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Research Article

Endophytic *Trichoderma citrinoviride* isolated from mountain-cultivated ginseng (*Panax ginseng*) has great potential as a biocontrol agent against ginseng pathogens

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ABSTRACT

Background: Ginseng (*Panax ginseng* Meyer) is an invaluable medicinal plant containing various bioactive metabolites (e.g., ginsenosides). Owing to its long cultivation period, ginseng is vulnerable to various biotic constraints. Biological control using endophytes is an important alternative to chemical control.
Methods: In this study, endophytic *Trichoderma citrinoviride* PG87, isolated from mountain-cultivated ginseng, was evaluated for biocontrol activity against six major ginseng pathogens. *T. citrinoviride* exhibited antagonistic activity with mycoparasitism against all ginseng pathogens, with high endo-1,4-β-D-glucanase activity.
Results: *T. citrinoviride* inoculation significantly reduced the disease symptoms caused by *Botrytis cinerea* and *Cylindrocarpon destructans* and induced ginsenoside biosynthesis in ginseng plants. *T. citrinoviride* was formulated as dustable powder and granules. The formulated agents also exhibited significant biocontrol activity and induced ginsenosides production in the controlled environment and mountain area.
Conclusion: Our results revealed that *T. citrinoviride* has great potential as a biological control agent and elicitor of ginsenoside production.

1. Introduction

Ginseng (*Panax ginseng*) is a highly valuable plant and has been used for medicinal purposes for thousands of years [1]. Ginseng produces bioactive metabolites such as ginsenosides, oleanic acids, and phenolic compounds, which have pharmacological properties, including anticancer, antiplatelet, and antimicrobial activities [2]. Mountain-cultivated ginseng (MCG) is propagated under natural conditions in the forest and requires a long period of cultivation [3]. Phytopathogens such as *Botrytis cinerea, Cylindrocarpon destructans, Pythium* spp., and *Rhizoctonia solani* cause serious problems in MCG cultivation [4,5]. Therefore, suitable biocontrol agents (BCAs) are urgently required to control ginseng pathogens.

Fungal endophytes dwell within plants without causing apparent disease symptoms [6]. They propagate in the intercellular or intracellular spaces without harming the plants. Previous studies have found that some fungal endophytes improve the resistance of plants to insects and pathogens [7] and tolerance to abiotic stresses such as drought, extreme temperature, and salinity [8]. Endophytes can also produce various metabolites that have a range of biological activities, such as anticancer, antimicrobial, and antiviral activities [9]. Therefore, fungal endophytes might have potential functions as BCAs against microbial pathogens.

Trichoderma spp. are the most prevalent soil fungi [10] and are opportunistic avirulent plant symbionts because they have beneficial effects on plants [10,11]. *Trichoderma* spp. are also used as BCAs against plant pathogenic fungi such as *B. cinerea*, *Fusarium* spp., *Pythium* spp., and *Rhizoctonia* spp. [12]. *Trichoderma* spp. use different modes of action as BCAs, including antibiosis, mycoparasitism, and induced resistance [11,13]. Furthermore, *Trichoderma*-

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based BCAs can promote plant growth and have soil remediation activity; therefore, they have greater ability than viruses, bacteria, and protozoa [14]. Among the different species, *T. citrinoviride* is one of the most widespread soil fungi of the longibrachiatum clade and is a well-known producer of strong cellulases [15]. Therefore, *T. citrinoviride* is a good BCA against phytopathogens.

In this study, we examined the antagonism of *T. citrinoviride*, which was isolated from MCG, as a BCA against major ginseng pathogens. We also confirmed the biocontrol activity of *T. citrinoviride* formulations. Furthermore, we determined the ability of *T. citrinoviride* and its formulations to induce ginsenoside synthesis and ginseng defense responses.

2. Materials and methods

2.1. Fungal endophyte, ginseng pathogens, and ginseng plants

T. citrinoviride PG87 was isolated from the roots of MCG plants, which were collected from 24 sites in Korea [16]. Ginseng pathogens were procured from the Rural Development Administration Genebank Information Center (Suwon, Korea): *Rhizoctonia solani* KACC40123 (Korea Agricultural Culture Collection), *Botrytis cinerea* KACC43521, *Alternaria panax* KACC42461, *Cylindrocarpon destructans* KACC44656, *Phytophthora cactorum* KACC40166, and *Pythium* spp. KACC40581. *B. cinerea* and *C. destructans* were used as above- (stem and leaf) and below-ground (root) pathogens, respectively.

Two-yr-old ginseng roots were purchased from Geumsan Insamjohap (Geumsan, Korea), planted into sterile artificial ginseng soil mix (Shinsung Mineral Co., Ltd., Seongnam, Korea), and grown in a growth room (23°C, 150 ± 10 μmol/m^2 sec, 16-h light) for 1–4 wk.

2.2. Mycoparasitism assay

T. citrinoviride was screened for mycoparasitic ability against ginseng pathogens using a microscope slide and dual-culture assays [5]. For the glass microscope slide assay, slides were covered with 50% potato dextrose agar (PDA) media and inoculated with PDA plugs (5-mm diameter) of *T. citrinoviride* and each of the six ginseng pathogens (3-cm apart). The pathogens alone were inoculated using PDA plugs as controls. Three replicate slides were used for each ginseng pathogen. After 3–7 d of incubation at 25°C, the contact area was observed under an Olympus BX51 TRF equipped with an Olympus digital Infinity1C camera at 1000 × magnification (Tokyo, Japan).

PDA plugs of both *T. citrinoviride* and each of the six ginseng pathogens were cocultured on a PDA plate at opposite edges of the petri dishes (6-cm apart) and incubated at 25°C. Antagonistic behavior was observed after 2–6 d of incubation. The pathogens alone were inoculated as controls. Mycelial growth inhibition (%) was calculated and compared with the control plates using the following equation: Mycelial growth inhibition (%) = [(growth of control − growth of treatment)/growth of control] × 100. Experiments were conducted with three sets of replication plates.

2.3. Dual culture of T. citrinoviride and B. cinerea

A *B. cinerea* PDA plug was inoculated on the edge of the PDA plate and incubated at 25°C for 4 d. Thereafter, a *T. citrinoviride* PDA plug was placed on the opposite edge of a PDA plate preinoculated with *B. cinerea* (6-cm apart) and incubated at 25°C for a further 3 d. *T. citrinoviride* and *B. cinerea* mycelia were harvested around the contact area using a sterile spatula. Fungal RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA and the GoScript Reverse Transcription System (Promega, Madison, WI, USA). The patterns of *T. citrinoviride* and *B. cinerea* gene expression were analyzed (primers sequences are shown in Table S1) as described previously [17]. The experiment was conducted twice with three biological replicates.

2.4. Activity assay of cell wall–degrading enzymes

T. citrinoviride was grown on PDA media at 25°C for 5 d. Then, five 5-mm plugs were inoculated into 50-mL basal media supplemented with one of the following carbon sources (1 g/L): cellulose microcrystalline, carboxymethy1 cellulose, *B. cinerea* cell wall, or *C. destructans* cell wall. Cell wall preparations of *B. cinerea* and *C. destructans* were prepared as previously described [18]. Each flask was incubated at 25°C with shaking at 120 rpm for 7 d to induce endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, β-1,3-glucanase, and β-glucosidase activity. The supernatants were collected after filtration through Whatman no. 1 filter paper (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 7,000 g at 4°C for 20 min. Protein concentrations were measured using the Bradford assay (Sigma-Aldrich) [19]. Endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, β-1,3-glucanase, and β-glucosidase activity was measured using 1% Avicel, carboxymethy1 cellulose, laminarin, and salicin as a substrate, respectively, and the reducing sugar was determined with dinitrosalicylic acid reagent (Sigma-Aldrich) [20]. One unit (U) of extracellular enzyme activity was defined as the amount of enzyme that produced 1 μmol reducing sugar/h under the above conditions. The experiment was conducted twice with three biological replicates.

2.5. Inoculation of ginseng plants with T. citrinoviride

To determine whether *T. citrinoviride* has any harmful effects on ginseng plants, we inoculated ginseng roots with *T. citrinoviride* potato dextrose broth (PDB) media and with the PDB media alone as a control. Briefly, 2-yr-old ginseng roots were surface-sterilized using 2% NaOCl for 10 min and rinsed three times with sterile distilled water. After sterilization, roots were inoculated with 1 mL 1×10^6 spores/mL of *T. citrinoviride* in PDB media by dipping for 3 h. After air-drying for 1 h on a clean bench, roots were planted in pots (5 × 5 × 11 cm) containing sterile artificial ginseng soil. Pots were placed in a growth room. After 3 wk of growth, ginseng roots were examined visually, and *T. citrinoviride* was reisolated. The ginseng plants (root, stem, and leaf) were surface-sterilized, cut into 1-cm pieces, and placed on a PDA plate. Plates were incubated for 10 d at 25°C, and fungal endophytes were identified using internal transcribed spacer sequences. The colonization frequency (CF%) was calculated as follows: CF (%) = (total number of *T. citrinoviride*-colonized segments/total number of segments) × 100.

After 1, 2, 3, and 4 wk of inoculation, the below- (root) and above-ground (stem and leaf) parts of ginseng plants were collected, and DNA, RNA, hormones, and ginsenosides were extracted. The experiment was conducted twice with three biological replicates.

