



Research Article

Pathogenesis strategies and regulation of ginsenosides by two species of *Ilyonectria* in *Panax ginseng*: power of speciation

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ABSTRACT

Background: The valuable medicinal plant *Panax ginseng* has high pharmaceutical efficacy because it produces ginsenosides. However, its yields decline because of a root-rot disease caused by *Ilyonectria mors-panacis*. Because species within *Ilyonectria* showed variable aggressiveness by altering ginsenoside concentrations in inoculated plants, we investigated how such infections might regulate the biosynthesis of ginsenosides and their related signaling molecules.

Methods: Two-year-old ginseng seedlings were treated with *I. mors-panacis* and *I. robusta*. Roots from infected and pathogen-free plants were harvested at 4 and 16 days after inoculation. We then examined levels of and expression of genes of ginsenosides, salicylic acid (SA), jasmonic acid (JA), and reactive oxygen species (ROS). We also checked the susceptibility of those pathogens to ROS.

Results: Ginsenoside biosynthesis was significantly suppressed and increased in response to infection by *I. mors-panacis* and *I. robusta*, respectively. Regulation of JA was significantly higher in *I. robusta*-infected roots, while levels of SA and ROS were significantly higher in *I. mors-panacis*-infected roots. Catalase activity was significantly higher in *I. robusta*-infected roots followed in order by mock roots and those infected by *I. mors-panacis*. Moreover, *I. mors-panacis* was resistant to ROS compared with *I. robusta*.

Conclusion: Infection by the weakly aggressive *I. robusta* led to the upregulation of ginsenoside production and biosynthesis, probably because only a low level of ROS was induced. In contrast, the more aggressive *I. mors-panacis* suppressed ginsenoside biosynthesis, probably because of higher ROS levels and subsequent induction of programmed cell death pathways. Furthermore, *I. mors-panacis* may have increased its virulence by resisting the cytotoxicity of ROS.

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1. Introduction

Root-rot diseases present the most devastating threat to many crops. At each stage of their development, infected plants produced fewer and/or smaller leaves, flowers, and fruits when compared with healthy plants. The responsible organisms are necrotrophic soil-borne oomycetous and fungal pathogens that inhabit the rhizosphere. Genera such as *Pythium*, *Phytophthora*, *Fusarium*, *Sclerotinia*, *Rhizoctonia*, *Verticillium*, and *Ilyonectria* affect a wide range of economically important hosts, including *Solanum tuberosum*, *Beta vulgaris*, *Spinacia oleracea*, *Brassica oleracea*, *Cucumis sativus*, *Cicer arietinum*, and *Panax ginseng* (ginseng) [1–8].

Investigations of the molecular mechanism(s) used by necrotrophic pathogens have shown that, at the early stage of infection, reactive oxygen species (ROS) are induced by fungal mycotoxins, serving as an antimicrobial agent to suppress those pathogens. However, prolonged exposure of plant cells to ROS suppresses their defense systems and activates programmed cell death (PCD). Therefore, ROS have a double role, acting as either defense stimulator or cell damaging agents depending on their concentrations [9–18]. Such molecular interactions have been investigated between *Fusarium* species and *Triticum aestivum*, as well as between *Botrytis cinerea* and *Arabidopsis thaliana* [19–21]. Although little is known about other plant–necrotrophic pathogen

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models, research results have indicated that an equilibrium is necessary between optimum ROS levels and the maintenance of signaling against abiotic and biotic stresses [9,11,22–24]

The generation of ROS is mediated dynamically by salicylic acid (SA) to stabilize plant tolerance against various environmental challenges, especially pathogen attacks [25–32]. Both ROS and SA induce the production of plant secondary metabolites, particularly terpenoids, which help confer stress tolerance [33–39]. This process is primarily mediated either directly by SA-induced ROS or indirectly through the induction of jasmonic acid (JA) production [35,39]. However, under oxidative stress conditions, ROS may not have a mediating role in signaling [39].

Panax ginseng, a member of the Araliaceae family, has long been used as a source of Asian medicines and is considered an important cash crop in Korea and China because of its pharmaceutical properties [40,41] that are derived by triterpenoid, dammarane-type saponins called ginsenosides [42,43]. Ginsenosides are thought to have a physiological role in protecting ginseng roots from attack by soil-borne pathogenic fungi. In the adventitious roots of those plants, ginsenoside accumulations are correlated with H₂O₂-mediated production of JA [35]. Furthermore, the addition of SA elicits those accumulations in a concentration-dependent manner [44], which then triggers the generation of ROS along with their antioxidant scavengers [38]. However, it is unclear whether high concentrations of ROS adversely affect the regulation of ginsenoside biosynthesis.

As a slow-growing perennial herb, ginseng requires several consecutive seasons of optimal growth to ensure that plants have the highest concentrations of ginsenosides. Therefore, these plants are vulnerable over time to several fungal diseases, including root rots [3]. Being the most devastating, root-rot diseases can reduce yields in ginseng cultivation fields by 20%. The causal agent is reported to be the necrotrophic fungus *Ilyonectria mors-panacis* [3,45,46], a pathogen that aggressively resulted in ginsenosides suppression. In contrast, infections by weaker species in that genus, e.g., *I. robusta*, can result in increased ginsenoside production. This has been demonstrated in pathogenicity experiments for both species where the levels of major ginsenosides were increased in *I. robusta*-infected roots but reduced in *I. mors-panacis*-infected roots at 28 days postinoculation (dpi). However, on media containing ginsenosides, the growth rate was higher for the less pathogenic species than for the more aggressive species. We also noted that *I. robusta*, but not *I. mors-panacis*, was able to partially degrade the ginsenosides, especially the protopanaxdiol type [47]. Based on these opposing responses, we hypothesized that the mechanism by which each of those species changes ginsenoside content determines the extent of their pathogenicity. Because the role of ginsenosides in biotic stress responses, including root-rot disease, and molecular mechanisms remains unclear, we performed transcriptional, metabolic, and biochemical analyses to examine how the regulation of ginsenosides and their signaling molecules (e.g., JA, SA, or ROS) is changed during the onset of root-rot disease.

