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Biocontrol of rusted root rot in *Panax ginseng* by a combination of extracts from *Bacillus amyloliquefaciens* YY8 crude protein and Enterobacteriaceae YY115 ethyl acetate

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Abstract

Background Rusted root rot is one of the most common root diseases in *Panax ginseng*, and *Cylindrocarpon destructans* is one of the main pathogenic fungus. The objective of this study was to screen and explore the extracts of biocontrol bacteria isolated from ginseng rhizosphere soil against *Cylindrocarpon destructans*.

Results Bacterial strains *Bacillus amyloliquefaciens* YY8 and Enterobacteriaceae YY115 were isolated and found to exhibit in vitro antifungal activity against *C. destructans*. A combination of crude protein extract from *B. amyloliquefaciens* YY8 and ethyl acetate extract from Enterobacteriaceae YY115 in a 6:4 ratio exhibited the strongest antifungal activity against *C. destructans*. Measurements of electrical conductivity, protein content, and nucleic acid content in suspension cultures of *C. destructans* treated with a mixture extracts indicated that the extracts disrupted the cell membranes of rusted root rot mycelia, resulting in the leakage of electrolytes, proteins, and nucleic acids from the cells, and ultimately inhibiting the growth of *C. destructans*. The combined extracts suppressed the infection of ginseng roots discs by *C. destructans* effectively.

Conclusion The extracts obtained from the two bacterial strains effectively inhibited *C. destructans* in *P. ginseng*. It can provide scientific basis for the development of new biological control pesticides, reduce the use of chemical pesticides, and promote the sustainable development of agriculture.

Keywords *Panax ginseng*, Rusted root rot, *Cylindrocarpon destructans*, Biological control, Membrane disruption

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Background

Ginseng (*Panax ginseng*), a perennial herb belonging to the Araliaceae has several medicinal and nutritional properties, and is extensively cultivated in China, Korea, and other countries [1, 2]. Compounds, such as saponins, peptides, and the amino acids found in roots, leaves, and fruits of ginseng, have been reported to have various beneficial properties, including anti-fatigue [3], anti-tumor [4], anticancer [5], anti-diabetic [6], analgesic [7], antioxidant [8], and cardiovascular protective effects [9]. Ginseng, as a perennial plant, is susceptible to soil-borne diseases caused by a variety of pathogenic microorganisms, that result in yield losses ranging from 30 to 60% [10]. Rusted root rot is a prevalent and severe fungal disease that significantly impacts the growth of ginseng, with the damage progressively increasing as the plant ages. Rusted root disease has a direct, negative impact on the yield and commercial value of ginseng, restricting the growth of the ginseng industry. Infected plants exhibit stunted growth, yellow or reddish leaves, and root discoloration in shades of red and rust, which eventually result in root rot and plant death [11]. The main fungal pathogen affecting ginseng rusted root rot is *C. destructans* [12]. *C. destructans* can persist in the soil over several years and also overwinters in diseased plant remnants, which then serve as the primary source of inoculum the following year. Thus, the fungal pathogen is particularly severe in continuously cultivated ginseng fields and remains a major problem in ginseng planting rotation practices.

Currently, chemical control is the primary approach for preventing fungal diseases in ginseng. However, the effectiveness of chemical control is insufficient due to the short efficacy period of chemical agents, the long growth period of ginseng, and the long persistence of rusted root rot pathogens in the soil. Additionally, the excessive use of synthetic chemical compounds can lead to environmental pollution, fungal resistance, and ecological imbalances [13]. In contrast, biological control represents a plant disease management strategy that offers several advantages, such as environmental protection, enhanced human and animal safety, and the prevention of pathogen resistance [14]. The use of a variety of microorganisms as biocontrol agents, including *Bacillus*, *Pseudomonas*, *Trichoderma*, and *Streptomyces*, have been extensively reported [15, 16]. *Streptomyces* [17], *Bacillus amyloliquefaciens* [18] have been reported to control rusted root rot in ginseng plants. The potential application of *B. amyloliquefaciens* extracts in preventing rust rot in ginseng plants, however, is still unclear, and the mechanism by which the extracts inhibit *C. destructans* has not been investigated.

In the present study, the bacterial strains YY8 and YY115 were isolated from the rhizosphere soil of *P.*

ginseng and shown to have potential for controlling rusted root rot. The strains were identified by 16 S rRNA sequencing, and they were from genus *Bacillus* and in the Enterobacteriaceae respectively. The stability of the fermentation supernatants from *B. amyloliquefaciens* YY8 and Enterobacteriaceae YY115 were evaluated under high temperature, high pressure, and proteinase K treatments. The response of *C. destructans* to the extracts was also characterized. Finally, the efficacy of the biocontrol strains were assessed by spraying them on fresh ginseng root discs inoculated with *C. destructans*. The main purpose of this study is to screen and investigate the effectiveness of mixed extracts from bacterial strains isolated from ginseng rhizosphere soil in controlling the rusted root rot: (1) Isolate strains with strong antagonistic effects against rust fungal pathogen; (2) Screen crude extracts with strong antagonistic effects from different extracts of the strains; (3) Preliminary exploration of the antibacterial activity and biocontrol mechanism of mixed bacterial strain extracts; (4) Evaluate the biocontrol effect against rusted root rot on ginseng. Our research will provide scientific basis for the development of new biological pesticides or biological control technology, reduce the use of chemical pesticides, and promote the sustainable development of agriculture.

Results

Isolation and screening of antagonistic bacteria against *C. destructans*

A total of 86 bacterial strains were isolated from ginseng rhizosphere soils. Among them above, six strains exhibited the inhibitory activity against the *C. destructans*. Furthermore, strains YY8 and YY115 showed the highest inhibition rates against *C. destructans* with 78.35% and 71.87%, respectively (Fig. 1). Therefore, strains YY8 and YY115 were used as the optimal antagonistic strains for subsequent experiments.

Molecular identification of YY8 and YY115

A phylogenetic tree was constructed (Fig. 2). YY8 had the highest similarity (99%) to *Bacillus amyloliquefaciens* (accession number OK037575.1), whereas YY115 had the highest similarity (98%) to bacteria belonging to the Enterobacteriaceae (accession number OK047733.1).

Stability of fermentation filtrates obtained from *B.*

Amyloliquefaciens YY8 and Enterobacteriaceae YY115

The stability of antifungal activity of fermentation filtrates obtained from the two strains were evaluated under conditions of high temperature and high pressure, and proteinase K treatments (Table 1). After high temperature and pressure and proteinase K treatments, the fermentation filtrates from the two strains still had good inhibitory activity, even though inhibitory activity was

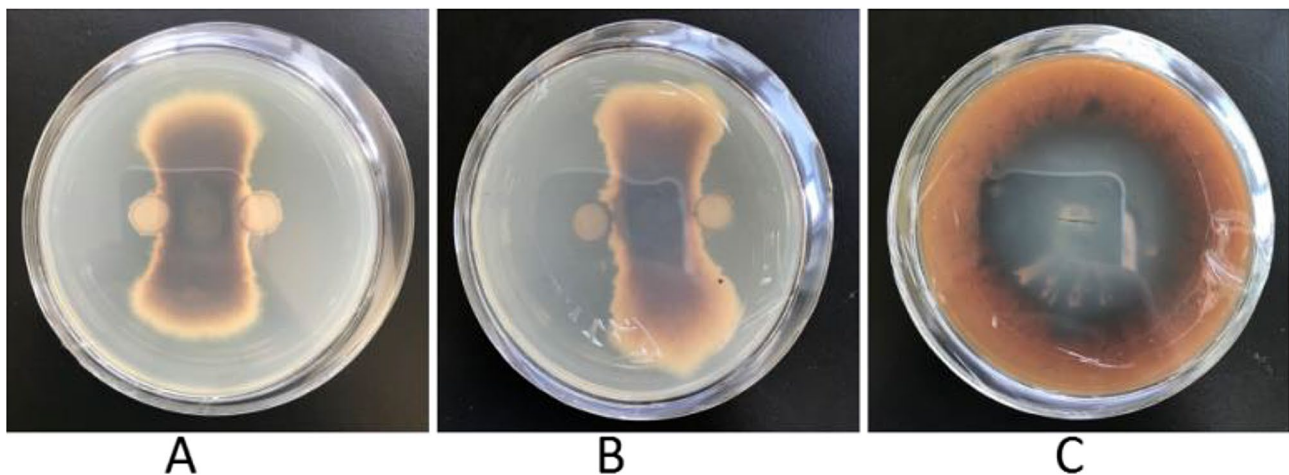


Fig. 1 Antifungal activity of YY8 and YY115 bacterial isolates against *C. destructans*. (A) antifungal activity of YY8, (B) antifungal activity of YY115, (C) Untreated (control)

