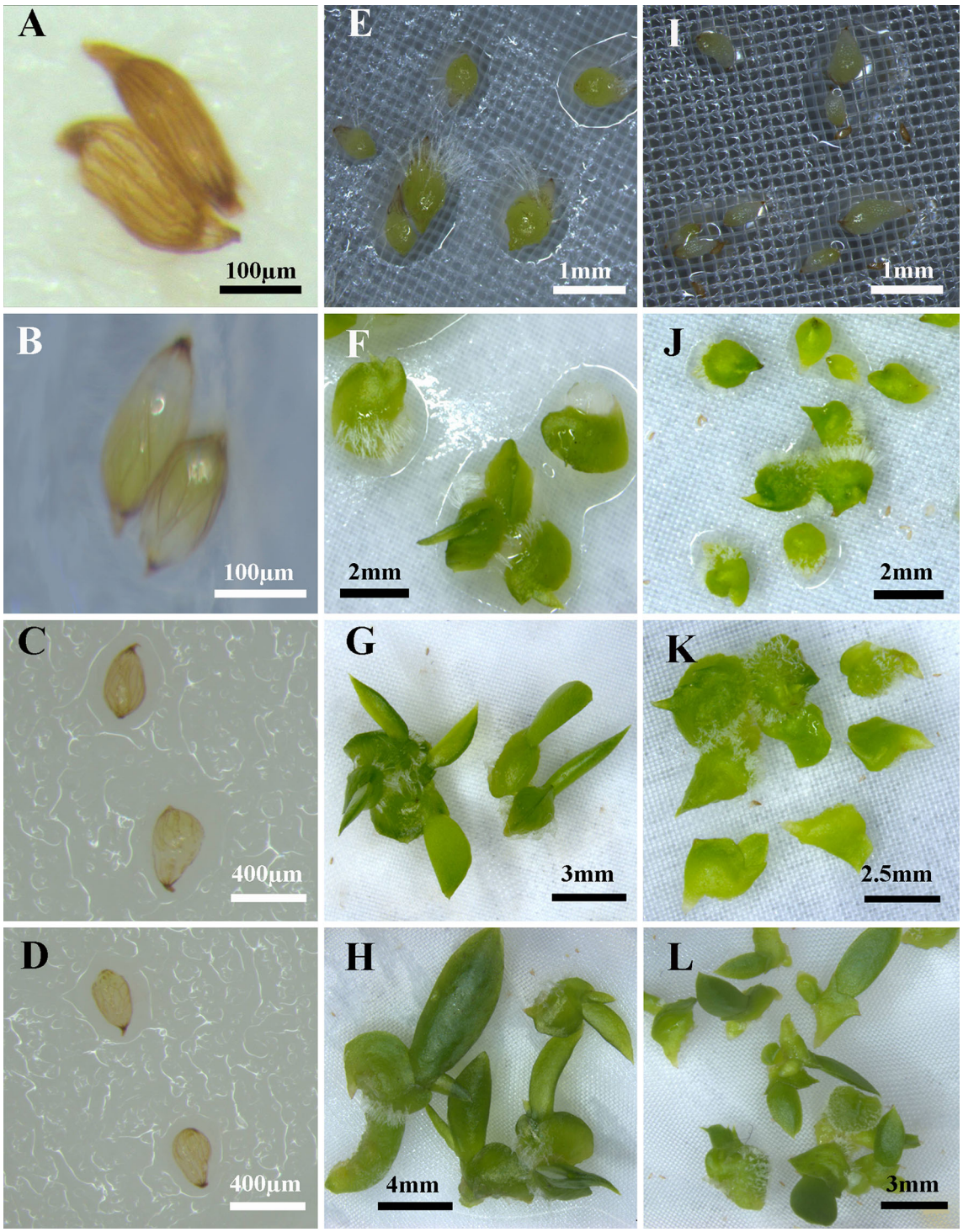

Fig. 3 Morphological characteristics of seeds, protocorms, and seedlings under the treatment of different culture mediums and developmental periods. **a–d** Seed morphology at 15, 30, 45, and 60 days after sowing on the OMA medium. **e–h** Protocorm or seedling morphology at 15, 30, 45, and 60 days after sowing on the OMA medium inoculated with the Epa-01 strain. **i–l** Protocorm or seedling morphology at 15, 30, 45, and 60 days after sowing on the AGS medium

seen (Wang et al. 2011; Zi et al. 2014; Cruz-Higareda et al. 2015). In the present study, seed packets were released to nature in rainy season, which provides optimal climatic conditions for many epiphytic orchid species including *P. teres*. So the in situ seed germination experiment got a high success rate.

