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SHORT COMMUNICATION

Effect of seed disinfection on Bakanae disease in *Ginkgo biloba* outer seed coat extract

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ABSTRACT

In research on the control of Bakanae disease using the *ginkgo biloba* outer seed coat, which currently is being discarded, it was found that *ginkgo biloba outer* seed coat extract can inhibit the growth of *Fusarium fujikuroi* at 60% or more at a concentration level of 400mg L^{-1} or more. Disaster-prevention experiments with Ilpumbyeo in which *F. fujikuroi* was inoculated with the *ginkgo biloba outer* seed coat extract as a water-dispersible powder were conducted. we found that prevent Bakanae disease for 36-48 hours with a treatment solution in which a *ginkgo biloba outer* seed coat control composition of 7.5% or more was mixed, a control effect of 80% can be used.

Keywords: Bakanae disease; *Fusarium fujikuroi*; *Ginkgo biloba* outer seed coat; Polysaccharide

Bakanae disease of rice is a seed infection disease which is caused by *Fusarium fujikuroi*, which was reported for the first time in Japan in 1898 (Kim, 1981). Bakanae disease occurs during the seeding period of the following year, after which *F. fujikuroi* winters inside of the seed (Park et al., 2008). The symptoms of Bakanae disease include succulent growth and seeding, after which the seed leaf does not emerge, followed by withering of the seed. Alternatively, no sprouted occurs after transplanting, it even if the seeds are not withered, leading to heavy losses in productivity. In addition, if an ascospore or large or small types conidia form in the diseased area at the time of sprouting to infect the seeds at the time of blooming, it is known that the degree of infectivity becomes high (Hemmi et al., 1931; Sun, 1975). At present, in order to prevent Bakanae disease, systemic fungicides such as hexaconazole, tebuconazole or prochloraz have been developed and distributed, and good disease control has been realized (Park et al., 2003). In addition, *F. fujikuroi*, with good resistance as a control insecticide, has also been investigated (Lee et al., 2010). Moreover, because the temperature of the Korean peninsula due to the impact of climate change has risen by approximately 1.5°C over the past 100 years, and spring and summer are prolonged with an increased number of high-temperature events during the farming period, it is considered that the occurrence of Bakanae disease will gradually increase. In order to prevent Bakanae disease, many fungicides are under developed (Korea Crop Protection Association, 2011).

The *Ginkgo biloba* outer seed coat is now completely disposed of due to its peculiar odor, but Lee et al. (2003) reported that extracts of the *Ginkgo biloba* outer seed coat have an effect on controlling aphids. In addition, Park et al. (2011) reported that the methanol extract of the *Ginkgo biloba* outer seed coat has antifungal activity.

In this study, the authors investigated the applicability and the method of application of a control agent pertaining to Bakanae disease, which uses the *Ginkgo biloba* outer seed coat, with research results reported.

MATERIALS AND METHODS

Extraction of *Ginkgo biloba* outer seed coat and separation of antibacterial substances

The *Ginkgo biloba* outer seed coats used in this experiment were sampled from the landscaping trees at the Industry and Science College of Kongju National University in October of 2013. Before use, they were kept in a cryogenic

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freezer set to -70°C after the separation of the *Ginkgo biloba* outer seed coat. The *Ginkgo biloba* outer seed coats were dried for five days at a temperature of -50°C and under a pressure of 1.5 MPA, respectively, using a freeze dryer. They were used as the samples for extraction and were ground with a grinder (HMF-340, Hanil, Korea) after lowering the moisture content of the *Ginkgo biloba* outer seed coat to 5%. Ethanol was used as an extraction solvent, and a powder sample of the *Ginkgo biloba* outer seed coat and ethanol were mixed at a ratio of 1:5 (w/v). They were then filtered with a filter paper (Whatman, No. 2) after stirring and extraction, and the filtrate was decompressed and concentrated by a rotary evaporator (N-1000, Eyela, Japan) in a water bath at a temperature of 45°C. The obtained concentrate of the *Ginkgo biloba* outer seed coat was 219 g, and in order to separate the polysaccharide, eight small fractions were obtained by column chromatography from 100% H_2O to 100% MeOH in a HP 20 column chromatograph. As a result, to determine the activity of each small fraction of Fr.3, which shows strong activity, an analysis by C18 column chromatography was carried out using an eluent (the mobile phase) MeOH-H_2O (0:100), after which Fr.3 was divided into two fractions again. As a result, analyzing the sugar composition of Fr.3-2, which shows strong activity at a flowrate of 1.0 ml/min with perfusate NaOH using Detection: ICS-5000 and Column: CarboPacPA10 (4.5×250 mm, Dionex, Sunnyvale, CA, USA) devices with a CarboPac PA10 cartridge (4.5×50 mm) in the Bio-LC ICS-5000 system found that Fr.3-2 was a polysaccharide mainly based on glucose.

