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ORIGINAL RESEARCH ARTICLE

Investigating the Role of Endophytic Fungi in *Gentiana scabra* bge. by Cross-Growth Period Inoculation

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Abstract *Gentiana scabra* Bge. (gentian) is a Chinese medicinal plant. Endophytic fungi from the roots of gentian were isolated and cross-growth period inoculation was performed to study the roles of three *Trichoderma* spp. strains (F1, F2, and F9) in their original host plant. In treatments inoculated with F1, F2, and F9, gentiopicroside content increased 33.6, 23.7 and 13% than that in the control. Strains F1, F2, and F9 could also improve polysaccharide content by more than 6.6, 18.7 and 30% compared to the control. The incidence of spot blight in gentian inoculated with F1, F2, and F9 decreased by 31.2, 26.7 and 8.5%. Inconsistent changes in the activity of the three enzymes (superoxide dismutase, catalase and peroxidase) were observed when the plants were attacked by pathogens or inoculated with fungi. High enzymatic activity did not reflect mild disease. Cross-growth period inoculation, which takes into account the original living environment (gentian plant as "substrate" and different microorganisms as symbionts) of endophytic fungi, provides a new idea for studying effects of endophytes on their original hosts. This is the first research about the role of endophytic fungi in *Gentiana scabra* bge. in vivo.

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Introduction

Numerous microorganisms promote growth by producing hormones and improving mineral nutrient uptake in plants by promoting fixation of nitrogen, solubilization of phosphorus, and mineralization of organic matter [1–3]. Microorganisms can also induce the production or accumulation of plant metabolites that can improve tolerance of plants to stresses [4–6]. Some studies have shown that microorganisms can mediate the interaction between host plants and pathogens by producing antimicrobial compounds and antioxidants or inducing systemic resistance [7–9].

Endophytes are indispensable mutualistic partners of higher plants that improve plant growth and disease resistance. Reports have shown that endophytic microorganisms in herbal medicine plants are capable of producing the natural products as their host plants [10, 11] and affecting the composition or levels of bio-active substances [12, 13], which have been major topics of interest in biomedical research.

Gentiana scabra Bge. (gentian) is a medicinal plant in China. Gentiopicroside, the secondary metabolite of gentian, possesses biological activities such as significant liver and gallbladder protection, anti-inflammatory, anti-pathogenic and analgesic properties [14, 15]. Gentian polysaccharides are known to impart hepatoprotective effects, serve as an anti-coagulants, reduce blood lipids, and improve immunity [16, 17]. However, gentian spot blight severely threatens gentian growth and results in major yield loss.

Du and Zhou [18] isolated 43 strains of endophytic fungi from gentian roots, of which four strains showed anti-fungal activity and six strains produced metabolites with antagonistic activities against the pathogen of gentian leaf blight. Another research group [19] studied the anti-fungal activity of endophytic fungi isolated from Gentiana in Yunnan by filter paper method. To date, attempts in elucidating the role of endophytic fungi from *G. scabra* Bunge on their original host remain limited.

The environment may alter plant–endophyte interactions [20, 21] and the effects of endophytes on their host. Soil and climate are important environmental factors in the field that influence growth of plants and the role of endophytic fungi. However, investigations on the effects of endophytes on their original host under complicated natural environmental conditions are limited [8, 22]. Furthermore, in most relevant research, the application of sterilized seeds, seedlings [2, 23], or adult plants and tissue culture seedlings [13, 24] in studying the roles of endophytic fungi did not consider either the complex environment in the field or the growth matrix (original host plant, coexisting microorganisms) for endophytic fungi. Therefore, the effects of fungal endophytes on plants varied among trials in the laboratory, greenhouse, and field [5, 25, 26].

The objectives of the present study were as follows: (1) to isolate and screen endophytic fungi from the roots of gentian for testing, (2) to design cross-growth period inoculation and assess the effect of endophytic fungi on their original host in the field, and (3) to analyze activities of defensive enzymes (catalase, peroxidase, and superoxide dismutase) to elucidate the mechanisms underlying disease resistance of endophytic fungi.