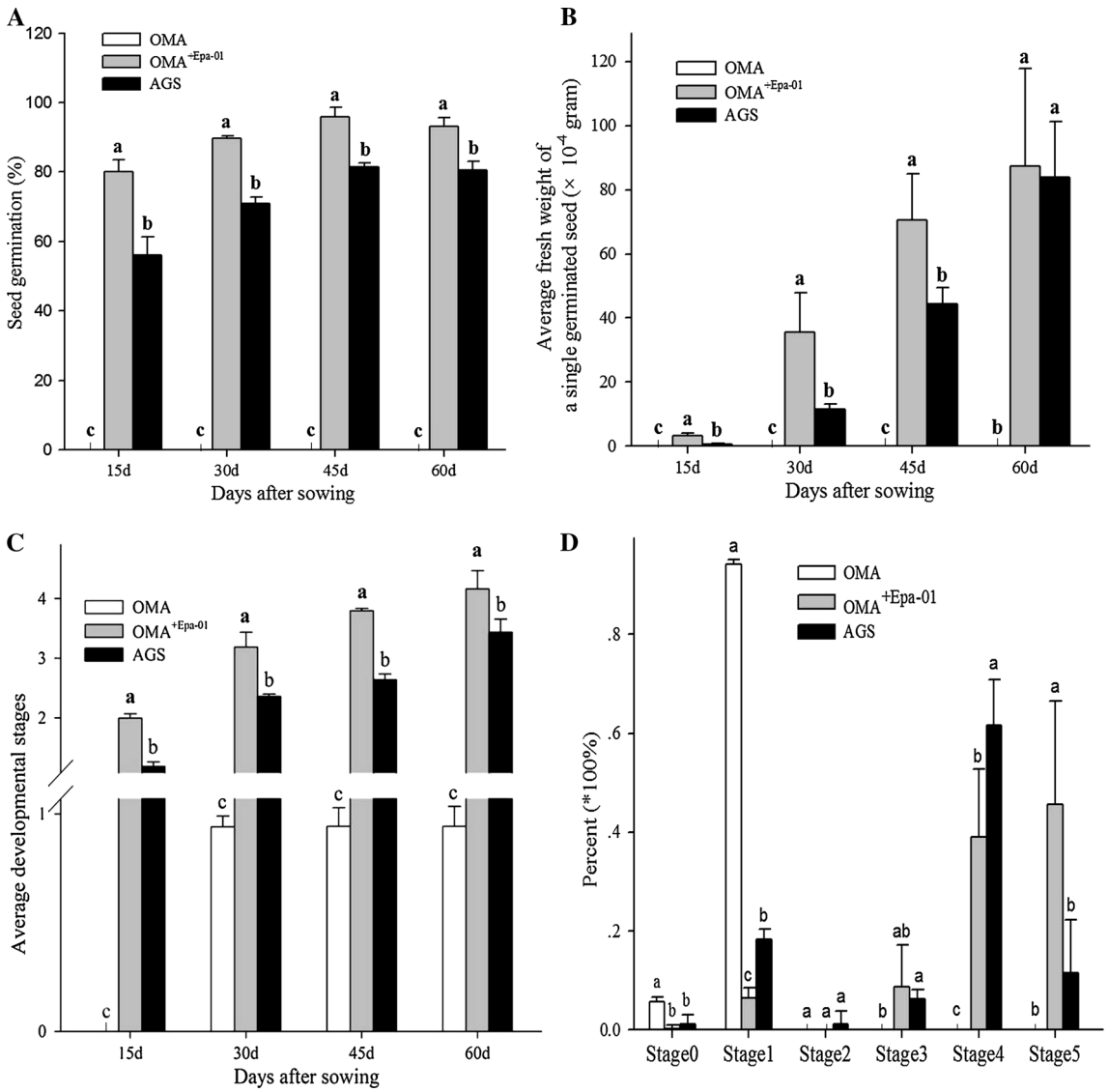

Fig. 4 Effects of culture mediums and developmental periods (15, 30, 45, and 60 days) on seed germination and protocorm development of *P. teres*. **A** Seed germination rate. **B** Average developmental stages. **C** Average fresh weight of a single germinated seed. **D** Percent of seed number with different developmental stages to total number of *P. teres* seeds at 60-day developmental period. The results are means ($n = 10$) ± standard error (SE). The *same* and *different letters* show that the differences are not significant ($P > 0.05$) and significant ($P < 0.05$), respectively

Previous studies have demonstrated that placing seed packets around the adult plants contributes to in situ symbiotic seed germination (Batty et al. 2001). Based on the research conclusion by Batty et al., seed packets were placed near the roots of *P. teres* in the present study. The germinated seeds were mostly found in the seed packets placed on the trunks covered with humus or bryophytes. Orchid mycorrhizal fungi are saprobiotic and heterotrophic (Brundrett et al. 2003), and utilize humus to obtain their nutrition. The location of seed packets determines the substrate type and has a profound effect on establishing associations between seeds and fungi. Bryophytes are important for orchid survival and growth (Crain 2012). They provide stable and homogeneous environment conditions for seed germination and seedling (Scheffknecht et al. 2010) and fungal growth (Osori-Gil et al. 2008), protect orchid plants from desiccation during dry periods (Venturieri and Arbieto 2011), and leach nutrients and other chemicals into water that may stimulate orchid growth (Clark et al. 1998). These studies together with the results of the present study suggest that selecting suitable locations to release orchid seeds contributes to in situ seed germination and seedling survival.

