Research paper

Resynthesis of Ericoid Mycorrhizae in Formosan Rhododendron (*Rhododendron formosanum* Hemsl.) with an Endophytic *Cryptosporiopsis* Species

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【Summary】

Formosan rhododendron (*Rhododendron formosanum* Hemsl.) is an indigenous species of the Ericales in Taiwan. The Rf32 fungal strain was isolated from the roots of wild Formosan rhododendron and identified as an endophytic species of *Cryptosporiopsis* based on its morphological characteristics. This identification was further confirmed by an internal transcribed spacer sequence analysis. This species is a new addition to the fungal flora of Taiwan. In an ericoid-mycorrhizal resynthesis experiment, Rf32-inoculated Formosan rhododendron seedlings exhibited vigorous growth, and the root association showed hyphal complexes in cortical cells. The results demonstrated that Formosan rhododendron can form ericoid mycorrhizae *in vitro* with *Cryptosporiopsis* sp. Rf32.

Key words: *Cryptosporiopsis*, ericoid mycorrhiza, ericoid mycorrhizal fungus, *Rhododendron formosanum*.

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研究報告

台灣杜鵑與內生菌*Cryptosporiopsis* species合成杜鵑類菌根

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摘 要

台灣杜鵑為台灣原生種杜鵑科植物。本研究利用自野生台灣杜鵑根系所分離純化之內生菌Rf32菌株，分析internal transcribed spacer序列，並與基因庫已知的杜鵑類內生菌資料比對，鑑定為*Cryptosporiopsis*菌株，此種真菌為台灣不完全菌類新紀錄種。以Rf32菌株接種台灣杜鵑無菌苗再合成菌根試驗發現，菌根合成苗生長旺盛，觀察顯示其根部皮層細胞中具有菌絲複合體，本研究顯示內生菌*Cryptosporiopsis* sp. Rf32能與台灣杜鵑形成典型的杜鵑類菌根。

關鍵詞：*Cryptosporiopsis*、杜鵑類菌根、杜鵑類菌根菌、台灣杜鵑。

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INTRODUCTION

Ericoid mycorrhizae (ERMs) are one kind of endomycorrhizae that have intracellular, septate hyphae and hyphal coils, but lack arbuscular hyphae, a mantle, a Hartig net, and vesicles (Harley 1969). Two types of ericoid mycorrhizal fungi (ERMFs), ascomycetes and hyphomycetes, have been identified. The well-known species *Pezoloma ericae* (Read) Baral belongs to the ascomycetes type (Baral and Krieglsteiner 2006); whereas *Oidiodendron maius* Barron (Couture et al. 1983, Dalpé 1986, Douglas et al. 1989), *Phialocephala fortinni* Wang and Wilcox (Stoyke and Currah 1991, Hambleton and Currah 1997), *Cryptosporiopsis ericae* Sigler, and *C. brunnea* Sigler (Sigler et al. 2005) are hyphomycetes.

Vohnik et al. (2005) indicated that ERMFs positively influence growth, survival, and competitiveness of host species by enhancing nutrient uptake (Read 1996, Read and Perez-Moreno 2003, Lin et al. 2010c, a), alleviating heavy-metal toxicity (Perotto et al. 2002), and secreting enzymes for saprobic ability (Rice and Currah 2001, Piercey et al. 2002, Lin et al. 2011). Our previous study demonstrated that the 3 endophytes, i.e., Rf9, Rf28, and Rf32, isolated from wild Formosan rhododendron (*Rhododendron formosanum* Hemsl.) (Lin et al. 2010b) were ERMFs, which have the ability to decompose organic matter (Lin et al. 2011). Among these 3 endophytes, the morphology and physiology of the Rf9 and Rf28 strains were studied and published (Lin et al. 2010c, a). The main objectives of this study were to determine the taxonomic status of the Rf32 strain by morphological, cultural characteristics, and internal transcribed spacer (ITS) of ribosomal (r)DNA sequence analyses. In addition, the nature of the association of the Rf32 strain with roots of Formosan rhododendron were examined by an axenic mycorrhizal resynthesis technique.

