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Identification of a novel broad-spectrum anti-fungal strain of *Pseudomonas aeruginosa* (SU8) and effect of its crude metabolites against *Rhizoctonia solani* and *Pyricularia oryzae*

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ABSTRACT

The objective of this study was to evaluate the potential applications of the antagonistic bacterium SU8 for biocontrol of plant diseases. In this study, the bacterial stain SU8 showed high anti-fungal activity and characterized using morphological, physiological, biochemical tests, sequencing of 16S rDNA genes, and phylogenetic analysis. Its crude metabolites as ferment filtrate was evaluated for its inhibitory effect on the pathogenicity of *Rhizoctonia solani* and *Pyricularia oryzae* on rice, and several verification experiments were conducted to evaluate its efficacy for controlling infection. The antagonistic bacteria SU8 was identified to be *Pseudomonas aeruginosa* and was registered in GenBank with a record number of HQ283487. Immersion treatment of *Rhizoctonia solani* and *Pyricularia oryzae* with SU8 crude metabolites in fermented filtrate demonstrated a superior pathogenicity-reducing effect on the pathogens infected rice, as demonstrated by a reduction in the plaque size on leaves infected with *Rhizoctonia solani* and *Pyricularia oryzae* by 0.4%-63.6%, 0.5%-58.3%, respectively. Furthermore, the lesion sizes were further reduced with increased soaking time and higher concentrations of the fermented filtrate. Spore germination of *P. oryzae* was inhibited (EC_{50} = 548.3457 μg/mL), but its toxicity was lower than that of Tricyclazole. The fermented filtrate reduced both rice sheath blight and rice blast up to 70% in pot cultures of rice plants by the concentration 120 μg/mL, showing better protection than that reported for other microbial biocontrol agents previously. The goals for utilizing SU8 were protection and therapy. Field experiment results showed that the SU8 fermented filtrate was able to effectively control rice sheath blight and rice blast, with reductions of 23.8-63.9% and 29.9-61.9%, respectively. However, the highest level of control achieved with the fermented filtrate was lower than that of Jinggangmycin and Tricyclazole. The antagonistic bacterial isolate SU8 showed superior potential as a bio-control agent in controlling rice sheath blight and rice blast. The high antifungal activity of its metabolites in fermented filtrate shows the possibility that it may be used to develop new fungicides.

Key words: *Pseudomonas aeruginosa;* Biocontrol; *Rhizoctonia solani; Pyricularia oryzae.*

INTRODUCTION

Rhizoctonia solani (*R. solani)* and *Pyricularia oryzae* (*P. oryzae)* are two major pathogens of rice in the world. *R. solani* and *P. oryzae* can be responsible for up to a 30% and 10% loss of yield every year, respectively (Prasad & Eizenga 2008, Castillo *et al.* 2010, Ye *et al.* 2010). *R. solani* is mainly controlled by Jinggangmycin at present (Shibata *et al.* 1982, Müller *et al.* 1995, Zhang 2007). However, Jinggangmycin resistance of *R. solani* is becoming an increasingly serious issue because of its high frequent usage (Baby & Hasussmann 1993). Since the early 1970s, the IRRI, China, and America have attempted to breed resistant rice varieties to *R.solani* (Bonman *et al.* 1992). However, screening for highly resistant varieties is challenging. because *R. solani* is strongly saprophytic and widely parasitic.

Chemical pesticides have been employed to limit *P. oryzae*, including tricyclazole, kitazine and iprobenfos (Hamer 1991, Causse *et al.* 1994, Wang *et al.* 1994). However, the applications of these pesticides are limited because of their toxicity and increasing resistance of the fungi (Sicard *et al.* 1997, Kongprakhon *et al.* 2010).

Although resistant varieties of rice were obtained, the diversity of pathogenicity and physiology of *P. oryzae* caused some difficulties in terms of rice breeding (Bonman *et al.* 1989).

Biological control is regarded as a safe and highly efficient way to control *R. solani* and *P. oryzae* infection to rice (Mew & Rosales 1986, Voisard *et al.* 1989, Cottyn *et al.* 2001, Kawamata *et al.* 2004). Thus, we sought to investigate the potential application of the broad-spectrum antagonistic crude metabolites of the isolate SU8 (*Pseudomonas aeruginosa*) as a biocontrol agent to control *R. solani* and *P. oryzae* infection.

The bacterial isolate SU8 was collected from the rice-duck ecosystem, and it was identified as *Pseudomonas aeruginosa* (GenBank: HQ283487). The potential control of this bacterium against rice sheath blight and rice blast was evaluated. The current study provide practical insights to enrich the bacterial pool present in soil and to prevent and treat *R. solani* and *P. oryzae*, infections. This study further explores the potential of high-efficient, low-toxic, environmental-friendly biological agents to substitute chemical pesticide in the future.

MATERIALS AND METHODS

Rice variety

Rice variety 'luliangyou996' was provided by Institute of Rice Research, Hunan Agricultural University, Changsha, China.

