Synergistic Effect of Dazomet Soil Fumigation and *Clonostachys rosea* Against Cucumber Fusarium Wilt

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ABSTRACT

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Soil fumigation and biological control are two control measures frequently used against soilborne diseases. In this study, the chemical fumigant dazomet was applied in combination with the biocontrol agent (BCA) *Clonostachys rosea* 67-1 to combat cucumber wilt caused by *Fusarium oxysporum* f. sp. *cucumerinum* KW2-1. When the mycoparasite *C. rosea* 67-1 was applied after dazomet fumigation, disease control reached 100%, compared with 88.1 and 69.8% for dazomet and 67-1 agent, respectively, applied alone, indicating a synergistic effect of dazomet and *C. rosea* in combating cucumber Fusarium wilt based on analysis of

Bliss Independence. To understand the synergistic mechanism, the effects of chemical fumigation on the colonization potential and activity of *F. oxysporum* f. sp. *cucumerinum*, and the interaction between the BCA and the pathogen were investigated. The results showed that growth of the pathogen decreased with increasing dazomet concentration subsequent to fumigation. When exposed to dazomet at 100 ppm, the fungal sporulation rate decreased by 94.4%. Severe damage was observed in fumigated isolates using scanning electron microscopy. In the greenhouse, disease incidence of cucumber caused by fumigated *F. oxysporum* f. sp. *cucumerinum* significantly decreased. Whereas germination of *C. rosea* 67-1 spores increased by >sixfold in fumigated soil, and its ability to parasitize fumigated *F. oxysporum* f. sp. *cucumerinum* significantly increased ($P = 0.014$).

Cucumber wilt, caused by *Fusarium oxysporum* f. sp. *cucumerinum* J. H. Owen, is a widespread soilborne disease, which can cause serious damage and great economic losses to cucumber production worldwide. In recent years, protected vegetable production has increased rapidly in China. However, long periods of continuous cropping accompanied by high-density propagation and intensified fertilization can trigger continuous cropping obstacles, which lead to changes in the soil microflora, accumulation of pathogens, and, eventually, disease outbreaks. At present, Fusarium wilt has become one of the major barriers to protected cucumber production in China.

Several measures have shown promising results in suppressing cucumber Fusarium wilt and improving overall yields (23,41,43), in which fumigation is frequently used in the greenhouse. Soil sterilization using chemical fumigants such as dichloropropene, dazomet, organic sulfur, and biofumigants can exterminate various pests and eliminate pathogenic soil microorganisms rapidly and efficiently. The fumigants volatilize soon afterward and do not contaminate the agricultural products (2,12,19,37,49). Dazomet (3,5-dimethyl-1,3,5-thiadiazine-2-thione) decomposes into methyl isothiocyanate (MITC) in moist soil, preventing soilborne diseases. Previous studies have shown that, by mixing soil or furrow applications, dazomet fumigation can suppress many soilborne diseases effectively and, thus, increase crop yield (21,25,29,48). Mao et al. (31) found that the population density of *F. oxysporum* decreased 93.1% after treatment with 98% dazomet prior to planting cucumber in a nursery garden. However, pathogens can potentially reproduce following soil fumigation, and a subsequent invasion could be serious (14). There is some evidence that

repeated use of chemical fumigants in the soil may result in a decreased ability to control soilborne diseases over time (15,47). Several studies have been conducted to analyze the effects of various fumigants on rhizospheric microorganisms, soil physicochemical and enzymatic characteristics, and different practices on microflora and microbial ecology in soil (13,24,33,38,54).

Biological control has played an increasingly important role in the suppression of plant fungal diseases and organic vegetable production. Several commercial biocontrol agents (BCAs)—*Trichoderma viride* Trieco, *Bacillus* GB03, MBI600, and FZB24—have been applied successfully to control Fusarium wilt (4,7). A promising mycoparasite, *Clonostachys rosea*, has also shown great potential in countering many plant fungal pathogens and increasing crop yield (8,9,26,35,36,56). However, inconsistent results have often been observed in field trials in different areas for BCAs, especially in seriously infected soil (25,42,44).