Materials and Methods

Isolation and Identification of Endophytic Fungi

On August 1 and September 1 of 2013, 80 plants of biennial and triennial of gentian (two locations with different year-old gentian × 20 individuals × 2 growth periods) were collected from the Good Agricultural Practices (GAP) base of gentian in Qingyuan, Liaoning province, China. Surface-sterilized root samples were cut into 1-cm thick pieces and placed in Petri dishes containing potato dextrose agar [PDA, containing benzylpenicill in sodium (50 mg L^{-1})]. The Petri dishes were incubated at 28 °C in the dark. When colonies developed, these were transferred to fresh PDA plates for purification. Strains thriving in either biennial or triennial plants were screened and identified further based on the internal transcribed spacer (ITS) region sequences of rDNA [27]. For long-term storage, all strains were maintained in cryovials layered with 15% (v/v) glycerol at − 70 °C.

Cross-Growth Period Inoculation of Endophytic Fungi on Plants

Gentian plants at the GAP base and three strains of endophytic fungi belonging to *Trichoderma* spp. (F1, F2 and F9) were selected for plant inoculation testing at the beginning of August in 2014.

The experiment consisted of five treatments replicated three times with 10 plants per replication. Culturing the endophytes in potato dextrose broth for 4 days and filtering out broth, we weighed 10 g of mycelia to daub roots of one plant, followed immediately by soil coverage and drip irrigation to keep the soil moist. F1 and F2, which were only detected in the biennial gentian plants, were inoculated separately onto the triennial gentian plants, and F9, which were only observed in triennial gentian plants, was inoculated onto the biennial plants. We named this type of inoculation as cross-growth period inoculation. Triennial and biennial gentian plants without inoculation were used as controls. The same strains that were obtained again from the inoculated plants indicated successful inoculation.

One month after inoculation, the incidence of gentian spot blight was assessed, followed by the collection of all test plants into plastic bags and transport to the laboratory within 24 h. The leaf samples were processed once back at lab. The root samples were washed, weighed, air-dried, milled, and sifted for chemical analysis.

Determination of Gentiopicroside and Gentian Polysaccharide Content

Gentiopicroside content was determined via reversed phase high-performance liquid chromatography (RP-HPLC) with external standard calibration curve method according to the pharmacopoeia of the People's Republic of China [28]. Gentian polysaccharides were analyzed by the anthrone-sulfuric acid method, using glucose as standard [29].

Investigation of Incidence of Gentian Spot Blight

To assess the effects of endophytic fungi on gentian spot blight, percent incidence before harvest was calculated using the following formula:

$$\text{Percent incidence}\,(\%) = \frac{\text{Number of plants exhibiting blight spots}}{\text{Total number of plants}} \times 100$$

Assay of Defense-Related Enzymes

One gram of fresh leaf sample was homogenized with 5 mL of 50 mM phosphate buffer containing 0.2 mol/L EDTA and 2% (w/v) polyvinylpyrrolidone at 4 °C. The homogenate was centrifuged at 10,000 rpm for 20 min, and the supernatant was used as crude enzyme extract [30].

Superoxide dismutase (SOD) activity was measured according to Bai et al. [31]. To suppress nitroblue tetrazolium (NBT) photo-reduction by 50% of control for one unit of enzyme activity (U), the SOD activity unit was expressed as U g^{-1} fw.

Peroxidase (POD) activity was measured according to Sofo et al. [32] with some modifications. The reaction solution (5 mL) in a tube contained phosphate buffer (2.9 mL, 50 mM; pH 5.5), guaiacol (1 mL, 50 mM), H_2O_2 (1 mL, 2% w/v), and crude enzyme extract (0.1 mL). The tube was put in a water bath at 34 °C for 3 min and immediately followed by double dilution. To 0.01 changes in A_{470} per minute for one unit of enzyme activity (U), the POD activity unit was expressed as U min^{-1} g^{-1} fw.

Catalase (CAT) activity was assayed by monitoring decomposition of H_2O_2 with a spectrophotometer at a wavelength of 240 nm, and enzyme activity was expressed as μmol (H_2O_2) min^{-1} g^{-1} fw.

Statistical Analysis

The experimental data were analyzed using SPSS 19.0 and Excel 2007. Differences among treatments were tested using a *t* test for independent samples. The significance of differences was set at a P value < 0.05.

