African Journal of Microbiology Research Vol. 6(15), pp. 3589-3593, 23 April, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1241
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Biological potential of *Pseudomonas* sp. BS1 in the control of *Phytophthora* root rot of soybean

Jumei Hou[1],Sining Bi[1], Lei Yan[1], Yuhu Zuo[1], Yanjie Wang[1,] Tong Liu[1]* and Jiewei Zhu[2]

[1]Institute of Plant Pathology and Applied Microbiology, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, 163319, P. R. China.
[2]School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, 200240, P. R. China.

Accepted 26 March, 2012

In this study, the biological potential of *Pseudomonas* sp. BS1 in the control of *Phytophthora* root rot of soybean was investigated. Fermentation filtrates of *Pseudomonas* sp. BS1 inhibited zoosporangium formation and zoospore germination of *Phytophthora sojae* (*P. sojae*), and the inhibitory effect gradually weakened with the increasing filtrate dilution. It had a relatively strong inhibition on hyphal growth of *P. sojae*, and the inhibition rate of stock solutions reach 95.9%; as dilution increased, the inhibition gradually weakened. The results indicated that *Pseudomonas* sp. BS1 can inhibit normal growth and development of *P. sojae*, and pathogen reproduction. Through investigating the controlling effect of *Pseudomonas* sp. BS1 fermentation filtrates on *Phytophthora* root rot of soybean under greenhouse conditions, we found that pre-treatment with fermentation filtrates had a preventive effect on disease caused by *P. sojae*, and post-inoculation treatment with fermentation filtrates had a therapeutic effect. Simultaneous treatment with fermentation filtrates and zoospore suspension had best controlling effects. These results will provide a theoretical basis for the biological control of *Phytophthora* root rot of soybean using *Pseudomonas* sp. BS1.

Key words: *Pseudomonas*, *Phytophthora*, zoosporangium, zoospore, biological control.

INTRODUCTION

Phytophthora root rot caused by *Phytophthora megasperma* Var. sojae is one of the most serious diseases of soybean around the world (Wrather et al., 2001; Wrather and Koenning, 2006). It causes serious losses to global soybean production, and is responsible for $1 billion to $2 billion in losses worldwide each year (Tyler 2007). *Phytophthora sojae* produces zoospores that move in water and are attracted to soybean roots, and germinate and infect the plant tissues, eventually kill the entire plant in wet conditions (Surhone et al., 2010). At present, the disease control is realized through combined approaches of plant quarantine, the cultivation of resistant varieties and chemical control (Dorrance et al., 2007; Schmitthenner 1985). Metalaxyl and other chemicals are major prevention methods, but long-term and large amount application of pesticide metalaxyl has caused drug-resistant pathogens, and brought many environmental and social problems (Cahill and Ward, 1987; Layton and Kuhn, 1988; Lamboy and Paxton, 1992). Therefore, vigorous development of microbial pesticides, and exploration of scientific and long-term biocontrol measures to replace chemical pesticides on *Phytophthora* prevention has become an urgent problem. Studies have shown that the rhamnolipids produced by *Pseudomonas aeruginosa* can lyse the zoospore of fungi such as *Pythium aphanidermatum* and *Pytophora capsici* (Stanghellini and Miller, 1997). Previously, our group isolated and identified *Pseudomonas* sp. BS1 producing rhamnolipid from oil pollution of soil (Liu et al., 2011) and found that *Pseudomonas* sp. BS1 has inhibitory effects on a variety of plant pathogens by dual culture, but its

*Correspondence author. E-mail: liutongamy@sina.com. Tel: 86-0459-8997858. Fax: 86-0459-8997856.

inhibition on *P. sojae* and related mechanism are unclear yet. Therefore, this study investigated the inhibition of *Pseudomonas* sp. BS1 on *P. sojae* zoosporangium formation, zoospore germination and hyphal growth, and performed greenhouse pot control experiments in an attempt to clarify biological control mechanism on *P. sojae*, so as to provide a practical basis on the biological control of soybean blight using *Pseudomonas* sp. BS1.

