Symbiotic seed germination and seedling growth promoted by *Rhizoctonia* fungi in *Cymbidium mastersii*, an endangered orchid species endemic to Southwest of China

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Abstract – The presence of appropriate fungal mycobiont for seedling recruitment and plant nutritional support is essential for the long-term survival of orchids in managed or restored habitats. In order to screen mycorrhiza fungus that can promote the seed germination and form the symbiosis with *Cymbidium mastersii* Griff. ex Lindl and scale produce high quality seedling, we first symbiosis cultivated seeds with different fungi in the medium of cortices symbiotic culture, then measured the biomass of seedlings, re-separation of fungus strain, studied the 3D hypha net by using the optical microscope and electron microscope, and determined the seed vitality with TTC method. The results showed that strain CLB111 and MLX102 that were separated from roots of *Cymbidium goeringii* Rchb f. and *C. sinense* Willd can promote the seed germination of *Cymbidium mastersii*. The differences of germination rates between treatments with strain symbiosises and control were significant. Re-separations from the culture medium confirmed the strains in symbiosis roots were the same as the inoculated strains. The optical microscope and electron microscope observation found that many fungus hypha infected into embryo cell and formed the 3D hypha net, and the embryo started differentiation.TTC measurement showed that the seed had high vitality. No strain was found by re-separation from culture medium, no hypha was observed and very low vitality under control treatment. Thus, it can be concluded that strain CLB111 and MLX102 can form the symbiosis and promote the seed germination of *Cymbidium mastersii*. The experiments found the fungus separated from adult orchids and the fungus that promote the seed germination were the same strains in *Cymbidium mastersii*. This phenomenon was different from the *Gastrodia elata* Blume. It was also confirmed that funguses that can form symbiosis with and promote the seed germination of *Cymbidium mastersii* were not absolutely specific. Under certain condition, different strains can significantly promote germination of one orchid species. It may need further investigation to verify the differences under different ecological conditions.

Key words – *Cymbidium mastersii* Griff. Ex Lindl, Mycorrhiza fungus, symbiosis system, 3D hypha net, seed activity.

INTRODUCTION

Cymbidium mastersii Griff. ex Lindl. produced in Yunnan and the Himalayan region. The flowers with almond fragrance bloom from September to December in each year. It is a traditional cultivated species in China with unique charm. Because of its high historical culture and ornamental value, it is still a popular collection and ornamental variety. However at present, plant division are still the only way of reproduction which is long growing period, low reproduction coefficient. The wild resources of the *C. mastersii* are on the verge of extinction because of the manual excavation and destruction. Therefore, it is urgent to study the effective germination and growth mechanism of *C. mastersii*. The orchids is a unique mycorrhizal plant. At present, almost all orchid plants need to form

mycorrhizal and symbiotic relationship with the partner corresponding fungi during their growth and development (Huijin *et al*., 2007). The seeds of Orchidaceae are tiny, the average thousand seeds are only 1-10 mg, and the seeds have no endosperm and only have the original undeveloped. The nutrient stored by the cells for seed germination is very little, so it is very difficult to germinate (Lu *et al*., 2005) under natural conditions. The results showed that only when infected by suitable fungi and symbiotic, could the seeds germinate (Wu *et al*., 2010; Arditti, 1995) under wild conditions. With the periodic dissolution of mycelium (Wu *et al*., 2005), the new mycelium infects and colonizes again, which maintains a good symbiotic relationship and provides essential nutrients for the seed germination of orchid without endosperm.

MATERIALS AND METHODS

Plant material

Seedlings of *C. mastersii* were collected from their native locality near Himalayan region of Baoshan district of Yunnan, China. Capsules were obtained through hand pollination in mother plants maintained in the field of Baoshan and collected after 12 months of pollination. At this time, the capsules were fully mature carrying green brown seeds.

