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Full Length Research Paper

Screening and evaluation of antiphytopathogenic activity of endophytic fungi from live foliages of *Ginkgo biobla* L.

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Twenty-four strains of endophytic fungi were isolated from 50 fresh leaves samples of *Ginkgo biobla* L collected using the cultivation-dependent method from the campus of Anhui agricultural university in Hefei of China. Out of 24 endophytes investigated, 12 strains can produce *in vitro* substances that are inhibitory to all or a few of tested phytopathogens whereas the rest yielded nothing active, while all fermented broths of endophytes showed activities against one and more tested phytopathogens. These results indicated that endophytic fungi could play an important role in protection of *G. biobla* L from disease as well as an excellent resource for searching for natural antifungal compounds.

Key words: Endophytic fungi, *Ginkgo biobla* L, antimicrobial activity.

INTRODUCTION

Plant diseases, particularly plant pathogenic fungi, are one of the most principal factors for decreasing food production. Application of a large amount of synthetic fungicides has been considered to be one of the cheapest and most common approaches for the control. However, these agrochemicals usually take long timelines to be degraded completely and then cause heavy toxicity to human being, animals, etc. Furthermore, phyto-pathogens have been developing huge resistances to frequent pesticides-using, sequentially the effectiveness of these pesticides are hugely decreased (Rosen et al., 1981). Accordingly, it is an urgent need to find safer antifungal substitutes which are expected to be renewable, naturally environment-friendly and easily obtainable (Liu et al., 2001).

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Abbreviations: PDA, Potato dextrose agar; **CFU,** colony-forming units; **CGMCC,** China general microbiological culture collection center.

Endophytic fungi, by definition, are the fungi which spend the whole or part of their lifecycle colonizing inter-and/or intracellularly inside the healthy tissues of the host plants, typically without causing apparent symptoms of disease (Azevedo and Araújo, 2007). Endophytes are mutualistic to their hosts, at least some of them are thought to be making returns from the plant by producing special substances, such as secondary metabolites to prevent the hosts from successful attack of fungi, pests and mammals (Bacon and White, 2000; Arnold 2007; Robinson et al., 2009). As a matter of fact, metabolites of endophytes have been reported to inhibit a number of microorganisms (Fisher et al., 1984; Gurney and Mantle, 1993; Kumar et al., 2004; Aly et al., 2010).

In the past decades, plenty of bioactive metabolites from these microorganisms have been tested to have application as medicinal and agrochemical candidates. Pioneeringly, a taxol-producing fungus was obtained from Pacific yew (Strobel et al., 1993). *Ginkgo biloba* L, is mainly distributed in mainland China, and is one of the most ancient plants on the earth with fossil records dating back more than 200 million years. It is scarcely infected with plant diseases and insect pests during a long life and has been widely used as an important and traditional

medicine for various ailments in China (Chen et al., 2007). As part of our on-going search for bioactive substances, it was found that endophytic fungi from the healthy and fresh foliage of *G. biloba* L showed antagonistic activities against plant pathogenic fungi. In this paper, we describe the isolation and assessment of antagonistic activity of the endophytic fungi.

MATERIALS AND METHODS

Sampling

Samples were collected in September 2010 from the campus of Anhui agricultural university in Hefei of Anhui province, People Republic of China (31°86'N and 117°25'E). The age of *G. biloba* L is approximately more than 30 years. The trees were chosen randomly and the healthy and fresh foliage were picked out and taken into the laboratory. Fungal endophytes were isolated within 24 h after sampling.

Isolation of fungal endophytes

The fifty foliage samples were firstly washed with tap water to remove soil and other debris, and were surface-sterilized by consecutive immersions for 1 min in 75% ethanol, 5 min in 2.5% sodium hypochlorite (NaClO), and then 1 min in sterile distilled water, the immersion in sterile distilled water were repeated three times. The materials were then surface-dried with sterile paper (Guo et al., 2000). Leaves were cut into 0.5 ×1 cm pieces in a laminar flow hood, and were inoculated on potato dextrose agar (PDA) plates containing streptomycin sulphate (the final concentration with ten thousand U, North China pharmaceutical group corporation, China) and penicillin sodium (the final concentration with eight thousand U, North China pharmaceutical group corporation, China) to suppress bacterial contamination, and then the Petri dishes were incubated under dark at 28±1°C until emergence of fungal hypha from inside the samples (Bayman et al., 1997). The growing mycelia were purified on PDA plates without antibiotics. The isolated pure cultures were stored in PDA slant tubes under dark at 4°C. The effectiveness of the surface sterilezation was confirmed by randomly imprinting sterilized sections on PDA plates. The surface was considered sterile when no microbial growth was observed on the imprinted plates after 3 to 5 days of incubation.