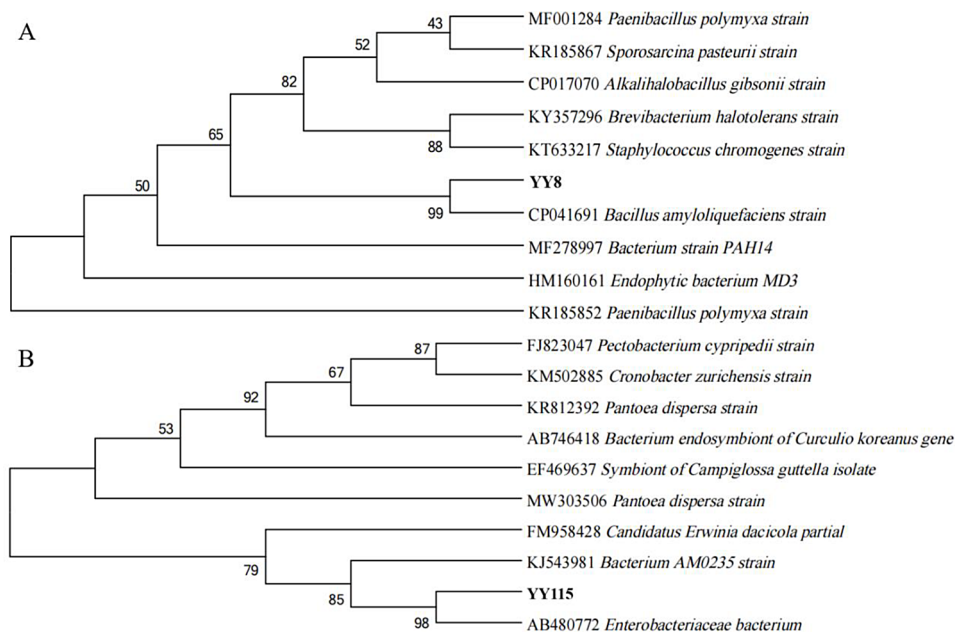


Fig. 2 Phylogenetic tree construction using 16S rRNA gene sequences of obtained from the YY8 (A) and YY115 (B) bacterial isolates and sequences of related taxa

reduced to some degree. This indicates that the antimicrobial compounds present in the fermentation filtrates of the two strains were very stable.

Assessment of the antifungal activity of individual extracts obtained from *B. Amyloliquefaciens* YY8 and Enterobacteriaceae YY115

Results indicated that the crude protein extract obtained from *B. amyloliquefaciens* YY8 and the ethyl acetate extract obtained from Enterobacteriaceae YY115 exhibited strong inhibitory activity against the ginseng rusted root rot pathogen (Fig. 3). Among the different extracts, the *B. amyloliquefaciens* YY8 crude protein extract had

the highest inhibition rate ($84.72\% \pm 3.78\%$) followed by the Enterobacteriaceae YY115 ethyl acetate extract ($81.51\% \pm 3.98\%$).

Inhibitory activity of different concentrations of *B. Amyloliquefaciens* YY8 crude protein extract and Enterobacteriaceae YY115 ethyl acetate extract on *C. destructans*

Results indicated that the inhibitory activity of the two strains extracts increased with increasing concentration (Fig. 4). The highest inhibition rate for both extracts was observed at a concentration of 20 mL/L. More specifically, the *B. amyloliquefaciens* YY8 crude protein extract

Table 1 Stability of fermentation supernatants obtained from YY8 and YY115 isolates exposed to high temperature and high pressure, and proteinase K

Antimicrobial substances	High temperature and high pressure	Proteinase K	Control
YY8 fermentation supernatants	74.31 ± 0.941 ^{ab}	72.48 ± 0.912 ^b	76.29 ± 1.063 ^a
YY115 fermentation supernatants	70.39 ± 0.908 ^b	71.39 ± 1.040 ^b	74.60 ± 1.086 ^a

The data represents percent inhibitory activity of single representative experiment

Data are presented as mean ± SD ($n=3$). Non-identical letters denote significant difference in the inhibitions of pathogen by different treatments at 0.05 significance level. Culture conditions for fermentation supernatants: YY8 and YY115 were inoculated in Nutrient broth, cultured at 28 °C, 180 r/min for 24 h

had an inhibition rate of 81.56%, while the inhibition rate of the Enterobacteriaceae YY115 ethyl acetate extract was 74.25%. Toxicity regression equations were calculated based on the inhibition rate of the two extracts at different concentrations. The calculated EC_{50} value for the *B. amyloliquefaciens* YY8 crude protein extract was 3.65 mL/L, and 5.39 mL/L for the Enterobacteriaceae YY115 ethyl acetate extract.

Biocontrol efficacy of different ratios of mixtures of *B. Amyloliquefaciens* YY8 crude protein and Enterobacteriaceae YY115 ethyl acetate extracts

The inhibitory effect of different ratios of mixtures of *B. amyloliquefaciens* YY8 crude protein and Enterobacteriaceae YY115 ethyl acetate extracts on the mycelial growth

of the ginseng rusted root rot pathogen was evaluated. Results indicated that the toxicity of the mixtures of the *B. amyloliquefaciens* YY8 crude protein extract and Enterobacteriaceae YY115 ethyl acetate extract at ratios of 7:3, 6:4, and 5:5, respectively, was significantly >1, indicating a synergistic interaction between the two antimicrobial extracts (Table 2). The 6:4 ratio exhibited the highest toxicity, indicating the strongest synergistic activity between the crude protein and ethyl acetate extracts.

The inhibitory effect of different dilutions of the 6:4 ratio of crude protein and ethyl acetate mixture on the mycelial growth of *C. destructans* were further evaluated. Results indicated that the inhibitory activity of the 6:4 ratio was directly proportional to its concentration, with the higher concentrations resulting in higher levels of inhibition (Fig. 5). The highest level of inhibitory rate of 94.25% was observed at 20 mL/L of the 6:4 ratio extract (Fig. 5A). A toxicity regression equation was calculated based on the inhibitory rate of the different concentrations of the 6:4 ratio mixture and used to obtain an EC_{50} value of 1.72 mL/L (Fig. 5B). Notably, the EC_{50} value of the 6:4 mixture was lower than that of the individual extracts, however, the mixture exhibited a higher inhibition rate than individual extracts. These results indicate that the *B. amyloliquefaciens* YY8 crude protein and Enterobacteriaceae YY115 ethyl acetate extract mixture had a greater inhibitory effect on the mycelial growth of *C. destructans* relative to the individual extracts.