Microorganisms and cultivation

The antagonistic bacterium isolate SU8 (*Pseudomonas aeruginosa*) was collected by dilution plate technique from the rice-duck paddy fields in Wulong village farm, Beisheng town, Liuyang city of Hunan province in 2010. The antagonistic bacterium SU8 inhibited plant pathogenic fungi by plate confrontation method (Zhang,2010). The antagonistic bacterium SU8 (Abbreviation SU8) is preserved in Chinese center for type culture collection (CCTCC NO: M2013178).

Plant pathogenic fungi *Rhizoctonia solani, Pyricularia oryzae, Phytophthora parasitica, Fusarium graminearum, Colletotrichum gloeosporioides, Alternaria alternata,* and *Ustilaginoidea oryzae* were retrieved from the safety monitoring center, College of Plant Protection, Hunan Agricultural University.

The antagonistic isolate SU8 was cultured in NA medium (3 g L^{-1} beef extract, 5 g L^{-1} peptone, 5 g L^{-1} sodium chloride, and 15-20 g L^{-1} agar, at pH 7.2-7.3) (Tiangen Biotech, Beijing) (Fang 2004), at 26-28°C for 48 h.

Six inoculating loops containing SU8 bacteria were transferred into an Erlenmeyer flask filled with NB medium (3 g L^{-1} beef extract, 5 g L^{-1} peptone, and 5 g L^{-1} sodium chloride, at pH 7.2-7.3) (Tiangen Biotech, Beijing) (Fang 2004), and shaken at 220 r min^{-1} at 28°C for 96 hours.

SU8 antimicrobial activity

All pathogenic plant fungi were cultured on Potato Dextrose Agar (PDA) (200 g L^{-1} potato, 20 g L^{-1} of glucose, and 15-20 g L^{-1} of agar) (Tiangen Biotech, Beijing) (Fang 2004) at 25-28°C for 72 h. The antimicrobial activity of antagonistic bacteria SU8 was determined using the pair culture method (Zhang *et al.* 2010) with seven types of plant pathogenic fungi. The size of the inhibition zone was recorded over time.

Morphological observation

The morphology and structure of SU8 was observed with a scanning electron microscope (JSM-6380 LV, JEOL., Japan; BCD-215KJM, Qingdao Haier Co. Ltd.,China). A gram stain was also performed (Zhang *et al.* 2010).

Physiological and biochemical characteristics

The optimal physiological and biochemical conditions of SU8 cultivation, including salinity range, aerobic and anaerobic respiration, the utilization and disintegration of carbon and nitrogen compounds, the disintegration of macromolecular compounds were determined according to the Common Bacterial System Identification Manual and Fang Zhongda's Plant Pathology Research Methods (Fang 2004).

Sequence analysis and construction of a phylogenetic tree

The genomic DNA of SU8 was extracted with a bacterial genomic DNA extraction kit (Qiagen, Germany). The primers for 16S rDNA were F: 5'-AGAGTTTGACCTGGCTCAG-3' and R: 5'-AAGGAGGTGATCCAGCCGCA-3'. PCR amplification of 16S rDNA was conducted with a Taq Platinum PCR MasterMix kit (Tiangen Biotech, Beijing), followed by sequencing of the PCR product. The sequence was analyzed and compared with BLAST GenBank. Finally, a polygenetic tree was constructed by MEGA4.0 software based on the sequencing results. A neighbor-joining algorithm and Jukes-Cantor model were used, and the exhibition number was set to 1,000. Bootstrap test results less than 50 were not shown.

Effect of SU8 fermented filtrate on the pathogenicity of *R. solani* and *P. oryzae*

SU8 bacteria were cultured in NA, shaken at 220 r min^{-1}, at 28°C for 48 hours. The culture was then centrifuged at 8,000 r min^{-1} for 15 min (5417R Small high-speed desktop centrifuge, Eppendorf, Germany). Fermented liquid from the bacterial culture was then filtered through a 0.22 μm filter and stored at 4°C.

The fermented filtrate was diluted 2-, 5-, 10-, or 20-fold with sterile water. Prosthecae of *R. solani* (5 mm in diameter, 3 mm thick) and *P. oryzae* hyphae blocks, which had been cultivated for 48 hours and 96 hours, respectively, were soaked in the various dilutions of fermented filtrate for 2, 4, 8, 16, or 24 h. Then, the soaked *R. solani* prosthecae were placed on a leaf sheath of tillering stage rice. Every treatment need 100 rice, with the repeated 3 times respectively. Meanwhile, the same experiment was repeated three times, and with the SU8 inactivation fermented filtrate (not diluted) as negative control.

Four days later, the diameters of the scabs on leaf sheaths and blades were calculated according to the following formulas: area of circular disease spot, $S = \prod (d/2)^2$, and area of oval disease spots,

$S = \prod ab$, where "S" represents area, "a" represents length, and "b" represents width.