MATERIALS AND METHODS

Fungal strain

The Rf32 strain used in this study was previously isolated from roots of Formosan rhododendron, located in the Sanlinchi Recreational Park (120°47'31.35"E, 23°38'9.85"N), Chushang Township, Nantou County, Taiwan (Lin et al. 2010b). The Rf32 culture was deposited at the Forest Mycobiont Laboratory of National Chiayi Univ. and the Bioresource Collection and Research Center (BCRC) (BCRC 34763). The ITS genomic sequence of Rf32 was also deposited in GenBank (with accession no.: HQ260955).

Morphology and growth of the colony

The Rf32 colony was transferred to 2% malt extract agar (MEA: 20 g L^{-1} malt extract, 15g L^{-1} agar, BD Bioscience PharMingen, San Jose, CA), potato dextrose agar (PDA: 39 g L^{-1}, BD), and oatmeal agar (OAT: 30 g L^{-1}, Himedia, Mumbai, India) plates and incubated in growth chambers at both 15 and 22℃. After 12 d, the growth rates of the colony were measured, while the cultural characteristics of the colony were observed every day (Stoyke and Currah 1991, Hambleton and Currah 1997, Lin et al. 2010a).

Morphology of hyphae

Cornmeal agar (CMA: 50 g L^{-1} corn meal and 15 g L^{-1} agar, Sigma, St. Louis, MO, USA) medium was used to culture the endophyte. After 20 d, plugs (5 mm in diameter) from different media were transferred to slides for further culture under a near-ultraviolet (nUV) growth light to induce sporulation of the endophyte. After 80 d of culture on slides, 1% aniline blue was applied to stain the hyphae, and the morphology of Rf32 was observed with a light microscope (Olympus BX51, Center Valley, PA, USA) (Hutton et al. 1994, Lin et al. 2010a).

DNA extraction and sequencing, and phylogenetic analysis

Methods described by Sigler et al. (2005) were followed. Mycelium for DNA extraction was scraped from the surface of PDA cultures. Genomic DNA was extracted using lysis buffer plus Puregene Proteinase K (Qiagen, St. Louis, MO, USA). Total fungal DNA was used as a template for amplification with primers ITS1-F and TW13 (Sigler et al. 2005). Polymerase chain reaction (PCR) products were sequenced by Genomics BioSci and Tech Company (Taipei, Taiwan). Sequences were assembled, and related sequences were searched using BLAST searches (Lin et al. 2010a).

Phylogenetic relationships were analyzed by MEGA (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2007). Bootstrapping was performed using a Neighbor-joining (NJ) method (Saitou and Nei 1987).

Resynthesis

To resynthesize ericoid mycorrhizae with *Cryptosporiopsis* sp. Rf32, the method of Dalpé (1986) was followed. After surface cleaning, seeds of Formosan rhododendron were sterilized with a 10% sodium hypochlorite solution for 15 min and rinsed 3 times with sterilized distilled water (dH_2O), then transferred to test tubes containing 1% agar (pH 6.2) for germination. Germinated seedlings were transplanted to modified Mitchell and Read (MMR) medium (32 mg L^{-1} NH_4Cl, 43.5 mg L^{-1} $CaCl_2 \cdot 7H_2O$, 10 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 5.5 mg L^{-1} KCl, 3.75 mg L^{-1} $FeCl_3$, 2 g L^{-1} sucrose, 210 mg L^{-1} KH_2PO_4, 100 μg L^{-1} pyridoxine, 100 μg L^{-1} thiamine, and 10 g L^{-1} agar). Seven days later, the aseptic seedlings were inoculated with the Rf32 fungal strain and grown in a growth chamber

at 22℃ and 16 h of light at 5000 lux (Lin et al. 2010a).

Morphology

After 2 mo of culture, roots of the inoculated seedling were sampled and cleaned with dH_2O in a supersonic oscillator (Upson et al. 2007). The morphology of the mycorrhizae was observed with a stereomicroscope (Usuki and Narisawa 2005).

