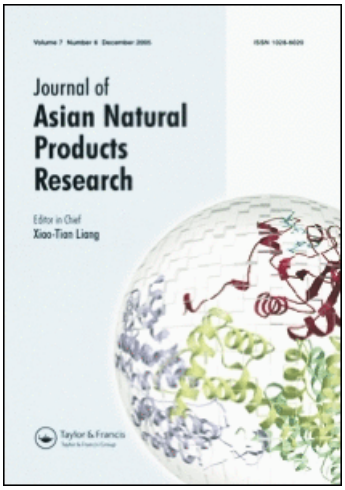

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ORIGINAL ARTICLE

Antifungal activity of the osthol derivative JS-B against *Phytophthora capsici*

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JS-B ($C_{12}H_{10}O_3$) is a derivative compound of osthol. The antifungal properties of JS-B were tested against 10 economically important plant pathogens. JS-B was effective in inhibiting the mycelial growth of *Phytophthora capsici*, and its inhibition on different stages of the life cycle of *P. capsici* was observed. The 50% effective concentration (EC_{50}) of JS-B on mycelial dry weight and zoospore germination of *P. capsici* was 43.74 and 86.03 μg/ml, respectively. The rupture of released zoospores induced by JS-B was reduced by the addition of 100 mM glucose. The ultrastructural study showed that JS-B caused destruction of most of the mitochondrions, the concentration of cell nuclear, and the existing vesicles. When compared with dimethomorph, the activity of JS-B on *P. capsici* was determined under pot conditions. The result showed that JS-B has a curative effect on pepper blight.

Keywords: antifungal; pepper blight; *Phytophthora capsici*; ultrastructure

1. Introduction

Phytophthora blight, caused by the oomycete pathogen *Phytophthora capsici* leonian, is one of the most important fungal diseases of pepper plants and is widely distributed in China and worldwide [1–5]. This disease causes serious economic losses of chilli peppers in more than 15 provinces of China [6]. This disease is very difficult to control, partly due to the pathogen's ability to survive for several years as thick-walled oospores in soil or as mycelium in plant residues. Then, dissemination and infection occur through the production of motile biflagellate zoospores from the oospores. Control of *Phytophthora* infection is most commonly accomplished by the application of fungicides, such as metalaxyl, chloropicrin, cymoxanil, mancozeb, etc. [7–10]. However, excessive and irrational uses can jeopardize fungicide effectiveness by exerting selection pressure on fungicide-resistant populations of *P. capsici* and can cause heavy toxicity to humans, domestic animals, etc. [11–14]. For instance, reduced metalaxyl efficacy in controlling Phytophthora blight is linked to the intensive use of this fungicide in China and elsewhere [15,16]. Hence, the development of fungicides with new modes of action is called for.

JS-B (Figure 1) is a novel fungicide patented by the Jiangsu Academy

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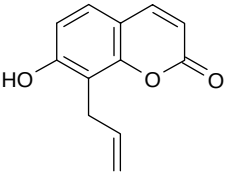

Figure 1. Chemical structure of JS-B.

of Agricultural Sciences (JAAS). JS-B is a derivative of osthol, which is a new fungicide developed by the JAAS to control powdery mildew [17]. In order to develop a sound recommendation for the use of a new fungicide, sufficient information must be available regarding its biological activity. Our objectives for this study were to: (1) assess the antifungal spectrum of JS-B *in vitro*, (2) test the efficacy of JS-B to interfere with various stages of the life cycle of *P. capsici in vitro*, and (3) determine the efficacy of JS-B in controlling Phytophthora blight of pepper under pot conditions.

2. Results and discussion

2.1 Antifungal spectrum

EC_{50} values of the effect of JS-B on the mycelial radial growth were tested to determine its fungicidal spectrum (Table 1). Of the 10 strains of phytopathogens examined, the mycelial growth of *P. capsici* was most sensitive to JS-B, with an EC_{50} value of 37.10 μg/ml. The mycelial growth of *Rhizoctonia solani* was less inhibited by JS-B, with an EC_{50} value of 56.08 μg/ml. The EC_{50} values of JS-B against *Fusarium oxysporum* and *Macrophoma kawatsuka* were nearly 100 μg/ml. The other seven strains showed weak sensitivity of JS-B, with EC_{50} values more than 100 μg/ml.

On potato dextrose agar (PDA) medium with pH values from 5 to 9, the inhibition of oligochitosan on the mycelial growth of *P. capsici* was not significantly affected (Figure 2).

2.2 Effect of JS-B on each life stage of P. capsici

A significant inhibition of JS-B on the mycelial dry weight of *P. capsici* was observed (Figure 3). JS-B inhibited the mycelial dry weight with an EC_{50} value of 43.74 μg/ml.