Hypha growth inhibition test of *Ginkgo biloba* outer seed coat extracts

Fusarium fujikuroi Nirenberg (KACC:44006) parceled out from the KACC (Korea Agricultural Culture Collection) was used as an official strain of Bakanae disease. In order to investigate the antibacterial activity of the *Ginkgo biloba* outer seed coat, the concentrate of the *Ginkgo biloba* outer seed coat was mixed such that the concentrate levels were 100, 200, 300, 400 and 500mg·L^{-1} before the PDA medium became hardened when the medium was manufactured. Subsequently, 10ml samples of the material were put into petri dishes 9 cm in diameter, and the solidification of the samples was assessed, the *F. fujikuroi* Nirenberg disc (diameter 5mm) was assessed and mounted at the center of the medium for culturing at 30°C for 5 days. After 5 days, the antibacterial activities of the *Ginkgo biloba* outer seed coats were measured by measuring the diameters based on the hypha growth rate, and the control plot was prepared mixing the water-dispersible powder of the triazole system at the same concentration as the *Ginkgo biloba* outer seed coat extracts, after which the antibacterial activities were compared.

Minimum inhibitory concentration (MIC) of polysaccharide

In order to examine the antibacterial activity of the polysaccharide, the MIC (minimum inhibitory concentration) was assessed by means of microdilution in 96-well plates.

An amount of 100ul of potato dextrose broth (PDB) was put into each well, and a total of seven treatment concentrations (50, 100, 150, 200, 400, 800 and 1600mg·L^{-1}) along with an untreated plot with a solution of triazoles diluted 500 times were prepared with polysaccharide processed by the serial dilution method, after which *F. fujikuroi* Nirenberg was cultured at a concentration of 10^6 spores/mL and inoculated at 30°C for 24 hours. The plot in which only *F. fujikuroi* Nirenberg was inoculated was used as the positive control group, and the negative control group had a triazole solution diluted 500 times. The absorbance was measured at 600nm with four-hour intervals using an Eon microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA) while incubating the sample. As a result, the minimum concentration at which the growth of bacteria did not appear was determined as the MIC.

Inoculation of Bakanae disease

Ilpumbyeo, known as a species susceptibility to Bakanae disease, was used as the test variety. In this case, the foreign matter was removed from the Ilpumbyeo by washing for 1 hour with running water after parceling out it from the Korea Seed & Variety Service in May of 2014.

F. fujikuroi Nirenberg (KACC:44006) parceled out from the KACC (Korea Agricultural Culture Collection) was used as an inoculum. The inoculum was prepared at a concentration of 1×10^3 ea/mL after recovering the spores by pouring 20 mL of sterile water after removing the air hyphae with a glass scrapper.