The time of seed packet retrieval from the field is important for fungal isolation. Earlier retrieval may not establish associations between seeds and fungi. With the variation in developmental stages, orchids form associations with different kinds of fungi (Rasmussen 2002). Germinated seeds and developing protocorms form limited association with several kinds of fungi as their fungal diversity is lower than that of seedlings and roots of adult plants (Bidartondo and Read 2008; Chen et al. 2012; Zi et al. 2014). Late retrieval may result in seeds in higher developmental stages (seedling or adult plants), making the isolation of germination-enhancing fungi difficult. In the present study, seed packets were retrieved after 4 months of their release. The germination-enhancing fungus Epa-01 strain was successfully obtained from protocorms with developmental stage 2 and stage 3.

Fig. 6 Effects of four bark substrates, *A. carambola*, *L. villosa*, *C. rigidus*, and *B. monosperma*, on seed germination and protocorm development of *P. teres* and the colony growth rate of the Epa-01 strain. **A–E** Effects of four bark substrates without (controls)/with (treatments) the inoculation of Epa-01 strain for 45 days on the development of *P. teres* seeds. **A** Seed germination. **B** Average fresh weight of a single germinated seed. **C** Average developmental stages. **D**, **E** Percent of seed number with different developmental stages to total number of *P. teres* seeds of controls and treatment groups. **F** The colony growth rate of the Epa-01 strain on the four bark substrates. The results are means ($n = 30$) ± SE. The *same* and *different letters* show that the differences are not significant ($P > 0.05$) and significant ($P < 0.05$), respectively

Most orchids have an obligate relationship with *Rhizoctonia*-like fungi during seed germination (Arditti 1992),

Table 2 Effects of media and a fungal isolate on seed germination and protocorm development of *P. teres* at different dates after sowing