JS-B exhibited a strong inhibition of zoosporangium formation and cystospore germination (Table 2). The zoosporangia

Table 1. Inhibition of the mycelial growth by JS-B against different phytopathogens.

	JS-B		Osthol	
Pathogenic fungi[a]	EC_{50} (μg/ml)[b]	*R*	EC_{50} (μg/ml)	*R*
Phytophthora capsici	37.10 ± 0.04	0.9967	41.78 ± 0.69	0.9926
Botrytis cinerea	183.09 ± 0.21	0.9822	149.40 ± 0.26	0.9940
Rhizoctonia solani	56.08 ± 0.18	0.9960	23.73 ± 0.48	0.9932
Fusarium graminearum	125.67 ± 0.20	0.9929	51.18 ± 0.09	0.9803
Colletotrichum capsici	100.72 ± 1.21	0.9992	127.46 ± 0.52	0.9969
Fusarium moniliforme	116.90 ± 1.56	0.9935	86.05 ± 0.15	0.9921
Fusarium oxysporum	95.68 ± 0.52	0.9907	88.73 ± 0.92	0.9746
Macrophoma kawatsuka	82.31 ± 0.42	0.9856	30.03 ± 0.63	0.9932
Sphaceloma ampelinum	186.68 ± 0.61	0.9978	36.13 ± 0.08	0.9661
Plasmopara viticola	142.69 ± 0.51	0.9657	–	–
Cercospora beticola	37.10 ± 0.26	0.9967	63.75 ± 0.45	0.9960

Note: –, not detected.
[a]All fungi were tested on PDA in the dark at 25°C.
[b]The concentration inhibiting by 50%.

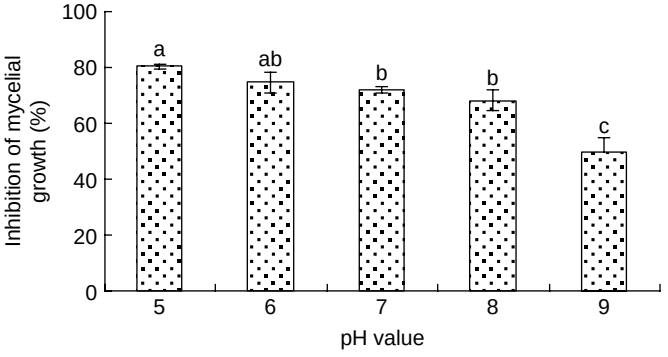

Figure 2. Effect of pH on the efficacy of JS-B against *P. capsici*. Measurement of the mycelial growth on pH-adjusted PDA amended with 100 μg/ml of JS-B was performed 5 days after inoculation with *P. capsici*. Letters indicate a significant difference ($P < 0.05$) with respect to pH.

production was strongly inhibited by JS-B with an EC_{50} value of 2.02 μg/ml.

After 1 h of incubation, cystospore germination in the control was c. 85%, while JS-B at 150 μg/ml inhibited cystospore germination completely. The EC_{50} value of JS-B against cystospore germination of *P. capsici* was 86.03 μg/ml.

Incubation with JS-B could induce the rupture of zoospores. After incubation for 60 min with 100 and 200 μg/ml of JS-B, the rates of rupture were 26.77 and 47.55%, respectively (Figure 4). When 100 mM glucose was added, the percentage of rupture decreased significantly to 18.60 and 39.11%, respectively.

2.3 Determination of the effect of JS-B on the hyphal morphology

Control mycelia of *P. capsici*, grown in the absence of JS-B (Figure 5(A)), showed cytological and ultrastructural features. Many organelles were observed, such as mitochondrion (M), endoplasmic reticulum (ER), lipid bodies (L), and dense body (DB).

When compared with the untreated cells, in the presence of 100 μg/ml of JS-B, a complete vacuolization of the mitochondrion was the most pronounced ultrastructural change observed in the hyphae (Figure 5(C)), while a little

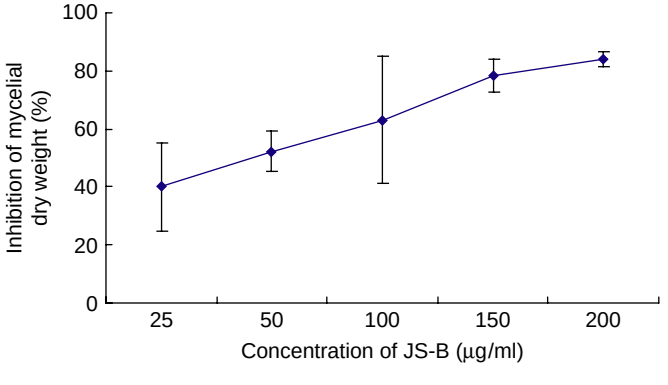

Figure 3. Inhibition of JS-B on the mycelial dry weight of *P. capsici*. Bars show the mean of mycelial growth ± SD for each treatment.