2.6. Quantitative real-time PCR (QPCR)

Quantitative real-time polymerase chain reaction (QPCR) was used to quantify the expression of target transcripts and DNA. Translation elongation factor alpha 1 (*TEF1*, KJ665454) was used to quantify *T. citrinoviride* using QPCR [21]. Primer 3 software from Biology Workbench (http://workbench.sdsc.edu/) was used to design primers (Table S1). Fungal genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and 100 ng of template DNA was used to amplify the TEF1 fragment

(approximately 250 bp) [22]. The amplified products were loaded onto 1.2% agarose gel and stained with ethidium bromide. The PCR products were purified from the excised gel using Wizard SV gel and a PCR Clean-up System (Promega) and processed for sequencing at Cosmo Genetech (Seoul, Korea). Generated sequences were analyzed using Clustal W (Biology Workbench). Specific primers (CDU1 and CDL1b) for *C. destructans* were designed from intergenic spacers as described previously [23]. The respective 152-bp product was sequenced and confirmed with the reported *C. destructans* sequence. Specificity of the primer sets for *T. citrinoviride* and *C. destructans* was confirmed by QPCR using a DNA template from ginseng root inoculated with the respective fungi. Ginseng root inoculated with PDB was used as a control.

Genomic DNA was extracted from inoculated ginseng roots using the DNeasy Plant Mini Kit (Qiagen). QPCR was then performed as described previously except for *C. destructans*, for which the following parameters were used: an initial denaturing period of 5 min at 94°C, followed by 30 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 1 min, followed by 10 min at 72°C. Genomic DNA from *T. citrinoviride* or *C. destructans* (10, 1, 0.1, 0.01, and 0.001 ng) was also included to generate calibration curves. Dilutions with five different concentrations, each with three replicates, were used, and QPCR was performed twice. Threshold cycle (C_t) values were obtained from reactions containing fungal DNA and were correlated with the amount of DNA.

2.7. Hormone analysis

Ginseng hormones, zeatin (ZA), abscisic acid (ABA), and jasmonic acid (JA), were analyzed as previously described [24,25]. Approximately 50 mg of frozen samples was extracted with 980 μL methanol:acetic acid (99:1, v/v) and 20 μL internal standard solution. The samples were centrifuged at 13,000 g for 5 min at 4°C. Supernatant (1 mL) was collected and filtered using a 0.45-μm nylon syringe filter (Sigma-Aldrich), and 10 μL of each sample was injected. The experiment was carried out using liquid chromatography–electrospray ionization–tandem mass spectrometry. The gradient profile was as follows: 0.01–2 min, 0–40%; 2–5 min, 40–60%; 5–13 min, 60–100%; 13–15 min, 100–20% eluent B. The flow rate was 0.5 mL/min, and the column was maintained at 40°C.

2.8. Ginsenoside analysis

Ginsenosides were analyzed as previously reported [26]. Methanol-dissolved samples were diluted 100 times to quantify major ginsenosides. Ginsenosides (Ro, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2, Rg3, Rh1, F1, and F2; Ambo Institute, Seoul, Korea) were used as standards. High-performance liquid chromatography–grade acetonitrile, methanol, and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid, for ionization, was obtained from Duksan Pure Chemicals Co., Ltd. (Ansan, Korea). All ginsenoside standards were dissolved in methanol to obtain 50 μg/mL stock solutions and stored at −20°C until use. To achieve the coefficient of determination, calibration curves of each standard were prepared with 11 different concentrations (25–20 mg/mL). liquid chromatography–electrospray ionization–tandem mass spectrometry analysis was performed using an LCMS-8040 tandem quadruple mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a degasser, binary pump, auto sampler, column oven, and UV detector. All ginsenosides were confirmed in negative electrospray mode. MS was operated in multiple reaction–monitoring mode using argon gas as the collision energy. Operating parameters were as follows: nebulizer gas flow 3 L/min, drying gas flow 15 L/min, desolvation line temperature 250°C, and heat block temperature 400°C. Data were analyzed by Labsolutions program (Shimadzu, Kyoto, Japan). The mobile phase consisted of formic acid in water ($1:10^3$, v/v; eluent A) and 100% acetonitrile (eluent B). Chromatographic separation was achieved on an ACE® UltraCore™ 2.5 Super C18 column (150 × 4.6 mm, 2.5 μm; Advanced Chromatography Technologies, Aberdeen, UK), and the gradient profile was as follows: 0.01–2 min, 25–35%; 2–6 min, 35%; 6–15 min, 35–90% B. The column was washed with 100% B for 5 min, followed by 25% B for 5 min after each injection. The flow rate and temperature were 0.5 L/min and 40°C, respectively, in all experiments, and 10 μL of each sample was injected. The ginsenosides contents are expressed in ng/g dry weight.

2.9. Biocontrol assay of B. cinerea and C. destructans in ginseng plants using T. citrinoviride

To study the induction of *B. cinerea* resistance, ginseng roots were inoculated with *T. citrinoviride* by root dipping as described and grown in a growth room for 2 wk. Then, a *B. cinerea* spore suspension (1 mL of 1×10^6 spores/mL) was sprayed on whole leaves of each plant. Ginseng plants were grown for 1 wk in a sealed plastic box (40 × 50 × 35 cm) with 100% humidity to aid disease progression. The relative infected area (RIA %) was measured, and leaves from ginseng plants inoculated with *T. citrinoviride* and control leaves (no *T. citrinoviride* root inoculation) were harvested, and the expression of ginseng and *B. cinerea* genes was examined.

To mimic field conditions, we inoculated ginseng shoots by spraying with *T. citrinoviride* spore suspension. After 12 h of spraying, plants were treated with *B. cinerea* spore suspension. For the control, PDB without *T. citrinoviride* was sprayed. After 1-week growth, the RIA (%) was measured, and leaf samples were harvested for subsequent expression analysis.

To investigate *C. destructans* suppression, ginseng roots were inoculated with *T. citrinoviride* spore suspension by pipetting into the soil within the pot. PDB was used as a control. After 24 h of inoculation, ginseng roots were pulled from the soil and wounded (1-mm depth, 0.25-mm diameter, 20 wounds) using sterilized needles [27]. A *C. destructans* spore suspension was poured into ginseng soil after wounding. After 3 wk of infection, ginseng roots were collected for fungal quantification and gene expression analysis.

2.10. Formulation of T. citrinoviride

T. citrinoviride was formulated in concurrence with Enbio (Gunpo, Korea). Two different formulations were selected for further tests: dustable powder (DP) and granule (GR) (Table S2). DP was formulated by mixing with white carbon, calcite, and pulverized *T. citrinoviride*. GR was formulated by mixing with white carbon, polyvinyl alcohol, corn starch, bentonite, calcite, and pulverized *T. citrinoviride*. The products were assembled through an extruder and dried with a floating dryer.

2.11. Application of DP and GR for the biocontrol of B. cinerea and C. destructans in ginseng

Biocontrol activity of the formulated DP and GR agents was tested against *B. cinerea* and *C. destructans*, respectively. After the growth of ginseng plants in soil for 2 wk, 0.5 g of DP was sprayed onto whole leaves in one set, and 0.5 g of GR was applied to the soil in another. After 1 wk, leaves from the set sprayed with DP were inoculated with *B. cinerea* spore suspension through foliar spraying. The second set of plants treated with GR was inoculated with *C. destructans* spore suspension by pipetting into the soil as described (20 wounds). Ginseng plants inoculated with *B. cinerea* and *C. destructans* were allowed to grow for a further 1 and 3 wk, respectively, after which tissue samples (above- and below-ground