Preliminary screening

The antifungal activities of endophytes against six tested phytopathogenic fungi were determined by a dual cultural method (Alvarez et al., 2001; Li et al., 2010). Briefly, a 6 mm diameter disc of fungal endophyte was inoculated in the one side of the PDA plates and a 6 mm diameter disc of the tested phytopathogens was cut from the periphery of four days old culture on PDA plates to place mycelia surface down on opposite edges of the test plates against the other side of the dish. The plates were incubated in the dark at 28±1°C for 4 days old. Three replicates were carried out in the experiment.

The antibacterial activities of endophytes against four tested pathogenic bacteria were also determined by agar block assays. 6 mm diameter discs of the endophytes were placed on the assay plates spread with tested bacterial strains with the concentration of approximately 1.0×10^6 colony-forming units (CFU). The tested strains were purchased from China General Microbiological Culture Collection Center (CGMCC). The tested strains were cultured according to the instructions of the provider. Three replicates were carried out in the experiment.

Test pathogenic fungi and bacteria

In our current study, the six crop-threatening pathogenic fungi were used as inhibitory indicators, such as *Phytophthora capasici* (PC) *Verticillium tricorpsis* (VT), *Fusarium graminearum* (FG), *Valsa mali* (VM), *Colletotrichum gloeosporioides* (CG) and *Venturia nashicola* (VN); as well, the four pathogenic bacteria, *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Xanthomonas oryzae* (XO) and *Escherichia coli* (EC), were used as inhibitory indicators in the experiment.

Bioassay of fermented broths of endophyte isolates

Antifungal activity

The obtained fermented broths of endophyte isolates were tested for the antifungal activity against test phytopathogenic fungi with the whole plate diffusion method (Nishioka et al., 1997). Briefly, the fermented broth was filtrated by 0.22 μm filters to remove contaminate cells, and then 1 ml sterile filtrate was fully mingled with 9 ml fused PDA to make a test plate. Once the agar had solidified, an agar block (6 mm) with plant pathogen fungi culture was inversely planted in the centre of each Petri dish, and the plates were incubated in an incubator at 28±1□ for 4 days old. Finally, the diameters of the tested fungi were measured. 80% carbendazim wet table powder (Hebei Guanlong Agrochemical Co. Limited) dissolving with sterile water was used as a positive control. Each inhibition experiment was replicated thrice. The inhibitory effect was calculated using following equation:

$$IR\ (\%) = \frac{Dc - Dt}{Dc} \times 100$$

Where IR = inhibitory rate (%), D_c = diameter of negative control (mm), D_t = diameter of treatment or positive control (mm).

RESULTS

Antagonistic action of isolates endophytic fungi

A total twenty-four endophytic fungi were isolated from the healthy and fresh leaves of *G. biobla* L. The same endophytic isolates were experientially eliminated on morphology characteristic by light microscope and naked eyes. The endophyte isolates were strikingly antagonized the tested pathogens, which was illustrated in Table 1. Among them, six endophytes (25%) could produce the substances antagonistic against *V. mali,* four (16.67%) against *V. nashicola*, two (8.33%) against *P. capasici*, three (12.5%) against *V. tricorpsis*, *F. graminearum* and *C. gloeosporioides*, respectively, the results demonstrated that some of the endophytes cultures were potent for inhibiting the growth of tested phytopathogenic fungi. However, it needs more investigation to disclose the possibility that those endophytes would be used for

biocontrol of the related plant diseases. As a conclusion

Table 1. Antagonistic action of isolates endophytes against tested pathogens.

Strains	Antagonistic activity									
	PC	VT	FG	VM	PM	VN	SA	BS	EC	XO
GBL01	−	−	−	+	−	−	−	+	−	+
GBL02	+	+	−	−	−	−	−	−	−	−
GBL03	−	−	−	+	−	−	−	−	−	−
GBL04	−	−	−	−	−	+	−	−	−	−
GBL05	−	−	−	−	−	−	−	−	−	−
GBL06	−	−	−	−	−	−	−	−	−	−
GBL07	−	−	−	−	−	−	−	−	−	−
GBL08	−	−	−	+	+	+	−	−	−	−
GBL09	−	−	−	−	−	−	−	+	+	−
GBL10	−	−	−	−	−	−	−	−	−	−
GBL11	−	−	−	−	−	+	−	−	−	−
GBL12	−	−	−	−	−	−	−	+	−	−
GBL13	−	−	−	+	−	−	−	−	−	−
GBL14	−	−	−	+	−	−	−	−	−	−
GBL15	−	−	+	−	−	−	−	+	−	−
GBL16	−	−	−	−	−	−	−	−	−	−
GBL17	−	−	−	−	−	−	−	−	−	−
GBL18	−	−	−	−	−	−	−	−	−	−
GBL19	+	−	−	−	−	−	−	+	−	−
GBL20	−	−	−	+	−	−	−	−	−	−
GBL21	−	−	−	−	−	−	−	−	−	−
GBL22	−	−	−	−	−	−	−	−	−	−
GBL23	−	−	−	−	−	+	−	−	−	−
GBL24	−	−	−	−	−	−	−	−	−	−

