

# Systemic Resistance against *Sphaerotheca fuliginea* in Cucumber by Cell Fungal Fragments of *Flammulina velutipes*

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**Abstract:** Cellular fragments of the fungus *Flammulina velutipes* (JZGP), extraction of *F. velutipes* fermentation solution (JZG), Benzothiadiazoles (BTH, BION®), and KangDuFeng (KDF) were studied as candidates of SAR inducers in the cucumber-*Sphaerotheca fuliginea* system. The results demonstrated that the treatments of all candidate inducers placed on the first leaf of cucumber plants six days before challenging inoculation on the upper leaves with *S. fuliginea*, induced systemic resistance. Disease incidence and index were significantly lower when compared with the water-treated control. The systemic acquired resistance (SAR) levels induced by the JZGP solution diluted 500 times and the JZG treatment were higher than that elicited by KDF alone, but lower than the BTH treatment. Treatments with these inducers led to an increase in activities of chitinase and beta-1,3-glucanase in cucumber leaves compared with control plants, indicating that these inducers triggered SAR in the cucumber plants examined. The results of this study will assist in the exploitation of biological inducers of plant resistance in cucumber.

**Introduction:** Powder mildew, caused by *Sphaerotheca fuliginea*, is a widely distributed and destructive disease of

greenhouse and field-grown cucumber plants. The disease is typically controlled by fungicide applications. As the problems of the fungicide resistance and the pollution to the environment have become increasingly serious, alternative methods of protection are essential. Resistance can be systemically induced in plants by abiotic or biotic elicitor(s) (1,4-6,11,12). Among the abiotic compounds, salicylic acid (SA) was found to induce systemic resistance (SAR) to fungal, bacterial and viral pathogens. Benzothiadiazole (BTH) mimics SA, and is capable of inducing SAR. In 1996, BTH was introduced in Germany, and is now available as the commercial product BION®. Resistance inducing effects of this product have been demonstrated in plants against different crop diseases (2,7).

Hijwegen and Verhaar demonstrated that three fungal culture filtrates were able to induce resistance to *Peronospora parasitica* in red cabbage reducing disease incidence by 52% (8). Based on our research investigating the active component of cellular fragment of *Flammulina velutipes* (JZGP), the active extracted component of *F. velutipes* fermentation is proteoglycan.

Biochemical changes associated with induced systemic resistance, including

enhancement of levels of chitinase and beta-1,3-glucanase in some plants, have been reported(2,3,10). However, the induction of systemic resistance to *S. fuliginea* by cell fragments of this fungus, however, has not been extensively investigated in cucumber. Therefore, the objectives of the research reported herein are to break the cells for releasing proteoglycan present in fungus cells, and to study the induced resistance in cucumber-*Sphaerotheca fuliginea* system.

In the present paper, four different resistance inducers were compared for their potential effect against powdery mildew (*S. fuliginea*) infection: 1) the synthetic inducer BTH (BION®); 2) biotic agents cellular fragment (JZGP); 3) biotic agents produced after extraction of fermentation (JZG) from *F. velutipes*, and; 4) a biotic product (KDF). We provide evidence for the effectiveness of fungal cell fragment's for inducing systemic protection against powdery mildew in cucumber plants. In addition, the levels of chitinase and beta-1,3-glucanase were investigated to evaluate their potential role in inducing systemic resistance against powdery mildew in the upper leaves of plants by foliar spray of fungal cell fragments.

**Materials and Methods:** *Pathogen.* *Sphaerotheca fuliginea* was obtained from cucumber plants grown in greenhouse. Inoculum was obtained from freshly infected leaves on which the fungus was sporulating. Conidia were gently brushed into distilled water resulting in a conidial suspension.