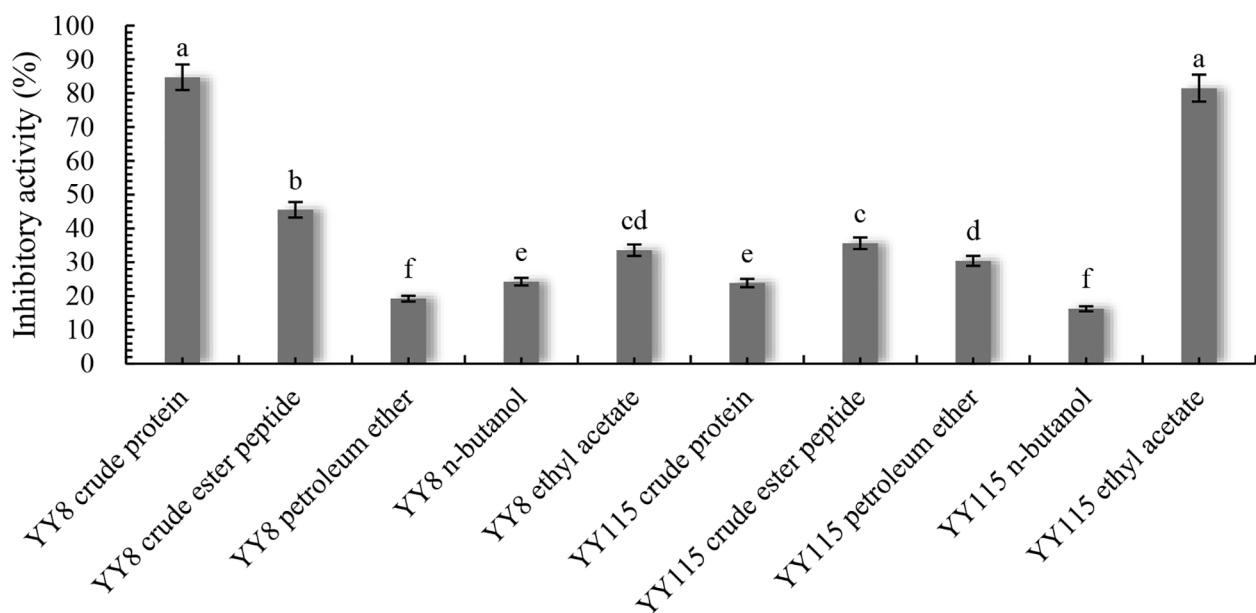


Fig. 3 Percent inhibitory activity of different individual extracts obtained from YY8 and YY115 isolates against *C. destructans*. Sterile fermentation broth was used as a control. Data presented are the mean ± SD ($n=3$). Different lowercase letters above the bars indicate a significant difference ($P < 0.05$) in the inhibition rate between the different extracts as determined by a Duncan's Multiple Range Test. Agar dilution methods, 100 μ l of extracts

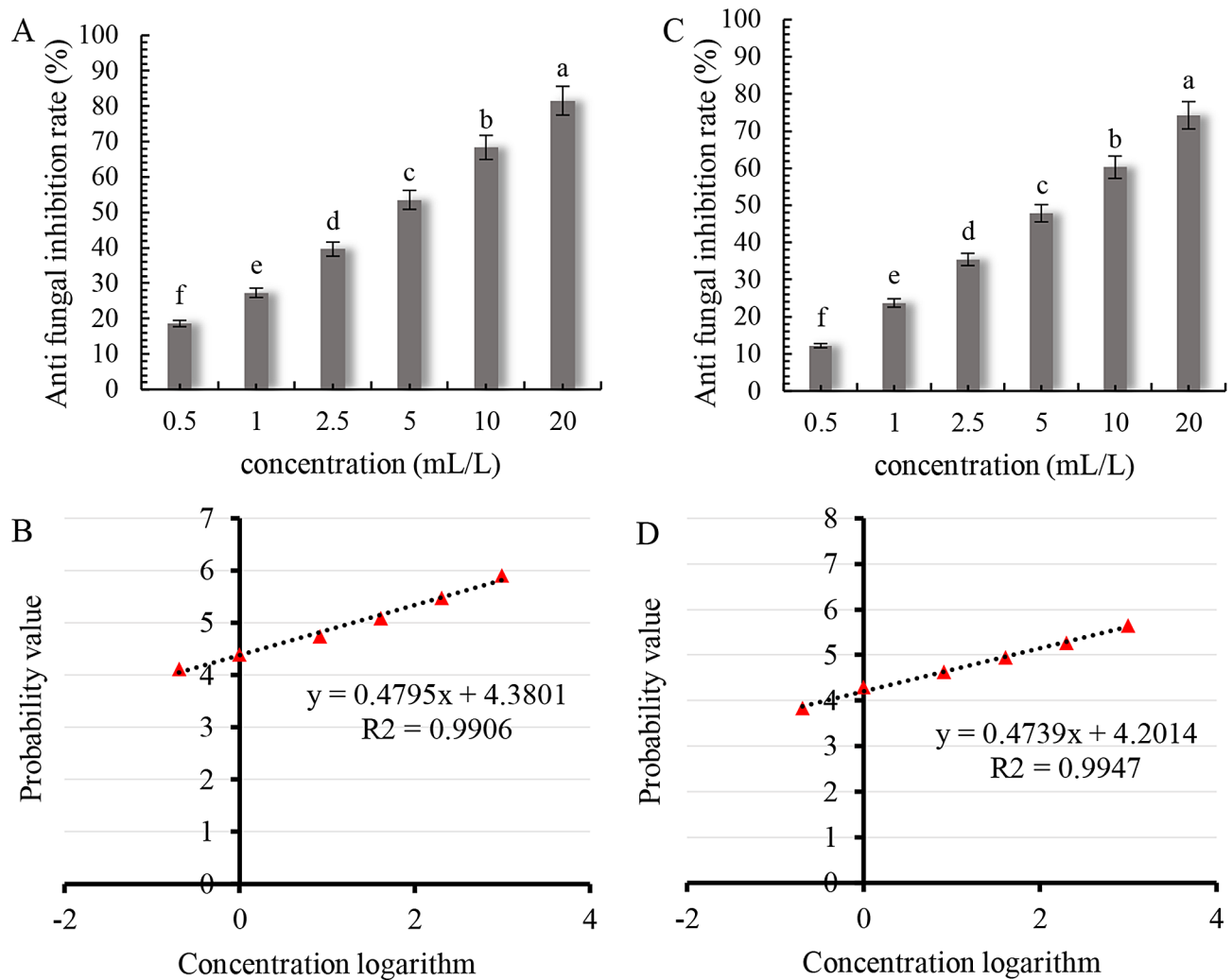


Fig. 4 Effects of different concentrations of individual extracts from strains YY8 and YY115 against *C. destructans*. The inhibitory rates (A) and toxicity regression equation (B) of different concentrations of YY8 crude protein extract against *C. destructans*, as well as the inhibitory rates (C) and toxicity regression equation (D) of different concentrations of YY115 ethyl acetate extract against *C. destructans*. Data are presented as mean \pm SD ($n=3$). Non-identical letters denote significant difference in the inhibitions of pathogen by different treatments at 0.05 significance level

Effect of YY8/YY115 extract mixture on the hyphal morphology of *C. destructans*

To gain a deeper understanding of the inhibitory effect of the combined extract mixture on *C. destructans*, changes in the hyphal morphology of the pathogen treated with the extract mixture were observed using light microscopy (Fig. 6). Observations indicated that, relative to untreated mycelia and mycelia treated with individual extracts, the hyphae of *C. destructans* treated with the extract mixture exhibited greater abnormalities, such as reduced branching, twisted hyphal structures, and unclear septa. These observations suggest that the YY8/YY115 extract mixture had a greater inhibitory effect on *C. destructans* than either of the individual extracts.

The effect of the YY8/YY115 extract mixture on the membrane permeability, protein content, and nucleic acid content of *C. destructans*

Results indicated that the conductivity exhibited a rapid increase from 0 to 1 h, suggesting that the damage occurred in the first hour of exposure for all extracts, and after 1 h, the conductivity continued to increase over time. The conductivity of *C. destructans* hyphal suspension treated with the extract mixture significantly increased compared to the untreated control (Fig. 7). The EC_{70} concentration of the extract mixture produced the highest increase in conductivity. These results indicate that the *B. amyloliquefaciens* YY8 crude protein and Enterobacteriaceae YY115 ethyl acetate extract mixture can disrupt the permeability of hyphal cell membranes of *C. destructans* resulting in an increased level of electrolyte leakage.