Table 2. Inhibitory effect of JS-B on different life stages of *P. capsici*.

	JS-B	
Stages in the life cycle of *P. capsici*	EC_{50} (μg/ml)	*R*
Mycelial dry weight	43.74 ± 1.42	0.9726
Zoosporangia production	2.02 ± 0.13	0.9945
Zoospore release	–	–
Cystospore germination	86.03 ± 0.18	0.9135

vacuolization of the mitochondrion was observed in the 40 μg/ml of JS-B-treated hyphae (Figure 5(B)).

2.4 *Effect of JS-B on* P. capsici *under pot conditions*

The anti-oomycete activity of JS-B for the control of Phytophthora blight in pepper plants at the fourth branch stage was examined under pot conditions (Table 3). At a concentration of 400 μg/ml, JS-B was as effective as dimethomorph in suppressing Phytophthora blight in pepper plants. At concentrations from 100 to 200 μg/ml, JS-B was less effective than the commercial fungicide dimethomorph (Table 3). The curative activity of JS-B was more effective than the protective activity against pepper blight.

2.5 *Discussion*

In the present study, we have studied the *in vitro* and *in vivo* antifungal activity of JS-B on the phytopathogenic fungus *P. capsici*. We demonstrated that JS-B was as effective as osthol in inhibiting the mycelial growth of the tested fungi (Table 1). The result showed that JS-B had a wider antifungal spectrum. Of the 10 strains of the phytopathogens tested in this study, the mycelial growth of *P. capsici*, a rapidly growing oomycete with no chitosan in the cell wall [18], was most sensitive to JS-B. This sensitivity might have a special relationship with the cell wall composition of *P. capsici*.

More interestingly, the investigation of the effect of JS-B on different stages in the life cycle of *P. capsici*, as well as the study on the ultrastructural alteration, may partially explain the different modes of the antifungal mechanism of JS-B. JS-B at low concentrations inhibited different stages in the life cycle of *P. capsici*, including mycelial growth, zoosporangia production, cystospore germination, and induced the rupture of released zoospores. It is well known that the zoospore rupture

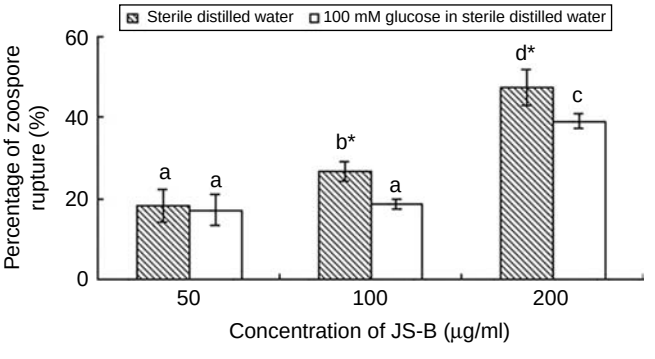

Figure 4. Effect of glucose on zoospore rupture caused by JS-B. Glucose (100 mM) in sterile distilled water was added 10 min before the addition of JS-B. Zoospore rupture was measured after 60 min of incubation. Vertical bars with different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

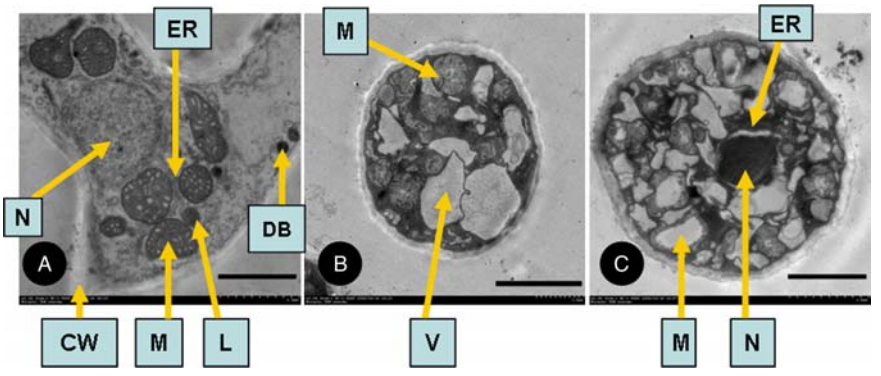

Figure 5. Transmission electron micrographs of *P. capsici* hyphae. Longitudinal (A) (control), showing normal cell wall (CW), lipid bodies (L), mitochondria (M), dense body (DB), endoplasmic reticulum (ER), and nucleus (N) (B,C) Hyphae treated with 40 and 100 μg/ml of JS-B. Note the disorganized vacuoles (V, arrowheads). Scale bar = 2 μm.