For observation of mycorrhizal colonization, root samples were cleared in 10% KOH for 24 h, washed in dH_2O, acidified in 10% HCl for 1 h, and transferred to 0.05% aniline blue (0.25 g aniline blue, 25 mL dH_2O, and 475 mL lactic acid) for 1 h and then destained with destaining solution (25 mL dH_2O and 475 mL lactic acid) for 2 h. Roots were then mounted on microscope slides in lactoglycerol (14: 1: 1, lactic acid: glycerol: dH_2O) for light-microscopic examination (Upson et al. 2007).

For the ultrastructural examination, root samples were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde fixative in a phosphate-buffered solution (PBS, 0.1 M, pH 7.0) for 4 h at room temperature, then rinsed with the PBS 3 times each for 15 min, followed by serial dehydration in 30, 50, 70, 85, 95, and 100% ethanol and 100% acetone, and finally dried in a critical-point dryer using liquid carbon dioxide (Hitachi HCP-2 Critical Point Dryer, Tokyo, Japan). Dried materials were mounted on an aluminum stub with twin adhesives, coated, and observed with a Hitachi S-2400 scanning electron microscope (SEM) (Nakamura et al. 2002, Lin et al. 2010a).

Statistical analyses

Differences in growth rates among treatments were analyzed using a one-way analysis of variance (ANOVA). Tukey's multiple-range test was applied for further analysis.

RESULTS AND DISCUSSION

Morphology of the strain

For the morphological study, the Rf32 endophyte was grown on MEA, OAT, and PDA plates at both 15 and 22℃. After 27 d, colonies of Rf32 cultured on MEA at 15℃ showed a light-yellow color (Fig. 1A). At 22℃, colonies had a light-brown color, the surface was covered by grayish-white hyphae in the early stage, and there was a pluriguttulate secretion after 27 d (Fig. 1B). The average growth rate of the colony at 22℃ (2.1±0.1 mm d^{-1}) was significantly higher than that at 15℃ (1.9±0.1 mm d^{-1}) (Table 1).

The center of colonies of Rf32, grown on OAT at 15℃, was grayish-white and light-brown in the early stage. After 27 d, they exhibited brown and white hyphae that covered the surface (Fig. 1C). At 22℃, colonies were brown in the early stage, and exhibited gray hyphae that covered the surface after 27 d (Fig. 1D). The average growth rate of the colony in 12 d at 22℃ (2.2±0.2 mm d^{-1}) was significantly higher than that at 15℃ (1.4±0.1 mm d^{-1}) (Table 1).

Table 1. Growth rates of the Rf32 strain on different media after 12 d of culture at 15 and 22℃

Medium	Temperature (℃)	Mean±SD (mm d^{-1})
OAT	15	1.40 ± 0.10^{d}
	22	2.20 ± 0.20^{b}
MEA	15	1.90 ± 0.10^{c}
	22	2.10 ± 0.10^{b}
PDA	15	2.80 ± 0.20^{a}
	22	2.90 ± 0.20^{a}

All values are reported as the mean±standard deviation of 4 replicates of culture.
Values with different superscript letters significantly differ at $p < 0.05$.
OAT, oatmeal agar; MEA, malt extract agar; PDA, potato dextrose agar.