Combining the two practices (fumigation and biological control) has been attempted to manage soilborne plant diseases in the field (17,20,44–46). When treated with high-efficiency, low-toxicity chemical fumigants, adverse factors in soil were found to be weakened or eliminated, and favorable conditions were created for the introduction and colonization of beneficial microorganisms (32,34). Slusarski and Pietr (46) found that, after using dazomet and *Trichoderma* sp. B35 continuously for 3 years, the incidence of Verticillium wilt on green pepper dropped sharply to 1.8% in the greenhouse. However, the relationship among fumigant, biocontrol fungus, and pathogen is unclear, and the synergistic mechanism of soil fumigation and biological control needs to be demonstrated. Paret et al. (39) studied the variation in cell structure and chemical components of *Ralstonia solanacearum* by using micro-Raman spectroscopy and electron microscopy, and indicated that fumigation could lead to the loss of nutrients and injury to the cell wall of the pathogen. Therefore, these authors theorized that fumigation might result in a decrease

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in the pathogenicity of *P. solanacearum*. However, no direct evidence of this was provided. Several biological traits were also regarded to be related to fungal pathogenicity. β -D-glucosaccharase is an important type of cellulase, and previous studies have shown that the enzyme activity and pathogenicity were closely correlated in *F. oxysporum* (5,6,52). When induced by host plants, the pathogen can secrete a series of cell-wall-degrading enzymes, including β -D-glucosaccharase, which enables *F. oxysporum* to penetrate and parasitize crop plants. Cumagun (11) found that the red pigment production gene *PIG1* in *Gibberella zeae* (anamorph *F. graminearum*) closely linked to disease severity. However, the impact of fumigation on these virulence-related characteristics is scarcely touched.

In order to evaluate the synergistic effect of fumigation and biological control in disease management, an efficient and low-toxicity chemical fumigant, dazomet, and a promising mycoparasite, *C. rosea* 67-1, were chosen to determine their effects on cucumber Fusarium wilt in the greenhouse. The effects of the fumigant on the colonization potential, the activity of the pathogen, and the interaction between the biocontrol isolate and the pathogen were studied. Understanding the synergistic effect of soil fumigants and beneficial microbes in countering plant pathogens will help establish a theoretical foundation for the effective use of BCAs against soilborne diseases.

MATERIALS AND METHODS

Isolates. *C. rosea* 67-1 was originally isolated from vegetable garden soil obtained from Ledong Farm in Hainan Province, China using a sclerotia baiting method (55) and identified by the Institute of Microbiology, Chinese Academy of Sciences based on its colony morphology and microscopic characteristics. The *F. oxysporum* f. sp. *cucumerinum* KW2-1 isolate used in these studies was isolated from a protected cucumber-growing facility in Langfang, Hebei Province, China. Both strains are maintained in the Biocontrol of Soilborne Diseases Lab of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS).

Plant materials. The cucumber ‘Zhongnong #6’ was provided by the Institute of Vegetables and Flowers, CAAS. This cultivar is rated as moderately susceptible to *F. oxysporum* f. sp. *cucumerinum* (30).

Agents. The soil fumigant, 98% dazomet powder, was provided by the Applied Microbiology Lab of the Institute of Plant Protection, Shanghai Academy of Agricultural Sciences.

Media. Millet-rice husk medium was prepared by mixing millet and rice husks (1:1, vol/vol) thoroughly. Water was added to maintain appropriate moisture (≈ 25 to 30%) and the substrate mixture was put into glass jars to fill one-third of the volume. Basal medium consisted of 25.0 g of glucose, 7.2 g of yeast extract powder, 0.5 g of NaCl, 1.0 g of K_2HPO_4 , 0.5 g of $MgSO_4 \cdot 7H_2O$, 50 mg of $ZnSO_4 \cdot 7H_2O$, 50 mg of $FeSO_4 \cdot 7H_2O$, and 1,000 ml of distilled water. The pH of the broth was then adjusted to 6.5. Both media were sterilized at 121°C for 30 min.

Preparation of soil infested with *F. oxysporum* f. sp. *cucumerinum*. A culture of *F. oxysporum* f. sp. *cucumerinum* KW2-1 was inoculated on potato dextrose agar (PDA) and incubated at 26°C for 7 to 10 days. The fungal culture was cut into 5-mm blocks with a sterile knife and inoculated into sterile millet-rice husk medium. Each plate was divided and transferred to two jars. The fungus and substrate were mixed thoroughly, and cultured in an incubator at 25 to 30°C for 10 days. KW2-1 inoculum was mixed into the soil collected from an uninfested vegetable yard at a rate of 3% (wt/wt), which corresponded to 6×10^5 CFU/g of soil, and put in 64-by-38-cm plastic boxes. Ten cucumber seedlings, preplanted in a nursery garden for 14 days, were transferred into the *F. oxysporum* f. sp. *cucumerinum*-infested soil in each box. After the symptoms of wilt fully

developed, the plants were pulled out. The soil in each box was remixed completely, dried, and passed through a 2-mm pore-size sieve to get rid of plant residues.