of *Phytophthora* is connected to the impairment of the energy generation system [19] and osmotic pressure [20]. The addition of glucose (an osmotic stabilizer) prior to treatment with a respiration inhibitor was able to reduce the inhibition on the rupture of zoospores [21]. It is interesting to note that the addition of 100 mM glucose prior to treatment with 50–200 μg/ml of JS-B was able to reduce zoospore rupture. This observation indicates that JS-B may act on the cell membrane by disturbing the osmotic pressure.

In order to understand the action of JS-B, an electron microscopic study has been carried out. Visible symptoms of the hyphae have been observed (Figure 5). JS-B induced marked structural alterations, including hyphae vacuolization, organelles deformity, etc. These findings indicate that the preventive action of JS-B against the mycelial growth is quite powerful and the unique modes of action may exist in the antifungal mechanism of JS-B. However, further study on the mechanism of JS-B against fungal pathogens at the molecular level is needed. The efficacy of JS-B on the *in vivo* control of *P. capsici* further supports its possible use in fungicidal treatment. The efficacy of JS-B on pepper plants was found to be concentration-dependent.

In conclusion, our results showed that JS-B has not only a potent *in vitro* antifungal and anti-oomycete activity against some plant pathogenic fungi, but also *in vivo* control efficacy against Phytophthora blight on pepper plants. Obviously, JS-B must conform to a stringent set of criteria on environmental and toxicological safety, and must be shown to be both effective and reliable under practical conditions. However, it is interesting to note that the study carried out on pepper plants suggests a crop tolerance

Table 3. The *in vivo* efficacy of JS-B on controlling Phytophthora blight in pepper plants.

Treatments	Concentration (μg/ml)	Protective effect (%)	Curative effect (%)
JS-B	100	26.11 ± 2.41	58.53 ± 1.42
	200	63.41 ± 2.19	65.85 ± 1.12
	400	71.95 ± 1.34	71.95 ± 1.60
Dimethomorph	550	75.61 ± 3.35	75.61 ± 0.79

for this compound. In addition, this compound is a derivative of osthol, which is regarded as safe and is currently used as an effective fungicide to control powdery mildew in China.

3. Materials and methods

3.1 Compound

Modified osthol derivative JS-B (minimum 96%) was provided and patented by the JAAS [22] and synthesized by the Nanjing University of Science and Technology.

3.2 Pathogens and cultures

P. capsici, *Botrytis cinerea*, *R. solani*, *Fusarium graminearum*, *Colletotrichum capsici*, *Fusarium moniliforme*, *F. oxysporum*, *M. kawatsuka*, *Sphaceloma ampelinum*, and *Cercospora bslicola* were provided by the Food Safety Research and Service Institute, JAAS. All the isolated pathogens were maintained on PDA (potato infusion from 200 and 20 g/l dextrose, and 20 g/l agar) in the dark at 25°C.

3.3 Antifungal spectrum

Mycelial disks (6 mm in diameter) of the test fungi grown on PDA plates were cut from the margins of the colony and were transplanted onto PDA plates containing different concentrations of JS-B (0, 12.5, 25, 50, 75, 100, 150, 200 μg/ml). A stock solution of JS-B was diluted with sterile distilled water and was added to sterile molten PDA to obtain the desired JS-B concentrations. The plates were placed in an incubation chamber in the dark at 25°C. When the mycelial growth on the control plate extended to more than two-thirds of the total diameter of the plate, growth measurements were taken using a ruler. The radial growth of each pathogen was measured (minus the diameter of the inoculation plug) by calculating the mean of the two perpendicular colony diameters. The EC_{50} values were determined for each isolated pathogen by calculating the percentage inhibition.

The effects of pH on the efficacy of JS-B on *P. capsici* were tested under the same conditions described above, except that medium pH values, from 5 to 9 with one unit interval, were adjusted with 1 M NaOH or HCl. All experiments were repeated twice with three replicates.