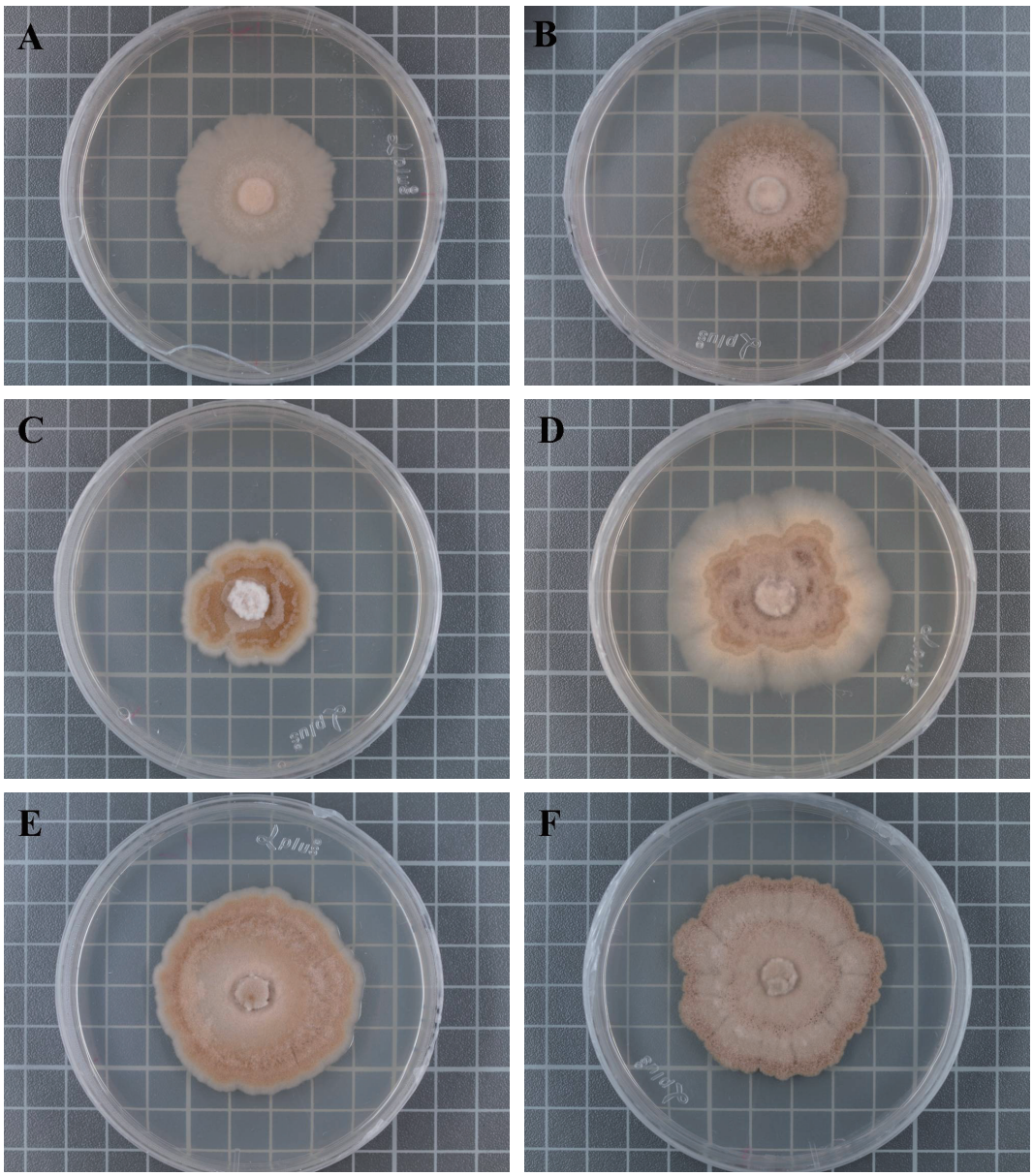

Fig. 1. Colonies of the Rf32 strain grown on malt extract agar medium (A and B), oatmeal agar medium (C and D), and potato dextrose agar medium (E and F) after 27 d of incubation at 15℃ (left column) and 22℃ (right column), respectively.

Colonies of Rf32, grown on PDA at 15℃, were white to light-brown in the early stage, and exhibited light-brown hyphae that covered the surface after 27 d (Fig. 1E). At 22℃, the colonies were brown, grayish-white hyphae covered the surface in the early stage, and the same color remained after 27 d (Fig. 1F). The average growth rates of the colony in 12 d at 22℃ (2.9 ± 0.2 mm d^{-1}) and 15℃ (2.8 ± 0.2 mm d^{-1}) did not significantly

differ from each other (Table 1). These results showed that the average growth rates of Rf32 on PDA medium in 12 d at 22 and 15℃ were significantly higher than those on the other media (Table 1).

Morphology of hyphae

After treating the slide cultures of CMA with nUV for 80 d, only monilioid, intercalary, catenulate chlamydospores (4.8~8.1 x 4.8~5.1 μm) had formed on the hyphal spread on the surface of the substrate (Fig. 2).

Molecular analyses

Although numerous sterile fungi were isolated from roots of the Ericaceae and Epacridaceae, they have not been identified to species level due to their inability to form teleomorphs and conidia (Burgeff 1961, Peretto et al. 1990, Stoyke and Currah 1991, Hutton et al. 1994, Xiao and Berch 1996, Hambleton and Currah 1997).