Effect of SU8 fermented filtrate on spore germination of *P. oryzae*

P. oryzae was inoculated onto sterile sorghum grains (121°C, 30 min) and fully shaken. The culture was incubated at 26–28°C for 5–6 d and then transferred to a metal sieve. The aerial hyphae were washed off with sterile water, and the reminder of the culture was transferred into an enamel dish spread with filter paper after the water naturally drained off. The dish was kept at 25–26% humidity for 12 h and covered with clean plastic wrap. A large amount of spores of similar maturity were formed within approximately 36 h. Sorghum grain fully covered with spores was transferred into a 100 mL Erlenmeyer flask and an appropriate amount of distilled water containing 2% glucose was added. The flask was shaken for 5 min, and the suspension was then filtered through sterile gauze. Spore density was examined by microscopy (magnification 15 × 10) and adjusted to ~60 spores per field of view.

The fermented filtrate of SU8 was regarded as the stock solution, *i.e.*, 1 mL ≈ 1 mg, which was diluted to 50, 100, 200, 400, and 800 μg/mL with sterile water. Solutions of the control drugs were prepared at 3.125, 6.25, 12.5, 25, and 50 μg/mL concentrations. For each concentration, 1 mL of the drug solution was mixed with 1 mL of spore suspension. One drop of the mixture was place onto a concave slide using a dropper. Each treatment was plated in triplicate. A blank control was prepared with clean water added to the spore suspension. The slides were placed in a petri dish furnished with moistened filter paper and incubated at 26–28°C with controlled moisture. When the spore germination rate of the control reached 95% or more, spore germination on the concave slides was examined by microscopy (magnification 15 × 10). The spore germination inhibition rate, regression equation, correlation coefficient and EC_{50} were calculated (Mu, 1994).

$$\text{Inhibition rate of spore germination (\%)} = \frac{\text{Spore germination rate of control treatment} - \text{Spore germination rate of experimental treatment}}{\text{Spore germination rate of control treatment}} \times 100$$

Determination of the optimal time and concentration of SU8 fermented filtrate required to achieve anti-fungal activity against *R. solani* and *P. oryzae*

SU8 bacteria were cultured in NA and shaken at 220 r min^{-1} at 28°C for 48 hours. The culture was then centrifuged at 8,000 r min^{-1}, for 15 min (5417R Small high-speed desktop centrifuge, Eppendorf, Germany). The fermented liquid was then filtered through a 0.22 μm filter and stored at 4°C.

The rice variety 'luliangyou996' was planted at the tillering stage. Then, 500 mL of either water (negative control) or SU8 fermented filtrate (400 μg/mL) was sprayed over plants infected with *R. solani P. oryzae*, *P. parasitica, F. graminearum, C. gloeosporioides, A. alternata,* or *U. oryzae* in triplicate, and samples were collected at 2, 4, or 8 days before inoculation and 1, 3 and 5 days after inoculation. After inoculation, nets were employed to shade plants and encourage moisture retention for 24 h. At day 7 after inoculation, the impact of SU8 fermented filtrate on plant viability was calculated.

Rice plants at the tillering stage were infected with *R. solani* and *P. oryzae*. SU8 fermented filtrate was prepared to 50, 100, 200, 400, and 800 μg/mL, and 500 mL of either the bacterial filtrate or water (negative control) was added to the infected plant in triplicate. For positive controls of anti-fungal activity, the *R. solani* inhibitor 20% Jinggangmycin WP (Zhejiang Tonglu bio-chemical co., ltd.) was added at 50 μg/mL and the *P. oryzae* inhibitor 20% Tricyclazole was added at 100 μg/mL (Hangzhou Nanjiao Chemical Co., Ltd.). After inoculation, nets were employed to shade plants and encourage moisture retention for 24 h. At day 7 after inoculation, the impact of SU8 fermented filtrate on plant viability was calculated.

Control effect of SU8 fermented filtrate in the field

An experimental field was selected from plots suffering severe rice sheath blight and rice blast. The soil type was red soil with a neutral pH. Rice was planted at a row spacing of approximately 13.32 cm × 19.98 cm in each experimental plot. Rice sheath blight and rice blast were treated with fermented filtrate of SU8 at concentrations of 50, 100, 200, 400, and 800 μg/mL. The positive controls for anti-fungal activity were 20% Jinggangmycin (50 μg/mL) and 20% Tricyclazole (100 μg/mL). The negative control was clean water. The plots were arranged in a randomized complete block design. Each plot covered an area of 66.7 m^2, and each treatment was repeated three times. The ferment filtrate was applied three times at the late tillering stage, before the rupturing stage, and at the full heading stage of rice plants. For each treatment, 5 L of fermented filtrate was sprayed with a DFH-16A sprayer. The incidences of rice sheath blight and rice blast were surveyed 7–10 d after treatment according to the criteria of Pesticide Guidelines for the Field Efficacy Trials (I) and the Ministry of Agriculture of the People's Republic of China. Then, the diseased plant rate, disease index, and control effect were calculated (Ministry of Agriculture of the People's Republic of China (GB/T 17980.20-2000), 2000; Ministry of Agriculture of the People's Republic of China (GB/T 17980.19-2000), 2000).

$$\text{Disease index} = \frac{\sum(\text{Number of diseased leaves at different levels} \times \text{relative level})}{\text{Total number surveyed} \times \text{highest level}} \times 100$$

$$\text{Control effect (\%)} = \frac{\text{Disease index of control treatment} - \text{disease index of experimental treatment}}{\text{Disease index of control treatment}} \times 100$$

Statistical Analysis

Statistical analysis was performed with Excel (Microsoft) and SPSS 11.5 (SPSS Inc., IL, USA). All results were calculated with Duncan's new multiple range method.