**Inducers:** For the JZGP solution, cell fragments of *Flammulina velutipes* (JZGP) was used at two concentrations (diluted with distilled water): 500 x and 1000 x. A solution of *F. velutipes* was designated JZG where a fermented solution was concentrated for about 1000 x as the base solution. The base solution was diluted 1000 x for experimentation. The benzothiadiazole (BTH, BION®) treatment was obtained from Novatis Agro-Chemistry

Company Ltd. The concentration of BTH used in the experiment was 75 µg/ml. The KangDuFeng treatment was produced by the Institute of Plant Protection, Beijing Academy of Agriculture and Forestry, Beijing, China.

*Plants, inoculation, and induction of systemic resistance.* Greenhouse grown cucumber plants (*Cucumis sativus* L. cv. Youza 8) were employed for experimentation when the sixth-true leaf was fully expanded. Treatment with inducers was made by daubing the third leaf from bottom into each treatment solution. Six days later, the upper three leaves of the plants were inoculated with the pathogen by spraying the conidial suspension of *S. fuliginea* onto non-infected leaves. Control plants were treated with tap water by spraying. Each treatment had nine replicates, and disease incidence and index were recorded.

*Assay of enzyme activity.* Sampling: The third cucumber leaf of each plant was treated with inducers JZGP, JZG, KDF and BTH. Control plants were treated with tap water. The 4th, 5th and 6th leaves were sampled at 2, 5, and 8 days after treatment and stored in refrigerator at -80°C for analysis.

The extraction of enzyme solution, preparation of colloidal chitin and detection of chitinase activity followed the methods of Boller(2). Total beta-1,3-glucanase activity was assayed by the method of Reuveni(8). For expression of chitinase, one unit of chitinase activity was defined as an amount capable of releasing 1 µg N-Acetylglucosamine (GlcNAc) from deacetyl glycol chitin in one hour, and was expressed as  $\mu\text{g}^{-1}$  fresh weight of tissue. The expression of beta-1,3-glucanase: was defined as the amount of enzyme required to release 1µg glucose of reducing lamminarin in one minute, and is expressed herein as  $\mu\text{g}^{-1}$  fresh weight of tissue.

**Results:** *Systemic induced resistance in cucumber seedlings.* Upper leaves of cucumber seedlings were more resistant to infection by *S. fuliginea* after being treated with different inducers on the third leaves (Table 1). Disease index and incidence were reduced greatly, showing significant difference from the control at  $P < 0.05$ .

After the third leaves were treated with BTH, the upper leaves of cucumber plants were inoculated with *S. fuliginea*, inducing systemic resistance. In the treated plants, the disease incidence (21.5%) and index (15.3) were reduced by 67.6% and 81.7% respectively compared with the control (66.4% and 83.5, respectively).

The disease incidence of cucumber plants inoculated with *S. fuliginea* after spraying with JZG and JZGP (500 x) in the third leaf was 25% (JZG) and 25.2% [JZGP (500 x)] on the upper leaves. This was a reduction by 62.3% (JZG) and 62.0% [JZGP (500 x)] when compared with control. Likewise, the disease index was 30.4 (JZG) and 26.4 [JZGP (500 x)], being reduced by 63.6% and 68.4% respectively, when compared with control.

Treatments with KDF and JZGP (1000 x) also induced resistance in the upper leaves of cucumber plants. The intensities, however, were lower than those of other inducers. Of the four inducers, BTH was the most effective at controlling *S. fuliginea*, followed by JZGP (500 x) and JZG. Treatments JZGP (1000 x) and KDF were similar and less effective than other treatments.

In addition to the reduction of disease index and incidence, the appearance of disease symptoms in treated plants was postponed by 3-4 days compared with the non-treated control.

*Activities of chitinase in leaves of treated plants.* The chitinase activity in leaves treated with inducers was higher than that in

control (Table 2). The chitinase activity increased beginning on the second day post-treatment. The enzymes activity continued to increase during the first eight days post-treatment. The chitinase activity was the highest in leaves treated with BTH. During the first five days, the chitinase activity in leaves treated with all agents was higher than control. These differences were significant ( $P < 0.05$ ) when compared to controls.