Preparation of inoculants of *C. rosea* 67-1. The *C. rosea* 67-1 isolate was cultured on PDA plates at 26°C. After 10 days, 5 ml of sterile distilled water was added to the plates to elute fungal spores with a sterile glass spatula. The spore suspension was inoculated into basal broth in a 500-ml flask, and cultured on a fermentation shaker at a speed of 180 rpm. The fungus was incubated at 26°C for 72 h, to prepare the inoculum of isolate 67-1.

Combined control of cucumber Fusarium wilt with dazomet and *C. rosea* in the greenhouse. Four treatments were included in the experiment: (i) fumigation with dazomet; (ii) biocontrol with *C. rosea* 67-1; (iii) *C. rosea* 67-1 inoculation after fumigation with dazomet; and (iv) the control, which received infested soil only. The soils with different treatments were put into plastic pots (15 cm in diameter), and cucumber seedlings that had been grown for 14 days were transferred into the pots and evaluated for the control efficiency of the four different treatments in the greenhouse. For fumigation, 98% dazomet powder was mixed with *F. oxysporum* f. sp. *cucumerinum*-infested soil (1:1,000, wt/wt) and put into the boxes. Water was added to reach 24% moisture content, and the boxes were sealed with 0.06-mm plastic film immediately and maintained at 25 to 28°C in the greenhouse for 10 days. The soil was turned twice during this period. After fumigation, the film was removed and the soil was loosened to release chemical gases. After aerating for 7 days, the soil was used as fumigated soil. For biocontrol, *C. rosea* agent was applied into the holes in *F. oxysporum* f. sp. *cucumerinum*-infested soil when transplanting. Three 5-cm-deep holes were dug in each pot and 67-1 culture broth together with a cucumber seedling and ≈ 20 g of growing substrates were transferred into the holes simultaneously. The concentration of 67-1 inoculum was 2×10^7 CFU/hole. For fumigation with biocontrol, the *C. rosea* 67-1 isolate was inoculated into fumigated soil at 2×10^7 CFU/hole during transplant. The growing temperature was 26 to 30°C during the day and 15°C at night. There were three seedlings per pot, and eight pots per treatment. Pots were arranged in a randomized complete block design with three replicates for each treatment.

All of the seedlings were investigated for the incidence and severity of Fusarium wilt on cucumber after 45 days, and the control efficiencies of the different treatments were evaluated. Disease indices were rated using a five-level grading scale (from 0 to 4) based on symptoms on leaves and stems (16). Grade 0 = no wilting symptoms observed; grade 1 = wilting only on a few leaves; grade 2 = slight wilting, necrotic spots appearing on the stem, and presence of chlorosis; grade 3 = moderate wilting, drooping leaves and chlorosis; and grade 4 = extensive wilting, lodging, and withering. The expected control efficacy for combined use of the two measures was estimated using the model of Bliss Independence (i.e., $E_{12} = E_1 + E_2 - E_1 \times E_2$, where E_1 and E_2 were the efficacy of the two individuals). The observed control efficacy for combined use (O_{12}) was compared with E_{12} , and the interactions among the two practices were judged as follows: if $O_{12} > E_{12}$, then synergistic interaction was indicated; if $O_{12} = E_{12}$, no interaction (i.e., additive effect) existed; and if $O_{12} < E_{12}$, an antagonistic interaction was implied (3).

Effect of fumigation on growth of *F. oxysporum* f. sp. *cucumerinum* KW2-1. Soil was collected from the top 0 to 20 cm of the ploughed layer in the experimental field at the Institute of Plant Protection of the CAAS in Langfang. The preceding crop was tomato. Soil was passed through a 1-mm sieve, and 500 g was put into a glass jar and dry-heat sterilized at 162°C in an oven for 5 h. Dazomet was added at the effective concentrations of 25, 50, 75, and 100 ppm. The soil was mixed thoroughly, and 120 ml of sterile water was added to keep the water content at 24%. From

the edge of the agar plate, a 5-mm disk of KW2-1 cultured for 10 days was transferred to a fresh PDA plate, and the petri dish was upended on the jar immediately. The upside-down plate was sealed at the bottleneck with Parafilm to avoid escape of the effective compounds from fumigation. The pathogenic *F. oxysporum* f. sp. *cucumerinum* culture was incubated at 26°C for 7 days, after which the morphology of the colony was observed and the growth rate was determined. Each treatment was replicated three times.