RESULTS AND DISCUSSION

Anti-fungal activity of SU8 fermented filtrate against pathogenic plant fungi

The antagonistic bacterium SU8 showed the strongest inhibitory effect on *R. solani* (width of inhibition zone = 37.14 ± 0.22 mm), followed by *P. oryzae* (34.22 ± 0.03 mm). The inhibitory effect exerted on the other pathogens was as follow (in order of decreasing effect): *P. parasitica* (29.11 ± 0.31 mm) > *F. graminearum* (27.07 ± 0.26 mm) > *C. gloeosporioides* (27.00±0.35 mm) > *A. alternata* (25.23 ± 0.21 mm) > *U. oryzae* (24.04 ± 0.43 mm) (Table 1). This experiment indicated that metabolites of SU8 bacteria inhibited the mycelial growth of many plant pathogens and has a wide anti-fungal spectrum.

Morphological observation

By electron microscopy, SU8 appears as long rods of regular size, ranging in length from 1 to 1.5 μm (Fig. 1A, 1B). SU8 rods can be aligned in pairs or short chains. After a gram stain, the thallus cells appeared red, indicating that this bacterial strain is a gram-negative bacteria (Fig. 1C).

Physiological and biochemical characteristics

SU8 may be cultured on multiple types of media. The colony shape and color of bacteria cultured on different media were slightly different. SU8 cultured on CMA, GYM, and GACM media did not produce any pigment (Supplement Table 1). According to the physiology and biochemistry of the antagonistic bacteria SU8 upon cultivation on PDA, NA, or King's media, it appears to be similar to *P. aeruginosa* (Supplemental Tables 1 and 2).

Sequence analysis and construction of a phylogenetic tree

As illustrated in Fig.1D, agarose gel electrophoresis of the PCR products amplified from the 16S rDNA fragment from SU8 bacteria revealed a band that was approximately 1,400 bp. The sequences of the amplified products were submitted to GenBank with the accession number HQ283487. According to a BLAST comparison, SU8 shared high homology with *P. aeruginosa*. The homology between SU8 16Sr DNA and *P. aeruginosa* SRDchr3 (EU724901) is 99%. We constructed a polygenetic tree with the HQ283487 sequences using the Neighbor-Joining module in MEGA4. SU8 and *P. aeruginosa* ATCC10145 map to the same system polygenetic branch, and their homology is up to 99% (Fig.2). Considering the sequence analysis, morphology, cultural characteristics, and physiological and biochemical characteristics, in concert with the *Common Bacterial Identification System Manual*, we conclude that SU8 belongs to *P. aeruginosa* genus and name it *P. aeruginosa* SU8.

Effect of SU8 fermented filtrate on the pathogenicity of *R. solani* and *P. oryzae*

The ferment filtrate of SU8 and its dilutions significantly reduced the pathogenicity of *R. solani* and *P. oryzae* (Table 2). Specifically, after soaking *R. solani* and *P. oryzae* in the ferment filtrate of SU8 and inoculating them onto the host plant, we observed smaller lesion sizes compared with the controls (reductions of 0.4% – 63.6% and 0.5 – 58.3%, respectively). After soaking the pathogens in the stock solution and 2-, 5-, 10-, and 20-fold dilutions, the pathogenicity of the two pathogens on rice plants decreased accordingly. Among those dilutions, the stock solution achieved the most significant pathogenicity reduction, whereas the 5-fold dilution still exhibited a good reduction of pathogenicity. However, the 10- and 20-fold dilutions did not strongly inhibit the pathogenicity of the fungi, as evidenced by the lack of a significant difference from the control in all treatment periods. When the pathogens were soaked for increasing time periods in the ferment filtrate of SU8, we observed even smaller lesion sizes induced by pathogenic infection and weaker pathogenicity. The stock solution and 2-fold dilution reduced the pathogenicity to the lowest level at 16 h of treatment and completely eliminated the pathogenicity at 24 h of treatment or more.

Effect of SU8 fermented filtrate on spore germination of *P. oryzae*

Table 3 shows that SU8 fermented filtrate has an inhibitory effect on rice blast fungus spore germination. The EC_{50} was 548.3457 μg/mL, which was significantly lower than the contrast agent of virulence (EC_{50} = 18.79 μg/mL). It is possible that the sterile fermented filtrate contains only a small amount of anti-fungal substances; however, the concentration of these agents can be improved in the future by utilizing a biosynthesis method to strengthen the anti-fungal effects.

Optimal treatment time and concentration of antagonistic bacteria SU8 fermented filtrate required to achieve anti-fungal activity against *R. solani* and *P. oryzae*

Spraying 400 μg/mL SU8 fermented filtrate on rice plants for different periods of time achieved good

Table (1): Anti-fungal activities of bacterial strain SU8 fermented filtrate against different plant pathogenic fungi

Pathogen	Inhibition width (mm)
R. solani	37.14±0.22[a]
P. oryzae	34.22±0.03[a]
P. parasite	29.11±0.31[b]
F. graminearum	27.07±0.26[bc]
C. gloeosporioides	27.00±0.35[bc]
A. alternata	25.23±0.21[c]
U. oryzae	24.04±0.43[c]

Note: Different small letters indicate significant differences and the same letters show no significant differences ($p < 0.05$), according to the Duncan's new multiple range method.