*Activities of beta-1,3-glucanase in leaves of treated plants.* There was a significant increase in beta-1,3-glucanase activity in cucumber leaves as a result of various treatments (Table 3). Only on the fifth day was the activity lower than control after foliar treatment with BTH. On the eighth day, the enzyme activities in all the treatments were dramatically ( $P < 0.05$ ) higher than controls.

**Discussion:** Four SAR inducer candidates were tested which included cell fragments of *F. velutipes* (JZGP), Benzothiadiazole (BTH), a fermented solution of *F. velutipes* (JZG) and KangDuFeng (KDF). Cucumber leaves treated with inducers systemically protected upper leaves against powdery mildew caused by *S. fuliginea*. Disease incidence and index were significantly reduced with all treatments. Of the four candidate inducers, JZGP (500 x) and JZG were higher than KDF in inducing resistance, but lower than BTH.

In order to find alternative resistance inducers, these treatments were selected from an array of about 20 potential agents (chemical and biological). Among the treatments, the effectiveness of the fermented solution of *F. velutipes* (JZG) was similar to cell fragments of *F. velutipes* (JZGP). However, the fungus solution producing procedure is time consuming, taking more than ten days to prepare. BTH has not been registered in China; and thus JZGP has the potential for practical application.

In the interaction between plant and fungus, beta-1,3-glucanase and chitinase are thought to play multiple roles in plant self-defense (9). The results of our study indicate that chitinase and beta-1,3-glucanase activity increased in inducer treated cucumber leaves, suggesting that the increased activity of chitinase and beta-1, 3-glucanase was related to induced resistance.

Time course of increased activity of chitinase and beta-1, 3-glucanase patterns indicated that the fungus-extract treatment probably is responsible for the triggering of defense responses, inducing the systemic resistance. Difficulties associated with the formulation of cell fragments of *F. velutipes* (JZGP) is an area that was addressed by this research. Thus, further research is necessary to define the action mode of the agents.

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Table1. Effects of inducers on the resistance of cucumber to powdery mildew.

Inducers	Concentrations	Date of symptom appeared	Disease incidence (%)	Disease index
Contol	Water	27 Dec.	66.4 a	83.5 a <sup>1</sup>
BTH	75µg/ml	31 Dec.	21.5 d	15.3 f
JZG	1000 x	31 Dec.	25 c	30.4 d
JZGP-1	500 x	31 Dec.	25.2 c	26.4 e
JZGP-2	1000 x	30 Dec.	33.5 b	50.3 b
KDF	400 x	30 Dec.	34.0 b	40.63 c

<sup>1</sup>Different letters indicate significant differences among the treatments (P < 0.05).

Table 2. Chitinase activity in leaves at various times after inducers treatments as measured by fresh weight (FW) changes (U/g FW).

Inducers	Concentrations	Days after treatment		
		2	5	8
JZGP	500 x	20.4 c	108.5 bc <sup>1</sup>	125.2 a
BTH	75µg/ml	23.4 b	129.0 a	132.8 a
JZG	1000 x	28.3 a	113.0 b	128.2 a
KDF	400 x	22.6 b	99.1 cd	117.0 b
Control		19.2 c	86.9 d	107.0 b

<sup>1</sup>Different letters indicate significant differences among the treatments (P < 0.05).

Table 3. Activities of beta-1,3-glucanase in leaves at various times after inducers treatment as measured by fresh weight (FW) changes (U/g FW).

Inducers	Concentrations	Days after treatment		
		2	5	8
JZGP	500 x	339.8 b	473.0 a <sup>1</sup>	650.4 c
BTH	75µg/ml	320.7 c	320.2 c	595.8 c
JZG	1000 x	521.0 a	457.9 ab	856.9 a
KDF	400 x	290.6 d	527.4 a	802.9 a
Control		288.5 d	369.4 bc	433.7 d

<sup>1</sup>Different letters indicate significant differences among the treatments (P < 0.05).