In recent years, the ITS of rDNA has successfully been used to clarify phylogenetic relationships and study the genetic diversity of ERMFs (Liu et al. 1998, Chambers et al. 1999, McLean et al. 1999, Monreal et al. 1999, Sharples et al. 2000, Usuki et al. 2003). Clearly, ITS analyses have contributed to research on ERMFs.

Taxonomic affinities were assigned to Rf32 based on a BLAST sequence similarity analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) including several very closely matching sequences. ITS sequences of Rf32 were matched to those of ericoid mycorrhizal sp. PPO-8 (AY599246) and *Cryptosporiopsis ericae* (AY540126), and these species were grouped (with 93% bootstrap support) in the NJ analysis (Fig. 3). Since the colony and hyphae of the Rf32 strain showed different features which contrasted to those of *C. ericae* (Sigler et al. 2005), the Rf32 strain appeared to be a new species belonging to the genus *Cryptosporiopsis*.

Pure resynthesis

After 2 mo of cultivation, seedlings of Formosan rhododendron inoculated with the Rf32 strain exhibited vigorous growth (Fig. 4A). The root associations exhibited distinct features under a stereomicroscope (Fig. 4B).

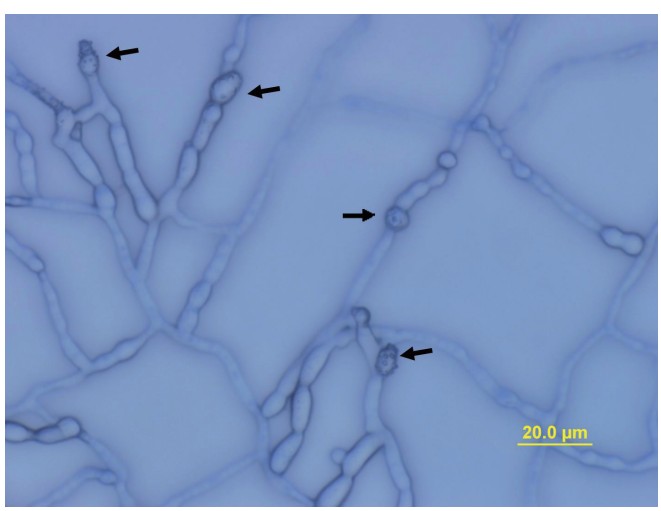

Fig. 2. Morphology of Rf32 hyphae on a cornmeal agar slide culture showing monilioid chlamydospores (arrows).