3.4 Effect of JS-B on each life stage of P. capsici

3.4.1 Mycelial dry weight

For the mycelial dry weight test, mycelial disks (6 mm in diameter) of *P. capsici* grown on PDA plates were cut from the margins of the colony and were inoculated into PDB (potato dextrose broth) in a triangular flask containing different concentrations of JS-B (0, 25, 50, 100, 150, 200 μg/ml). Erlenmeyer flasks were placed in a swing bed at 25°C, 120 rpm. The mycelia were harvested at the end of 5 days, filtered through pre-weighed filter-paper, and followed by drying to a constant weight at 80°C for about 24 h in an oven, after which the yield of mycelial biomass was measured. All the runs were replicated twice and the averaged values are presented in this work.

3.4.2 Sporulation

For the sporulation test, three mycelial disks (5 mm in diameter) were cut from the edge of the actively growing culture and were immersed in 25 ml sterile Petri solution ($Ca(NO_3)_2$: 0.4 g; KH_2PO_4: 0.5 g; $Mg(NO_3)_2$: 0.15 g; $CaCl_2$: 0.06 g; distilled water: 1000 ml) containing 0, 1.5625, 3.125, 6.25, 12.5, 25 μg/ml JS-B in plates. After 3 days of incubation in the dark at 25°C, the zoosporangia along the margins of each mycelial disk were observed with a light microscope (Nikon COOLPIX4500, Nanjing Nikon Jiangnan Optical Instrument Co. Ltd, Nanjing, China).

3.4.3 Zoospore release

The effect of JS-B on zoospore release was tested in Eppendorf tubes. Zoosporangia suspension (2×10^4 zoosporangia/ml) was obtained by the method of Zheng [23]. A total of 10 μl of the JS-B solution was added to the tube containing 2 ml of the zoosporangia suspension to make the final concentrations of JS-B of 0, 6.25, 12.5, 25, 50, 100 and 150 μg/ml. After the incubation at 25°C for 30 min, approximately 100 zoosporangia were observed with the light microscope and the inhibition percentages of zoospore release of *P. capsici* were calculated. The experiments were repeated twice with three replicates.

3.4.4 Cystospore germination

For the determination of the effect of JS-B on the cystospore germination of *P. capsici*, zoospore suspension (1×10^5 zoospore/ml) was obtained by the method of Ozgonen and Erkilic [24]. JS-B was added to the suspension from the start of the incubation at 25°C. Approximately 100 cystospores were observed with a light microscope (Nikon COOLPIX4500, Nanjing Nikon Jiangnan Optical Instrument Co. Ltd) and the percentages of cystospore germination were calculated.

The effect of glucose on the zoospore rupture of *P. capsici* induced by JS-B was tested under the same conditions described above, except that glucose (an osmotic stabilizer) in water was added to the zoospore suspension at 10 min before the addition of JS-B to give a final concentration of 100 mM. All the experiments were repeated twice with three replicates.

3.5 Determination of the effect of JS-B on the hyphal morphology

P. capsici mycelial tips (5 mm) from the margins of an actively growing colony on the PDA medium amended with 0 and 100 μg/ml of JS-B were cut down and fixed with 1% w/v OsO_4 solution. After fixation, the samples were dehydrated with ethanol and embedded in Epon 112. Thin sections were cut and double-stained with uranyl acetate and lead citrate. The grids were examined with a HITACHI H-600 electron microscope at 75 kV [25].

3.6 Effect of JS-B on P. capsici under pot conditions

For the pot experiments, zoospore suspension (1×10^5 zoospore/ml) was obtained by the method of Ozgonen and Erkilic [24]. The plants were wounded by making 1 cm slits approximately 1 cm above the soil surface. The sterile cotton soaked in zoospore suspensions (10^5 zoospores/ml) was enclosed around the wounded position. The inoculated sites were covered with plastic tapes to maintain moisture.

To evaluate the protective and curative activity of the fungicides tested, sprays were applied to 'run-off' with a hand sprayer at 24 h before (protective treatments) and after (curative treatments) the inoculation of the pathogen. JS-B was applied at 100, 200, and 400 μg/ml, while the commercial fungicide dimethomorph was applied at 550 μg/ml as a positive control. Control seedling plants were sprayed with sterile tap water 24 h before the inoculation. Fifteen plants were used per fungicide treatment and each inoculation time and the experiment was repeated in triplicate. Disease severity was measured daily after the inoculation based on a scale of 0–5: 0 = no visible disease symptoms, 1 = leaves slightly wilted with brownish lesions beginning to appear on stems, 2 = 30–50% of the entire plant diseased, 3 = 50–70% of the entire plant diseased, 4 = 70–90% of the entire plant diseased, and 5 = plant dead [18]. The efficacy percentage (%) was determined by the following formula: (disease index of Ck_1 − disease index of Pt_1/disease index of Ck_1) × 100.

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