Table 2 Toxicity of different ratios of mixtures of YY8 crude protein and YY115 ethyl acetate extracts against *C. destructans*

Concentration ratio (v/v)	Actual inhibition rate (%)	Expected inhibition rate (%)	Toxicity ratio (TR)
10:0	47.15 ± 0.07 ^b	47.15 ± 0.04 ^a	1.00 ± 0.05 ^d
9:1	48.77 ± 0.06 ^a	46.46 ± 0.08 ^b	0.95 ± 0.07 ^e
8:2	44.45 ± 0.06 ^c	45.77 ± 0.03 ^c	1.03 ± 0.04 ^c
7:3	38.10 ± 0.05 ^f	45.08 ± 0.06 ^d	1.18 ± 0.03 ^b
6:4	32.37 ± 0.61 ^k	44.39 ± 0.14 ^e	1.37 ± 0.05 ^a
5:5	36.94 ± 0.16 ^g	43.70 ± 0.13 ^f	1.18 ± 0.04 ^b
4:6	37.60 ± 0.10 ^j	43.01 ± 0.21 ^g	1.14 ± 0.08 ^b
3:7	48.50 ± 0.17 ^a	42.32 ± 0.09 ^h	0.87 ± 0.06 ^e
2:8	42.59 ± 0.06 ^d	41.63 ± 0.07 ⁱ	0.98 ± 0.09 ^d
1:9	42.71 ± 0.04 ^d	40.94 ± 0.02 ^j	0.96 ± 0.09 ^d
0:10	40.25 ± 0.03 ^e	40.25 ± 0.02 ^k	1.00 ± 0.02 ^d

Data are the mean ± SD ($n=3$). Different lowercase letters within a column indicate a significant difference ($p=0.05$) between the different ratio mixtures as determined by a Duncan's Multiple range test

The protein and nucleic acid contents of the extract mixtures showed an increasing trend gently during the time range of 0 to 5 h (Fig. 8). The effect of the extract mixture on the leakage of macromolecules (proteins and nucleic acids) from *C. destructans* was also assessed. Relative to the untreated control, protein (Fig. 8A) and nucleic acid (Fig. 8B) content in the supernatant of hyphae exposed to the extract mixture significantly increased. These data provide confirmation that the structure of the cell membrane in hyphae of *C. destructans* was damaged, resulting in the leakage of nucleic acids and proteins from the hyphal cells. Hyphae treated with the EC_{70} concentration of the extract mixture exhibited a higher level of protein and nucleic acid leakage than was observed in the other treatment concentrations. These data suggest that the *B. amyloliquefaciens* YY8 crude protein and ethyl Enterobacteriaceae YY115

acetate extract mixture had a synergistic inhibitory effect on *C. destructans*.

Biocontrol efficacy of the YY8/YY115 extract mixture against *C. destructans* on ginseng root discs

Results of the in vitro experiment (Fig. 9) indicated that ginseng root discs inoculated with *C. destructans* exhibited discoloration and a small amount of hyphal growth beginning on the second day following inoculation of the pathogen. Hyphal growth significantly increased by day three and by day four, the surface of root discs were covered with hyphae and exhibited distinct symptoms of decay. By day six, the root discs were completely covered with hyphae, and by day eight, they were severely decayed. In contrast, *C. destructans* inoculated root discs that were sprayed with YY8/YY115 extract mixture at three days after pathogen inoculation did not exhibit and further signs of mycelial growth. There were no signs of discoloration or decay until the 8th day. These results indicate that the extract mixture of *B. amyloliquefaciens* YY8 crude protein and Enterobacteriaceae YY115 ethyl acetate (6:4), according to which the biocontrol preparation of *C. destructans* can be obtained.

Discussion

In recent years, the use of natural microorganisms isolated from the soil or plants as biocontrol agents for the management of plant diseases has gained significant attention in the field of sustainable agriculture as an alternative to the use of traditional synthetic chemical pesticides [19]. Biological control using microbes that are associated with plants was an efficient and environmental-friendly approach for controlling disease [20]. *Bacillus amyloliquefaciens* strain S917 has been reported to have significant antagonistic activity against soft rot pathogens [21]. Wang et al. [18] reported that *B. amyloliquefaciens* FG14 can effectively suppress rusted root disease in

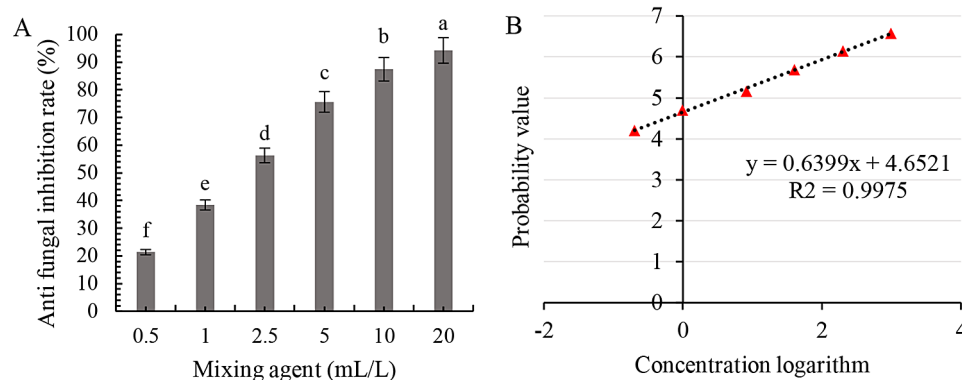


Fig. 5 The inhibitory rates (A) of different concentrations of the 6:4 ratio mixture of YY8 crude protein and YY115 ethyl acetate extracts against *C. destructans*, and the calculated toxicity regression equation (B). Data in A are the mean ± SD ($n=3$). Different lowercase letters above the bars indicate a significant difference ($P<0.05$) in the inhibition rate between the different concentrations of the 6:4 ratio extract mixture as determined by a Duncan's Multiple Range Test