2. Materials and methods

2.1. Plant and microbial materials

Two-year-old ginseng seedlings, obtained from the Ginseng Bank (Kyung-Hee University, Yongin, Korea), were used to test the time-dependent effects on ginsenoside regulation after plants were inoculated with either the highly aggressive *I. mors-panacis* HB11 or the weaker *I. robusta* HB3. Those two species and strains were chosen for comparative analysis based on the results from previous experiments [47].

2.2. Pathogen infections

Similarly sized, healthy roots containing rhizomes were infected by *I. mors-panacis* HB11 or *I. robusta* HB3, as described previously [47], but with some modifications. In brief, each isolate was grown on potato dextrose agar (PDA) in the dark at 25°C. After 10 days, equally sized plugs were taken from colonies of each strain and transcultured for another 14 days in clarified V8 broth (10 plugs/flask) in the dark at 25°C with shaking (150 rpm). Each mycelial mat was then filtered, blended with 50 mL of sterilized water, and thoroughly mixed with sterilized artificial soil to a final concentration of 10%. The same concentration of sterilized water-mixed soil was prepared as the mock control. Infected soil samples were transferred to 15-cm pots, in which 2-year-old ginseng roots (six per pot) of similar length, width, and weight were cultivated for 28 days under greenhouse conditions (22 ± 2°C, 12-h photoperiod). At two time points during this experimental period—4 dpi and 16 dpi—roots showing similar symptoms were harvested from each treatment group for estimations of ginsenoside, SA, and H₂O₂ concentrations; RNA extractions; and measurements of catalase activity. Our mock control was pathogen-free (noninoculated) roots. Samples collected for ginsenoside analysis and RNA extraction were stored at –80°C, while freshly harvested roots were used for catalase and H₂O₂ determinations.

2.3. RNA extraction and quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA was extracted from frozen samples using TRI Reagent (Molecular Research Center, Inc, Cincinnati, USA) according to the manufacturer's instructions. Samples (1 µg each) were reverse-transcribed with RevertAid™ HMinus M-MuLV Reverse Transcriptase (Fermentas, Massachusetts, USA) according to the manufacturer's instructions. Reverse transcriptase polymerase chain reaction (PCR) was performed with a MyCycler™ thermal cycler (Bio-Rad, California, USA), using a reaction volume of 15 µL that comprised 1 µL of synthesized cDNA, 10 µM of forward and reverse primers for each target gene (Table S1), 5.5 µL of Premix Taq™ DNA Polymerase (Takara Bio USA, Inc., California, USA), and water to make up to the final volume. Each reaction involved an initial denaturation at 96°C for 5 min and then 30 cycles at 95°C for 10 s, 56°C for 10 s, and 72°C for 20 s, followed by a final elongation at 72°C for 5 min. The PCR products were visually analyzed on 1% agarose gels.

Quantitative real-time PCR was conducted with a 10 µL reaction volume that consisted of 1 µL of cDNA, 10 µM of forward and reverse primers for each target transcript (Table S1), and 2X iQ™ SYBR® Green Supermix (Bio-Rad, California, USA). The reactions were performed with a CFX Connect™ Real-Time System (Bio-Rad, California, USA) in Hard-Shell® 96-well PCR plates (Bio-Rad, California, USA). The housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as a control, as recommended previously [48]. Thermal cycler conditions recommended by the manufacturer included 95°C for 3 min and then 39 cycles at 95°C for 10 s and 56°C for 30 s, followed by a final 95°C for 10 s and 65°C to 95°C for 5 s, with 0.5°C increments for melt curve analysis. The fluorescent product was detected during the final step of each cycle. Amplification, detection, and data analysis were performed with CFX Manager™ Software, version 3.1 (Bio-Rad, California, USA). To determine relative fold differences in template abundance for each sample, we normalized the C_T value for each expressed gene to the C_T value for *GAPDH*, using the formula $2^{-\Delta\Delta C_T}$.