MATERIALS AND METHODS

Strains, culture condition and soybean seedlings

P. sojae race 3 were provided by Institute of Plant Pathology and Applied Microbiology (Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang) and cultured on carrot cultivation medium (CA) at 25°C. *Pseudomonas* sp. BS1 was stored at -80°C. A soybean seed HeFeng 3 susceptible to *P. sojae* were gifted by Heilongjiang Academic of Agricultural Sciences and were grown in pots in a growth chamber at 25°C and with a 14 h photoperiod and 60% relative humidity.

Preparation of fermentation filtrates from *Pseudomonas* sp. BS1

Pseudomonas sp. BS1 was maintained on *Pseudomonas* isolation agar at 37°C for 24 h. A colony was selected and grown in 10 ml minimal salt medium (MSM) in a 50 mL Erlenmeyer flask. Inoculation was performed with shaking (180 rpm) at 37°C for 7 d. 2.5 mL of culture filtrates was transferred into fresh MSM at 37°C for 5 d with shaking at 250 rpm. The culture filtrate was centrifuged at 8,000 × g for 10 min at 25 °C to remove the cells and the debris. The supernatant was obtained and was used as fermentation filtrates.

Effect to the formation of zoosporangium

P. sojae was cultured on carrot cultivation medium (CA) at 25°C until the growing edge of the hyphae reached the edge of the plates. Ten pieces of *P. sojae* cakes (9 mm diameter) was taken from the edge of culture and transferred into a 90 mm sterile Petri dish, each dish containing 10 mL of sterile water, which was changed once every 30 min. After changing the water four times, 10 mL different concentrations of filtrate fermentation were added (seven concentrations of filtrate fermentation were set up: no dilution, 10, 20, 30, 40, 50 and 100 fold dilution) using a blank fermentation mediums in corresponding concentrations as control. They were placed in 25°C under darkness and static culture conditions for 12 h. Then, the number of zoosporangium was examined under an optical microscope with 10 × 10 magnification. Five fields were randomly examined for each mycelia block, and each treatment was repeated three times. The inhibitory effect of fermentation filtrates was measured by the number of zoosporangium/field.

Effect to the germination of zoospore

Phytophthora zoospore suspension was prepared according to the method by Zuo et al. (2002). The prepared zoospore suspension was examined under an optical microscope with 10 × 10 magnification to 30 to 50 zoospores/field in average for use. Seven concentrations of filtrate fermentation were set up: no dilution, 10, 20, 30, 40, 50, and 100 fold dilution. Each concentration was prepared with sterile water mixed with *P. sojae* zoospore suspension in a ratio of 1:1, using a blank fermentation medium as control. They were placed at 25°C under a moisturized condition for 12 h, and the zoospore germination was examined under an optical microscope with 10 × 10 magnification. Using germ tube length exceeding half of spore diameter as standard, the number of zoospore germination was recorded to calculate zoospore germination rate: germination rate (%) = the number of zoospore germination/the total number zoospore examined. Each treatment had 200 spores, and the experiment was repeated three times.

Effect to growth of mycelium

Fermentation filtrates was added to carrot cultivation medium that has been cooled to about 45°C and was diluted to 10, 20, 30, 40, 50 and 100 times. They were quickly mixed to make the drug-containing plates. When medium was cooled, *P. sojae* cakes in 5 mm diameters were inoculated into the middle of plates using an inoculation knife. *P. sojae* cakes were inoculated into CA plates with corresponding dilutions of the blank fermentation medium as control. Each treatment was repeated three times. The colony diameter was measured by criss cross method after inoculation of 7 days on dish.