+, antagonistic activity; −, no antagonistic activity.

to above observation, it was found that the growths of 12 endophyte isolates were antagonized by at least one phytopathogen. Especially, the growth of endophyte GBL08 was strongly antagonistic to *V. mali*, *C. gloeosporioides* and *V. nashicola.*

Antimicrobial activities of endophytic fungi fermented broths

The antimicrobial activities of fermented broths were investigated after filtrating by 0.22 µm filters and removing contaminate cells. The results were illustrated in Table 2. Regarding the antifungal potential of the endophytes fermented broths, the growth of *P. capasici* could be more strongly inhibited by eight endophytes cultures (GBL01, GBL08, GBL09, GBL11, GBL13, GBL14, GBL15 and GBL23) than by the positive control carbendazim with the concentration of 200 µg/ml, that of *V. tricorpsis* by twenty-three (except GBL03), *F. graminearum* by six (GBL06, GBL07, GBL11, GBL12, GBL20 and GBL24), *V. mali,* by twenty-one (except GBL06, GBL08 and GBL19), *C. gloeosporioides* by ten (GBL01, GBL02, GBL06, GBL11, GBL13, GBL14, GBL17, GBL19 and GBL23) and *V. nashicola* by eleven (GBL01, GBL02, GBL08, GBL10, GBL11, GBL13, GBL15, GBL18, GBL19, GBL23 and GBL24). Especially, the fermented broths of GBL11 more strikingly antagonized six tested phytopathgenic fungi (Figure 1) than carbendazim. And none of endophytic fungi cultures stimulated the growth of tested pathogens. It is highly desired to obtain from the fermented broths of endophyte GBL11 new antifungal substances which could be used as lead compound(s) for the development of new bio-fungicide(s) necessitated for the control of crop-threatening diseases.

DISCUSSION

Table 2. Antagonistic action of fermented broths of endophyte isolates against tested pathogens.

Strains	Antagonistic activity					
	PC	VT	FG	VM	PM	VN
GBL01	7.22±1.10	17.14±0.18	9.80±4.22	2.86±1.86	20.98±0.21	48.54±11.87
GBL02	2.78±1.41	7.35±2.00	0.85±0.40	4.07±1.55	4.32±0.63	33.76±0.77
GBL03	1.72±1.31	0.78±0.41	3.78±1.22	2.28±1.25	1.78±1.14	4.78±1.31
GBL04	-1.78±0.19	12.24±3.61	9.51±0.13	20.50±0.89	1.49±1.06	22.15±0.14
GBL05	-0.68±1.35	8.80±0.18	6.88±2.31	26.58±7.70	-2.80±2.37	37.80±7.79
GBL06	1.50±0.19	20.03±5.95	15.22±3.59	-3.23±3.01	14.93±3.69	19.88±21.44
GBL07	-4.23±0.19	9.82±1.98	24.55±2.43	6.15±3.57	-2.24±0.53	18.01±2.64
GBL08	6.89±1.57	10.31±1.09	-7.10±6.83	-4.01±4.58	4.17±0.42	46.72±0.52
GBL09	8.47±0.39	26.15±1.26	10.24±0.64	62.30±8.03	-1.12±0.53	19.00±11.27
GBL10	1.09±0.77	17.60±2.53	12.14±9.48	5.68±0.22	4.10±5.28	46.46±17.26
GBL11	38.00±3.14	1.75±0.36	13.64±1.61	61.43±3.42	22.32±0.84	40.51±10.32
GBL12	2.23±2.90	19.13±1.08	27.72±1.54	24.37±4.80	2.05±1.32	1.28±0.97
GBL13	21.00±0.79	11.21±0.18	-20.03±2.21	10.82±0.54	6.10±0.21	34.12±2.84
GBL14	15.78±0.31	6.70±2.19	-6.96±9.44	16.37±2.18	5.36±2.10	16.24±3.35
GBL15	27.44±0.16	7.35±1.64	1.99±1.21	10.49±1.32	2.53±0.21	44.89±4.13
GBL16	1.23±0.75	3.60±1.53	2.14±2.48	5.18±1.22	2.10±1.28	6.48±3.26
GBL17	0.41±0.19	19.64±1.08	7.52±1.67	7.13±5.58	4.48±1.06	22.74±11.00
GBL18	1.64±1.55	8.55±3.07	11.41±4.61	3.71±2.79	-5.60±4.75	25.00±1.39
GBL19	2.78±0.47	4.77±0.18	2.13±2.61	0.82±0.39	4.46±0.84	47.08±3.61
GBL20	1.64±1.16	32.02±0.90	22.28±2.56	46.53±1.12	-2.43±1.32	9.06±10.30
GBL21	1.41±1.19	4.64±1.34	5.52±1.54	4.15±1.29	1.48±1.65	6.74±4.25
GBL22	2.41±1.25	5.35±1.56	4.52±1.35	3.13±1.32	-1.48±1.15	5.43±3.21
GBL23	25.89±2.99	11.08±2.19	-26.28±7.43	9.62±2.56	8.18±3.16	58.76±7.23
GBL24	1.50±0.19	14.67±0.90	21.20±0.51	88.96±1.34	2.61±0.53	44.88±3.34
Carbendazim	5.33±3.14	1.68±2.37	12.64±7.03	1.01±1.04	2.68±1.26	24.09±4.65