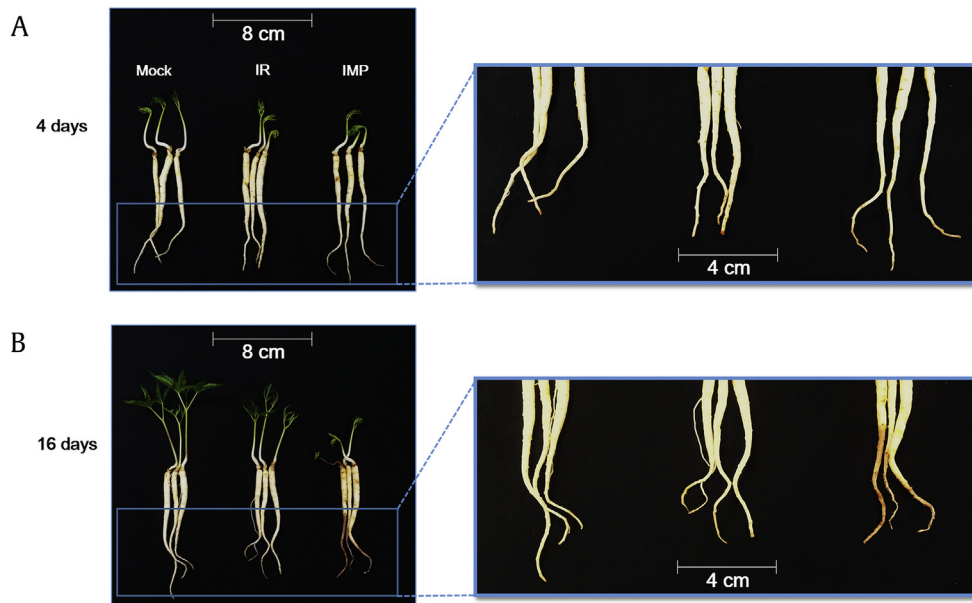


Fig. 1. Morphology of roots from 2-year-old ginseng seedlings infected by *Ilyonectria robusta* HB3 (IR), *I. mors-panacis* HB11 (IMP), or no pathogen (mock), at 4 dpi (A) and 16 dpi (B), days postinoculation.

2.4. Ginsenoside extraction and quantification by HPLC analysis

Total ginsenosides were extracted, purified, and estimated by HPLC, as described previously [47], with some modifications. In brief, the root samples were frozen in liquid nitrogen and then ground into powder with a mortar. The powder was placed in a round flask, mixed with 40 mL of 80% methanol, and heated at 80°C in a Soxhlet water bath. After 2 h, the methanol extract was collected and rotary-evaporated at 45°C. After the dried samples were redissolved in 20 mL of distilled water, their ginsenosides were extracted using 20 mL of water-saturated *n*-butanol. The butanol layers were collected and rotary-evaporated at 55°C. Crude ginsenosides from those dried samples were redissolved in 2 mL of methanol and passed through 0.2- μ m filters before being subjected to HPLC for quantification, as described previously [49].

2.5. Estimation of root SA concentrations via LC-Electrospray Ionization (ESI)-MS/MS

Levels of SA in the roots were determined after extraction and quantification were performed as described previously [50], with some modifications. Frozen samples (500 mg each) were grounded with liquid nitrogen and then mixed with 5 mL of extraction solution [2-propanol/H₂O/conc. HCl (2:1:0.002, v/v/v)]. Extraction efficiency was enhanced by shaking the mixture (100 rpm for 30 min) at 4°C. This phytohormone was isolated from the extract by adding 10 mL of dichloromethane and then shaking (100 rpm for 30 min) at 4°C. Afterward, the samples were centrifuged (1000 g at 4°C for 5 min), and the down layer of each was carefully collected and evaporated at 40°C before each dried residue was dissolved in 400 μ L of HPLC methanol before LC-ESI-MS/MS analysis [50].

2.6. Estimation of H₂O₂ production

Fresh root samples (1 g each) were ground to powder with liquid nitrogen and then extracted using 2 mL of cold acetone. The extract was collected by centrifugation (5,000 g at 4°C for 5 min) and then allowed to interact with 20% titanous tetrachloride in concentrated HCl (1:10, v/v) for 5 min. As described previously

[38,51], this process enabled us to determine the concentration of H₂O₂, which was based on the formation of the yellow-colored precipitate of the H₂O₂–titanium complex. For the blank samples, only water was mixed with the titanium solution. All reactions were stopped by the addition of concentrated NH₄OH. The supernatant was discarded by centrifugation (10,000 g for 5 min), and the precipitate was dissolved in 2 N H₂SO₄. Absorbance of the eluted precipitate was evaluated spectrophotometrically (415 nm wavelength), and the concentration was estimated according to the standard curve made by colored precipitates that consisted of different known concentrations of H₂O₂ and titanium.

2.7. Estimation of catalase activity

Activity of the antioxidant enzyme catalase was assayed as described previously [38]. For each treatment, 1 g of fresh root sample was powdered using liquid nitrogen. Total enzymes were extracted with 4 mL of extraction buffer [50 mM of potassium phosphate buffer (pH 7) containing 1% (w/v) insoluble polyvinylpyrrolidone and 1 mM phenylmethylsulfonylfluoride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and 0.2% (v/v) Triton X-100]. After collection via centrifugation (5,000 g at 4°C for 10 min), an amount of crude enzyme equivalent to 20 mg per sample was mixed with 500 μ mol of H₂O₂ in 10 mL of 100 mM phosphate buffer (pH 7) and kept for 5 min. For the blank, the same volume of water was mixed with the H₂O₂ solution. The reactions were stopped by the addition of 5 mL of 2 N H₂SO₄. Decomposition of H₂O₂ after a 5-min reaction was estimated by reading the absorbance at 240 nm. The blanks were maintained by adding 5 mL of 2 N H₂SO₄. Catalase activity was expressed as units per milligram protein.

2.8. In vitro assay of pathogen sensitivity to H₂O₂

Sensitivity of the two pathogens to H₂O₂ was estimated as follows. The H₂O₂ was filter-sterilized, and 8, 16, and 24 mM was added to autoclave-warmed PDA media before being poured into Petri plates. After solidification, discs were taken, using a cork borer, from the edges of 10-day-old colonies of each fungus and

transferred to the middle of H₂O₂-amended PDA plates. For the control, discs were placed on H₂O₂-free PDA plates. For each strain, the growth rate on H₂O₂ was calculated according to the following formula:

$$100 - [(dc - dt) / (dc) \times 100]$$

Where dc is the diameter of the fungal colony from the control, and dt is the diameter of the fungal colony from the H₂O₂ treatment.