Isolation of the fungus

Mycorrhizal fungi were isolated from the collected roots according to the method described in Warcup and Talbot (1967), with slight modifications. Briefly, the orchid root surfaces were first washed with tap water to get rid of soil particles and organic debris. The roots were then sterilized by immersing them in 70% ethanol for 1 min, followed by submerging them in a sodium hypochlorite solution containing 1% chlorine for 1 min, and finally by immersing them in mercuric chloride (0.1%) for 5-6 min. A piece of the sterilized root from each plant, approximately 5-6 mm in length, was put into 1 mL of sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse intracellular fungal hyphal coils (pelotons). About 20 mL of sterilized potato dextrose agar (PDA, pH 5.6) medium was cooled to 40-45 C, poured into the Petri dish, and mixed with the crushed roots through gentle shaking. The mixture was incubated at 25° C in the dark for 3-4 d. To prevent bacterial growth, the antibiotic chloramphenicol (final concentration 50 mg/mL) was added to the culture medium. Fungal colonies of consistent appearance that grew from a peloton were transferred onto fresh PDA medium using a sterilized scalpel and cultured individually. To further purify the cultures, small pieces of agar containing hyphal tips from each isolate were subcultured two more times. These cultures were then observed under a light microscope (400×) (Figure 1a, b) and putatively identified on criteria established for other orchid mycorrhizal fungi using hyphal morphology in culture (Sneh *et al*., 1991; Currah *et al*., 1997).

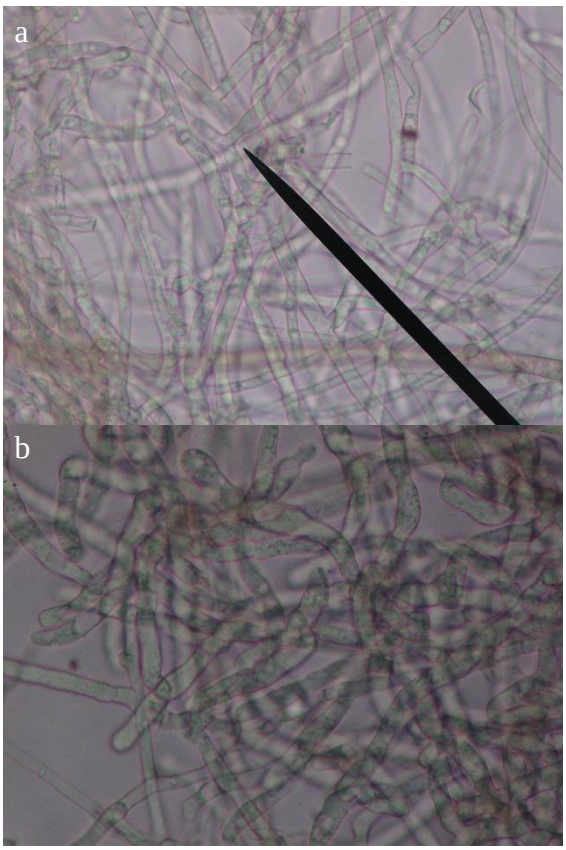

Figure1. a, **b**: The morphological feature of MLX102 and CLB111.

Characterization and identification of fungus

The fungal isolates were first characterized morphologically using the methods outlined by Currah *et al*. (1997). The fungal isolates were cultured on 1/5th PDA at 25 ± 3° C. For fast-growing fungal isolates, diameters of three colonies were measured

every 2 days until it reached 9 cm. For slow growing fungal isolates, diameters of three colonies were measured every 3 days for at least 2 weeks. Cultural characteristics (e.g., colony color, colony zonation, and types of hyphae) were observed over one month period and monilioid cells developed during the period were observed under a phase contrast microscope and photographs were taken.