Control effects of *P. sojae* under a greenhouse conditions

To detect whether fermentation filtrates of *Pseudomonas* sp. BS1 had a therapeutic effect on *P. sojae*, zoospore suspension ($I \times 10^5$ cfu/ml) of *P. sojae* (10 ml) was first prepared and poured into the hole around the soybean seedling using injector. Then the soybean seedling was treated with fermentation filtrates of *Pseudomonas* sp. BS1 with no dilution (10 ml) after inoculation of 0h, 24, 48 and 72 h. For prevention experiment, fermentation filtrates of *Pseudomonas* sp. BS1 with no dilution (10 ml) was first injected into the root of soybean seedlings. Then zoospore suspension ($I \times 10^5$ cfu/ml) of *P. sojae* (10 ml) was inoculated with corresponding root of soybean seedlings after treatment of 0h, 24, 48 and 72 h. Each treatment had three pots of soybean seedlings. Each pot included ten seedlings which were used for inoculated when seedlings had reached the five-leaf stage. The incidence of *Phytophthora* root rot of soybean was investigated using the mortality rate. When leaf water lost and wilts, soybean seedlings stem base became brown, and the whole plant fell down, the plant was recorded as death. The controlling effect = 100%-mortality rate.

RESULTS

The inhibition on the formation of *P. sojae* zoosporangium

The results of *P. sojae* inhibition are shown in Figure 1. Different concentrations of fermented filtrates can suppress the formation of *P. sojae* zoosporangium at certain levels. The inhibition rate could reach 100% for stock fermented filtrate, and was still above 50% when the fermentation filtrates was diluted to 30 times. These indicated that the fermentation filtrates of *Pseudomonas* sp. BS1 had a relatively strong inhibition on the formation of *P. sojae* zoosporangium. Although the inhibition rate gradually decreased as the dilution of fermdifferent concentrations of fermented filtrates can inhibit *P. sojae*

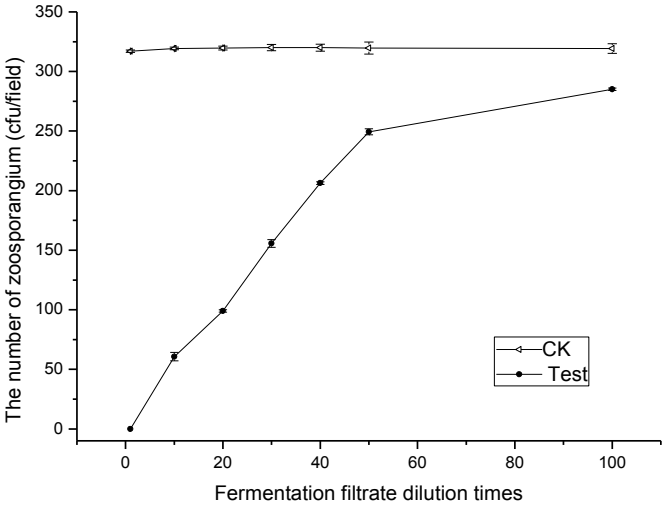

Figure 1. Inhibition of different concentration fermentation filtrates on the formation of *P. sojae* zoosporangium. Treatment with the fermentation filtrates; Ck: treatment without the fermentation filtrates.

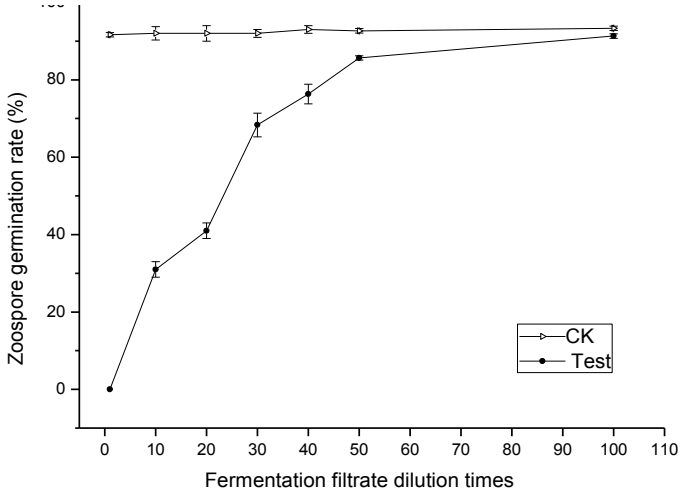

Figure 2. Inhibition of different concentration fermentation filtrates on the germination of *P. sojae* zoospore. Treatment with the fermentation filtrates; Ck: treatment without the fermentation filtrates.

zoospore germination at certain levels. The stock fermented filtrates showed the strongest inhibition on *P. sojae* zoospore germinentation filtrates increased, the inhibition rate was still up to 10.75% when diluted to 100 times.