Morphology of spores and hypha of the fumigated *F. oxysporum* f. sp. *cucumerinum* KW2-1-F isolate. The KW2-1 isolate treated with dazomet was transferred to a fresh PDA plate and marked as KW2-1-F. After growing at 26°C in an incubator for 7 days, 5 ml of sterile distilled water was added to elute fungal spores with a sterile glass spatula, and the spore suspension was collected. Fungal mycelia on another agar plate were also collected. The samples were processed as described by Pisi et al. (40), and photos were taken with a scanning electron microscope (Hitachi S-570).

Sporulation of *F. oxysporum* f. sp. *cucumerinum* KW2-1-F. A 5-mm agar disk of the dazomet-treated isolate KW2-1-F was inoculated onto the center of PDA plate and incubated at 26°C for 7 days. The spores were eluted with 5 ml of sterile distilled water, and the suspension was collected and diluted. The number of spores was counted under a microscope using a hemocytometer. The original strain KW2-1 was used as a control. Three replicates were used for both isolates.

Pigment production by KW2-1-F. The concentration of the KW2-1-F spore suspension obtained from the agar plate was adjusted to 1×10^6 spores/ml by dilution with sterile water. The spore suspension (5 ml) was inoculated into 50 ml of pigment-producing liquor in a 250-ml flask and cultured on a shaker at a speed of 200 rpm. After incubating at 28°C for 5 days, the pigment in the fungal cells was extracted by ultrasonic disruption, and the color value was determined at 520 nm with an ultraviolet spectrophotometer (10). Three replicates were used.

Activity of β -D-glucosaccharase activity in KW2-1-F. β -D-glucosaccharase was extracted from *F. oxysporum* and its activity was determined. A spore suspension of the fumigated strain was prepared as described above, and 1 ml of the suspension was inoculated into 100 ml of potato sucrose broth. The culture was then shaken at 25°C at a speed of 120 rpm for 7 days, filtered through a double layer of cheesecloth to remove fungal hyphae, and centrifuged at 10,000 rpm for 20 min to obtain a crude enzyme preparation. The activity of β -D-glucosaccharase was determined by a spectrophotometer according to Che et al. (5).

Effect of KW2-1-F on germination of cucumber seed and shoot growth. Plump cucumber seed were selected and heated at 60°C in an oven for 5 h. A spore suspension (1.5 ml) of KW2-1-F (1×10^6 spores/ml) was applied to coat the seed, and the seed were stirred intermittently during the presoaking. After 3 h, the cucumber seed were placed on wet sterile filter paper in a petri dish and cultured at 25°C in a lighted incubator. Germination rates were recorded. Seed soaked in a spore suspension of KW2-1 and water were used as controls. Thirty seeds were included in each treatment, and five replicates were used.

Pathogenicity of KW2-1-F on cucumber. Cucumber seed were preheated as described above and soaked in sterile water for 3 h. The seed were then placed on wet filter paper in sterilized culture dishes to accelerate germination. After 12 h, the germinated seed were sown in a 50:50 mixture of nutrient soil and vermiculite that had been dry-heat sterilized at 163°C for 5 h. Ten days after sprouting, the cucumber seedlings were pulled out, the soil attached to the roots was washed off, and the seedlings were then immersed into the KW2-1-F and KW2-1 spore suspensions (1×10^6 spores/ml) for 15 min. The seedlings were transplanted into sterile nutrient substrate in paper cups (10 cm in diameter),

two seedlings per cup. The plants were grown in a lighted incubator with 12 h of illumination at 26°C. There were five cups per treatment, with five repeats per treatment. Fourteen days after planting, the incidence of Fusarium wilt on cucumber in both treatments was determined. The disease severities were graded on a scale of 0 to 4 where grade 0 = no browning; grade 1 = slight infection, brown areas covering <25% of the root and the basal part of the stem; grade 2 = brown areas covering 25 to 50% of the root and the basal part of the stem; grade 3 = brown areas covering 50 to 75% of the root and the basal part of the stem; and grade 4 = extensive wilting, with browning covering >75% of the root and the basal part of the stem.

Effect of soil fumigation on fungistasis to the biocontrol fungus. Soil collected from the vegetable yard was mixed thoroughly with 0.1% (wt/wt) dazomet powder. The mixture was put into plastic trays (15 by 10 by 5 cm), watered, and covered with a plastic mulch film. After fumigating for 5 days, the soil was uncovered and air dried. The soil was then screened through a 0.38- μ m sieve, and 20 g was put into a sterile plate (90 mm in diameter) and moistened with 10 to 20 ml of sterile distilled water. Two pieces of sterile cellophane, 30 by 30 mm and 10 by 10 mm, were placed on the surface of the soil in succession. Spores of *C. rosea* 67-1 were collected and washed twice by centrifugation with sterile water to remove nutrients. The spores were dropped in the center of the upper cellophane with a concentration of ≈ 30 to 50 spores per microscope field, and were then covered with a piece of 30-by-30-mm cellophane and a layer of 0.5 to 1 cm of fumigated soil. The soil was kept moist and incubated at 25°C for 8 h; the cellophane with the droplet of 67-1 spores was then removed and examined under a microscope for spore germination. PDA medium and untreated soil were used as controls, and three replicates were used.