Their molecular identifications were based on their DNA sequences at the internal transcribed spacer (ITS) regions of the nuclear ribosomal rRNA gene cluster. Briefly, the strains were first cultured on YEPD agar medium (per litre of medium: 10 g yeast extract; 20 g peptone; 20 g dextrose; 20 g agar; in 1 L of water) for 7 d. Mycelia from the top of the agar were harvested and ground into a fine powder in liquid nitrogen using a micropipette tip in a 1.5 mL microcentrifuge tube. Subsequent steps of the DNA extraction followed those described by Xu *et al*. (2000). The ITS regions were amplified from the extracted DNA by the polymerase chain reaction (PCR) with primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3', Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', White *et al*., 1990). A typical PCR reaction contained 5 µL of template DNA solution (*20 ng), 0.75 U of the Taq DNA polymerase, 0.25 mmol/L of each primer, 200 mmol/L of each deoxyribonucleotide triphosphate, and 3 µL of PCR buffer in a total volume of 30 µL. The reaction was performed using the following conditions: an initial denaturation step at 94 °C for 5 min, a subsequent step of 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, and a final elongation step at 72° C for 5 min. PCR products were purified using the MicroCLEAN kit 2MCL-10 following the manufacturer's instructions (DiaMed, Mississauga, Ontario, Canada). The purified DNA fragments were sequenced with an ABI 3700 DNA analyzer and an ABI BigDye3.1 terminator cycle sequencing kit (Huada gene, Shenzhen). The same ITS1F and ITS4 primers described above for PCR were used for the sequencing reactions. The sequences from the four strains were compared with those in the GenBank through BLAST searches. Our BLAST searches identified that their close matches were all from the broad *Rhizoctonia* fungi.

Symbiotic seed germination

The effects of fungal isolates on promoting *C. mastersii* symbiotic seed germination *in vitro* were evaluated using a modified method of Stewart and Kane (2006). Indehiscent mature capsules of *C. mastersii* (12 months after pollination) produced through hand pollination in mother plants maintained in the field of Baoshan were collected, brought to the laboratory and processed without any storage. The capsules were washed thoroughly in running tap water using a commercial detergent and surface sterilized thrice by dipping in spirit followed by flaming. The capsules were split open and the seeds transferred to 50-100 mL sterile distilled water to obtain 50-100 seeds per drop of seed suspension (OMA: Hollick, 2004) (pH 5.8). The seeds were sown onto the surface of Nylon netsterile of sterile OMA medium. The plates were inoculated the fungal inoculums (MLX102 and CLB111) taken from the actively growing hyphae edge 10 days after culturing on PDA. Uninoculated plates were used as a control. Ten replicates were maintained for each treatment and the whole experiment was repeated thrice. Petri dish plates were sealed with cling film and stored at the room conditions with day light (8:30 am to 5:30 pm) at 25 °C for 25 weeks. The cultures were examined after 2 weeks initially and further at monthly intervals under stereo microscope to assess germination and progress of protocorm and seedling development. Seed germination and seedling development were scored on 0–7 increment scale (modified from Stewart and Kane, 2007). Percentage of seed germination and protocorm development for each treatment was calculated using the relation: Number of seeds in each developmental stage /Total number of seeds with fully developed embryos*100.

Seed vigor determination and observation on mycelium infection

The optical microscope and electron microscope observation found that many fungus hypha infected into embryo cell and formed the 3D hypha net, and the embryo started differentiation. TTC measurement showed that the seed had high vitality.

No strain was found by re-separation from culture medium, no hypha was observed and very low vitality under control treatment (Figure 2a, b).

RESULTS

Fungal isolation and identification

Natural populations of *C. mastersii* possessed significant numbers of seedlings with endomycorrhizal colonization in their roots. Two *Rhizoctonia* sp. endophytic fungal isolates were recovered from their roots and one of them triggered symbiotic germination and supported enhanced growth of *C. mastersii* seedlings, as revealed in a preliminary examination. Molecular characterization of the isolate was obtained by PCR amplification of the ITS region using the primer pair ITS4 and ITS5 and sequencing of the 550 bp amplified product. This was followed by identification through comparing the sequence obtained with already available sequence in NCBI Genbank database using BLAST search tool (Wu *et al*., 2010).