2.9. Statistical analyses

For each tested pathogen, four pots (each containing eight roots) were set up for the isolate and the control treatments, and independent experiments were conducted three times. At each time point—4 dpi and 16 dpi—pairs of roots showing similar symptoms were collected to examine their concentrations of ginsenosides, SA, and H₂O₂; RNA expression; and catalase activity. For the sensitivity assays, each strain was incubated on two plates with or without H₂O₂, and the experiments were conducted three times. All results were presented as mean values of the three experiments \pm standard error (SE). Data for concentrations and growth rates were statistically analyzed via analysis of variance, using Tukey's tests to compare among treatments. The results from expression analysis were investigated with Student's *t*-tests. Differences with *P*-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Effects of *Ilyonectria* infection on ginseng seedling physiology

To determine how different species of pathogens vary in their effects on ginsenoside biosynthesis, we inoculated ginseng seedlings with either the highly aggressive *Ilyonectria mors-panacis* or the weaker *I. robusta*. Biochemical and molecular changes in the plants were monitored for up to 16 days, and the results were compared with the performance of mock (pathogen-free) seedlings. During the early stage (4 dpi), rhizome development was similar between the infected and the control seedlings, and their growth rates were constant. However, their root morphologies were slightly altered, especially for plants infected by the more aggressive *I. mors-panacis*. Those symptoms included a brown discoloration at the tip of the tap root. Roots infected with the weaker species were morphologically similar to those from the mock samples (Fig. 1A). At 16 dpi, the morphology of the infected seedlings, especially those receiving the *I. mors-panacis* treatment, was drastically different from that of noninfected roots. Furthermore, growth of the foliar portions was inhibited in the *I. mors-panacis* group, and some of those seedlings displayed discoloration on their stems and leaves. Their roots were softened and dark brown discoloration extended from the tip of the tap root to the midportion. In contrast, the foliage and roots from seedlings infected by *I. robusta* were not drastically changed but were similar to the mock samples. For the *I. robusta* treatment group, foliar growth was only slightly reduced from the control rate but was not affected as much when compared with the *I. mors-panacis*-infected seedlings (Fig. 1B).

3.2. Regulation of ginsenoside production

Because RNA was not intact at the time point of 28 dpi, which showed a decreased level of ginsenosides of *I. mors-panacis* infection [47], we analyzed earlier infect point. Quantitative analysis indicated no significant differences in ginsenoside levels at 4 dpi among roots from the infected and mock treatments. At 16 dpi, however,

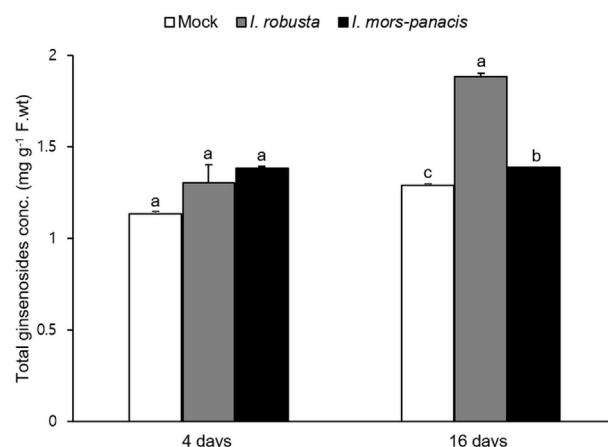


Fig. 2. Quantification of total ginsenosides in roots infected by *Ilyonectria robusta* or *I. mors-panacis* compared with pathogen-free roots (mock). Measurements were made at 4 dpi and 16 dpi. Values are expressed as means \pm standard error. For each time point, data not labeled with same letter are significantly different ($P < 0.05$, Tukey's test; $n = 3$). F.wt, fresh weight.

ginsenoside concentrations were significantly increased over mock levels in roots infected by that weaker pathogen. In contrast, the amount of ginsenoside did not change significantly in *I. mors-panacis*-infected roots between 4 to 16 dpi (Fig. 2). At both time points, expression of genes for ginsenoside biosynthesis—*P. ginseng farnesyl pyrophosphate synthase* (PgFPS), *P. ginseng squalene synthase 1* (PgSS1), *P. ginseng squalene epoxidase 1* (PgSE1), and *P. ginseng dammarenediol synthase* (PgDDS)—was significantly upregulated in *I. robusta*-infected roots when compared with the mock treatment. For the *I. mors-panacis* group, expression at 4 dpi was similar to that determined from the mock samples but was significantly reduced from control levels at 16 dpi in those infected roots (Fig. 3).

Our results experimentally validated an earlier hypothesis that (1) the total major ginsenosides may intervene in the defense mechanism against weakly aggressive species of *Ilyonectria* and (2) the reduction in the synthesis of those ginsenosides is not caused by detoxification activity when ginseng plants become infected with *I. mors-panacis* but is instead a result of downregulated expression of transcripts that control ginsenoside production [47]. One of the possibilities of unchanged level of ginsenosides even biosynthetic genes were decreased at 4–16 dpi is that the movement of metabolites from leaves to roots to complement at earlier defense response, which needs further test to know how ginsenosides move for defense mechanisms [52].

3.3. Regulation of ginsenoside signaling molecules

Because JA and SA participate in the induction of ginsenoside biosynthesis, we monitored the expression of *lipoxygenase 6* (PgLOX6) and *phenylalanine ammonia-lyase 1* (PgPAL1), which positively regulate JA [53] and SA, respectively. At 4 dpi, expression of the former gene was not significantly increased in roots infected by either pathogen when compared with the mock roots. However, at 16 dpi, its expression was significantly increased in roots infected only by *I. robusta* but was not changed in those infected by *I. mors-panacis* (Fig. 4). In contrast, expression of PgPAL1 was significantly increased in infected roots, regardless of the pathogen species, with transcript levels being highest in the *I. mors-panacis* treatment group (Fig. 4). To confirm that SA is synthesized via the pathway for phenylalanine ammonia lyase, we determined the concentration of the SA metabolite and investigated the expression of two SA-triggered pathogenesis-related (PR) genes, PgPR2 and PgPR5. SA