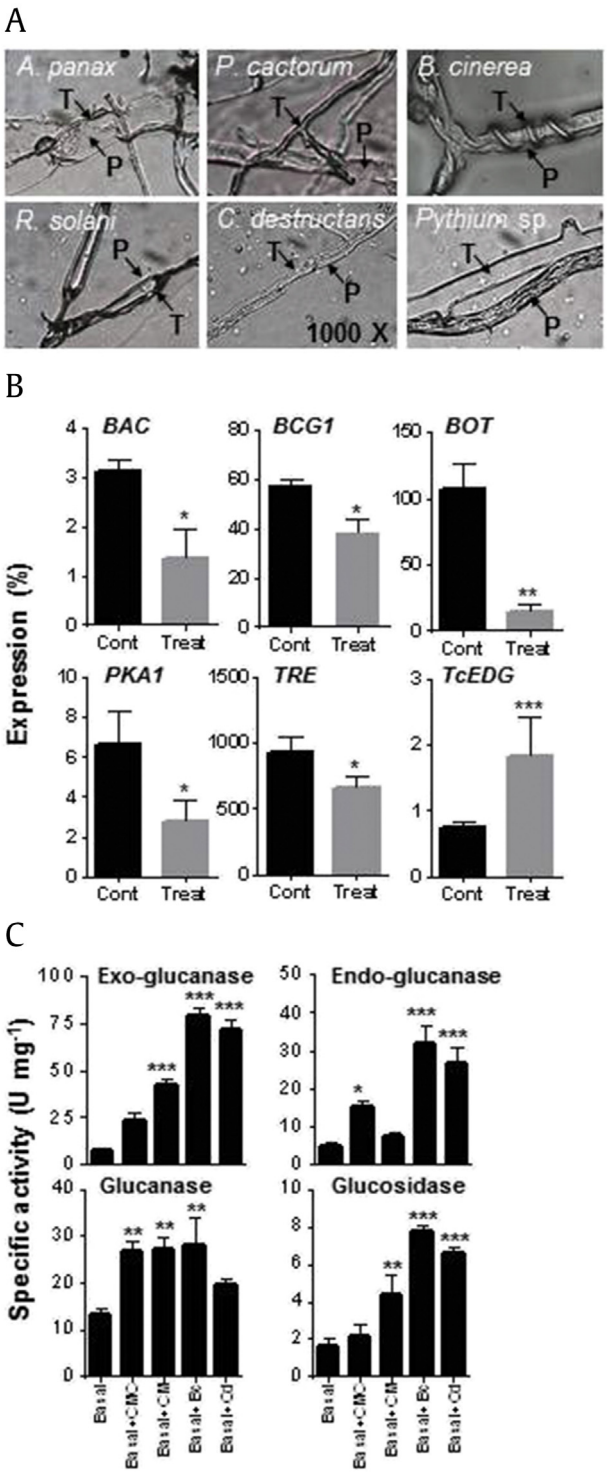

Fig. 1. Biocontrol activity of *Trichoderma citrinoviride* PG87 against ginseng pathogens. (A) *T. citrinoviride* was screened for mycoparasitic ability against ginseng pathogens on potato dextrose agar (PDA) slides. Glass slides covered with 50% PDA were inoculated with PDA plugs of *T. citrinoviride* and each of the six ginseng pathogens (3-cm apart). The contact area was observed under the microscope (1000 × magnification). T, *T. citrinoviride*; P, pathogen. **(B)** Quantitative real-time PCR (QPCR) analysis of *Botrytis cinerea* (*BAC*, *BCG1*, *BOT*, *PKA1*, and *TRE*) and *T. citrinoviride* (*TcEDG*) genes in dual culture. A *B. cinerea* PDA plug was inoculated on the edge of the PDA plate and incubated at 25°C for 4 d. Thereafter, a *T. citrinoviride* PDA plug was placed on the opposite edge of a PDA plate preinoculated with *B. cinerea* (6-cm apart) and incubated at 25°C for a further 3 d. *T. citrinoviride* and *B. cinerea* mycelia were harvested around the contact area, and fungal RNA was extracted for QPCR analysis. *B. cinerea* and *T. citrinoviride ACTINs* were used to normalize the relative expression levels. The experiment was conducted twice with three biological replicates. **Cont**, Control; **Treat**, Treatment. **(C)** Activity assay of cell wall–degrading enzymes (CWDEs) for *T. citrinoviride*. *T. citrinoviride* was grown for 7 d in basal media plus one of the following carbon sources: carboxymethy1 cellulose (CMC), cellulose microcrystalline (CM), *B. cinerea* cell wall (Bc), or *C. destructans* cell wall (Cd). The culture filtrates were assayed for endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, β-1,3-glucanases, and β-glucosidases. The experiment was conducted twice with three biological replicates. Vertical bars indicate the means with standard error from six biological replications. $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.
PCR, polymerase chain reaction.

parts, respectively) were harvested to analyze RIA (%), quantification, and gene expression.

2.12. Application of DP and GR in a ginseng field

A field test was carried out at the MCG cultivation site (Jecheon, Korea) with the formulated *T. citrinoviride* agents. Three-yr-old MCG plants were grown under the same environmental conditions. During the growth period (May–June, 2016), DP and GR agents were applied to foliage five times weekly at 10 g/m^2. Morbidity for each plant was recorded until harvest (August, 2016). The harvested MCG plants were prepared for experimentation as described. These experiments were conducted with six biological replicates.

2.13. Statistical analyses

Mean values were obtained from three to six replicates, and the standard errors are indicated by error bars. Means and significant differences were calculated using analysis of variance followed by Duncan's multiple range test at $p = 0.05$ with SAS v9.4 (Cary, NC, USA). Graphs were prepared using GraphPad Prism 6 project (San Diego, CA, USA).

3. Results

3.1. Mycoparasitism

T. citrinoviride showed the highest inhibitory effect on the mycelial growth of six ginseng pathogens with rapid colonization and profuse sporulation (Fig. S1). Thus, the mycoparasitic ability of *T. citrinoviride* was evaluated against ginseng pathogens. The results revealed the unrestricted growth of *T. citrinoviride* along the hyphae of the pathogens in the contact area in agar slides (Fig. 1A). *T. citrinoviride* hyphae directly penetrated *A. panax* and *P. cactorum* hyphae without coiling. However, *T. citrinoviride* coiled around and did not penetrate *B. cinerea* and *R. solani* hyphae. A parallel association with appressorium formation was observed without coiling or penetration in *C. destructans* and *Pythium* spp.

3.2. Molecular interaction between T. citrinoviride and B. cinerea in dual culture

B. cinerea genes related to growth and virulence were analyzed in PDA dual culture with *T. citrinoviride* by QPCR (Fig. 1B). Five *B. cinerea* genes were analyzed using RNA extracted from the mycelia around the contact area. All five *B. cinerea* genes were significantly downregulated in the presence of *T. citrinoviride*. In contrast, *TcEDG* was strongly induced by *B. cinerea*.

3.3. Production of cell wall–degrading enzymes involved in mycoparasitism

The activities of cell wall–degrading enzymes (CWDEs) were determined in the PDB liquid culture filtrates of *T. citrinoviride*

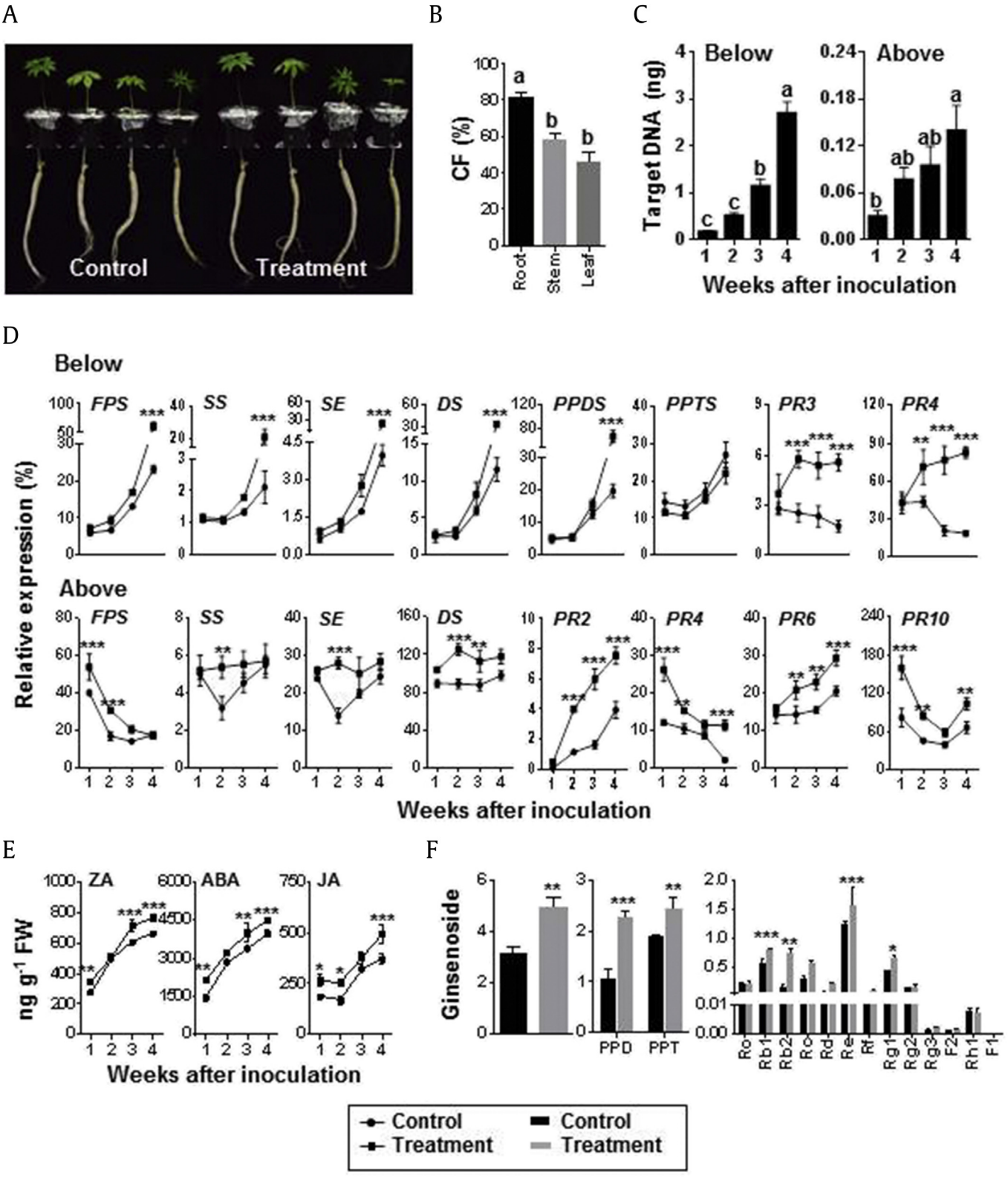

Fig. 2. Endophytism of *Trichoderma citrinoviride* PG87 in ginseng plant. Ginseng roots were inoculated with *T. citrinoviride* spore suspension by dipping. Inoculated roots were planted into sterile artificial ginseng soil and grown for 1–4 wk. After 1, 2, 3, and 4 wk of inoculation, below- (root) and above-ground (stem and leaf) parts of ginseng plants were collected to extract DNA, RNA, hormones, and ginsenosides. **(A)** Photographs showing the absence of disease symptom in ginseng plants inoculated with *T. citrinoviride* for 3 wk. **(B)** Colonization frequency (%) of *T. citrinoviride* in three different tissues. After 3 wk, ginseng plants (root, stem, and leaf) were surface-sterilized, cut into 1-cm pieces, and placed on PDA plates for 10-d growth. Fungal endophytes were identified using internal transcribed spacer (ITS) sequence. **(C)** Quantification of *T. citrinoviride* colonization in below- and above-ground parts of ginseng plants using QPCR. Target DNA = ng of target DNA in 50 ng of total genomic DNA. **(D)** Expression of genes involved in ginsenoside biosynthesis (*FPS*, *SS*, *SE*, *DS*, *PPDS*, and *PPTS*) and defense (*PR2*, *PR3*, *PR4*, *PR6*, *PR10*) in the below- and above-ground parts of ginseng plants. Ginseng *ACTIN* was used to normalize the relative expression levels. **(E)** Alterations in hormone levels in ginseng roots. **(F)** Ginsenoside contents of ginseng roots after 4 wk of *T. citrinoviride* inoculation. The total ginsenoside