The inhibition on *P. sojae* zoospore germination

As shown in Figure 2, *P. sojae* has an inhibition rate of

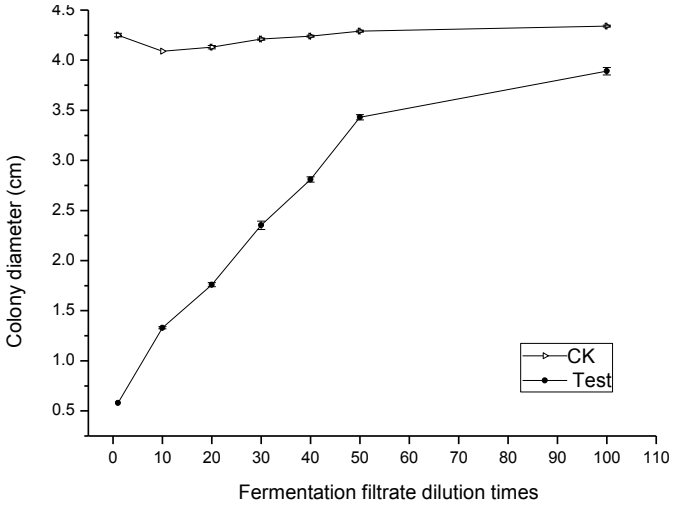

Figure 3. Inhibition of different concentration fermentation filtrates on hyphal growth of *P. sojae* Treatment with the fermentation filtrates; Ck: treatment without the fermentation filtrates.

100%. As the dilution of fermentation filtrates increased, the inhibition rate gradually reduced; when diluted to 30 times, the inhibition rate could still achieve 31.83%, indicating that the *Pseudomonas* sp. BS1 fermentation filtrates had strong inhibitory effects on *P. sojae* zoospore germination.

The inhibition on *P. sojae* mycelium

As shown in Figure 3, different concentrations of fermentation filtrates can inhibit hyphal growth of *P. sojae* at certain levels. The stock fermented filtrate showed strongest inhibition on *P. sojae* mycelium, reaching an inhibition rate of 95.93%. As the dilution increased, the inhibition of bacteria-free fermentation filtrates on *P. sojae* mycelium gradually decreased, while at 30 time dilutions, the inhibition was still able to achieve 51.33%, indicating that *Pseudomonas* sp. BS1 fermentation filtrates had a relatively strong inhibition on *P. sojae* mycelium. When the fermentation filtrates was diluted to 100 times, the inhibition was weakest, with an inhibition rate of 10.95%.

Greenhouse controlling effect

As shown in Figure 4. When zoospore suspension was first inoculated, and fermentation filtrates was root-poured at 24, 48 and 72 h, the controlling effects were 29.17, 11.20 and 6.67%, respectively. These results indicated fermentation filtrates of *Pseudomonas* sp. BS1 had a certain therapeutic effect compared with the control. When fermentation filtrates was first inoculated, and zoospore suspension was poured at 24, 48 and 72 h, the