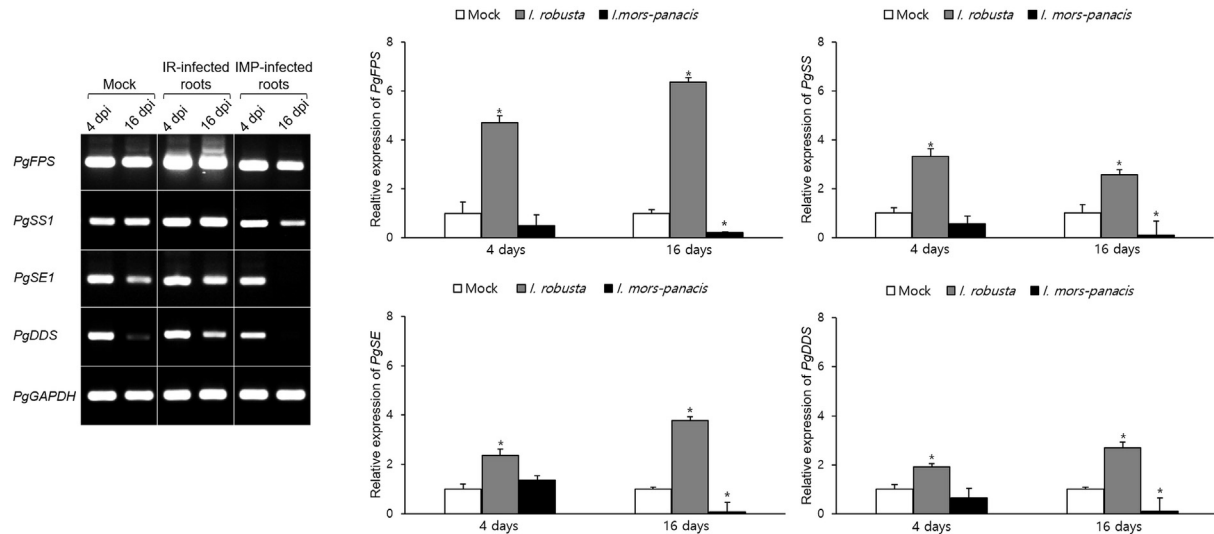


Fig. 3. Relative expression profiles of genes related to ginsenoside biosynthesis in ginseng roots infected by *Ilyonectria robusta* (IR) or *I. mors-panacis* (IMP) versus pathogen-free roots (mock). *PgFPS*, farnesyl pyrophosphate synthase of *Panax ginseng*; *PgSS1*, squalene synthase 1 of *P. ginseng*; *PgSE1*, squalene epoxidase 1 of *P. ginseng*; *PgDDS*, dammareniol synthase of *P. ginseng*; *PgGAPDH*, glyceraldehyde 3-phosphate dehydrogenase of *P. ginseng*; dpi, days postinoculation. Values are expressed as means \pm SE, based on Student's *t*-tests. *Differences between treatments are statistically significant ($P < 0.05$; $n = 3$).

levels were positively correlated with the expression pattern of *PgPAL1* (Fig. 5A), whereas they were not correlated with the expression of those PR genes. For example, expression of *PgPR2* and *PgPR5* was significantly increased in *I. robusta*-infected roots, particularly at 4 dpi. In contrast, expression of both genes was drastically reduced in the *I. mors-panacis*-infected roots over time, especially at 16 dpi (Fig. 6). We also calculated the concentrations of H_2O_2 , the second messenger that regulates ginsenosides upstream [35]. For both pathogen treatments, H_2O_2 was more abundant in the infected roots than in the mock samples. In particular, the increment was significantly greater in the *I. mors-panacis* group at both time points. However, for the *I. robusta*-infected roots, the amount of H_2O_2 was significantly increased over mock levels only at 4 dpi (Fig. 5B).

All these data indicated that the genes for ginsenoside biosynthesis were upregulated in response to infection by *I. robusta*, probably through the induction of their mediators H_2O_2 and JA. We noted that stimulation of ginsenoside production by SA was in a concentration-dependent manner. In fact, previous study has shown that higher levels of that phytohormone can inhibit such biosynthesis in ginseng hairy roots [44]. Furthermore, a high

concentration of SA is associated with the degradation of NPR1, an activator of SA-dependent PR genes in *Arabidopsis thaliana*, which ultimately leads to the suppression of those genes and the induction of PCD [54]. Those earlier findings support our conclusion that the downregulation of PR genes in *I. mors-panacis*-infected roots was mediated by the high concentration of SA. An excess accumulation of ROS also has harmful effects, causing oxidation to major cellular components such as lipids, proteins, and DNA, which finally induces PCD [13–18] and cannot also induce further activity by pathways for secondary metabolites [39]. Based on those reports, we believe our results suggest the presence of a similar induction mechanism in *I. mors-panacis*-infected roots.