Results and Discussion

Identification of Predominant Strains of Endophytic Fungi and Selection of Fungi for Subsequent Testing

Except for strains isolated from both biennial and triennial gentian plants, there were nine strains (designated F1–F9) that inhabited either the biennial or triennial gentian. Strains F1–F5 remained alive only in biennial gentian plants, whereas F6–F9 only survived in triennial plants. Their morphologies are shown in Fig. 1 and the identification results are listed in Table 1. *Trichoderma* spp. has generally been exploited for the biocontrol of plant diseases [33] and plant growth promotion [34]. Hence, three strains (F1, F2, and F9) belonged to *Trichoderma* spp. were selected for cross-growth period inoculation.

Endophytes that only harbor in biennial gentian plants were inoculated onto triennial plants at a suitable stage (without the inoculated endophytes in vivo) and endophytes in triennial plants were inoculated on biennial following the same rules. This type of inoculation considers both the matrix (that is the host plant) for endophytes and other coexisting endogenous microorganisms, furthermore, it avoids the effects of the same fungi (because the same fungi as tests didn't exist in the host at the stage of study proceeding).

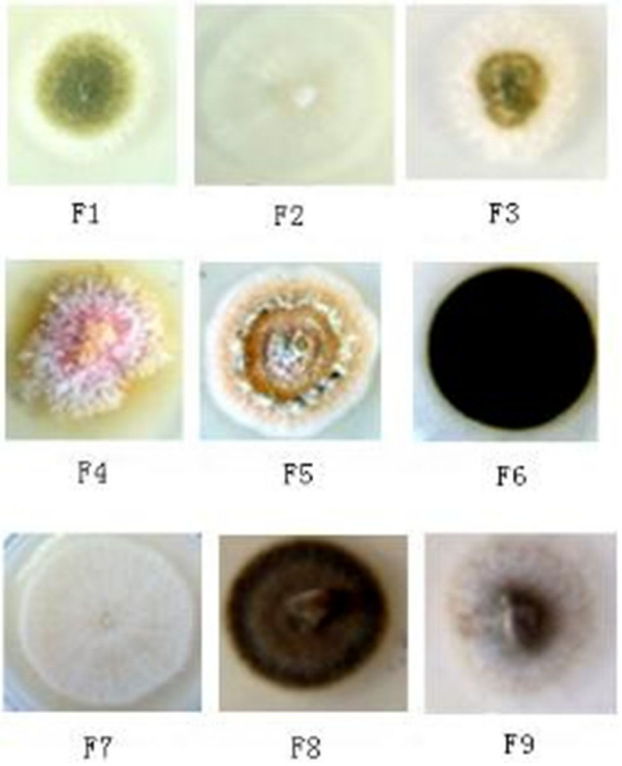

Fig. 1 Morphologies of F1–F9

Effect of Inoculation on the Metabolites of Gentian

A typical chromatogram for the determination of gentiopicroside is shown in Fig. 2, and the measurement data are presented in a bar chart (Fig. 3). Gentiopicroside content in the biennial gentian plants inoculated with F9 were 13% higher than that in the control (biennial gentian without inoculation, CK1). Treatments with F1 and F2 resulted in a significant increase in gentiopicroside production in triennial gentian plants by 33.6% and 23.7%, respectively, compared to that in the control (triennial gentian without inoculation, CK2). The subsequent thin-layer chromatography and high performance liquid chromatography analysis of broth extracts of the three stains indicated that F1, F2, and F9 were likely to produce gentiopicroside as their host plants. We had detected indoleacetic acid (IAA) in cultures of F1, F2, and F9. The results showed that IAA levels peaked after 2 days of liquid culture and were secreted by F1 (84.63 mg/L), F2