Days after sowing (days)	Treatment	Percentage of seeds and protocorm (mean ± SD)					
		Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
15	OMA	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	$OMA^{+Epa-01}$	16.36 ± 2.21	3.56 ± 2.39	44.05 ± 11.17	36.03 ± 9.40	0.00 ± 0.00	0.00 ± 0.00
	AGS	37.41 ± 2.55	6.60 ± 4.18	55.99 ± 5.46	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
30	OMA	5.98 ± 0.50	94.02 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	$OMA^{+Epa-01}$	0.59 ± 0.87	9.32 ± 1.03	4.75 ± 5.41	44.32 ± 16.45	41.02 ± 19.27	0.00 ± 0.00
	AGS	1.68 ± 1.58	27.28 ± 2.61	4.44 ± 2.58	66.60 ± 2.63	0.00 ± 0.00	0.00 ± 0.00
45	OMA	5.82 ± 0.87	94.18 ± 0.87	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	$OMA^{+Epa-01}$	0.29 ± 0.66	3.83 ± 2.50	0.00 ± 0.00	7.90 ± 7.96	87.98 ± 5.73	0.00 ± 0.00
	AGS	1.10 ± 1.00	17.45 ± 1.85	5.79 ± 3.43	67.44 ± 7.79	8.22 ± 7.48	0.00 ± 0.00
60	OMA	5.73 ± 0.91	94.27 ± 0.93	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	$OMA^{+Epa-01}$	0.30 ± 0.68	6.50 ± 2.06	0.00 ± 0.00	8.62 ± 8.46	38.97 ± 13.83	45.61 ± 20.82
	AGS	1.18 ± 1.78	18.26 ± 2.11	1.21 ± 2.68	6.21 ± 2.02	61.64 ± 9.24	11.50 ± 10.84

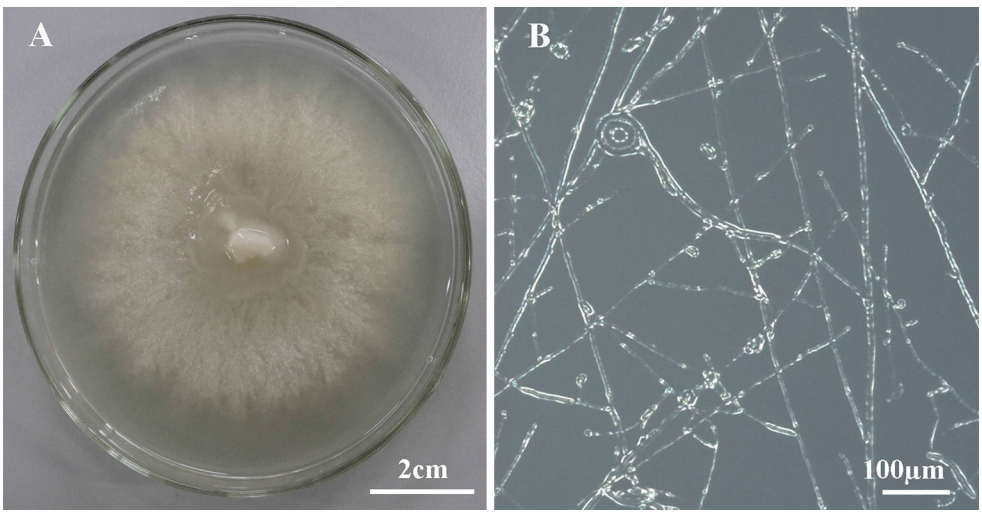

Fig. 5 Culture and morphological characters of the Epa-01 strain. **a** Morphology of isolate Epa-01 at 8 days on the PDA medium. **b** Microscopic characteristics of mycelia of the Epa-01 strain