Manufacturing of *Ginkgo biloba* outer seed coat control agent, and seeding test

Because the *Ginkgo biloba* outer seed coat contains a fat component which does not dissolve well in water at low temperatures, the control agent containing it was prepared as a water-dispersible powder. The powder was prepared by mixing the *Ginkgo biloba* outer seed coat extracts, a surfactant and methanol at a ratio of 6:2:2, with this water-dispersible powder used as a Bakanae disease control agent. The control liquid was prepared by mixing the seed coat control agent at concentrations of 10%, 7.5%, 5%, 2.5% and 1% in 500ml of water, and the Ilpumbyeo in which the *F. fujikuroi* Nirenberg was inoculated depending on the concentration level, as noted above, was sown after soaking in the control liquid at 30°C for 48 hours. Ilpumbyeo was sown with 220 particles in a flowerpot of 15 cm (dia) x

13 cm (height) in size, and the test plot was created by three-fold repetition of a completely randomized design. The growth temperature in the greenhouse was held at 30°C. The investigation of the occurrence of Bakanae disease after sowing was conducted on the 18th day and the 25th day after sowing, and at the time of the investigation on the 18th day, only the seeds undergoing succulent growth were separated, noting the occurrence of Bakanae disease; during the investigation of the 25th day, the succulent growth seeds and the seeds for which foliage did not emerge, as well as withered seeds were investigated and considered as examples of the occurrence of Bakanae disease. The control plot, treated with a liquid which dilutes triazole to a 500-fold solution, was used as a comparison group by sowing using the same method as the test plot.

Statistical processing

Duncan's test was used with SAS 8.0 (Statistical Analysis System) as the test for the hypha growth inhibition of the *Ginkgo biloba* outer seed coat and the occurrence rate of Bakanae disease.

RESULTS AND DISCUSSION

Antibacterial Activity of the *Ginkgo biloba* outer seed coat extract

Because *Ginkgo biloba* outer seed coats, which are currently discarded, contain ginkgolic acid, they have the antibacterial activity against tubercular bacillus (Park et al., 2011). It is known that polysaccharide exists in the *Ginkgo biloba* outer seed coat, and such compositions of sugar have high antibacterial activity levels for pathogenic microorganisms and viruses. In this study, we investigate the antibacterial activities of Bakanae disease using extracts of *Ginkgo biloba* outer seed coats. The results are shown in Table 1.

In the untreated plot, it was identified that the hypha growth diameter was 54mm on the 5th day after cultivating. For the control plot, *F. fujikuroi* Nirenberg did not grow at all at the treatment concentration level, becoming completely extinct. Thus, was found that the antibacterial activity of *Ginkgo biloba* outer seed coat extracts is high.

It showed the tendency that the more the mixing level of the *Ginkgo biloba* outer seed coat extracts is high, the more the growth of the hypha of Bakanae disease is inhibited, and in the level of 500 mg·L^{-1}, the growth diameter of hypha showed the highest antibacterial activity as 22mm, and in the level of 400 mg·L^{-1}, the growth diameter was 40% level of the untreated plot as 25mm, so it was confirmed that the growth of *F. fujikuroi* Nirenberg was effectively inhibited. However, at the level of 300 mg·L^{-1} or less, the hypha growth diameter showed a growth inhibition ratio of 50% or less in comparison with the untreated plot at 32mm. At the level of 200 mg·L^{-1} or less, the growth diameter was 41mm or more, indicating that the antifungal activity was low.

In the research results of Choi et al. (2013), it has reported that the fermented extract of the *Ginkgo biloba* outer seed coat has high antibacterial activity against mold of the types which cause various plant diseases. It was also identified that in this experiment, the extract with a concentration level of 400 mg·L^{-1} or more can inhibit 60% or more of the growth of Bakanae disease.

The *Ginkgo biloba* outer seed coat contains a glucose-based polysaccharide which is now widely used in the medical field. This type of polysaccharide has glucose, rhamnose and mannose; or mannose, glucose and galactose linkages. It is known that the polysaccharide extracted from mushrooms has anti-cancer activity for various cancer cells (Tong et al., 2009; Yang et al., 2005). Zhu et al. (2012) reported that polysaccharide separated from mushrooms has antibacterial activity against certain types of bacteria. Fig. 1 shows results which indicate that the polysaccharide extracted from the *Ginkgo biloba* outer seed coat has antibacterial activity against *F. fujikuroi* Nirenberg, as found with the MIC (minimum inhibitory concentration) method. It was found that spores

Table 1: Effect of antifungal activity on Bakanae disease according to the added concentration of the *Ginkgo biloba* outer seed coat extract