Carbendazim at the concentration of 200 µg/ml.

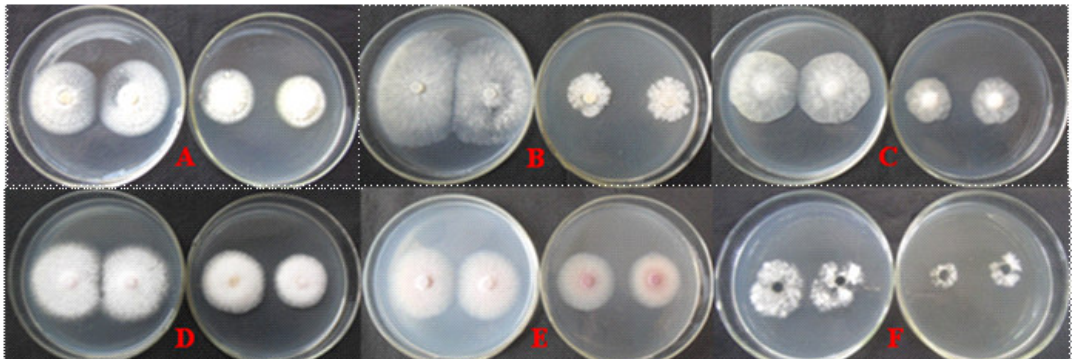

Figure 1. The inhibitory activities of endophyte GBL11 fermented broths against six tested pathogenic fungi. (A against PM, B against VM, C against PC, D against FG, E against VT and F against VN; the left are CK and the right is tested).

On the one hand, the necessary energy and nutrition requirement of endophytes are supplied by host plants; on the other hand, endophytes affect the early evolution of host plants and impose selective pressure on the plants by means of their metabolites or signal transduction. Furthermore, plant endophytes are natural

constituents in plant-microbe ecosystem; they not only might promote adoptability of host plants in different

environments, but also enhance the balance of plant-microbe ecosystem (Liu et al., 2010a, b; Ezra et al., 2004; Hyde and Soytong, 2008). The plants have an enormous diversity of endophytes that make bioactive metabolites, some of which are inhibitory or lethal, to various pathogenic organisms. Recent reports also claimed that endophytic microbes play a major role in the therapeutic properties of plants (Mahesh et al., 2008; Qiu et al., 2010). The advantage of the cultivation-dependent method is effective for rapid discovery of a large number of endophytic fungal species from plant tissues.

In our present study on endophytes of *G. biobla* L, twenty-four endophyte isolates were obtained using this method, and two bioassay methods were carried out to avoid the endophyte isolates producing bioactive metabolites omitting. Strain GBL08 showed strong activities against *V. mali*, *C. gloeosporioides* and *V nashicola* using a dual cultural method, while the fermented broth of strain GBL11 revealed sparkly activities against tested pathogenic fungi *P. capasici*, *V. tricorpsis*, *F. graminearum*, *V. mali*, *C. gloeosporioides* and *V. nashicola.* These results presented in this study suggested that strain GBL08 and GBL11 could be valuable candidates for the discovery of new drugs or agents for anti-phytopathogenic fungi. The major endophytes isolated in this current study were not sporulating, and the mycelia sterilia obtained were not identified using traditional morphological observation method. So, in the present study, the same endophytes were experientially eliminated only based on morphology characteristic by light microscope and naked eyes, the endophyte isolates obtained were not identified or classified. Nowadays, the techniques of molecular biology are widely used for the identification of microorganisms (Arnold et al., 2000; Okane et al., 2001; Baayen et al., 2002; Qiu et al., 2010; Bridge and Newsham, 2009; Duong et al., 2009), especially, the ITS region has typically been most useful for molecular systematics at the species level, and even within species. Because of its higher degree of variation than other genic regions of rDNA, sequencing the ITS region can sometimes identify the fungi to species and subspecies level. Future studies should be to identify all endophytic fungi isolated and to purify the bioactive constituents of endophytic fungi from *G. biloba* L.

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