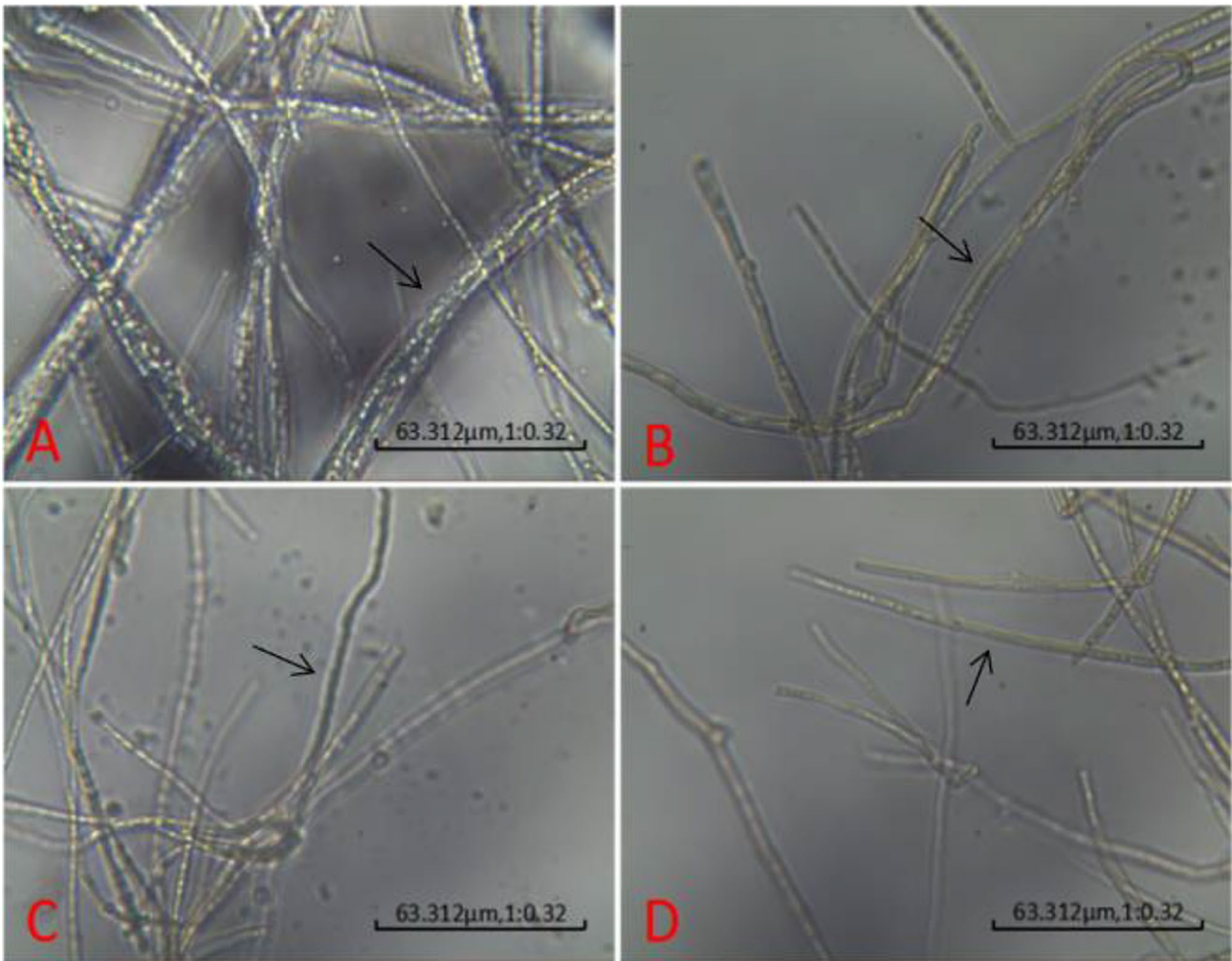


Fig. 6 Microscopic observations of the effect of the individual and combined YY8 crude and YY115 ethyl acetate extracts on hyphae of *C. destructans*. **A:** Untreated (control), **B:** effect of YY8 crude protein extract, **C:** effect of YY115 ethyl acetate extract, **D:** effect of the YY8/YY115 extract mixture (100X)

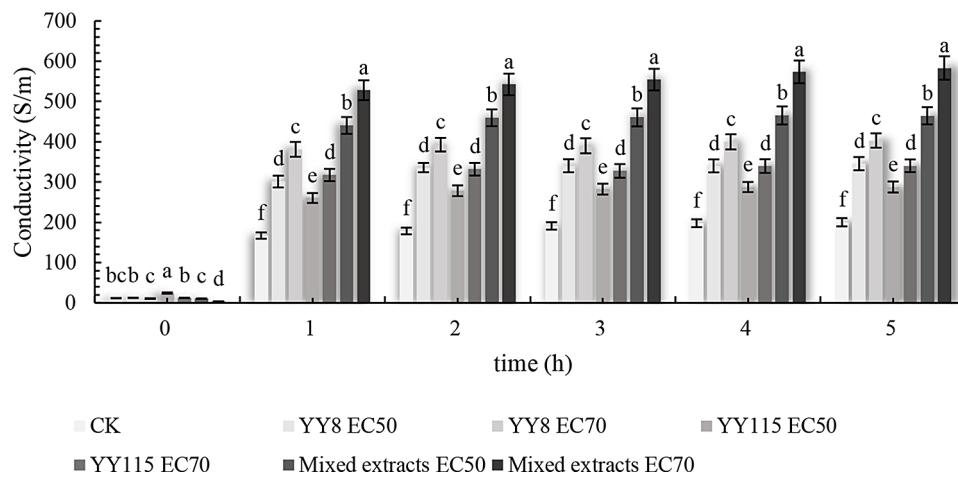


Fig. 7 Temporal effect (5 h) of EC₅₀ and EC₇₀ concentrations of individual and combined YY8 crude protein and YY115 ethyl acetate extracts on the electrical conductivity of hyphal suspensions of *C. destructans*. Sterile fermentation broth was used as a control. Data are the mean ± SD (n = 3). Different lowercase letters above the bars indicate a significant difference (P < 0.05) in conductivity between the different extract concentrations (EC₅₀ and EC₇₀) of the individual and combined YY8 and YY115 supernatant extracts within each timepoint as determined by a Duncan's Multiple Range Test

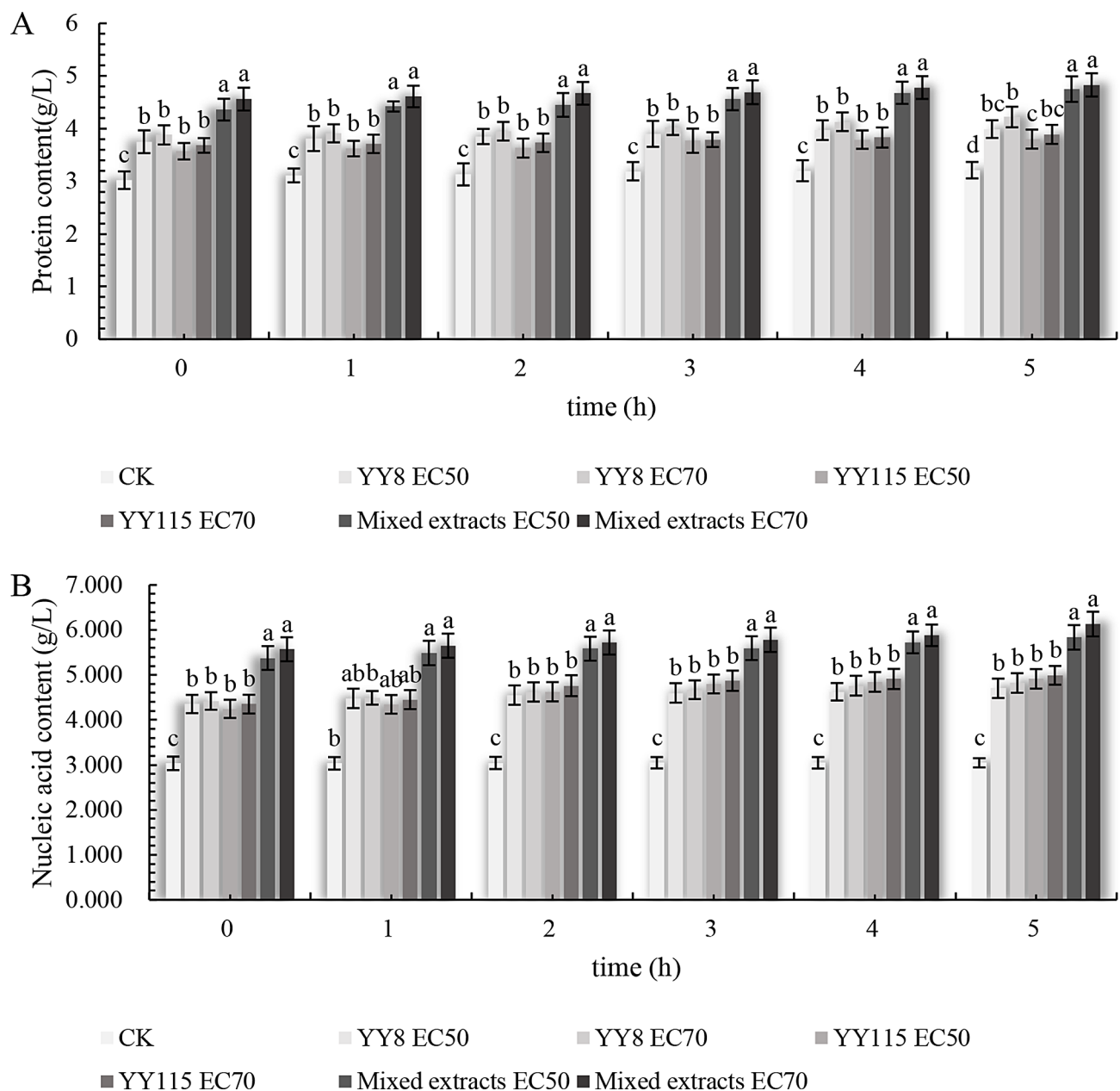


Fig. 8 Temporal effect (5 h) of EC₅₀ and EC₇₀ concentrations of individual and combined YY8 crude protein and YY115 ethyl acetate extracts on the protein content (A) and nucleic acid content (B) of hyphal suspensions of *C. destructans*. Sterile fermentation broth was used as a control. Data are the mean \pm SD ($n=3$). Different lowercase letters above the bars indicate a significant difference ($P < 0.05$) in protein or nucleic acid content between the different extract concentrations (EC₅₀ and EC₇₀) of the individual and combined YY8 and YY115 supernatant extracts within each timepoint as determined by a Duncan's Multiple Range Test