Concentration (mg L^{-1})	Growth diameter of hypha (mm)
Control[a]	-[b]
Untreated[c]	54±3.9a[d]
100	43±2.8b
200	41±1.5b
300	32±2.7c
400	25±1.1d
500	22±2.3d

[a]Triazole water-dispersible powder, [b]Hyphae did not grow and became extinct, [c]PDA Medium with control medicine not mixed (Untreated plot), [d]Mean separation within columns according to Duncan's multiple range test ($P \leq 0.05$)

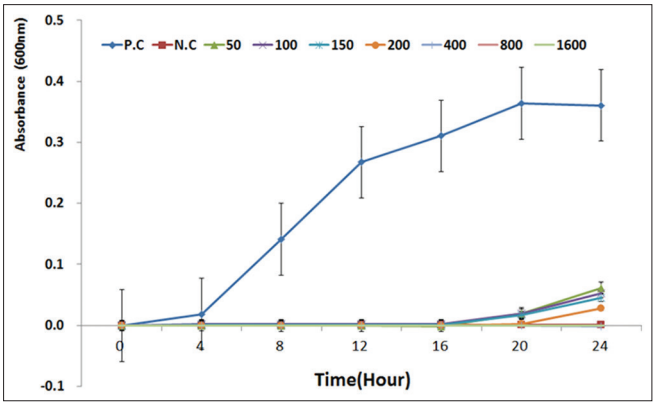

Fig 1. Minimum inhibitory concentrations (MIC) of *Fusarium fujikuroi* Nirenberg according to the treatment concentration of *Ginkgo biloba* outer seed coat extract.

are vigorously generated in the untreated plot, but it could be confirmed that the higher the added concentrations of polysaccharide become, the greater the antibacterial activity for *F. fujikuroi* Nirenberg is. It was also identified that spore generation of *F. fujikuroi* Nirenberg at all treatment concentrations was effectively inhibited in comparison with the untreated plot and that spore generation at 50 $mg \cdot L^{-1}$ is significantly lowered.

These results suggest that the polysaccharide contained in the *Ginkgo biloba* outer seed coat has high antibacterial activity despite the fact that only small amounts exist and that the availability as a control agent against Bakanae disease is high.

Ginkgo biloba outer seed coat extract field test of Bakanae disease

The genus Fusarium is one of the most important groups of phytopathogenic fungi. They infect a broad spectrum of crops worldwide and are responsible for huge economic losses due to yield reductions and mycotoxin contamination (Philipp Wiemann etal 2013). For the seeds infected with *F. fujikuroi* Nirenberg, they showed succulent growth during the seeding period, after which they withered before being transplanted. Although the transplants were accomplished, they withered before a proper formation; consequently, the infection of seeds has a direct effect on the yield (Ou, 1985). In order to control Bakanae disease, as a result of testing the antibacterial activity against *F. fujikuroi* Nirenberg, the causative organism of Bakanae disease, it was identified that the *Ginkgo biloba* outer seed coat has antibacterial activity. The results of the investigation of the control effect against Bakanae disease by the seed disinfection method with the prepared *Ginkgo biloba* outer seed coat as a water-dispersible powder are shown in Table 2.

Polysaccharide exists in the *Ginkgo biloba* outer seed coat extracts, but it is known that phytotoxicity exists in polysaccharide at a high concentration level (Strobel, 1967). However, the treatment liquid with the 10% mixture of the *Ginkgo biloba* outer seed coat control agent used in this experiment also showed a high seeding stand rate of 89%. At all concentrations, statistical significance of the seeding stand rate was not found, in comparison with the control plot and the untreated plot, and it was judged that the problems of toxicity and damage from agricultural chemicals at the time of the disinfection of the seed will not arise in this case.