3.4. Regulation of catalase activity

The signaling properties of ROS are usually associated with elevated antioxidant activity [38]. Because catalase specifically acts in detoxifying H_2O_2 , we monitored the levels of this antioxidant enzyme in infected roots and those collected from mock-treated plants. Although catalase was detected in all samples, activity was highest in roots infected by the weakly aggressive *I. robusta*,

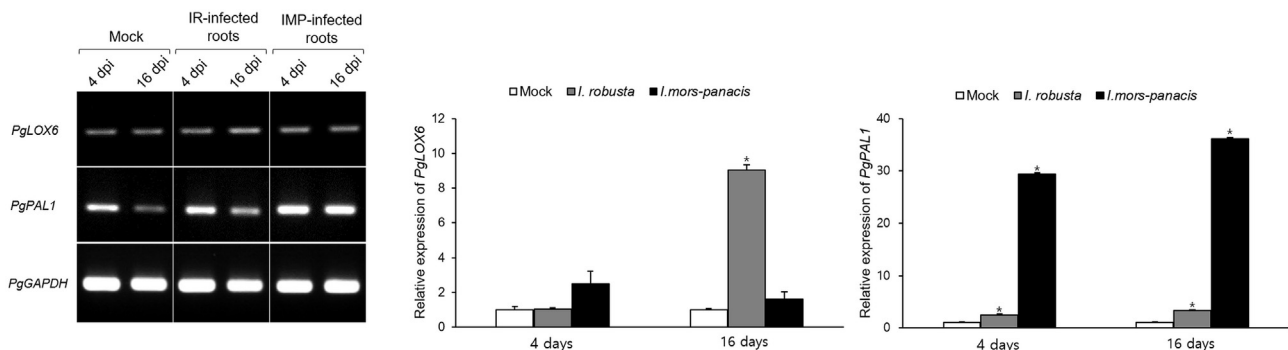


Fig. 4. Relative expression profiles of genes related to JA and SA biosynthesis in ginseng roots infected by *Ilyonectria robusta* (IR) or *I. mors-panacis* (IMP) versus pathogen-free roots (mock). SA, salicylic acid; JA, jasmonic acid; dpi, days postinoculation; *PgLOX6*, lipoxygenase 6 of *Panax ginseng*; *PgPAL1*, phenylalanine ammonia lyase 1 of *P. ginseng*; *PgGAPDH*, glyceraldehyde 3-phosphate dehydrogenase of *P. ginseng*. Values are expressed as means \pm SE, based on Student's *t*-tests. *Differences between treatments are statistically significant ($P < 0.05$; $n = 3$).

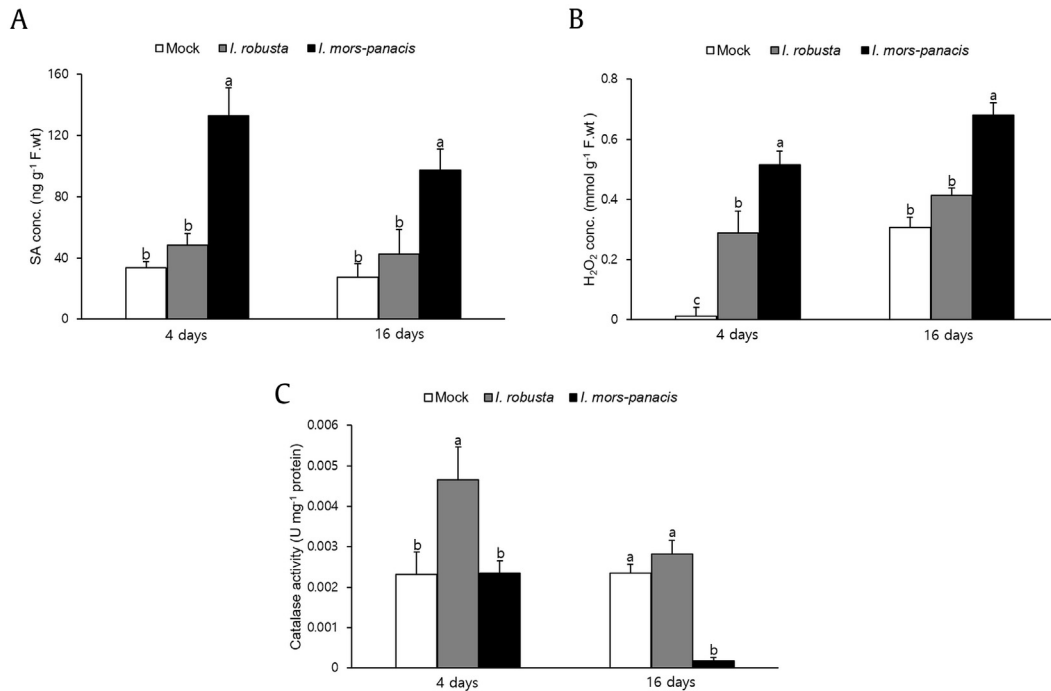


Fig. 5. Evaluations of SA (A), H₂O₂ (B), and catalase (C) in ginseng roots infected by *Ilyonectria robusta* or *I. mors-panacis* versus pathogen-free roots (mock). Values are expressed as means \pm SE, based on Tukey's tests. For each time point, data not labeled with same letter are significantly different. SA, salicylic acid; F.wt, fresh weight.

particularly at 4 dpi. The activity was reduced at 16 dpi but was still higher than that measured in the mock (Fig. 5C). Expression of a related gene, *P. ginseng catalase 1* (*PgCAT1*), matched the trend found for enzyme activity in our assay (Fig. 7). In contrast, activity at 4 dpi did not differ between pathogen-free roots and those infected by the highly aggressive *I. mors-panacis*. However, at 16 dpi, activity in the infected roots was drastically decreased to a level that was lower than that in the mock roots (Fig. 5C). For those samples, the expression pattern of the related gene matched with the enzyme activity at each time point (Fig. 7).

Induction of either the signaling or oxidative effects of ROS depends on an equilibrated balance between the levels of ROS and antioxidant scavengers. Signaling can be introduced if those scavenging agents are produced in quantities sufficient to reduce the toxicity of the generated ROS. If not, then those oxidative properties will become dominant and lead to PCD. High activity by antioxidant

enzymes is crucial for successful tolerance against ROS-generated pathogen attacks [55,56]. Furthermore, SA- or ROS-based accumulation of ginsenosides is accompanied by strong antioxidant activity [33,38]. Therefore, we suggest that the elevated catalase activity in *I. robusta*-infected roots maintained those ROS signaling effects, which then stimulated the production of ginsenosides and, subsequently, retarded the fungal attack. In contrast, catalase activity was suppressed in response to infection by *I. mors-panacis*, which might explain the continued oxidative effects by the accumulated ROS.