(Fig. 1C). Enzymatic activity was measured by the amount of reducing sugars released from different substrates. Enzymatic activity was substrate dependent, which could induce the activity of multiple enzymes. The highest activity was observed for endo-1,4-β-D-glucanase. Fungal cell wall from *B. cinerea* was the best substrate to induce enzymatic activity: 79, 32, 28, and 8 U/mL for exo-1,4-β-D-glucanase, endo-1,4-β-D-glucanase, β-1,3-glucanase, and β-glucosidases, respectively. As a substrate, *C. destructans* cell wall also induced high enzymatic activity, similar to *B. cinerea* cell wall, with the exception of β-1,3-glucanase.

3.4. Endophytism of *T. citrinoviride* in ginseng plants

No disease symptoms or growth inhibition were observed in ginseng roots after 3-wk preinoculation with *T. citrinoviride*, as noted for both below- and above-ground parts (Fig. 2A). CF% varied significantly among different tissues. CF was higher in the roots than in the stem and leaf tissues (81.6 vs. 58 and 46.0%, respectively) (Fig. 2B). No negative effect was imparted by *T. citrinoviride* in ginseng plants, with the best colonization observed in root tissue. After the inoculation of ginseng plants with *T. citrinoviride* for 1, 2, 3, and 4 weeks, *T. citrinoviride* was quantified using QPCR using *TEF1* primers. The quantities of standard DNA versus their respective C_t for *T. citrinoviride* were obtained, and calibration curves were constructed (Fig. S2). The amount of *T. citrinoviride* target DNA was presented as nanograms target DNA in 50 ng of total genomic DNA. Target DNA increased significantly with inoculation period (ranging from 0.17 ng to 2.69 ng) in the below-ground part (Fig. 2C). However, the increment in target DNA in the above-ground part was relatively lower (ranging from 0.03 ng to 0.14 ng) than in the root. Thus, *T. citrinoviride* colonized the below-ground part more than the above-ground part (5.30 and 0.28% per total DNA in 4 wk, respectively).

3.5. In planta study of ginseng-induced disease resistance, hormones, and ginsenosides

Preinoculation of ginseng roots with *T. citrinoviride* by root dipping upregulated genes involved in ginsenoside biosynthesis (*FPS, SS, SE, DS, PPDS*, and *PPTS*) and defense (*PR2*, *PR3, PR4*, *PR6*, and *PR10*). The expression of five ginsenoside biosynthetic genes (*FPS, SS, SE, DS*, and *PPDS*) significantly increased 4 wk after inoculation compared with the controls in the below-ground parts, except for *PPTS* (Fig. 2D). Defense-related genes (*PR3* and *PR4*) were significantly upregulated 2 wk after inoculation; induction was maintained up to 4 wk in below-ground parts, and a slight reduction was detected in the control. In contrast, *FPS*, *SS*, *SE*, and *DS* were significantly upregulated in above-ground parts after 1 and/or 2 wk of inoculation, with no significant changes observed after 4 wk (Fig. 2D). *PR2* and *PR6* expression was gradually upregulated in *T. citrinoviride*-inoculated plants compared with that in the control, except at 1 wk. *PR4* and *PR10* were significantly upregulated 1, 2, and 4 wk after inoculation compared with the control.

Phytohormones (ZA, ABA, and JA) were significantly upregulated in *T. citrinoviride*-inoculated ginseng roots (Fig. 2E). Notably, phytohormones were significantly increased at each time point, except at 2 or 3 wk after inoculation. The highest induction of phytohormones was observed 4 wk after inoculation.

Ginsenoside contents were evaluated in the *T. citrinoviride*-inoculated ginseng roots 4 wk after inoculation. The total ginsenosides in treated roots were significantly increased (1.57-fold) compared with those in the control (Fig. 2F). Protopanaxadiol (PPD)-type ginsenosides were induced more than protopanaxatriol (PPT)-type ginsenosides, which was mainly due to Rb2 induction. The highest induction was detected in ginsenoside Rb2. Ginsenosides Rc, Re, and Rg1 were also significantly induced.

3.6. Induced disease resistance against *B. cinerea* by *T. citrinoviride* inoculation

Ginseng roots were inoculated with *T. citrinoviride* by root dipping and grown for 2 wk before *B. cinerea* infection. *T. citrinoviride*-inoculated roots showed significantly reduced RIA 1 wk after *B. cinerea* foliar infection, with 100% and 11.5% RIA in the control and treatment groups, respectively (Fig. 3A and B). The expression of six genes involved in the growth and virulence of *B. cinerea* (*BAC*, *BCG1*, *BOT*, *CHS3*, *PKA1*, and *TRE1*) was significantly downregulated in the above-ground parts compared with that in the control (Fig. 3C). Conversely, the expression of ginseng defense-related genes (*PR2, PR4, PR5*, and *PR10*) was significantly upregulated in leaf samples from preinoculated plants (Fig. 3D).

3.7. Suppression of *B. cinerea* by *T. citrinoviride* preinoculation

Ginseng leaves were inoculated with *T. citrinoviride* by leaf spraying and dried for 12 h before *B. cinerea* infection. *T. citrinoviride*-inoculated leaves showed significant resistance to *B. cinerea* (Fig. 4A). Minor infection was detected in the preinoculated leaf samples (5.7% RIA), whereas severe infection was observed in the control (Fig. 4B). Both *B. cinerea* (*BAC*, *BCG1*, *BOT*, *CHS3*, and *PKA1*) and ginseng (*PR2, PR3, PR4, PR6*, and *PR10*) genes were expressed at significantly lower levels in *T. citrinoviride* preinoculated ginseng plants than in the control (Fig. 4C and D).

3.8. Suppression of *C. destructans* by *T. citrinoviride* preinoculation

Ginseng roots were inoculated with *T. citrinoviride* by pipetting in soil. After 24 h, roots were pulled, wounded, replanted in soil, and infected with *C. destructans* by pouring the culture into soil. *T. citrinoviride*-inoculated roots showed a slight yellowish color without any rot symptoms 3 weeks after *C. destructans* infection (Fig. 5A). The above-ground parts of the control plants wilted, and the below-ground parts exhibited severe rot. *T. citrinoviride* colonization was confirmed by QPCR in treated roots (2 ng of target DNA per 50 ng of total DNA), whereas *T. citrinoviride* was not detected in control roots (Fig. 5B). A high amount of *C. destructans* DNA (3.7 ng) was detected in control roots, whereas a small amount (0.29 ng) was detected in treated roots. Ginseng defense-related genes (*PR2, PR3, PR4*, and *PR10*) were expressed at significantly lower levels in the treatment group than in the control (Fig. 5C).

3.9. Formulation of *T. citrinoviride*

DP and GR agents were formulated and maintained at three different temperatures (4, 24, and 40°C) to confirm the survivability of *T. citrinoviride* (Fig. S3). After 8 wk at 40°C, the GR agent was highly preserved with a slight reduction in the *T. citrinoviride* concentration (10^8 to 10^7 cfu/g) compared with DP (10^8 to 10^2 cfu/g).

contents and ginsenoside contents of PPD (Rb1, Rb2, Rc, Rd, Rg3, and F2) and PPT type (Re, Rf, Rg1, Rg2, Rh1, and F1). The experiment was conducted twice with three biological replications. Vertical bars indicate the means with standard error from six biological replications. Significance of the relative values is indicated by different letters on the means as per Duncan's multiple range test ($p < 0.05$). $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.

ABA, abscisic acid; FW, fresh weight; JA, jasmonic acid; PPD, Protopanaxadiol; PPT, protopanaxatriol; QPCR, quantitative real-time polymerase chain reaction; ZA, zeatin.