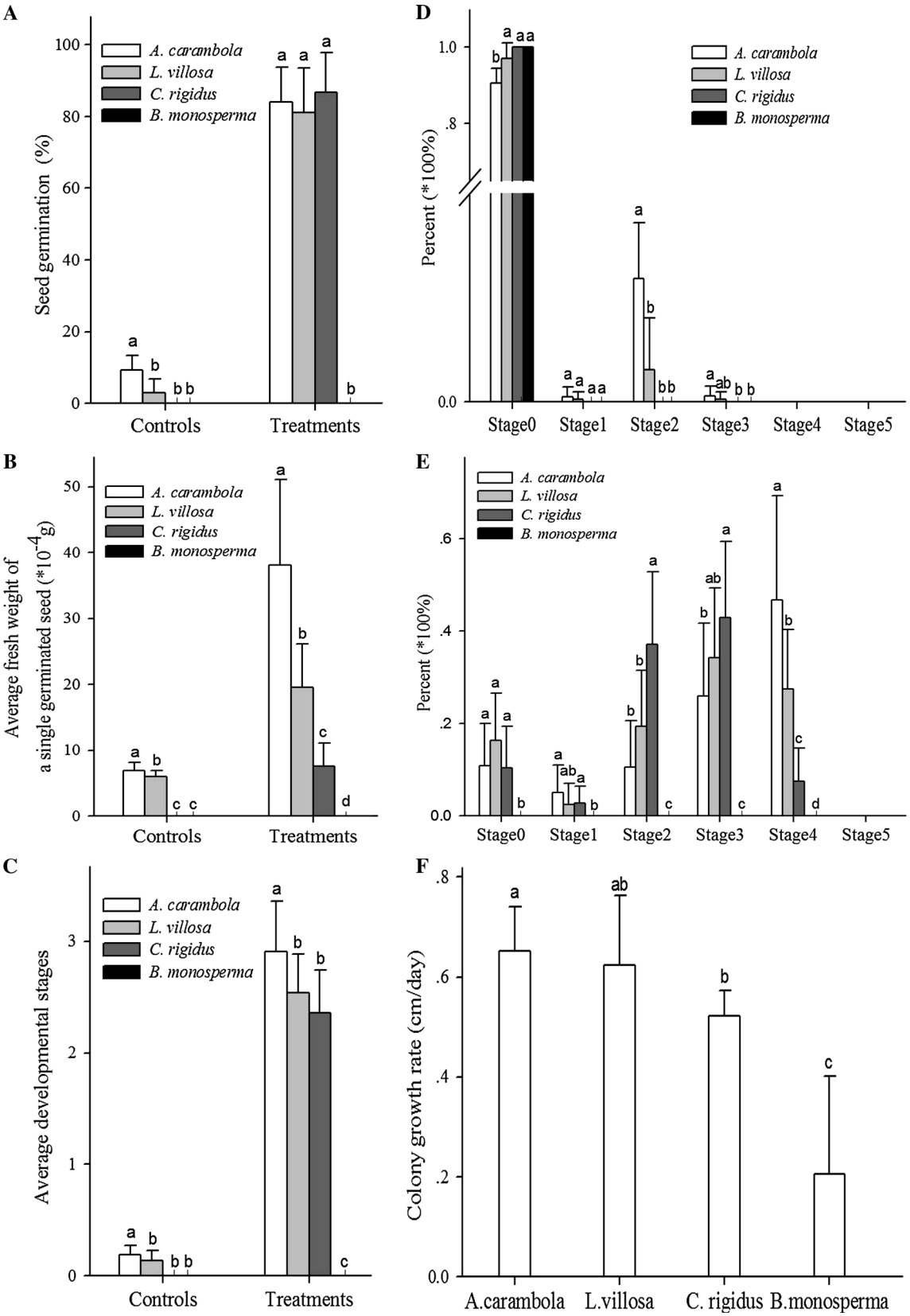
A
Seed germination (%)
A. carambola
L. villosa
C. rigidus
B. monosperma
Controls
Treatments
B
Average fresh weight of a single germinated seed (*10^-4g)
C
Average developmental stages
D
Percent (*100%)
Stage0
Stage1
Stage2
Stage3
Stage4
Stage5
E
F
Colony growth rate (cm/day)
A.carambola
L.villosa
C. rigidus
B.monosperma

Table 3 Effects of different bark substrates inoculated with Epa-01 strain on seed germination and protocorm development of *P. teres* at 45 days after sowing

Treatments	Types of bark substrates	Percentage of seeds and protocorm (mean ± SD)					
		Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
No inoculation of Epa-01 strain	*A. carambola*	90.40 ± 3.83	0.36 ± 0.75	8.82 ± 4.03	0.42 ± 0.70	0.00 ± 0.00	0.00 ± 0.00
	L. villosa	97.32 ± 4.13	0.18 ± 0.56	2.32 ± 3.67	0.18 ± 0.56	0.00 ± 0.00	0.00 ± 0.00
	C. rigidus	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	B. monosperma	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Inoculation of Epa-01 strain	*A. carambola*	10.92 ± 9.15	5.04 ± 6.06	10.50 ± 10.14	26.05 ± 15.56	47.49 ± 21.57	0.00 ± 0.00
	L. villosa	16.33 ± 10.27	2.55 ± 4.52	19.39 ± 12.03	34.18 ± 15.13	27.55 ± 12.80	0.00 ± 0.00
	C. rigidus	10.36 ± 9.12	2.86 ± 3.59	37.14 ± 15.65	42.86 ± 16.55	6.78 ± 7.13	0.00 ± 0.00
	B. monosperma	100 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