ginseng while promoting root system growth. Sallam et al. [22] reported that the used *Enterobacter* strain was able to colonize root tissues of maize and induce maize growth as well as SA-dependent SAR, which allowed pre-bacterial colonized plants to resist *Fusarium* infection as the disease severity reduced to a marked level. Jiang et al. [23] reported that two *Bacillus velezensis* strains 5YN8 and DSN012 were screened out with high antagonistic and hydrolase activity from 100 strains by

co-culture of bacteria and fungi, which could significantly control pepper gray mold disease and promote the pepper growth. The *B. amyloliquefaciens* strain YY8 and an *Enterobacteriaceae* strain YY115 screened in this study had strong antifungal activity against *C. destructans*, and it showed an efficient biocontrol effect on rusted root rot in ginseng.

The efficacy of biocontrol agents may be influenced by various environmental factors, which can either weaken

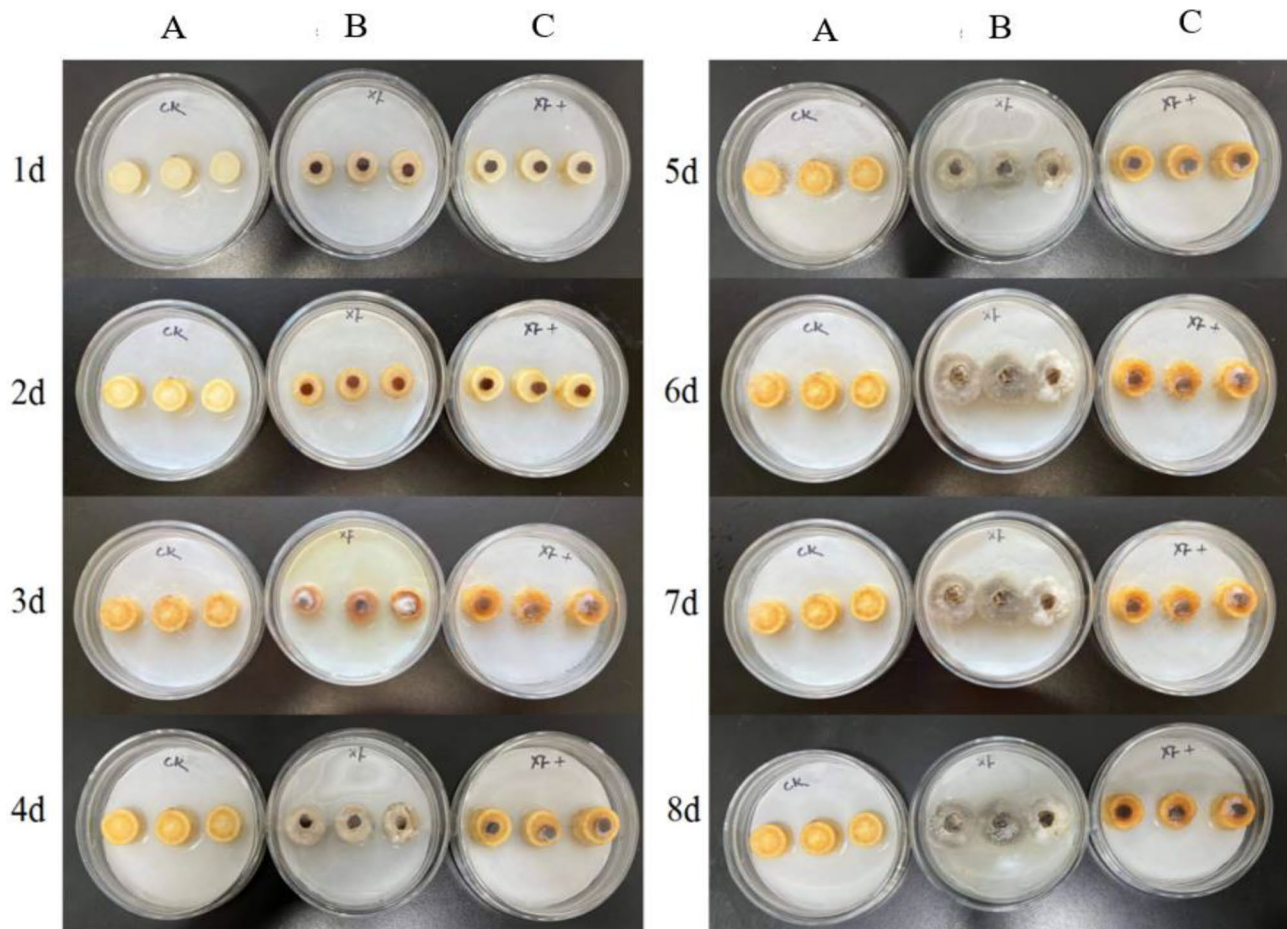


Fig. 9 Symptoms of rusted root rot on ginseng root discs and biocontrol activity of the combined YY8/YY115 extract. **A:** ginseng root discs only treated with sterile fermentation broth (control), **B:** ginseng root discs inoculated with *C. destructans*, **C:** root discs inoculated with *C. destructans* followed 3 days later with the combined YY8/YY115 at 6:4 ratio extracts

or enhance their antagonistic effects [24]. Therefore, in this study, antifungal metabolites produced by the two strains were treated with high temperature, high pressure, and proteinase K. In the present study, the antifungal metabolites in the fermentation supernatants of *B. amyloliquefaciens* YY8 and Enterobacteriaceae YY115 were thermally stable, which is similar to the results obtained by Myo et al. [25]. Previous studies have reported that antimicrobial proteins extracted from *Streptomyces sanguinis* treated with proteinase K showed 92.4% of the inhibitory activity of the control, indicating that the antimicrobial proteins were not sensitive to proteinase K [26]. This is consistent with the results of the present study, where the fermentation supernatants of the two strains still had stable inhibitory activity after proteinase K treatment. We speculate that the inhibitory activity may originate from non-protein substances, antimicrobial proteins or peptides that are insensitive to proteinase K. Yan et al. [27] reported that *B. subtilis* crude protein constantly maintained antimicrobial activity even treated with proteases K exposure. Metabolites with such

stability can exhibit antifungal activity under various environmental conditions, making them more reliable and effective biocontrol agents.

Numerous studies have been conducted on the antimicrobial properties of compounds secreted by antagonistic bacteria and the secondary metabolites produced by plants. Abdel-Aziz et al. [28] found that an ethyl acetate extract obtained from the fermentation broth of *Streptomyces* EGY-S6 effectively controlled tomato wilt disease caused by *Fusarium oxysporum*. Crude lipopeptides extracted from *B. amyloliquefaciens* ET-1 exhibited significant control of powdery mildew [29]. Gerayeli et al. [30] noted that whole cultures of *B. subtilis* and *B. pumilus* strains harboring lipopeptide genes (for surfactants, iturins, fengycins and bacillomycin L) inhibited the growth of *P. carotovorum* subsp. *carotovorum* in vitro. *B. amyloliquefaciens* S917 with the potential for production of various antimicrobial metabolites (bacteriocins, bacillaene, macrolactin H, bacilysin, and bacillibactin) showed antagonistic activity against *P. carotovorum* subsp. *brasiliense* by reducing the soft rot lesion diameters in

chilli peppers [31]. In the present study, the inhibitory effect of extracts obtained from the fermentation broth of the two strains cultures on *C. destructans* were evaluated in vitro. Results indicated that a crude protein extract obtained from *B. amyloliquefaciens* YY8 cell free and an ethyl acetate extract obtained from Enterobacteriaceae YY115 culture medium had significant antifungal activity against *C. destructans*. Identifying the monomeric components in these extract mixtures that are responsible for the antifungal activity will be the focal point of our subsequent research endeavors.