Supplement Table (1): Cultivating characteristics of strain SU8

Culture medium	Cultural characteristics	Soluble pigment
PDA	Bacterial colony likes mountain, wetting. Its surface is rough, and its edge is smooth. It appears faint yellow without metal luster. Cultivating for a long time, it will produces dark green precipitation. It is fluorescent under the excitation of UV light.	+
NA	Bacterial colony is round and wetting. Its surface is rough with small point-like projections. It is yellow-green at its early stage, and reddish brown in the late period. It is not fluorescent under the excitation of UV light.	+
CMA	Bacteria colony is round and wetting. Its surface and edge are smooth. It is colorless and transparent.	–
GYM	Bacteria colony is round and wetting. Its surface is smooth. It is colorless and transparent.	–
GACM	Bacteria colony is round and wetting. Its surface and edge are smooth. It is colorless and transparent	–
King	Bacterial colony likes mountain, wetting. Its surface is rough with small point-like projections. Its edge is smooth. It has copper green metallic luster. It is fluorescent under the excitation of UV light.	+

Table (2): Effect of SU8 fermented filtrate on the pathogenicity of *R. solani* and *P. oryzae*

Dilution Ratio of crude extract	Scab area of different treatments (cm^2)									
	R. solani					*P. oryzae*				
	2 h	4 h	8 h	16 h	24 h	2 h	4 h	8 h	16 h	24 h
0	7.35[c]	6.11[c]	3.18[c]	1.80[d]	1.03[c]	4.89[d]	5.01[c]	3.24[d]	2.53[d]	1.10[c]
2	9.45[b]	8.73[b]	4.23[b]	2.12[c]	1.12[c]	5.65[c]	5.58b	5.11[c]	3.19[c]	1.15[c]
5	11.07[a]	8.81[b]	4.21[b]	4.13[b]	3.57[b]	8.05[b]	7.91[a]	6.01[b]	5.15[b]	5.02[b]
10	11.17[a]	10.39[a]	10.07[a]	10.19[a]	11.52[a]	8.10[a]	7.96[a]	8.00[a]	8.15[a]	7.78[a]
20	11.16[a]	10.44[a]	10.07[a]	10.11[b]	11.56[a]	8.05[b]	7.98[a]	7.92[a]	8.22[a]	7.79[a]
CK	11.18[a]	10.45[a]	10.05[a]	10.20[a]	11.63[a]	8.11[a]	8.01[a]	7.99[a]	8.32[a]	7.71[a]

Note: Different small letters indicate significant differences and the same letters show no significant differences ($p < 0.05$), according to the Duncan's new multiple range method.. CK: inactivition fermented filtrate.

Supplement Table 2 Biological properties of bacteria strain SU8 compared to those of *P. aeruginosa* (/)

Testing Item	Reaction Type	Results	Reaction Type	Results
Utilization and Metabolized of Carbon Compounds	Lactose	- / -	Loose charitableness	+ / -
	Algum aldose	- / -	Dextrin	- / -
	Ankylose	+/+	Maltose	- / -
	L rhamnose	- / -	Glycerol	+/+
	Glucose	+/+	Erythroblast	+/+
	Inositol	- / +	Arab	+/+
	Mannitol	+/+	Sorbitol	- / -
	Galactose	- / -	Salicin	- / -
	D fructose	- / -	Citrate	+/+
	Sucrose	- / -	MR	+/+
	Raffinose	- / -	Esculin .	- / -
Utilization and Metabolized of Nitrogen Compounds	Nitrate	+ / +	hydrosolic	- / -
	Ammonia	- / -	Indole	- / -
Metabolized of Macromolecular compounds	Gelatin	+/+	Fat Catabolism	+/+
Other tests	Litmus Milk	- / -	Peroxidase	+/+
	Oxidase	+/+	Aerobic Test	+/+
	Growth at 4°C	- / -	Growth at 41°C～	+ / +
	Plastocyanin	+ / +	Ornithine decarboxylase	+/+
	Catalase	+ / +		

Note: In the result, the content of the left of oblique line is characteristics of bacteria strain SU8, and the one of the right is characteristics of standard bacteria strain (*P. aeruginosa*).

Table (3): Capability of SU8 fermented filtrate to inhibit spore formation of *P. oryzae* (24 h)

Treatment	Concentr-ation (μg/mL)	Spore number	Spore germination number	Germination rate (%)	Inhibition of germination Rate (%)	Regression equation	Correlation coefficient	EC_{50} (μg/mL)
SU8 fermented filtrate	50	132	121	91.66	7.9d	y = 1.4524x + 1.0218	0.968	548.3457
	100	126	111	88.1	11.49^{c}			
	200	153	119	77.78	21.85^{b}			
	400	132	62	46.97	52.81^{a}			
	800	131	58	44.27	55.51^{a}			
Tricyclazole	3.125	136	133	97.79	1.74^{e}	y = 2.5807x + 1.7120	0.9917	18.79
	6.25	142	121	85.21	14.38^{d}			
	12.5	130	94	72.31	27.35^{c}			
	25	133	41	30.83	69.03^{b}			
	50	138	22	15.94	83.98^{a}			
CK		211	210	99.53				

Note: Different small letters indicate significant differences and the same letters show no significant differences ($p < 0.05$), according to the Duncan's new multiple range method. CK: Sterile water.