which includes the anamorphic (asexual) genera, including *Ceratorhiza*, *Epulorhiza*, *Moniliopsis*, and *Rhizoctonia* (Moore 1987), and a variety of teleomorphs (sexual stages), including *Ceratobasidium*, *Thanatephorus*, *Tulasnella*, and *Sebacina* (Warcup and Talbot 1966, 1971). The present study confirms that the fungal isolate Epa-01 strain belongs to the *Epulorhiza* genus and effectively supports seed germination and seedling development of *P. teres*. In our previous study, FCb4 strain was isolated from ex situ seed baiting and confirmed to effectively promote seed germination and protocorm development of *C. mannii* (Sheng et al. 2012). Though the two strains (Epa-01 and FCb4) both belong to *Epulorhiza* genus, they are completely different species as their sequence identity of ITS and 5.8S rDNA sequence only reaches 88 %. *Epulorhiza* has been frequently found in association with tropical epiphytic and terrestrial orchids (Ma et al. 2003; Pereira et al. 2003; Sharma et al. 2003; Athipunyakom et al. 2004; Bonnardeaux et al. 2007; Illyés et al. 2009; Tan et al. 2012), and could promote seed germination of many orchid species (Nontachaiyapoom et al. 2011b; Swangmaneecharern et al. 2012). These results imply that species of *Epulorhiza* may be non-host specific and can associate and function across a wide range of orchid life forms (Sathiyadash et al. 2014). However, whether the Epa-01 strain promotes seed germination and seedling development of the other orchid species with the same distribution as *P. teres* on a host plant requires further study.

The isolation of only one common germination-enhancing fungus from protocorms in seed packets suggested that *P. teres* mainly depended on the Epa-01 strain to promote seed germination at study sites under natural conditions. Low sampling and low encounter probability of seed germination-enhancing fungi in the wild (Wang et al. 2011) may be the reasons for not obtaining germinated seeds from seed packets placed on the trunks of *B. monosperma*. The Epa-01 strain did not promote seed germination of *P. teres* on the bark substrate of *B. monosperma* (Figs. 2h, 6A), and its growth rate on this bark substrate was significantly lower compared with that on the other three host plants (all $P < 0.01$) (Fig. 6F). Field investigation showed that natural populations of *P. teres* occurred on the trunks of the four host plants including *B. monosperma* (data not shown), indicating that the Epa-01 strain was not the only seed germination-enhancing fungus at the study sites. The species of seed germination-enhancing fungi on the host plant *B. monosperma* requires further study.

Symbiotic seed germination may be a more desirable means of producing orchids if symbiotic seedlings develop more rapidly than asymbiotic seedlings (Johnson et al. 2007). It is important for orchids to reach higher developmental stage faster under natural conditions to enhance its survival and resistance to disadvantageous environmental and climate conditions (Smith et al. 2007; Stewart 2008). Although successful asymbiotic seed germination for *P. teres* was previously reported, the method was time and money consuming under laboratory conditions (Chen et al. 2007). The presence of mycorrhizal association is essential for successful colonization and establishment of an orchid in nature (Rasmussen 2002; Leake 2004). Seedlings of *P. teres* produced by the asymbiotic method require reestablishment of fungal association and possibly have higher mortality after being released to nature. This is a novel study documenting the specific symbiotic seed germination for the species *P. teres*. The growth and development speeds of *P. teres* seeds under the treatment of $OMA^{+Epa-01}$, such as seed germination rate (Fig. 4A), average fresh weight of a single germinated seed (Fig. 4B), and developmental stage (Fig. 4C), were more rapid than under the treatment of AGS medium. These results suggest that promotion effects of the Epa-01 strain in $OMA^{+Epa-01}$ on seed germination and seedling development are superior to effects of the AGS medium. Releasing mycotrophic

seedlings in situ results in the release of suitable fungi in such areas, and these fungi could then potentially spawn additional seedlings once established (Rasmussen 1995). Therefore, in vitro and ex vitro symbiotic seed germination is an effective method for *P. teres* seedling production and their reintroduction into natural habitats.