Yu et al. [32] reported a 1:1 mixture of *Streptomyces* strains (NEAU-D88 and NEAU-31) exhibited better biocontrol efficacy than the use of individual strains alone. Yang et al. [33] found that a mixture of *B. amyloliquefaciens* strains (Fy11 and Zy44) showed synergistic enhancement in controlling *Phytophthora* blight in pepper. Although these represent mixtures of the actual bacterial strains, these findings highlight the value of combining the active properties of different antagonistic strains to enhance disease suppression. Studies on the use of combinations of antimicrobial substances from different antagonistic strains, however, are limited. Yu et al. [32] reported that a 1:1 mixture of *Streptomyces* strains, (NEAU-D88 and NEAU-18) was the most effective against tomato wilt disease. Marian et al. [34] found that a 2:1 mixture of strains *Mitsuaria* sp. TWR114 and nonpathogenic *Ralstonia* sp. TCR112 exhibited optimal control of tomato wilt disease. These studies indicate that the efficacy of disease control varies depending on the ratio of mixed antagonistic strains. We also calculated an EC_{50} value of 1.72 mL/L for the 6:4 mixture of *B. amyloliquefaciens* YY8 crude protein extract and Enterobacteriaceae YY115 ethyl acetate extract, a value that was lower than the EC_{50} value of the individual extracts. This finding further confirmed that the 6:4 mixture of extracts had greater efficacy against *C. destructans* than the individual extracts.

Biocontrol agents control plant diseases by inhibiting pathogen hyphal growth. The cell-free filtrate of *B. amyloliquefaciens* AK-0 effectively inhibits the hyphal growth of *C. destructans*, the causal agent of rusted root rot in ginseng [35]. In our study, hyphae of the pathogen treated with a mixture of the extracts exhibited the greatest abnormalities, such as reduced branching, twisted hyphal structures, and unclear septa, relative to the level of alterations induced by the individual extracts.

Changes in the conductivity of hyphal suspensions can reflect alterations in cell membrane permeability and the leakage of electrolytes [36]. Zhang et al. [37] found that extracts obtained from cone root tubers (*Cyperus rotundus*) disrupt the membrane permeability of *Staphylococcus aureus* cells via a membrane-mediated apoptosis pathway. In the present study we assessed the

conductivity, protein content, and nucleic acid content in *C. destructans* suspension cultures treated with YY8/YY115 extract mixture to investigate the mechanism responsible for inhibiting the growth of the pathogen. Our results showed a significant increase in conductivity, protein content, and nucleic acid content in the hyphal suspensions of the pathogen treated with the extract mixture compared to the untreated control. The mixture damaged the hyphal cell membranes of the rusted root rot pathogen, leading to the leakage of intracellular electrolytes, proteins, and nucleic acids. These findings are consistent with the results reported by Kong et al. [38] and support the premise that the YY8/YY115 extract mixture inhibits the growth of *C. destructans* by disrupting hyphal cell membranes.

Results of the ginseng root disc inoculation experiment indicated that the YY8/YY115 extract mixture effectively suppressed the growth of *C. destructans* and also prevented infection and decay of the ginseng root discs inoculated with the pathogen. Results indicated that the hyphae of *C. destructans* ceased to grow on root discs treated with the extract mixture. Additionally, no signs of discoloration or decay were observed on the treated root discs, even at 8 days after inoculation with the pathogen. In contrast, ginseng root discs inoculated with *C. destructans* alone were completely covered with hyphae and exhibited discoloration and significant decay. Based on these results, we suggest that the mixture of *B. amyloliquefaciens* YY8 crude protein extract and Enterobacteriaceae YY115 ethyl acetate extract has potential application for use in controlling rusted root rot in ginseng.

Conclusion

Bacillus amyloliquefaciens YY8 and Enterobacteriaceae YY115, isolated from the rhizosphere soil of ginseng, exhibit significant inhibitory activity against *C. destructans*. After treatment with high temperature, high pressure, and proteinase K, the metabolites produced by the two strains exhibited stable antifungal activity. *B. amyloliquefaciens* YY8 crude protein extract and Enterobacteriaceae YY115 ethyl acetate extract obtained from fermentation filtrates effectively suppressed the growth of *C. destructans*. Also, the mixture of YY8/YY115 extract (6:4) effectively inhibited the hyphal growth of the pathogen on ginseng root discs and prevented discoloration and decay of the root discs. These findings provide a foundation for the development of biocontrol strategies for the management of rusted root rot disease in ginseng.

Methods

Soil sample collection and bacterial isolation

C. destructans CGMCC3.3590 used in this study was purchased from the Institute of Microbiology, Chinese

Academy of Sciences. *C. destructans* was cultured on potato dextrose agar (PDA).

Panax ginseng rhizosphere soil samples were collected from a ginseng cultivation site in Fusong County, Baishan City, Jilin Province, China. Samples were collected using the five-point sampling method described by Sun et al. [39]. All soil samples were placed in sterile bags and stored at 4 °C until further processing.

Bacterial isolation and purification were performed by serial dilution as described by Ding et al. [40]. The Petri plates were incubated upside down in an incubator at 37 °C until colonies were visible. After the colonies appeared, the different forms of colonies were inoculated into fresh NA. The pure strains were then preserved in 15% glycerol and stored at -80 °C.

Screening bacterial isolates for antifungal activity

The bacteria were shaken by 180 rpm/min for 24 h at 37 °C, and the liquor was subsequently subjected to centrifugation (6,000 g, 4 °C) for 10 min. The bacterial suspension were obtained after washing the precipitate three times with sterilized deionized water [39]. Fungal plugs of *C. destructans* were obtained from culture plates using a puncher, and placed in the center of 90 mm diameter potato dextrose agar (PDA) plates. Two sterilized filter paper discs (1 cm diameter) were then placed equidistantly around each plate positioned 25 mm away from the center. Two filter paper discs were inoculated with 20 µL of a bacterial suspension (10^8 CFU/ml) of the isolates, while two filter paper discs of the separate plates served as a control and inoculated with 20 µL of sterile water. Each treatment was set to repeat three times. The plates were incubated at 28 °C for 10 d. After incubation, colony diameters of *C. destructans* were measured and recorded to determine the level of inhibition produced by each of the bacterial isolates bacteria [41]. Screening of strains with an inhibition rate greater than 70% effect on *C. destructans* were used for subsequent studies.

Inhibition rate was calculated using the following formula: inhibition rate = $[(A - B) / A] \times 100\%$, where A represents the colony diameter of the pathogenic fungi in the control group, and B represents the colony diameter of the pathogenic fungi exposed to the bacterial isolates [42].

Molecular identification and phylogenetic analysis of antagonistic bacteria

Molecular identification of the selected antagonistic bacterial strains was performed by analyzing the sequence of the 16 S rRNA gene of each isolate. DNA was extracted from the isolates using a DP336 DNA extraction kit. To amplify the 16 S rRNA gene, polymerase chain reaction was performed with the primers 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and

1492R (5'-GGYTACCTTGTTACGACTT-3') [43]. The amplification products were analyzed by 1% agarose gel electrophoresis and sequenced at the Beijing Genomics Institute (BGI). The obtained sequences were queried against the NCBI database using BLAST [44] to identify their sequence similarity to known species in the NCBI data base. A phylogenetic tree was constructed using MEGA software (version 7.0) to determine phylogenetic relationships of the obtained isolates.