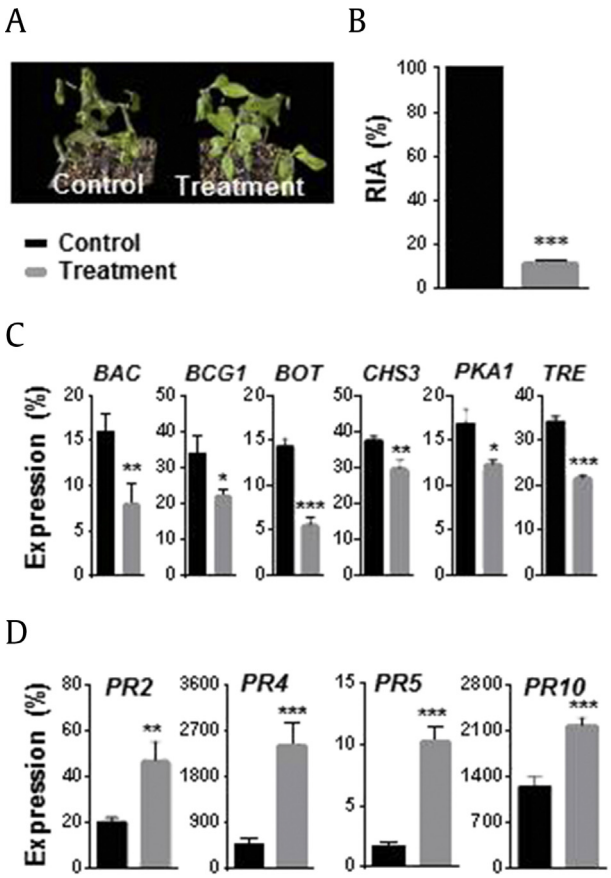

Fig. 3. Induced disease resistance in ginseng plants. Ginseng roots were inoculated with *Trichoderma citrinoviride* PG87 by root dipping and grown for 2 wk. Thereafter, *Botrytis cinerea* spore suspension was sprayed onto whole leaves. Ginseng plants were grown for 1 wk. Relative infected area (RIA %) was measured, and leaves were harvested to analyze the expression patterns of ginseng and *B. cinerea* genes. **(A)** Photographs showing the effect of induced disease resistance in ginseng leaves. **(B)** Relative infected area (RIA %). **(C)** Expression analysis of *B. cinerea* genes involved in growth and pathogenicity. **(D)** Expression analysis of ginseng pathogenesis-related (*PR*) genes. *B. cinerea* and ginseng *ACTINs* were used to normalize the relative expression levels. Vertical bars indicate the means with standard error from three biological replications. $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.

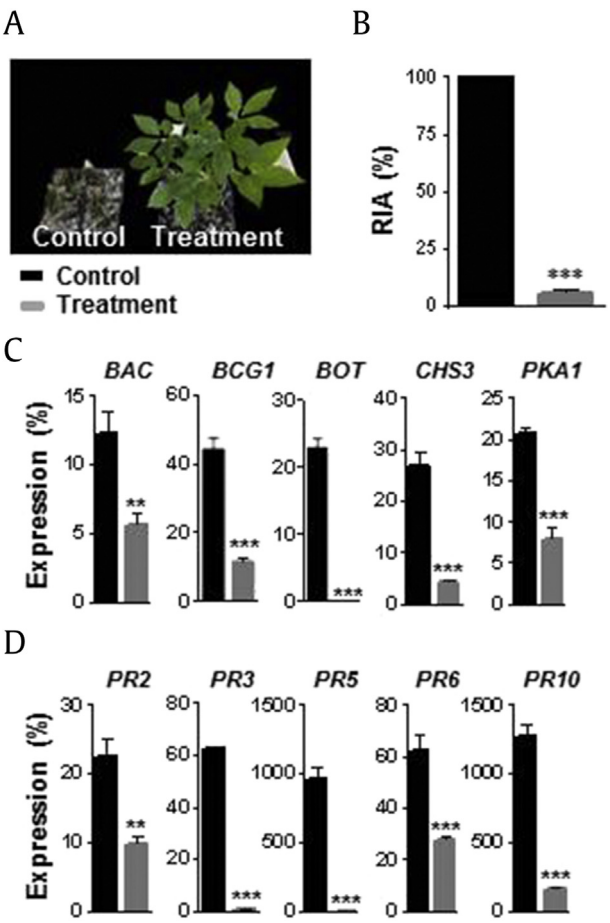

Fig. 4. Biocontrol activity of *Trichoderma citrinoviride* PG87 on leaf spot disease caused by *Botrytis cinerea*. *T. citrinoviride* spore suspension was sprayed onto ginseng leaves. After 12 h of spraying, *B. cinerea* spore suspension was sprayed onto ginseng leaves. After 1 wk of growth, relative infected area (RIA %) was measured, and leaf samples were harvested for expression analysis. **(A)** Photographs showing the biocontrol activity of *T. citrinoviride* against *B. cinerea*. **(B)** Relative infected area (RIA %). **(C)** Expression analysis of *B. cinerea* genes. **(D)** Expression analysis of ginseng *PR* genes. *B. cinerea* and ginseng *ACTINs* were used to normalize the relative expression levels. Vertical bars indicate the means with standard error from three biological replications. $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.

3.10. Suppression of *B. cinerea* by *T. citrinoviride* DP

Application of the DP agent significantly reduced disease symptoms (Fig. 6A). The RIA of control plants was 60.50%, compared with 4.15% in DP-treated ginseng leaves (Fig. 6B). The expression levels of *B. cinerea* (*BAC*, *BCG1*, *BOT*, *CHS3*, and *PKA1*) and ginseng (*PR2, PR3, PR4, PR6*, and *PR10*) genes were significantly reduced in DP-treated ginseng leaves (Fig. 6C and D).

3.11. Suppression of *C. destructans* by *T. citrinoviride* GR

The GR agent showed greater antagonistic activity against *C. destructans* root rot disease than the control (Fig. 7A). No root rot symptoms were observed in roots treated with GR before *C. destructans* infection. *T. citrinoviride* target DNA was detected only in GR-treated ginseng roots (3.89 ng of target DNA per 50 ng of total DNA) (Fig. 7B). *C. destructans* colonization was confirmed in control roots (3.66 ng per 50 ng of total DNA) and was not detected in GR-treated ginseng roots. Ginseng genes involved in defense (*PR2, PR3, PR4*, and *PR10*) were expressed at significantly lower levels in GR-treated ginseng roots than in the control (Fig. 7C).

3.12. Application of DP and GR agents to MCG fields

DP and GR agents were sprayed onto foliage in the field. The GR agent was dropped instantaneously into soil, while most of the DP agent remained in aerial plant parts. During treatment, *Alternaria* blight disease, caused by *Alternaria panax*, occurred naturally in aerial parts of control plants (94% *Alternaria* incidence), whereas blight disease was significantly inhibited in DP- and GR-treated plants (30 and 28%, respectively) (Fig. 8A). Two months from the last application, DP- and GR-treated MCG roots showed no disease symptoms or growth inhibition.

CF% in DP-treated plants was invariable among the different tissues and was much lower than that in GR-treated plants (Fig. 8B). In GR-treated plants, CF in roots was higher (43%) than in other tissues (leaf, 24%; stem, 16%). *T. citrinoviride* target DNA was observed at higher levels in GR- versus DP-treated plants, with 2.70 and 0.11 ng in below- and above-ground parts, respectively (Fig. 8C).

In below-ground parts, genes involved in ginsenoside biosynthesis (*FPS, DS, PPDS*, and *PPTS*) were expressed at significantly higher levels in GR-treated plants (Fig. 8D). Notably, *DS* was

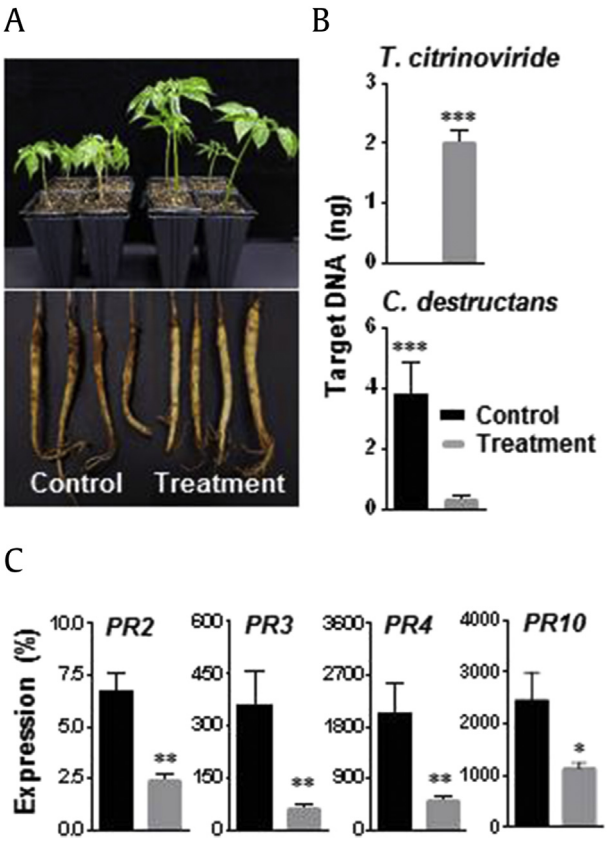

Fig. 5. Biocontrol activity of *Trichoderma citrinoviride* PG87 on root rot disease caused by *Cylindrocarpon destructans*. Ginseng roots were inoculated with *T. citrinoviride* spore suspension through pipetting into the soil within the pot. After 24 h of inoculation, ginseng roots were pulled out from soil, wounded, and infected with *C. destructans* spore suspension. After 3 wk of infection, ginseng roots were collected for fungal quantification and gene expression analysis. **(A)** Photographs showing the biocontrol activity of *T. citrinoviride* against *C. destructans*. **(B)** Quantification of *T. citrinoviride* and *C. destructans* in ginseng roots using QPCR. Target DNA = ng of target DNA in 50 ng of total genomic DNA. **(C)** Expression analysis of ginseng *PR* genes. The ginseng *ACTIN* was used to normalize the relative expression levels. Vertical bars indicate the means with standard error from three biological replications. $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.
QPCR, quantitative real-time polymerase chain reaction.