The Epa-01 strain had a wide distribution at study sites as it could be obtained from protocorms coming from the three host plants. But it showed differences in abundance and vitality on the bark substrates of the four host plants. For instance, the colony growth rate on bark substrates of *A. carambola* was significantly higher compared with that of *C. rigidus* and *B. monosperma* (all $P < 0.01$), suggesting that the growth vitality of the Epa-01 strain was affected by bark substrate species. Ex situ seed symbiotic germination showed that the seed germination rate (Fig. 6A), average fresh weight of a single germinated seed (Fig. 6B), and developmental stage (Fig. 6C) on the bark substrate of *A. carambola* were significantly higher than those of the other three host plants (all $P < 0.05$). These results confirmed a high abundance of seed germination–enhancing fungi on the host plant *A. carambola* under natural conditions. Although ex vitro symbiotic seed germination showed no difference in seed germination rates (all $P > 0.05$) (Fig. 6A), a significant difference was found in the growth and development speed (Fig. 6B, E) among the three bark substrates (*L. villosa*, *A. carambola*, *C. rigidus*) (all $P < 0.01$). This indicates that the Epa-01 strain exhibited different promotion efficiency of seed germination and seedling development on the bark substrates of the three host plants. Perhaps the difference in physicochemical properties of different bark substrates, for example, bark chemicals (Frei and Dodson 1972), pH (Adhikari et al. 2012), and carbon and nitrogen content, cause such differences.

Accumulating studies have revealed that some epiphytic orchids may also have strong preferences for their host trees (Crain 2012; Gowland et al. 2013; Otero et al. 2011). Possible mechanisms for the preferences involve microclimate (Callaway et al. 2002), propensity for exfoliation (bark sloughing), presence of certain bark chemicals (Frei and Dodson 1972) and other bark characteristics (Benzing 1981), and distribution of mycorrhizal fungal symbionts. Field investigation shows that natural populations of *P. teres* is inclined to occur on the trunks of *A. carambola* and *L. villosa*, followed by *C. rigidus* and *B. monosperma* (data not shown). Ex situ and ex vitro symbiotic seed germination indicated that the distribution preference of *P. teres* for its host trees was possibly and partly related to the abundance and vitality of the Epa-01 strain on the host plants. The Epa-01 strain showed higher growth speed (Fig. 6F) and promotion efficiency of protocorm development on bark substrates of *A. carambola* and *L. villosa* (Fig. 6B, C, E) compared with those of the other two host plants. Hence, selecting *A. carambola* and *L. villosa* as epiphytic host plants of *P. teres* contributes to improving in situ seed germination and seedling development, and enhancing the success rate of its reintroduction.

The current study presents the detection of a highly compatible mycorrhizal fungus for promoting seed germination and seedling development of *P. teres* by the in situ seed baiting technique; the advantages of symbiotic method; and the effects of seed packet location, and releasing and retrieval time on in situ seed germination and of four bark substrates on symbiotic seed germination and seedling development. The threatened status of *P. teres* for its very narrow distribution and habitat degradation in China necessitates the development of an efficient plant production protocol. The information referring to the in situ, in vitro, and ex vitro seed germination and the determination of the fungal growth rate on the four bark substrates of host plants will be critical for the future plant production and reintroduction efforts aimed at the conservation of *P. teres* into its natural habitats. Besides, the present study will provide a successful example for the conservation of other tropical epiphytic orchids at the current study sites. To efficiently produce the seedling of *P. teres*, only elucidating the effects of different bark substrates on seed germination and seedling development of *P. teres* is possibly not enough. The effects of other testable ecological factors, such as temperature, humidity, photoperiod, pH, and carbon and nitrogen content, on seed germination and seedling development of *P. teres* require further study.

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