Table (4): Control effect of strain SU8 fermented filtrate against *R. solani* and *P. oryzae*

Disease type	Pesticide	Concentration (μg/mL)	First control effect			Second control effect			Third control effect		
			Diseased plant rate (%)	Disease index	Control effect (%)	Diseased plant rate (%)	Disease index	Control effect (%)	Diseased plant rate (%)	Disease index	Control effect (%)
R. solani	SU8 fermented filtrate	50	55.6	6.32	35.84^{eE}	56.65	8.88	23.91^{fF}	57.45	11.25	32.88^{fF}
		100	48.48	4.51	54.21^{dD}	51.22	6.45	44.73^{eE}	52.12	9.64	35.35^{eE}
		200	45.67	4.51	54.21^{dD}	46.72	6.4	45.16^{dD}	47.86	8.73	47.96^{dD}
		400	36.56	4.05	58.88^{cC}	41.61	6.1	47.73^{cC}	41.6	7.98	54.51^{cC}
		800	23.54	3.65	62.94^{bB}	33.52	4.21	63.92^{bB}	34.51	6.54	61.07^{bB}
	Validamycin	50	19.11	3.22	67.31^{aA}	23.35	4.06	65.21^{aA}	24.61	5.89	64.57^{aA}
	CK		58.94	9.85		61.21	11.67		61.28	15.66	
P. oryzae	SU8 fermented filtrate	50	49.61	7.15	29.97^{fF}	52.64	7.88	32.88^{fF}	52.65	10.22	37.42^{eE}
		100	43.61	6.99	31.54^{eE}	44.44	7.59	35.35^{eE}	44.44	10.22	37.42^{eE}
		200	35.55	5.24	48.68^{dD}	37.17	6.11	47.96^{dD}	39.87	9.34	42.8^{dD}
		400	39.89	4.01	60.72^{bB}	40.02	5.34	54.51^{cC}	41.29	6.43	60.62^{cC}
		800	25.35	3.99	60.92^{aA}	26.83	4.57	61.07^{bB}	28.17	6.21	61.97^{bB}
	Tricyclazole	100	46.56	3.89	51.19^{cC}	48.84	4.16	64.57^{aA}	49.13	5.37	67.12^{aA}
	CK		58.64	10.21		59.91	11.74		60.04	16.33	

Note: Different small letters indicate significant differences and the same letters show no significant differences ($p < 0.05$), according to the Duncan's new multiple range method. CK: Sterile water.

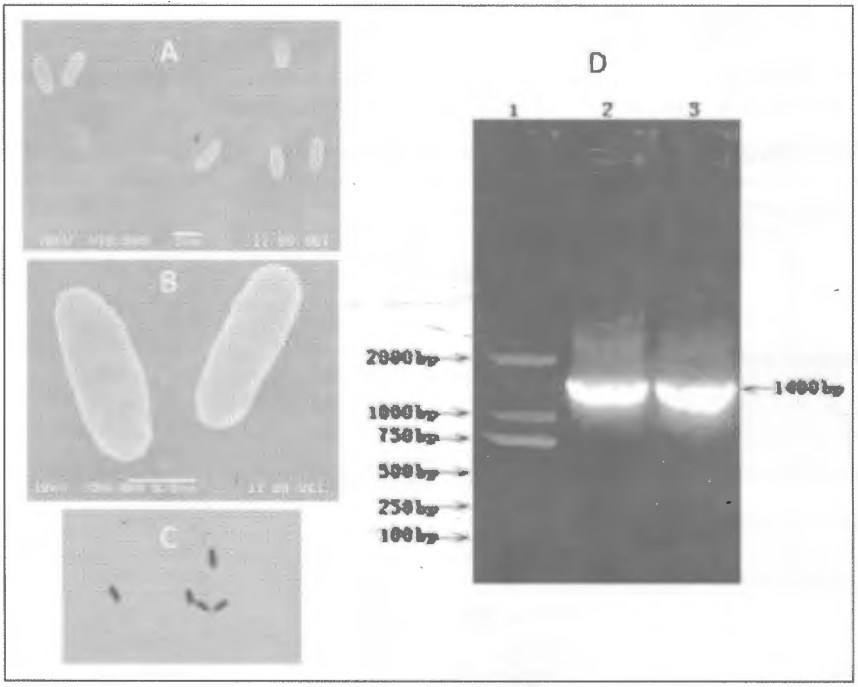

Fig. (1): The SEM (A, B) and Gram Stain (C) of SU8 bacteria (magnification: ×10,000 (A), × 50,000 (B) and × 160 (C)). D, Agarose gel electrophoresis of the PCR products from the amplification of 16S rDNA of SU8 bacteria.