Evaluation of stability of the fermentation supernatant obtained from the bacterial isolates

To assess the stability of the fermentation supernatant obtained from the investigated bacterial isolates under conditions of high temperature, high pressure [24], and proteinase K treatment [45], the bacterial isolates were inoculated into Nutrient Broth (NB) medium and incubated at 37 °C for 24 h. The culture was then centrifuged at 6000 g for 10 min at 4 °C, and the supernatant was collected for stability testing. For the combined high-temperature and high-pressure evaluation, the fermentation supernatant was first sterilized at 121 °C (high temperature) and then subjected to high pressure for 30 min. Evaluations were performed in triplicate. For the stability test involving proteinase K treatment, 5 mL of the fermentation supernatant was mixed with proteinase K (1 mg/mL). The proteinase K- treated samples were then incubated at 37 °C for 2 h. The antifungal activity of the fermentation supernatants was evaluated using the filter paper disc method [23]. Sterile fermentation broth was used as a control. Each treatment was set to repeat three times.

Antifungal activity assay of individual extracts from antagonistic bacterial isolates

Supernatant of NB cultures of the antagonistic bacterial isolates were subjected to crude protein [46], crude peptide [29], petroleum ether, n-butanol, and ethyl acetate extraction [47]. The inhibitory effect of these extracts on *C. destructans* was evaluated using the Oxford cup diffusion method [48]. Briefly, 100 µL of *C. destructans* suspension was evenly spread on the surface of a PDA plate. Oxford cups (5 mm) were created in the center of each culture plate using a cork borer, and 100 µL (1500 µg/mL) of each extract was added to each cup. Sterile fermentation broth was used as a control. Each treatment was set to repeat three times. The plates were incubated at 25 °C for 10 d. The formation of transparent inhibition zones around the Oxford cups indicated antifungal activity and the diameter of the inhibition zones were measured to calculate specific levels of inhibition.

Biocontrol efficacy of different concentrations of the individual extracts obtained from antagonistic bacteria cultures

Obtained from bacterial isolates that exhibited significant inhibitory activity were subsequently analyzed to determine the optimal inhibitory concentration of the extracts using the agar diffusion method [49]. The extracts were diluted with sterile water to obtain different solution concentrations (0.5, 1, 2.5, 5, 10, and 20 mL/L). The diluted extracts were then thoroughly mixed with PDA medium at approximately 45 °C in a 1:99 ratio. A 7 mm plug of *C. destructans* was then placed in the center of each plate, while PDA agar plug was used as control. Treatments were repeated in triplicate. The inoculated plates were then incubated at 25 °C for 10 d. Sterile fermentation broth was used as a control. Each treatment was set to repeat three times. Subsequently, the colony diameter of *C. destructans* was measured using the cross method, and the inhibition rate was calculated. A toxicity regression equation was calculated based on the inhibitory level of *C. destructans* at different extract concentrations and used to determine the EC_{50} value of each extract.

Biocontrol efficacy of mixtures of different ratios of combined extracts obtained from antagonistic bacterial isolates

The calculated EC_{50} value of each extract was used to construct mixtures of combined extracts at different ratios: 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 (v/v). The antifungal activity of the different mixture ratios was evaluated using the agar diffusion method. A toxicity ratio was calculated based on the inhibition rates of the extract mixtures at different ratios. A toxicity ratio of 1 indicates that the toxicity effect of the mixture was similar to the individual extracts. A toxicity ratio greater than 1 indicates that the toxicity effect of the combined extract at the given ratio was higher than the inhibitory effect of the individual extracts, thus, indicating a potential synergistic interaction between the extracts. A toxicity ratio less than 1 indicates that the toxicity of the given mixture at the indicated ration was lower than the effect of the individual mixtures, thus, indicating a potential antagonistic interaction between the substances.

The best-performing ratio of extract mixture was further diluted with sterile water to obtain different solution concentrations (0.5, 1, 2.5, 5, 10, and 20 mL/L). The antimicrobial activity of these extract mixtures was also evaluated as suggested earlier in section. A toxicity regression equation was calculated based on the inhibition rates of the extract mixture at different concentrations, and the EC_{50} value of the extract mixture at the optimum ratio was calculated.

Effect of antagonistic bacterial extracts on the hyphal morphology of *C. destructans*

The effect of antagonistic bacterial extracts on the hyphal morphology of *C. destructans* was investigated using the filter paper disc method. A 7 mm plug of *C. destructans* was placed in the center of a PDA plate. Two 1 cm diameter paper discs were placed 20 mm from the center of the plug. Five microliters of single and mixed extracts were separately added onto the paper discs. Sterile fermentation broth was used as a control. Each treatment was set to repeat three times. The plates were incubated at 25 °C for 10 d, fungal hyphae were taken from the edge of the inhibition zone, and the hyphal morphology of *C. destructans* was observed under a light microscope.

Effect of antagonistic bacterial extracts on *C. destructans*

Analysis of cell membrane permeability

The effect of prepared bacterial extracts on the permeability of mycelial cell membranes of *C. destructans* was determined using the method described by Wang et al. [50]. Agar blocks (5 mm) of *C. destructans* on PDA were added to 250 ml flasks containing 100 ml of Potato Dextrose Broth (PDB) and cultured at 25 °C on a rotary shaker at 150 rpm for 3d. Single and mixed extracts were diluted with sterile water and EC_{50} and EC_{70} concentration and added to the PDB medium to create a 1.0 mM solution. Sterile fermentation broth was used as a control. Each treatment was set to repeat three times. After incubation at 25 °C on a rotary shaker at 150 rpm for 2 h, fungal mycelia were recovered by filtered through two layers of sterilized cheesecloth, and washed three times with double-distilled water with centrifugation between each wash. After the last centrifugation, the volume of the mycelial suspension was adjusted to 10 ml with double-distilled water and electrical conductivity was measured at 0, 1, 2, 3, 4, and 5 h using a portable conductivity meter (Hach, Loveland, CO, USA).

Determination of protein and nucleic acid content in treated mycelia of *C. destructans*

The culture of *C. destructans* and different concentrations of antagonistic bacteria extracts were prepared as in the previous section. Cultures were incubated in a constant temperature incubator and 10 mL samples were collected at 0, 1, 2, 3, 4, and 5 h. The collected samples were then centrifuged at 10,000 rpm for 5 min. Absorbance of the resulting supernatant was measured at 260–280 nm respectively using a spectrophotometer to determine both nucleic acid and total protein content in the supernatant.

Efficacy of mixed extracts obtained from antagonistic bacterial isolates in controlling rusted root rot in ginseng

Roots from freshly harvested, healthy, disease-free ginseng plants of a uniform size were harvested and immersed in a 2% (v/v) sodium hypochlorite solution for 3 min and then rinsed with sterile distilled water. The roots were then air dried for 2 h on a sterile bench. The roots were then cut into discs 0.5 cm in length with a surgical scalpel. Three ginseng root discs were placed in a culture dish lined with two layers of sterile filter paper, and an appropriate amount of sterile water was added to maintain the moisture and keep the root discs from drying out. The root discs were exposed to three treatments: a negative control, in which 20 µl of sterile fermentation broth was spotted on the root discs; a positive control, in which 5 mm agar blocks of the rusted root rot pathogen were inoculated onto the ginseng root discs; and one in which the agar blocks of the rusted root rot pathogen were inoculated onto the ginseng root discs for 3 d followed by 20 µl of the mixed extract obtained from antagonistic bacterial isolates was spotted on the root discs. The plates were incubated at 25 °C, and the level of rot in the ginseng root discs was observed and recorded daily.

Data analysis

Data analysis was conducted using IBM SPSS Statistics software (version 25.0; IBM Corporation, Armonk, NY, USA). The data was subjected to a one-way analysis of variance (ANOVA) and individual treatment means were subjected to a Duncan's multiple range test to determine significant differences ($P < 0.05$) between treatments. Data obtained from the repeated experiments are presented as the mean ± standard deviation ($n = 3$).

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Author contributions

SQF, YCZ: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. QYW, JYZ, XL, ZYF: Investigation, Data curation, Writing – review & editing. WWD, YJL: Project administration, Supervision, Validation, Writing – review & editing. WXJ: Conceptualization, Methodology, Funding acquisition, Resources, Project administration, Supervision, Validation, Writing – review & editing. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited in the NCBI with the accession number OK037575.1 and OK047733.1.

Declarations

Ethics approval and consent to participate

The study protocol must comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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