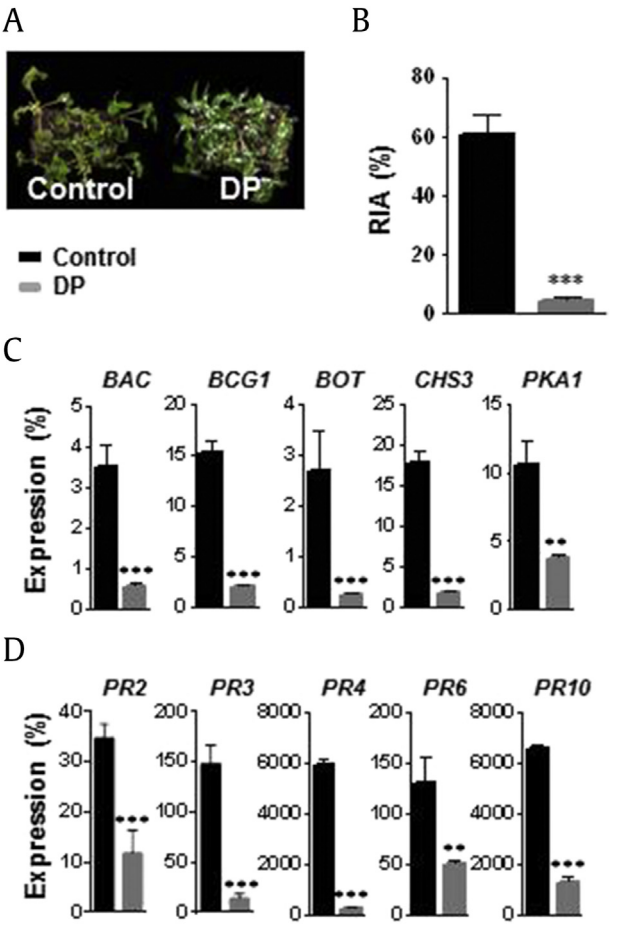

Fig. 6. Biocontrol activity of dustable powder (DP) agent of *Trichoderma citrinoviride* PG87 on leaf spot disease caused by *Botrytis cinerea*. After 1 wk of DP spraying, leaves were inoculated with *B. cinerea* spore suspension through foliar spraying. After 1 wk of inoculation, leaf samples were harvested for the analyses of relative infected area (RIA %), quantification, and gene expression. **(A)** Photographs showing the efficacy of DP agent in biocontrol of the leaf spot disease. **(B)** Relative infected area (RIA %). **(C)** Expression analysis of *B. cinerea* genes. **(D)** Expression analysis of ginseng *PR* genes. *B. cinerea* and ginseng *ACTINs* were used to normalize the relative expression levels. Vertical bars indicate the means with standard error from three biological replications. $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.

induced twofold compared with the control. The expression of all *PR* genes was significantly higher in GR-treated plants, except for *PR3*, which was most highly induced in DP-treated plants. In above-ground parts, only *FPS* and *PPTS* were significantly induced by DP or GR (Fig. 8E). Most *PR* genes were significantly upregulated by GR application in aerial parts, except for *PR6*.

Application of DP and GR significantly induced ZA, ABA, and JA in below-ground parts (Fig. 8F). ZA was highly induced by DP, whereas JA was significantly induced by GR.

The GP agent significantly induced ginsenosides in below-ground parts (Fig. 8G). Both PPD and PPT types were notably increased in GR-treated roots compared with those in DP-treated roots and the control. Ginsenosides Rb1, Rb2, Rc, Re, Rg1, and Rg3 were significantly induced after GR treatment, resulting in high amounts of total ginsenosides. The DP agent showed no effect, except for the induction of Rg1.

4. Discussion

Fungal endophytes living in host plants are valuable natural resources that can be exploited as BCAs because of their beneficial effects on host plant development, growth, and fitness [6,28]. In agriculture, BCAs are recognized as environmental-friendly alternatives to chemical pesticides to control plant pathogens [29]. Although ginseng is an important medicinal plant, research exploring its endophytic community remains in its infancy. Endophytes can induce phytochemical production and defense resistance against pathogens. Herein, we investigated the biocontrol activity of *T. citrinoviride* isolated from MCG. We found that treatment of ginseng plants with *T. citrinoviride* induced disease resistance and ginsenoside accumulation. We conducted *in vitro* and *in vivo* experiments and confirmed the beneficial effects of *T. citrinoviride* in ginseng plants. Several *Trichoderma* spp. are being widely used in agriculture as BCAs against phytopathogens [11]. We observed significant antagonistic behavior of *T. citrinoviride* against ginseng pathogens on pathogen growth through a dual-culture assay. Coiling and/or penetration of *T. citrinoviride* mycelia were observed around or into the hyphae of different ginseng pathogens in the contact area of the dual culture. Coiling is a common response of mycoparasitic *Trichoderma* [30].

BAC stimulates the formation of cyclic adenosine monophosphate (cAMP), and regulates pathogenicity, morphogenesis, and differentiation [31]. Induced cAMP is bound to the regulatory

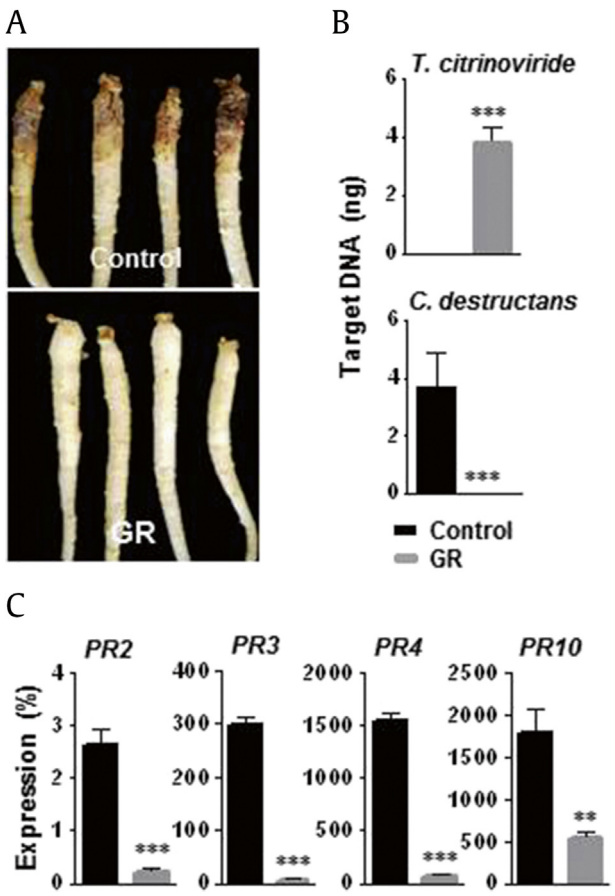

Fig. 7. Biocontrol activity of granule (GR) agent of *Trichoderma citrinoviride* PG87 on root rot disease caused by *Cylindrocarpon destructans*. After 1 wk of GR application into soil, roots were inoculated with *C. destructans* spore suspension by pipetting into the soil. After 3 wk of inoculation, root samples were harvested for the analyses of quantification and gene expression. **(A)** Photographs representing the efficacy of GR agent in biocontrol of the root rot disease. **(B)** Quantification of *T. citrinoviride* and *C. destructans* in ginseng roots using QPCR. **(C)** Expression analysis of ginseng *PR* genes. The ginseng *ACTIN* was used to normalize the relative expression levels. Vertical bars indicate the means with standard error from three biological replications. $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.
QPCR, quantitative real-time polymerase chain reaction.

subunits of *PKA1*, leading to the phosphorylation of target molecules, and this catalytic subunit controlled the growth and virulence of *B. cinerea* [32]. *BCG1* is important for signal transduction and is involved in the regulation of pathogenicity and vegetative growth of *B. cinerea* [33]. Consistent with this, the *BCG1* mutant was shown to lose its pathogenicity and could not produce the phytotoxin botrydial [34]. *BOT1* is involved in botrydial biosynthesis and in the generation of *B. cinerea* disease symptoms and is controlled by signal transduction [35]. During spore germination, *TRE1* regulates carbohydrate metabolism through the decomposition of intercellular trehalose [36]. QPCR confirmed a significant reduction in the expression of *B. cinerea* genes involved in growth and virulence in ginseng plants pretreated with *T. citrinoviride* compared with that in the control. The findings of previous studies strongly support the downregulation of *B. cinerea* genes by fungal endophyte metabolites [37].

Mycoparasitism requires increased levels of CWDEs, and consistent with this, *T. citrinoviride EDG* was highly induced in the dual-culture assay. These results indicated that endoglucanase contributes to the antagonistic activity of *T. citrinoviride* against *B. cinerea*. The results of the enzyme assay revealed that *T. citrinoviride* produces large amounts of enzymes with high β-1,3-glucan-, β-1,4-glucan-, and β-glucoside-degrading activity. These enzymes may attack the cell wall of pathogenic fungi [38]. *T. citrinoviride* produced enzymes with strong activity in the presence of *B. cinerea* and *C. destructans* cell wall components. The lowest level of β-glucosidase activity was observed with all carbon sources compared with other enzymes. The activity of endo-1,4-β-D-glucanase was higher than that of other enzymes when the cell wall components of pathogens were used as a carbon source. This suggests that endo-1,4-β-D-glucanase is important in the degradation of *B. cinerea* and *C. destructans* cell walls. In addition, β-1,3-glucanase might contribute to inhibit *P. cactorum* and *Pythium* spp., because 80–90% of the oomycetes cell wall is β-1,3-glucan [39]. Strong enzyme activity of *T. citrinoviride* is an advantageous property of BCA.