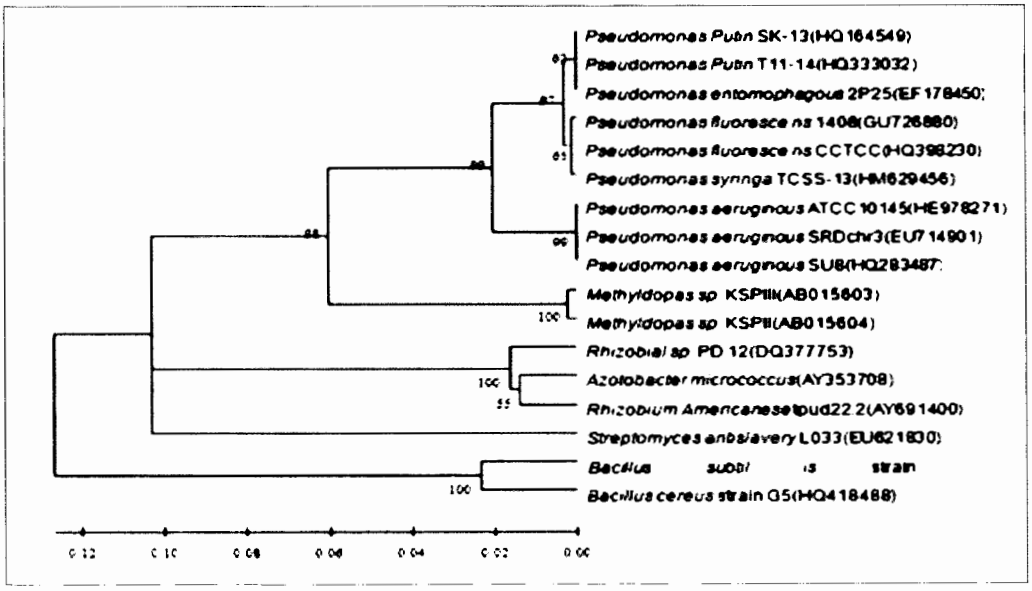

Fig. (2): Phylogenetic relationship of strain SU8 with other related strains.

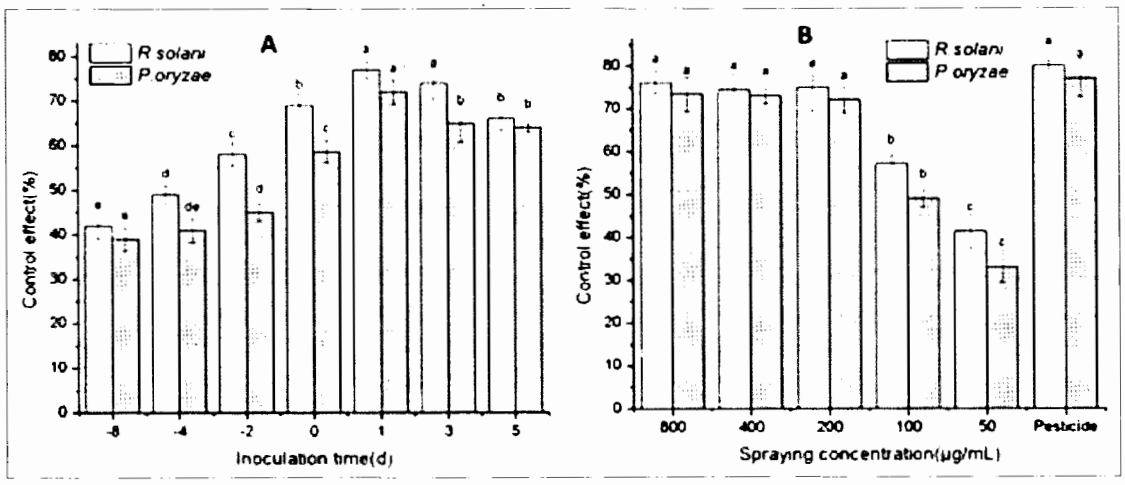

Fig. (3): Control efficacies of strain SU8 fermented filtrate against *R. solani* and *P. oryzae* infection in different treatment periods (A) and different inocula concentrations (B). a-e indicate a significant difference at 5% a confidence interval according to the test of Duncan's new multiple range method. The same letters indicate no significant difference.

control of *R. solani* and *P. oryzae* (Fig. 3A). The best control of *R. solani* infection was obtained by spraying plants 1 and 3 d after inoculation (>70%), and for protection against *P. oryzae*, the best effect was achieved by spraying plants 1 d after inoculation (>70%). The SU8 fermented filtrate-mediated protection against these two diseases was consistently better when the treatment was sprayed on the same day after pathogen inoculation than when it was sprayed in the days after the pathogen inoculation. The lowest control effect (<40%) was observed in the treatment with SU8 fermented filtrate sprayed at 8 d after pathogen inoculation. In each treatment condition, SU8 fermented filtrate exhibited stronger control of *R. solani* than *P. oryzae* infection. In the later case, the ability of SU8 fermented filtrate to control infection exhibited a decreasing trend at 3 d after pathogen inoculation. These results indicated that the ability of SU8 fermented filtrate to control these fungal infections is associated with both the development and progression of plant diseases and the mechanism of SU8 action.