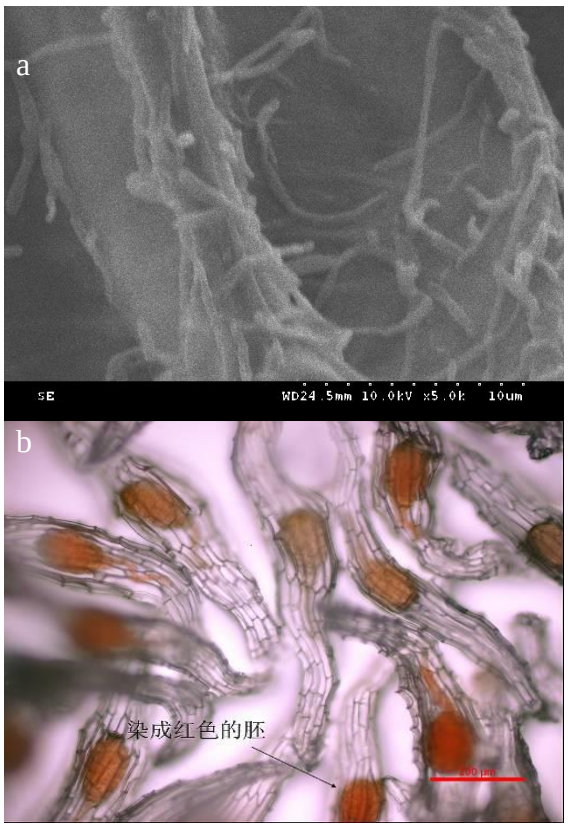

Figure 2. a, **b**: Hypha of CLB111 infected into the seeds and formed the 3D hypha ent (×5000), the seed became red with TTC stained seeds of *C. mastersii.*

Symbiotic seed germination

Seeds of *C. mastersii* germinated on OMA medium were previously inoculated with the symbiotic fungus CLB111. Early symptom of germination as swelling of embryos and rupture of seed coat occurred in 7 days and later they progressed to stage 2 in another 7 days. By 30 days, most of the germinated seeds were transformed into stage 3 with rhizoid formation and development of chlorophyll. Upper portion of the protocorm became bulged to form globular structure and leaf primordia were also developed during this stage. In another 30 days, 48% of the protocorms were progressed into stage 4 with the development of first leaf. However, majority of them died or did not grow further without symbiotic fungus CLB111 and MLX102. As the data was not promising for symbiotic seedling production, detailed data was not gathered (Figure 3a-h).

DISCUSSION

Propagation through symbiotic approaches is the most preferred option of threatened and endangered orchid species for their restoration into native habitats. Such methodology has already been applied in a series of terrestrial orchids and thus become a popular method for resto ration programs (Aggarwal and Zettler, 2010; Chutima *et al*., 2011; Sathiyadash *et al*., 2014). Even though extensive studies are not available, symbiotic propagation has also been proved or recommended as an effective method in a few epiphytic orchids (Khamchatra *et al*., 2016). Nevertheless, there is no study reporting symbiotic seed germination of *C. mastersii*, an endemic orchid of Himalayan region, China endangered due to habitat destruction. Thereby, this is the first report of *in vitro* symbiotic germination in this endangered terrestrial orchids.