3.5. *In vitro* assays of pathogen sensitivity to H₂O₂

Because we confirmed that H₂O₂ was responsible for the enhanced oxidative stress in *I. mors-panacis*-infected roots, we hypothesized that this pathogen was better able to tolerate those

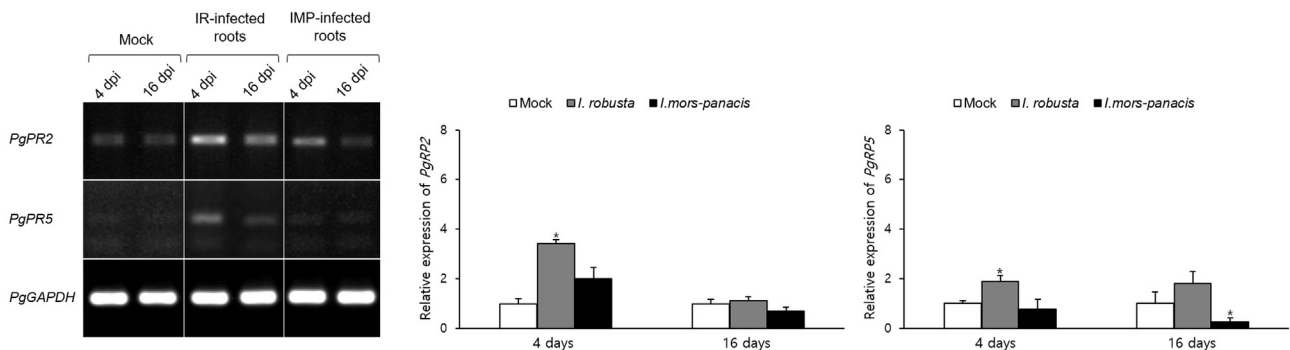


Fig. 6. Relative expression profiles of SA-dependent PR genes in ginseng roots infected by *Ilyonectria robusta* (IR) or *I. mors-panacis* (IMP) versus pathogen-free (mock) roots. *PgPR2*, pathogenesis related 2 of *Panax ginseng*; *PgPR5*, pathogenesis related 5 of *P. ginseng*; *PgGAPDH*, glyceraldehyde 3-phosphate dehydrogenase of *P. ginseng*; SA, salicylic acid; PR genes, pathogenesis-related genes; dpi, days postinoculation. Values are expressed as means \pm SE, based on Student's *t*-tests. *Differences between treatments are statistically significant ($P < 0.05$; $n = 3$).

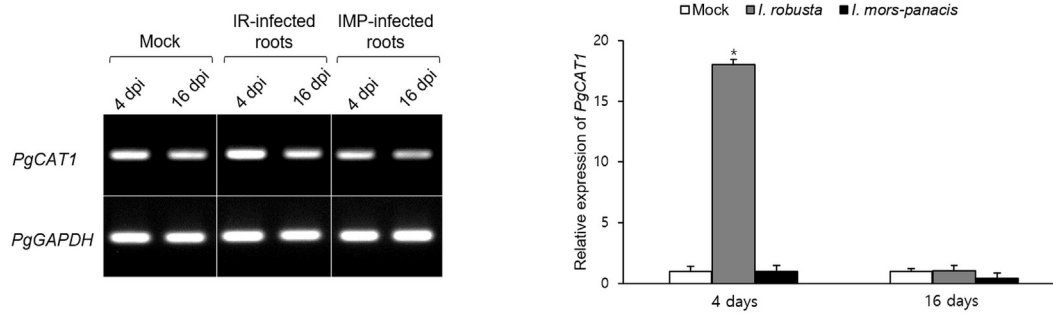


Fig. 7. Relative expression profile of catalase-related gene *PgCAT1* in ginseng roots infected by *Ilyonectria robusta* (IR) or *I. mors-panacis* (IMP) versus pathogen-free roots (mock). *PgCAT1*, catalase 1 of *Panax ginseng*; *PgGAPDH*, glyceraldehyde 3-phosphate dehydrogenase of *P. ginseng*; dpi, days postinoculation. Values are expressed as means ± SE, based on Student's *t*-tests. *Differences between treatments are statistically significant ($P < 0.05$; $n = 3$).

oxidative effects when compared with *I. robusta*. We investigated sensitivity to oxidative toxicity by calculating the 7-d growth rates of both pathogens. At concentrations of 8, 16, and 24 mM H₂O₂, those rates were significantly higher in the *I. mors-panacis* than in the *I. robusta* (Fig. 8).

The necrotrophic pathogen *Botrytis cinerea* is responsible for mold diseases in many plants. This agent triggers the plant to induce the ROS pathway while simultaneously producing anti-apoptotic agents to overcome the toxicity of the ROS that are then

generated [21]. Furthermore, the hemibiotrophic fungus *Cercospora nicotianae*, which causes leaf spots in *Nicotiana*, induces ROS-based oxidative stress via production of cercosporin, a photosensitizer mycotoxin, while also reducing the toxicity of ROS through the production of pyridoxine, a ROS-quenching molecule [57]. Our results suggest that, when compared with *I. robusta*, the greater tolerance of *I. mors-panacis* to H₂O₂ stress might be beyond its ability to increase its virulence by resisting the toxicity of the generated ROS.