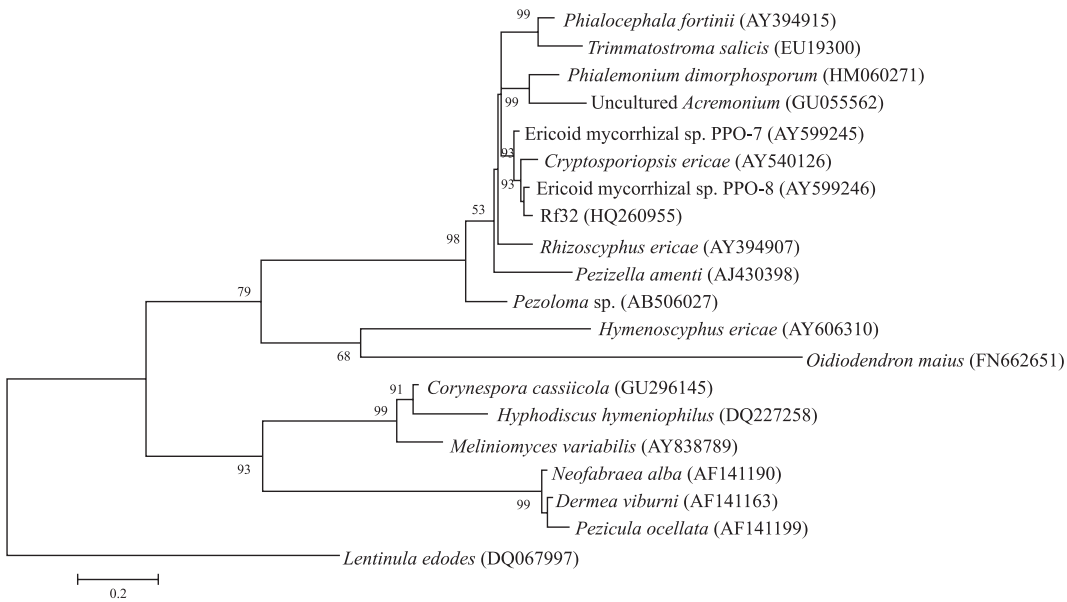

Fig. 3. Neighbor-joining phylogenetic tree based on ribosomal (r)DNA internal transcribed spacer (ITS) sequence data from endophytes of Rf 32 isolated from the root systems of *Rhododendron formosanum* with known ericoid endophytes and selected fungal species from GenBank with high sequence similarities. Numerical values above the branches indicate bootstrap percentiles from 1000 replicates. Only bootstrap values of ＞50% are indicated. Horizontal branch lengths are proportional to the scale of basepair substitutions.

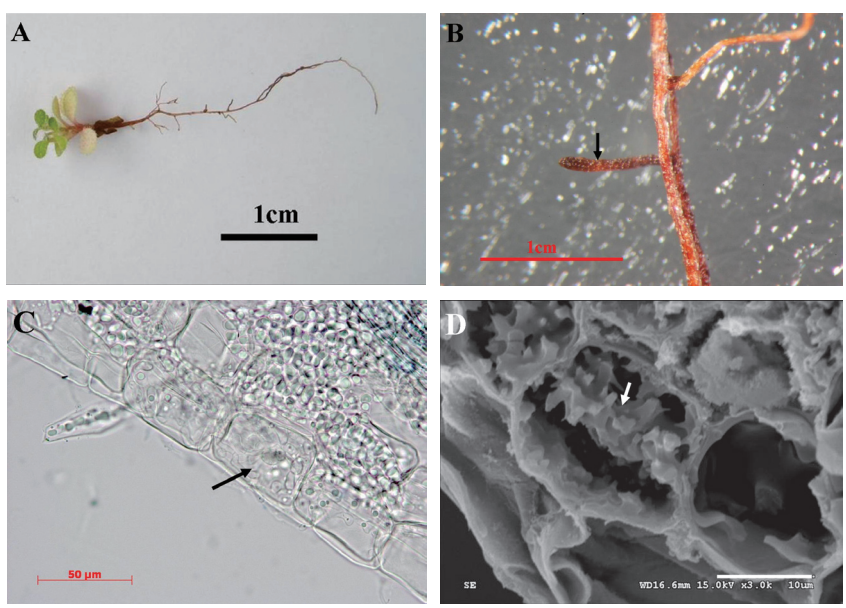

Fig. 4. Root association of a *Rhododendron formosanum* seedling inoculated with the Rf32 strain. A, Seedling; B, root-fungus association (arrow); C, hyphal complex (arrow); D, ultrastructure of the hyphal complex in cortical cells (arrow).

Meanwhile, features of hyphal complexes of ERMFs were observed in cortical cells of root associations in resynthesized seedlings under light microscopy (Fig. 4C) and SEM (Fig. 4D). These results demonstrated that Formosan rhododendron can form ericoid mycorrhiza in vitro with *Cryptosporiopsis* sp. Rf32.

CONCLUSIONS

In this study, the molecular analysis classified the endophyte Rf32 strain into the genus *Cryptosporiopsis* (Fig. 3). In the mycorrhizal resynthesis study (Fig. 4), hyphal complexes of ERMFs were discovered in cortical cells of root associations of seedlings inoculated with Rf32. These results support the notion that Formosan rhododendron can symbiose with endophytic fungal *Cryptosporiopsis* spp. to form ericoid mycorrhizae. Furthermore, the Rf32 strain is an ERMF. Endophyte Rf32 performs most significantly in decomposing organic matter in its natural habitat, regardless of self decomposition or symbiosis with Formosan rhododendron (Lin et al. 2011). Hence, the Rf32 strain plays a key role in the adaptation of Formosan rhododendron to harsh environments.

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