No harmful effect of *T. citrinoviride* inoculation was observed in ginseng plants. This suggests that *T. citrinoviride* functions as a beneficial endophyte in the host. We confirmed the endophytic nature of *T. citrinoviride* by reisolation after inoculation. *T. citrinoviride* was also detected in the above-ground part of the ginseng plant (stem and leaf) after inoculation through root dipping. This is also an advantageous property of a BCA. QPCR confirmed the existence of *T. citrinoviride* as an endophyte in all ginseng tissues.

We analyzed the various plant responses to *T. citrinoviride* inoculation, including the levels of ginsenoside and hormones and the expression patterns of genes involved in ginsenoside biosynthesis and defense. The major pharmacologically active compounds in ginseng are triterpene ginsenosides. Ginsenosides have many pharmacological activities, such as anticancer, neuroprotective, antioxidant, and antidiabetic properties [40]. Based on aglycones, ginsenosides are classified into two groups, namely dammarane and oleanane types. Dammarane-type ginsenosides are further classified into PPD type (Rb1, Rb2, Rc, Rd, Rg3, and F2) and PPT type (Re, Rf, Rg1, Rg2, Rh1, and F1) [36]. Elicitors (methyl jasmonate, salicylic acid, and biotic factors) increase the expression or activities of these enzymes [41,42]. In the present study, *T. citrinoviride* inoculation by root dipping significantly induced the expression of genes involved in ginsenoside biosynthesis (*FPS, SS, SE, DS*, and *PPDS*) in the below-ground parts of ginseng plants, with a slight induction observed in the above-ground parts. This indicates that *T. citrinoviride* functions as an elicitor to stimulate ginsenoside production, resulting in a 1.57-fold induction compared with the control. The PPD type was more highly induced than the PPT type. *T. citrinoviride* inoculation also significantly induced phytohormones (ZA, ABA, and JA) in the below-ground parts 4 wk after root inoculation. ZA and ABA contribute to root development through cell differentiation or elongation and plant immune signaling [43]. ABA acts synergistically on the JA response pathway on wounding or herbivore attack [44]. JA plays a major role in the response to wounding and in systemic acquired resistance [44]. ZA and JA also induce ginsenoside biosynthesis [45,46]. Furthermore, we observed the induction of genes responsible for disease resistance, including *PR2*, *PR3*, *PR4*, *PR5*, *PR6*, and *PR10*. Pathogenesis-related proteins (PRs) exhibit antimicrobial activity and contribute toward plant resistance against pathogen infection. PR proteins constitute β-1,3-glucanases, chitinases, thaumatin-like proteins peroxidases, and ribosome-inactivating proteins [47]. Among these, β-1,3-glucanases and chitinases are the most important in many plant species as they degrade the major component of pathogen cell walls, β-1,3-glucan and chitin [48]. PR2 is composed of β-1,3-glucanases and is rapidly activated and accumulated in response to pathogen infection [49]. As chitinases, PR3 and PR4 are specialized in the degradation of fungal cell walls and exhibit

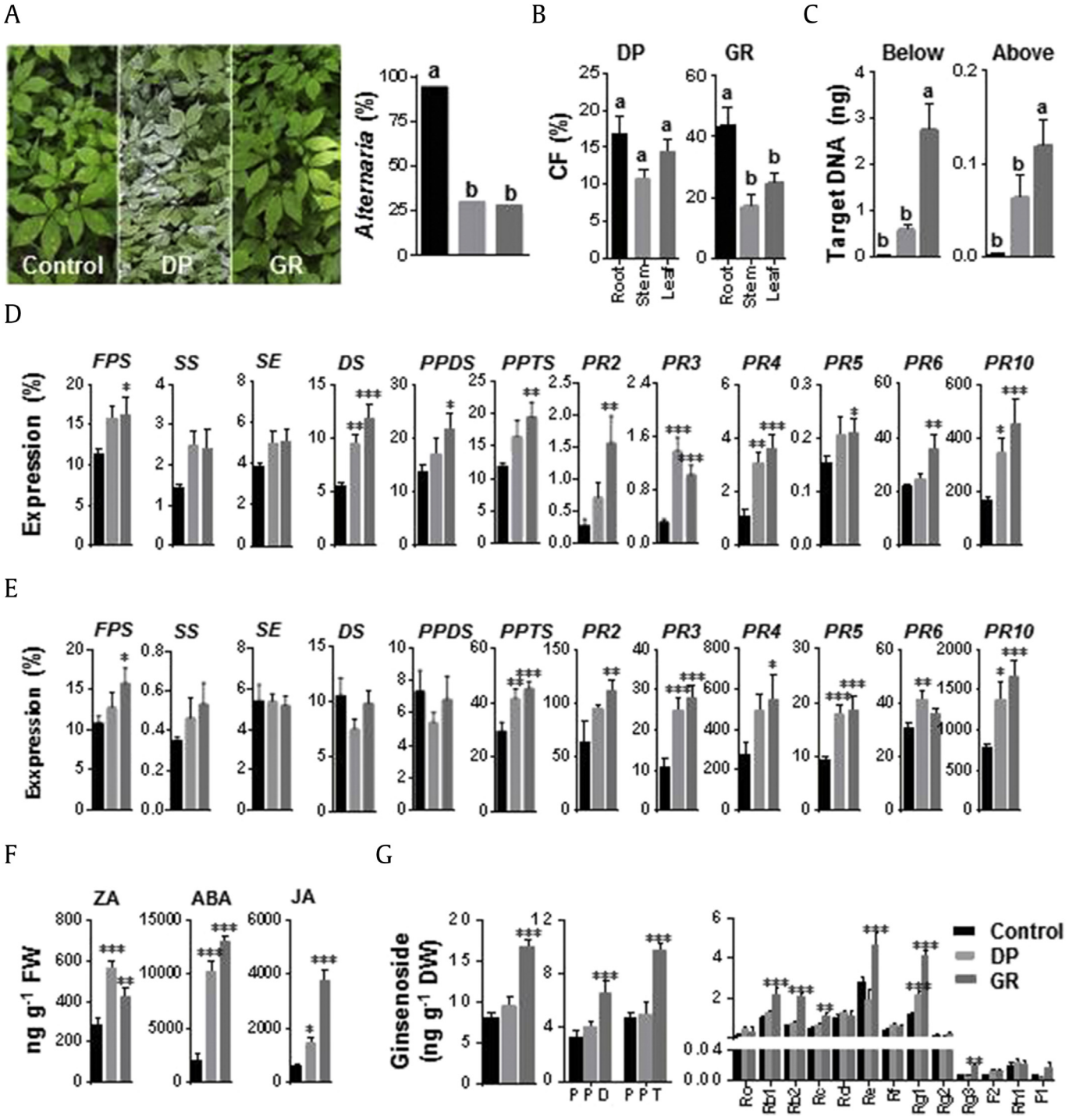

Fig. 8. Biocontrol activity and endophytism of *Trichoderma citrinoviride* PG87–formulated agents (dustable powder, DP and granule, GR) in mountain-cultivated ginseng (MCG) field. **(A)** Photographs showing the efficacy of DP and GR agents in biocontrol of *Alternaria panax* disease and *Alternaria* incidence (%) in MCG leaves. **(B)** Colonization frequency (%) of *T. citrinoviride* in three different tissues of DP- and GR-treated MCG plants. **(C)** Quantification of *T. citrinoviride* colonization in below- and above-ground parts of MCG plants using QPCR. **(D)** Expression of ginseng genes involved in ginsenoside biosynthesis (*FPS*, *SS*, *SE*, *DS*, *PPDS*, and *PPTS*) and defense (*PR2*, *PR3*, *PR4*, *PR5*, *PR6*, and *PR10*) in below-ground parts of ginseng. **(E)** Expression of ginseng genes involved in ginsenoside biosynthesis (*FPS*, *SS*, *SE*, *DS*, *PPDS*, and *PPTS*) and defense (*PR2*, *PR3*, *PR4*, *PR5*, *PR6*, and *PR10*) in above-ground parts of ginseng. Ginseng *ACTIN* was used to normalize the relative expression levels. **(F)** Changes of hormone levels in ginseng roots. **(G)** The total and each ginsenoside content of PPD (Rb1, Rb2, Rc, Rd, Rg3, and F2) and PPT type (Re, Rf, Rg1, Rg2, Rh1, and F1). These experiments were conducted with six biological replications. Vertical bars indicate the means with standard error. Significance of the relative values is indicated by different letters on the means as per the Duncan's multiple range test ($p < 0.05$). $^{*}p < 0.1$, $^{**}p < 0.05$, and $^{***}p < 0.01$ represent significant difference with respect to control.

ABA, abscisic acid; JA, jasmonic acid; PPD, Protopanaxadiol; PPT, protopanaxatriol; QPCR, quantitative real-time polymerase chain reaction; ZA, zeatin.