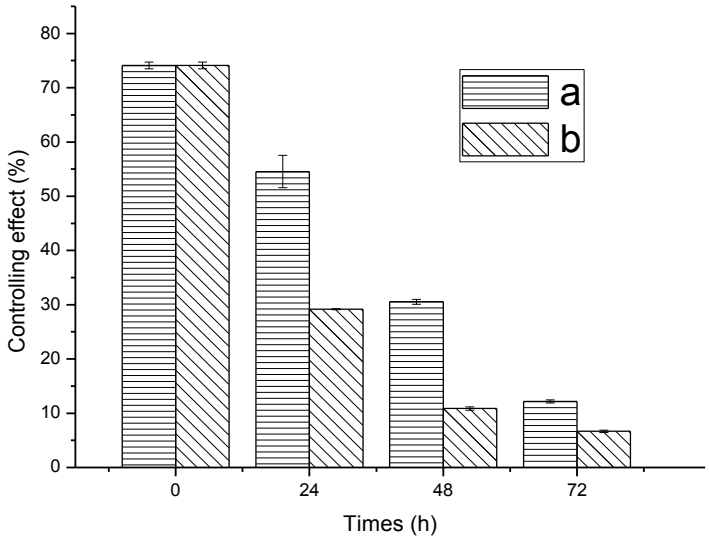

Figure 4. The controlling effect of fermentation filtrates with different inoculation times on *P. sojae* a: Therapeutic effect of *Pseudomonas* sp. BS1 on *P. sojae* with 0h, 24, 48 and 72 h inoculation times respectively under greenhouse; b: Prevention effect of *Pseudomonas* sp. BS1 on *P. sojae* with 0h, 24, 48 and 72 inoculation times respectively under greenhouse.

controlling effects were 54.55, 30.55 and 12.17%, respectively, which *Pseudomonas* sp. BS1 had a better preventive effect compared with the blank group. Simultaneous inoculation of *Pseudomonas* sp. BS1 fermentation filtrates and zoospore suspension showed that there had the best controlling effect, up to 74.12%.

DISCUSSION

For biological control of plant pathogenic fungi, potential of rhamnolipids produced by Pseudomonas was recently recognized (Soo et al., 2005). For example, Stanghellini and Miller, (1997) suggested that rhamnolipids produced by *P. aeruginosa* can lyse zoospore of aphanidermatum and *P. capsici* at 5 to 30 µg/mL concentrations. Kim et al. (2000 a, b) also demonstrated that rhamnolipids have not only zoosporicidal activity, but also inhibit spores germination and hyphal growth of several pathogens. Similarly, De Souza et al. (2003) confirmed that rhamnolipids can lyse zoospores of multiple oomycetes, including *Pythium* sp., *Albugo candida* and Phytophthora. So, biofungicide containing rhamnolipids was used to control zoosporic plant pathogenic fungi, including downy mildew, *Pyhtium* and *Phytophthora* sp. (Nielsen et al., 2006). On a variety of crops, including tubers and vegetables, citrus fruits, ornamental plants, trees, shrubs, bedding plants and turf grasses. The mechanism of controlling was considered that rhamnolipids can insert into the cell plasma membrane and destruct the membrane structure, ultimately achieving the inhibition of pathogens (Kulkarni et al., 2007). But till now, few studies investigated the inhibitory effects on *P. sojae*. Previous experiments demonstrated that *Pseudomonas* sp. BS1 identified can produce dirhamnolipids (Liu et al., 2011). In this study, our results clearly indicated that fermentation filtrates of *Pseudomonas* sp. BS1 had relatively strong inhibitory effects on the hyphal growth of *P. sojae*, the formation of zoosporangium and the germination of zoospore, suggesting that fermentation filtrates of *Pseudomonas* sp. BS1 may inhibit the normal growth and development of pathogens by affecting *P. sojae* mycelium growth, zoosporangium formation, zoospore germination, etc. Greenhouse control experiment also showed that simultaneous inoculation of *Pseudomonas* sp. BS1 fermentation filtrates and zoospore suspension had best controlling effects. It is possible that the fermentation filtrates and *P. sojae* zoospore had full interaction, which allows them to play better inhibitory effect and reduce soybean seedling mortality. But, whether dirhamnolipids is the substance to inhibit *P. sojae* or some other metabolites are playing mainly inhibitory roles is required to in-depth study. In summary, the experimental results provide a theoretical basis for the use of *Pseudomonas* sp. BS1 on the biological control of soybean blights, which points a new research direction for the biological control of soybean blights.

ACKNOWLEGEMENTS

This work was supported by NSFC (Grant Nos.

31171807, 31101407 and 31100006), Doctoral Fund of Ministry of Education of China (20112305120001), Scientific Research Fund of Heilongjiang Provincial Education department (11551327, 12511326). open project of State Key Laboratory of Crop Stress Biology in Arid Regions (CSBAA2011-11, CSBAA2011-18).

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