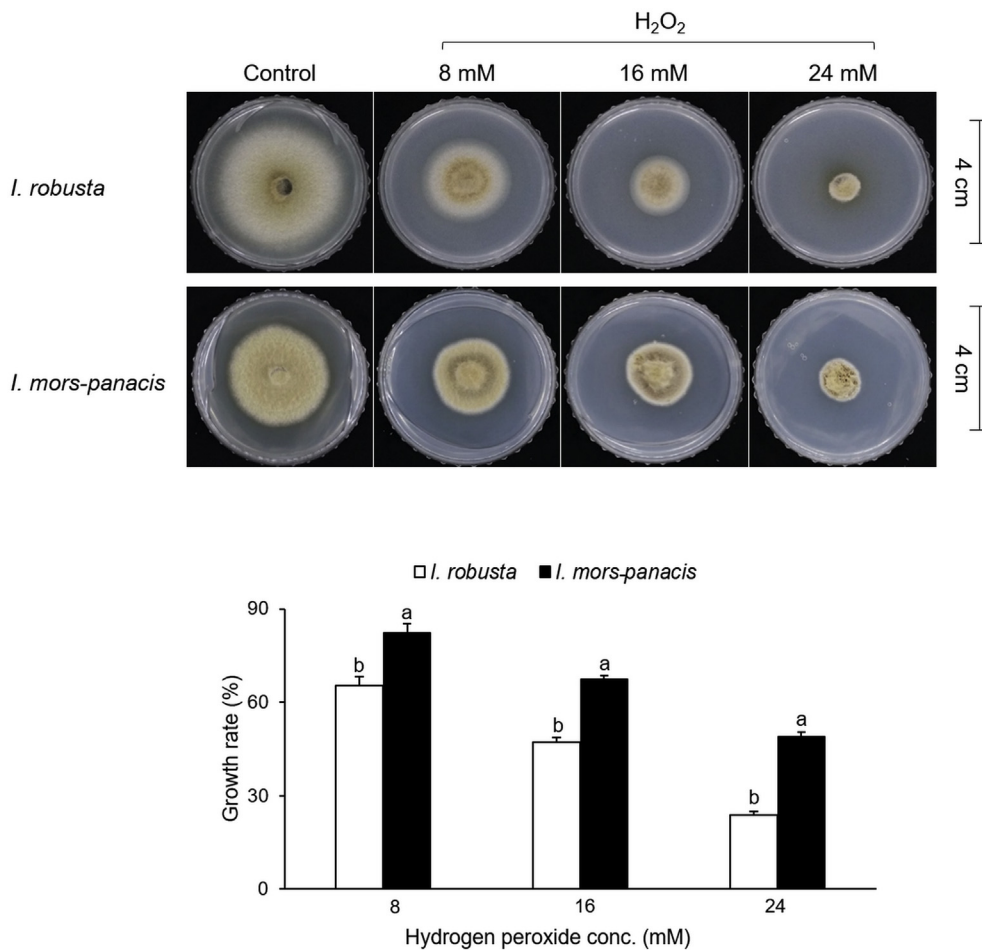


Fig. 8. Growth rates by *Ilyonectria robusta* and *I. mors-panacis* on plates containing PDA plus 0, 8, 16, or 24 mM H₂O₂. For each tested concentration, data not labeled with same letter are significantly different, based on Tukey's tests. PDA, potato dextrose agar.

3.6. Proposed mechanism for ginsenoside regulation in plants upon infection by weak and aggressive species of *Ilyonectria*

Based on all these results, we can elucidate the possible mechanism by which ginsenoside production is mediated through infection by weakly or highly aggressive species of *Ilyonectria*. When the weaker *I. robusta* invades a ginseng root, the first barrier might be the generation of H₂O₂ in the apoplast via the activity of NADPH oxidase enzyme. This H₂O₂ upregulates endogenous SA, which then stimulates further H₂O₂ production and expression of PR genes. In parallel, the plant cells are induced to synthesize an adequate amount of catalase to suppress the negative effects of H₂O₂ and maintain it at a low level. Finally, that small quantity of H₂O₂ regulates ginsenoside biosynthetic genes, probably through JA biosynthesis, which leads to the accumulation of more ginsenosides. Such a scenario has been reported previously, in which the antioxidant activity of terpenoids, including saponins, reduces the oxidative burst of generated ROS [58–60]. Therefore, we postulate that the accumulated ginsenosides help scavenge the ROS to maintain the signaling effects. All these highly defensive responses can successfully retard the pathogen invasion (Supplementary Fig. S1A).

By comparison, when the highly aggressive *I. mors-panacis* attacks a ginseng root, H₂O₂ in the apoplast has no effect because that pathogen is either less sensitive to the cytotoxic activity of H₂O₂ or might produce an unknown effector. In plant cells, generation of a high quantity of H₂O₂ is triggered through the strong upregulation of SA. This in turn leads to downregulations of catalase, expression of SA-dependent PR genes, and ginsenoside biosynthesis, which ultimately causes PCD induction (Supplementary Fig. S1B).

4. Conclusion

Regulation of ginsenosides is affected differentially by the infection of highly and weakly aggressive species of *Ilyonectria*. This is probably due to the amount of ROS that is generated, which then determines the effectiveness of other plant defense responses. Future studies should investigate how ginsenoside is influenced by elevated levels of ROS. In doing so, we may learn how to enhance antioxidant production in ginseng roots so that ROS concentrations are reduced while maintaining their signaling effects on defense mechanisms. Moreover, if the more aggressive *I. mors-panacis* can override the oxidative effects of ROS, this organism could increase in virulence. Therefore, we must also explore the antiapoptotic mechanism by which a pathogen survives against ROS if we are to identify a way in which to eliminate it.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2019.02.001>.

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