Table 1 BLAST results of dominant fungi rDNA ITS sequences

Strain no.	Most closely related species	Maximum identity (%)
F1	*Trichoderma hamatum* SZMC 20785	100
	T. asperellum SCGA5008	100
F2	*T. hamatum* HBJZ1001	100
	T. asperellum SCGA5008	100
F3	*Aspergillus flavus* YLJ-65	100
	A. flavus ATCC 11489	100
	A. oryzae SZ8 M-20	100
F4	*Fusarium tricinctum culture-collection* WAC:12337	100
	F. acuminatum IBE000006	100
F5	*Epicoccum nigrum* NMG_16	100
	Coniothyrium aleuritis HLJ_7	100
F6	*Cladosporium cladosporioides* YS-23	100
	Cladosporium gossypiicola ATCC 38026	100
	Cladosporium coralloides ATCC 16160	100
F7	*Leptodontidium* sp. C_2_BESC_319 g	98.76
	Leptodontidium orchidicola PA 077	98.75
F8	*Acephala* sp. WGS11780	98.96
F9	*T. asperellum* SCGA5008	100
	T. hamatum HBJZ1001	100

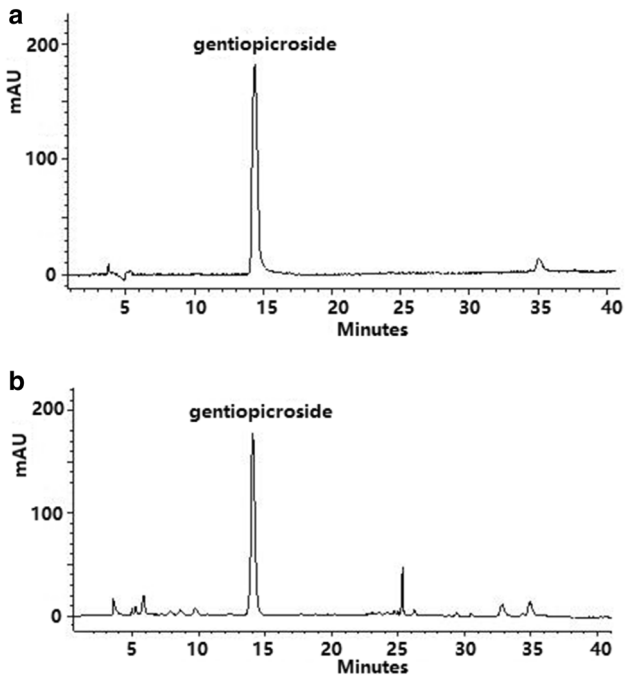

Fig. 2 Representative chromatograms in quantification of gentiopicroside by external standard calibration curve method (**a**—standard solution, **b**—sample solution)

(45.08 mg/L), and F9 (18.22 mg/L). IAA production was apparently the mechanism underlying the increase in fresh weight of gentian roots and then improvement of gentiopicroside content.

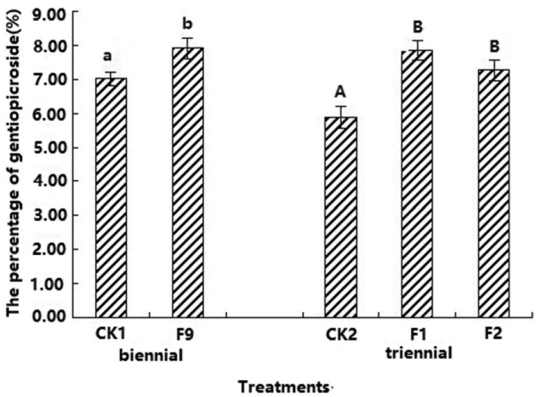

Fig. 3 Relative percentages of gentiopicroside in different treatments. Different letters (use the same case: significant difference between CK1 and F9 in lowercase letter, significant difference between CK2 and F1, F2 in capital letter) indicate statistically significant differences at $P < 0.05$ between inoculated and non-inoculated plants

Strains F1, F2, and F9 also improved the polysaccharide content in gentian plants by more than 6.6, 18.7 and 30% compared with corresponding controls (Fig. 4). Carbohydrates are products of photosynthesis. To explore the effects of endophytic fungi on gentian polysaccharides, chlorophyll *a* and *b* contents were determined, however, no content differences between inoculation and non-inoculation plants were observed.