Spraying different concentrations of SU8 fermented filtrate onto rice plants 1 d after pathogen inoculation achieved good control of *R. solani* and *P. oryzae* infection (Fig. 3B). The higher the SU8 fermented filtrate concentration, the better the control effect. Among different treatments, spraying SU8 fermented filtrate at 200, 400, and 800 μg/mL achieved the best control effect against the pathogens (70%), without significant differences from spraying 20% Jinggangmycin or 20% Tricyclazole. However, the control effect of the SU8 ferment filtrate declined with decreasing concentrations of the fermented filtrate. At a concentration of 50μg/mL, the control

effect was 40% against *R. solani* and 30% against *P. oryzae*. Among different sprayed concentrations, SU8 exhibited stronger control capability against *R. solani* than against *P. oryzae*. The effective concentration required to control both diseases was at least 200 μg/mL.

Control effect in the field

SU8 fermented filtrate was effective at inhibiting both *R. solani* and *P. oryzae* infection in the field (Fig. 4). The concentration applied was inversely proportional to the incidence and severity of the two rice diseases but positively proportional to the control effect. It was able to reduce infections of *R. solani* and *P. oryzae* between 23.8–63.9% and 29.9–61.9%, respectively. The best effect was achieved at 800 μg/mL of SU8 fermented filtrate, but the amount of control was less than that observed with the Jinggangmycin and Tricyclazole as controls. In terms of control effect, the first control effect was generally better than the second and third control effects. However, there were a few treatments with the second and third control effects weaker than the first control effect, possibly due to the influence of the environmental conditions. Additionally, none of the treatments had adverse effects on rice growth or phytotoxicity to rice booting and heading.

This study indicates that SU8 fermented filtrate can inhibit several pathogenic plant fungi in a dose dependent manner and that this broad-spectrum antagonistic bacterial strain is likely to be closely related to *P. aeruginosa*.

Recently, it was reported by Kuo & Kim (2001) that bacterial PR3 inhibits *P. oryzae* It was reported by Kumar *et al.* (2005) that PUPA3 could prevent and control *R. solani*, and Xie *et al.* (1998) found that *P. aeruginosa* isolated from rice field soil inhibited *R. solani*. Results of the present studies are in line with those observations.

We found that the sterile fermented liquid from SU8 cultures reduced the pathogenicity of *R. solani* and *P. oryzae*, indicating that SU8 can produce active secondary metabolites during the fermentation period (All of the filtrate was filtered through 0.22μm bacterial filters). The fermented filtrate contained secondary metabolites that significantly inhibited spore germination of *P. oryzae*. This result indicated that the filtrate could reduce the primary infection source by inhibiting spore germination, thus reducing the incidence of rice blast. These secondary metabolites included phenazines, 2, 4-diacetyl-chloramphenicol, DAPG, trinitrin, ern, pyrolusite, alt, plastocyanin, and mostly possessed anti-fungal properties (Voisard *et al.* 1989, Thomashow & Weller 1996, Slininger *et al.* 2000, Seveno *et al.* 2001). The purification, characterization, and structural identification of the precise proteins or chemicals crucial for SU8's anti-fungal properties is necessary to determine the mechanism by which this type of bacteria inhibits the growth of *R. solani* and *P. oryzae*.

This study revealed that SU8 fermented filtrate prevents and controls *R. solani* and *P. oryzae* infection in potted plants and that spraying this fermented filtrate one day after inoculation with pathogenic fungi can prevent disease by up to 70%. However, the application of the fermented filtrate after a fungal infection was already established as not effective. Field data demonstrated that *R. solani* and *P. oryzae* infections were reduced between 23.8–63.9% and 29.9–61.9%, respectively, after treatment with SU8 ferment filtrate. These values were lower than those of the control agents, Jinggangmycin and Tricyclazole. It is possible that the SU8 fermented filtrate has a low content of antimicrobial substances, which needs to be further concentrated for improved control of fungal infection.

When pathogenic bacteria invade host plants, SU8 fermented filtrate can inhibit their growth and prevent infection. Thereby, SU8 fermented filtrate protects plants from invasion. Still, a careful characterization of the most effective application period and formulation, an analysis of the control mechanism, potential resistance genes (Wang *et al.* 1995), and the impact on plant nutrition and environment are still required for most effective use of this anti-fungal agent. Although *P. aeruginosa* was not found to inhibit the growth of the plants, it belongs to a group of conditioned pathogens that can cause harm to people and animals. Direct application of living bacteria SU8 as agent controls plant disease, is not feasible. Thus, application of SU8 fermented filtrate is the best alternative way for controlling plant disease. The waste materials produced from SU8 cultivation, including the culture medium, pipette tips, petri dishes, test tubes, beakers, and culture broth, must be disinfected by immersion in 75% alcohol and then autoclaved to avoid environmental contamination. All waste materials should be discharged to a specialized waste disposal station. Further study is needed to provide a theoretical foundation for widespread use of this bacterial product.

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