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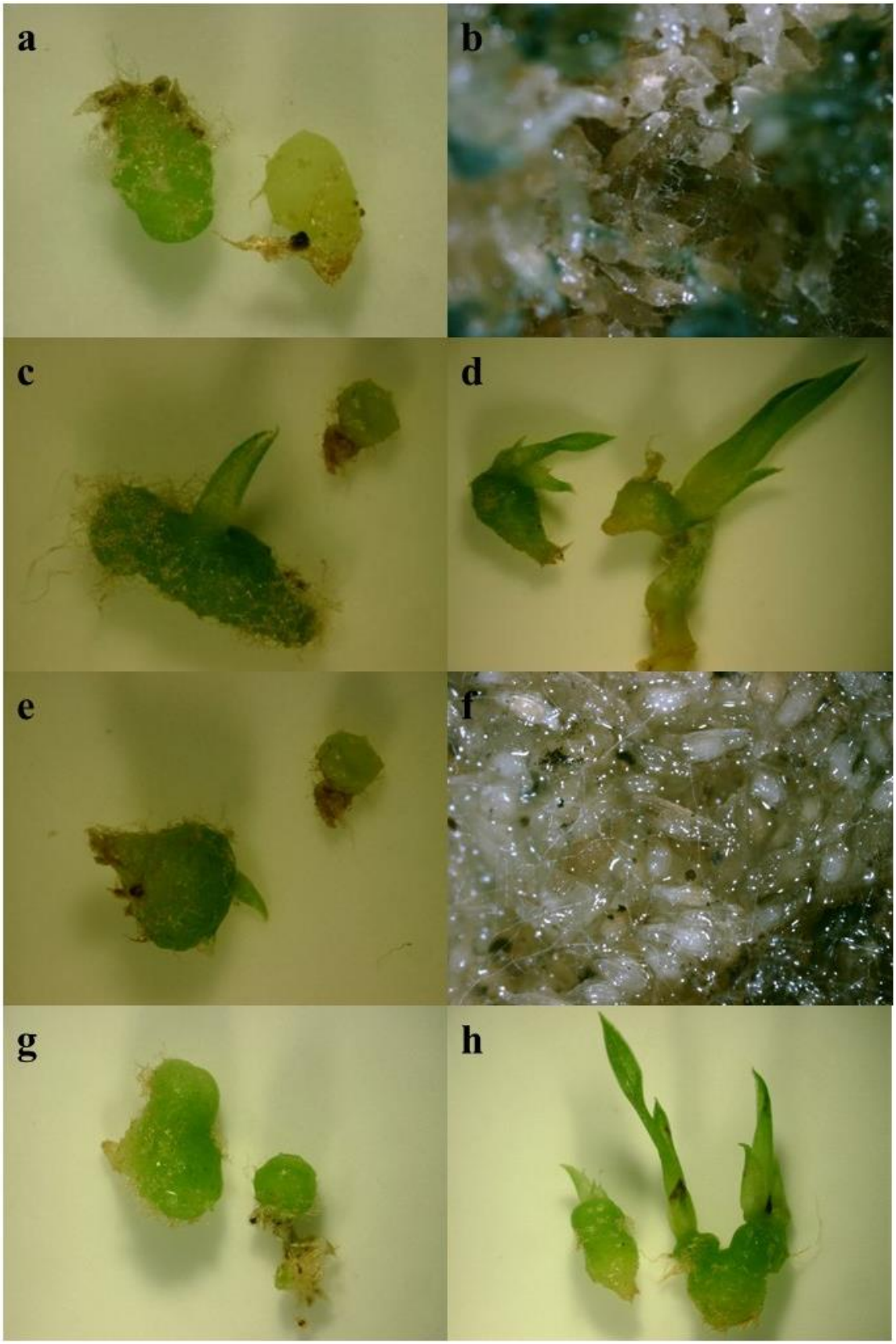

Figure 3. Germination of seeds of *C. mastersii*. **a**, **b**: Germination stages 1 of symbiotic seed with CLB111 and CK; **c**, **d** : germination stages 2 (left) and stages 3 (right) of symbiotic seed with CLB111 and CK; **e**, **f** : germination stages 1 of symbiotic seed with MLX102 and CK; **g**, **h**: germination stages 2 (left) and stages 3 (right) of symbiotic seed with MLX102 and CK.

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