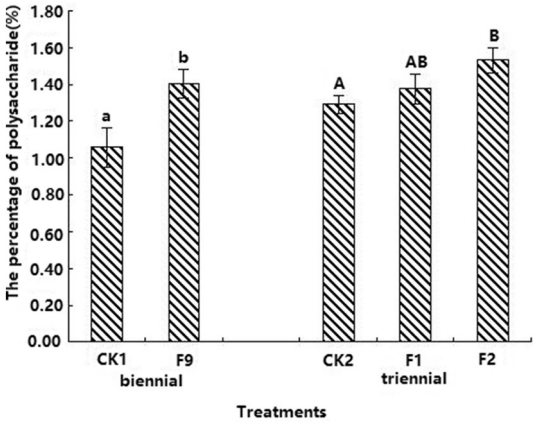

Fig. 4 Relative percentages of gentian polysaccharide in different treatments. Different letters (use the same case: significant difference between CK1 and F9 in lowercase letter, significant difference between CK2 and F1, F2 in capital letter) indicate statistically significant differences at $P < 0.05$ between inoculated and non-inoculated plants

Efficacy of Endophytic Fungi Against Gentian Spot Blight

Compared to non-inoculation, the incidence of spot blight in gentian inoculated with F1, F2, and F9 decreased by 31.2, 26.7 and 8.5% (data not shown). Continuous synthetic chemical application results in the development of resistant pathogens and the accumulation of residual fungicides in the natural ecosystem, which eventually become human or animal health risks. Biological control through the use of microorganisms is considered a safe and efficient control strategy for plant disease [35, 36]. Strains F1 and F2 showed potential disease resistance and deserved further research.

The Activities of Defense Enzymes

Biotic and abiotic stresses may cause the formation of reactive oxygen species (ROS) [37]. Enzymes such as SOD, CAT, and POD are involved in eliminating ROS [38]. Inoculated with strain F9, SOD and CAT activity increased by 1.2- and 1.4-fold separately compared to CK1, whereas, no statistically significant difference in POD activity between inoculation and non-inoculation was observed (Table 2). Compared to CK2, the individual inoculation with strains F1 and F2 resulted in significant improvements in SOD and POD activity, particularly with F1, which displayed a 40% increase in SOD activity. Conversely, CAT activity decreased after inoculation of strain F1 to 5.14 ± 0.15 μmol min^{-1} mg^{-1} fw and increased with strain F2 to 12.78 ± 0.16 μmol min^{-1} - mg^{-1} fw (Table 2).

Spot blight in CK2 with higher SOD and CAT activities was more severe compared to that in CK1 which suggests that higher enzymatic activity is not associated with lower disease incidence. Infection by fungal pathogens results in an increase in ROS and elevated ROS levels are accompanied by changes in enzymatic activity that decrease ROS toxicity to plant cell structures. Hence, higher enzymatic activities coupled with higher ROS levels sometimes indicate more necrotization and more severe disease. Otherwise, when pathogens attack plants and cause ROS formation, the inoculated endophytic fungi can increase enzymatic activity but this may not be adequate to eliminate ROS thus, the disease still progresses. Hence, changes in enzymatic activity may be opposite to that of disease severity.

In this study, cross-growth period inoculation may provide a feasible and meaningful method for elucidating the roles of endophytic fungi on their original host in vivo

Table 2 Efficacy of endophytic fungi on defense enzymes activity (mean ± SE) in biennial and triennial gentian

Treatments	SOD (U g^{-1} fw)	CAT (μmol min^{-1} mg^{-1} fw)	POD (U min^{-1} g^{-1} fw)
CK1 (biennial gentain)	50.33 ± 5.50a	6.00 ± 2.53a	5.00 ± 0.29a
F9	62.84 ± 2.53b	8.75 ± 2.53b	4.82 ± 0.15a
CK2 (triennial gentian)	61.44 ± 3.19A	9.03 ± 0.20B	4.21 ± 0.22A
F1	85.48 ± 3.25C	5.14 ± 0.15A	5.65 ± 0.18B
F2	73.86 ± 1.73B	12.78 ± 0.16C	5.57 ± 0.26B

Different lower case letters denote statistically significant differences ($P < 0.05$) between treatments of CK1 and F9; different capital letters denote statistically significant differences ($P < 0.05$) among treatments of CK2, F1 and F2

and in the field. In different plant growth stages with various environment or abiotic stress, interactions between plant and endophyte may vary [5, 39] which requires further investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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