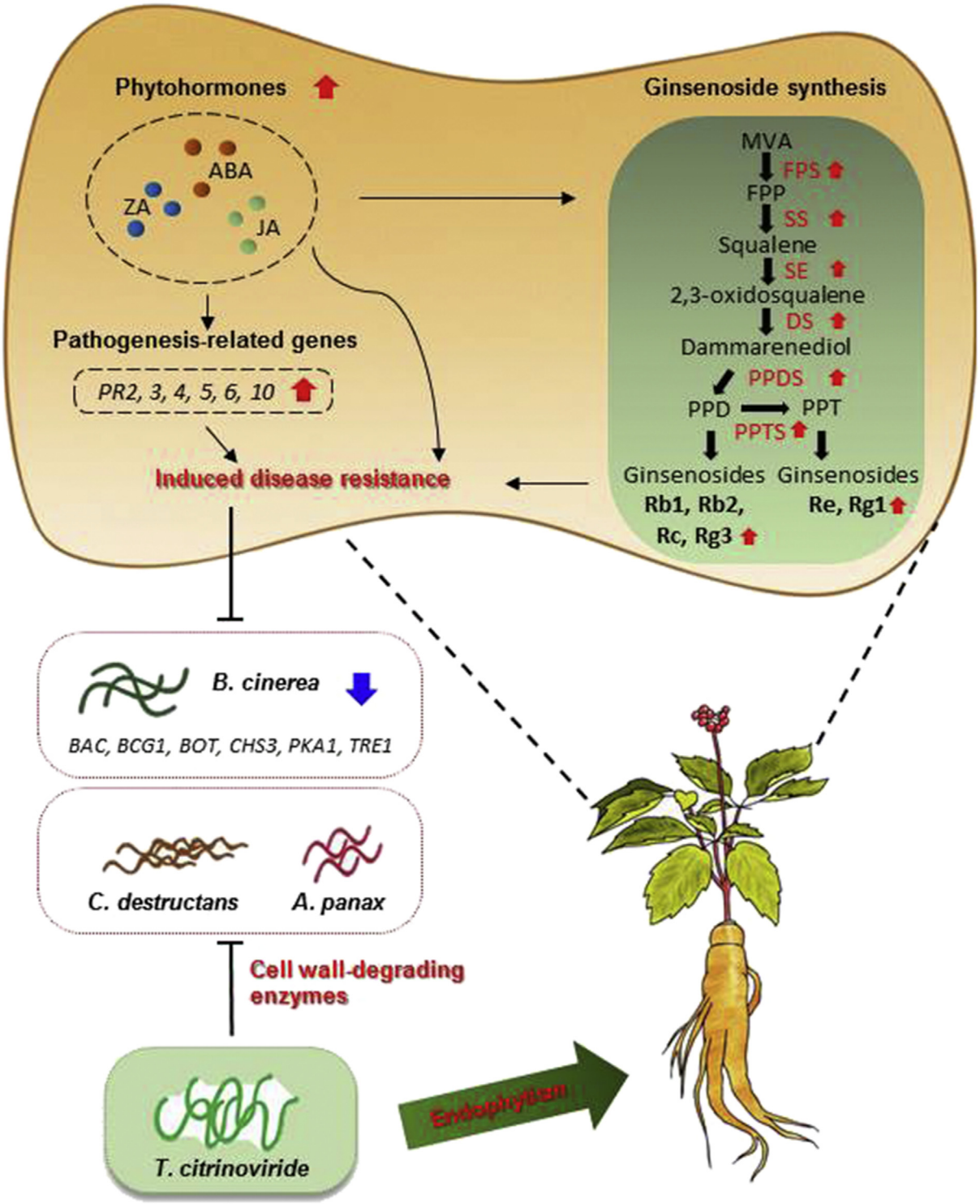

Fig. 9. Summary of the efficacy of *Trichoderma citrinoviride* PG87 in biocontrol of ginseng pathogens. *T. citrinoviride*, fungal endophyte of mountain-cultivated ginseng, has great potential as biocontrol agent against ginseng pathogens. *T. citrinoviride* can heavily colonize ginseng plants as a beneficial symbiont. *T. citrinoviride* can directly inhibit the growth of ginseng pathogens through mycoparasitism (e.g., cell wall–degrading enzymes) and induce disease resistance and ginsenoside accumulation in ginseng plants. Various mechanisms might be involved in biocontrol activity against ginseng pathogens: downregulation of *B. cinerea* genes involved in growth and virulence, upregulation of ginseng genes involved in pathogenesis-related proteins (PRs), production of cell wall-degrading enzymes and phytohormones, priming effect, and so forth.
ABA, abscisic acid; FPP, farnesyl pyrophosphate; JA, jasmonic acid; MVA, mevalonate; PPD, Protopanaxadiol; PPT, protopanaxatriol; ZA, zeatin.

synergism with β-1,3-glucanases [50]. PR5 consists of thaumtin-like proteins, which have roles in the plant defense against various stress conditions and pathogen infection [51]. PR6 and PR10 act as a protease inhibitor and a ribonuclease, respectively, with essential roles in plant defense, development, and disease resistance [52]. We found that PR2, PR5, PR6, and PR10 were significantly upregulated in the above-ground parts of plants, whereas only PR3 and PR4 were induced and retained constantly in the

below-ground parts after *T. citrinoviride* inoculation by root dipping. These results support the notion that *T. citrinoviride* inoculation can induce ginseng plant immunity against pathogens through *PR* gene induction. Therefore, *T. citrinoviride* inoculation has a "priming effect". Priming occurs when pretreatment with a mild stress or chemical improves the efficiency of plant defense mechanisms. The priming effect of fungal endophytes is well known in diverse plant species [53]. The priming effect and induction of disease resistance were confirmed using an *in planta* assay against *B. cinerea*. Leaves of ginseng plants preinoculated with *T. citrinoviride* by root dipping had significantly reduced symptoms on subsequent infection with *B. cinerea* (100 vs. 11.5% RIA in the control and treatment groups, respectively). Furthermore, genes involved in defense (*PR2, PR4, PR5*, and *PR10*) were significantly induced in ginseng leaves preinoculated with *T. citrinoviride* by root dipping compared with the control. In addition, the expression of *B. cinerea* genes (*BAC*, *BCG1*, *BOT*, *CHS3*, *PKA1*, and *TRE1*) was significantly reduced in ginseng leaves preinoculated with *T. citrinoviride* by root dipping. *CHS3*, which encodes chitin synthase 3, is important for hyphal growth [54]. The expression of *CHS3* was inhibited in preinoculated plant leaves, indicating the suppression of *B. cinerea* growth. Previous studies have shown induced systemic resistance to *B. cinerea* through enhanced levels of PR proteins or phytohormones in plants preexposed to *Trichoderma harzianum* [55,56]. These results confirmed that the enhanced levels of phytohormones and *PR* genes after *T. citrinoviride* preinoculation may explain the improved disease resistance against *B. cinerea* via priming.

In addition to a priming effect, *T. citrinoviride* could directly inhibit *B. cinerea* growth and pathogenicity on ginseng leaves. Preinoculation of ginseng leaves with *T. citrinoviride* induced significant resistance against *B. cinerea*. Significant downregulation of *B. cinerea BOT1* also confirmed the direct antimicrobial activity of *T. citrinoviride* against *B. cinerea*. *BOT1* deletion significantly reduced *B. cinerea* virulence in plants [35]. Interestingly, ginseng defense genes were significantly downregulated in *T. citrinoviride*-treated leaves. This is probably because the growth and pathogenicity of *B. cinerea* were directly suppressed by *T. citrinoviride* at an early time point. Therefore, priming is not necessary. This result also demonstrates the potential of *T. citrinoviride* as a BCA. Similar direct inhibitory activity of *T. citrinoviride* was found in ginseng root against *C. destructans*. Preinoculation of ginseng roots with *T. citrinoviride* significantly downregulated ginseng defense-related genes.

Several species of *Trichoderma* (*T. harzianum*, *T. hamatum*, and *T. viride*) have been exploited as BCAs with various mechanisms, including mycoparasitism, antibiosis, induced resistance, and competition for nutrients. However, this is the first report to show the potential of endophytic *T. citrinoviride* as a BCA. Generally, BCAs are grown in their natural habitat and combat both aerial and below-ground diseases [57]. BCA formulation is a key process for functional success [58]. Effective formulation requires the consideration of various components, such as the biocontrol organism, pathogen, environment, application practices, and equipment. Typically, dry formulations (powder or granule) provide a long shelf life and convenience for storage and treatment [59]. Therefore, we developed DP and GR agents for application onto aerial and below-ground tissues of MCG, respectively. The GR agent showed consistent stability for microbe survival and physical properties in the field. Application of formulated *T. citrinoviride* exhibited a greater antagonistic effect on *B. cinerea* and *C. destructans* compared with the application of *T. citrinoviride* spore suspension. The DP agent was superior for foliar disease caused by *B. cinerea*, whereas the GR agent was better for root disease caused by *C. destructans*.

The formulated BCAs were also tested in the MCG field under natural conditions. The GR agent applied to the ginseng plant eventually reached the soil and more heavily colonized the plant compared with the DP agent. The incidence of foliar disease caused by *A. panax*, which occurred naturally during the application period of formulated *T. citrinoviride* agents, was significantly reduced by DP and GR agents. The GR agent exhibited better colonization in all ginseng tissues. These results indicate that the GR agent has greater potential as a BCA for the control of ginseng pathogens both in the aerial and below-ground parts of MCG. In addition, GR treatment induced a significant accumulation of ginsenosides, which were induced more than twofold compared with the control root.

In summary, the fungal endophyte isolated from MCG, *T. citrinoviride*, has great potential as a BCA against ginseng pathogens (Fig. 9). *T. citrinoviride* can grow heavily inside ginseng plants, where it lives as a beneficial endophyte. *T. citrinoviride* can directly inhibit the growth of ginseng pathogens and induce disease resistance and ginsenoside accumulation in ginseng plants. Various mechanisms might be involved in biocontrol activity against ginseng pathogens, such as inhibition of *B. cinerea* genes involved in growth and virulence, production of CWDEs and phytohormones, and priming. Taken together, our results revealed that *T. citrinoviride*-colonizing MCG has great potential as a BCA and an elicitor of ginsenoside production. In accordance with green agriculture, this would represent a sustainable model of MCG cultivation.

Conflicts of interest

The authors declare that there are not conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2018.03.002.

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