In the untreated plot, succulent growth seeding, which is a symptom of Bakanae disease, was observed, and it was confirmed that the occurrence rate of Bakanae disease was 44.1%. In the control plot, the control effect at the time of the investigation on the 18th day showed an excellent control effect of 96.6%. The *Ginkgo biloba* outer seed coat control agent showed a control effect at a lower level than the control plot, but a control effect of 80% could be confirmed at a mixed level of 10% and 7.5%. However, at a mixing level with less than 5% of the *Ginkgo biloba* outer seed coat control agent, the occurrence rate of Bakanae disease exceeded 22.4%. Thus, it was judged that there is almost no control effect because the occurrence rates of Bakanae disease at the levels of 2.5% and 1% were 40% or more.

Moreover, 25 days after sowing, the same trend noted on the 18th day was noted. For the untreated plot, the ratio of withered seeding was also being increased. Also, at the levels at which the 2.5% and 1% *Ginkgo biloba* outer seed coat control agents were mixed, withered seeding and other effects were generated, and the control efficiency was low, at 40%. The 5% mixing level of the *Ginkgo biloba* outer

Table 2: Control effect of *Ginkgo biloba outer* seed coat control agent on Bakanae disease after sowing

Day	Mixing Level Treatment (%)	Seedling stand rate (%)	Diseased plant (%)	Control value (%)
Sowing after 18 days	Untreated[a]	92±1.5a[c]	44.1±5.8a	-
	Control[b]	90±2.4a	2.8±0.7d	96.6±2.1a
	10	89±3.7a	14.3±0.9c	85.0±1.8b
	7.5	90±1.4a	15.4±2.1c	84.3±2.5b
	5	93±2.5a	22.4±5.5b	74.3±3.9c
	2.5	92±1.2a	41.4±6.7a	47.9±6.5d
	1	90±2.1a	43.9±7.8a	45.6±6.8d
Sowing after 25 days	Untreated	53±6.7c	48.1±2.7a	-
	Control	88±2.5a	4.1±1.1d	95.4±1.0a
	10	87±3.4a	16.3±0.5c	83.5±0.5b
	7.5	88±2.8a	17.9±0.5c	81.3±1.1b
	5	81±5.7a	31.7±3.7b	65.6±4.8c
	2.5	68±4.8b	49.4±4.1a	48.6±2.6d
	1	72±7.2b	47.1±5.8a	40.9±3.4d

[a]Untreated experimental plot not inoculated with *Fusarium fujikuroi* Nirenberg, [b]Experimental plot disinfected with the triazole water-dispersible powder solution 500 times, [c]Mean separation within columns by Duncan's multiple range test ($P \leq 0.05$)

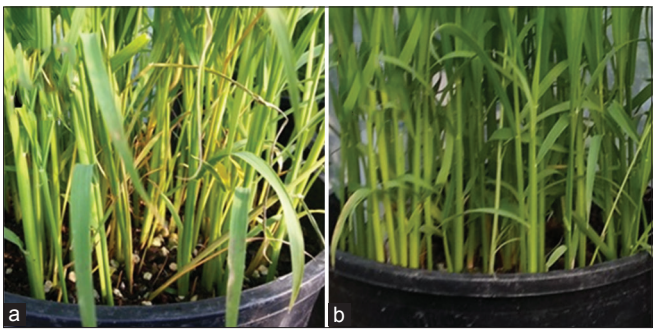

Fig 2. State of rice growth of treated *Ginkgo biloba* outer seed coat. (a) Disinfection of *Ginkgo biloba* outer seed coat extracts showing withering rice. (b) *Ginkgo biloba* outer seed coat extracts treated with 7.5%.

seed coat control agent showed a stronger control effect at 65.6% than the untreated plot or the low concentration level, while at 10% and 7.5% mixing levels of the *Ginkgo biloba* outer seed coat control agent, a high control effect of 80% or more was confirmed, as shown in Fig. 2. As a result, it is considered that to prevent Bakanae disease using the *Ginkgo biloba* outer seed coat control composite, disinfection of the seed should be conducted with the liquid at a mixing level exceeding 7.5%.

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Authors' contribution

Tae Seok Oh and Youn Jin Park was the main researcher and author of this manuscript. Chang ho Kim participated in the laboratory experimentation,Young Koo Cho performed the field experimentation, Myung Jun Jang contributed significantly to the research effort and writing, editing specific sections and approved the final version of the manuscript.

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