Mycoparasitism of the biocontrol isolate *C. rosea* 67-1 on *F. oxysporum* f. sp. *cucumerinum*. A piece of a sterile microscope slide was placed on two glass bars in a 9-cm-diameter petri dish. A nutrient-deficient medium (1/4 $\times$ PDA) was melted, and 10 μ l was dropped in the center of the slide glass with a pipette (Gilson, France) and smeared evenly with a sterile glass scraper to form a 20-by-20-mm thin film. Small agar blocks (2 mm) containing *C. rosea* 67-1 and *F. oxysporum* f. sp. *cucumerinum* KW2-1-F were placed on opposite sides of the film. The plate was sealed with Parafilm to maintain moisture and incubated at 25°C. When the colonies of the biocontrol fungus and pathogenic fungus grew into each other, the slides were lifted and mounted on a microscope with a 10-by-10 net eyepiece micrometer (Olympus BX51, Japan). The microstructure and interaction between the two fungi were observed under a microscope at $\times 400$ magnification.

The numbers of *F. oxysporum* hyphae at each intersection of the micrometer grid were determined, and the mycelia of *C. rosea* 67-1 that were attached to or bound to the *F. oxysporum* f. sp. *cucumerinum* hyphae were regarded as parasitic. Three random fields were examined. The original pathogenic strain KW2-1 with *C. rosea* 67-1 was the control. Three replicates were used for each treatment.

Statistical analysis. SPSS Statistics V17.0 software (IBM Corporation) was used for statistical comparisons of the treatments. Analysis of variance and *t* tests were used for comparisons of the means from each treatment, and regression was used for the study of a quantitative factor.

RESULTS

Integrated control of cucumber Fusarium wilt by dazomet and *C. rosea* in the greenhouse. Dazomet fumigation was an efficient practice for suppressing Fusarium wilt disease on cucumber caused by *F. oxysporum*, as was application of the biocontrol fungus *C. rosea* 67-1. Both methods significantly reduced the population of the pathogen in soil ($P < 0.01$). Dazomet

fumigation followed by *C. rosea* 67-1 application decreased the density of *F. oxysporum* in soil by 98.8%, and provided a control efficiency of Fusarium wilt of 100% (Table 1). Based on the respective control efficiency of soil fumigation and biocontrol with three replicates, the expected control efficacy for combined use of the two measures was calculated ($E_{12} = E_1 + E_2 - E_1 \times E_2$). The expected measurement of 96.4% was lower than the actual measurement ($P = 0.02$), indicating that there was a synergistic effect of dazomet and *C. rosea* in combating cucumber Fusarium wilt according to the analysis of Bliss Independence.

Effect of fumigation on growth of *F. oxysporum* f. sp. *cucumerinum* KW2-1. After dazomet fumigation, the growth of *F. oxysporum* f. sp. *cucumerinum* KW2-1 was increasingly suppressed with increasing concentrations of the effective components in the fumigant. The regression equation was $y = -0.0618x + 9.676$, with the correlation coefficient (R^2) of 0.986. When the pathogenic fungus was exposed to soil mixed with dazomet at 100 ppm, the extension of fungal hypha decreased by 67.1% (Fig. 1). In this study, the fungus treated with dazomet at 100 ppm was considered to be the fumigated isolate and was named KW2-1-F.

Morphology of the fumigated isolate KW2-1-F. Isolate KW2-1 grew rapidly, forming thick hyphae and an obvious purple zone. However, KW2-1-F that had been treated with dazomet displayed slow, weak growth. There were fewer aerial hyphae and no distinct pigment zone was present. The microstructures of the pathogens with and without fumigant treatment showed significant differences under scanning electron microscopy. The cells of KW2-1 spores and mycelia were active, with intact cell walls and visible lipid droplets. Intracellular structures were distinct, with clear and smooth boundaries (Fig. 2A and C). In contrast, cells of KW2-1-F appeared to be distressed. Most of the cells were shrunken and deformed, and some appeared empty. Lysis of organelles and release of cell contents were observed, which meant that the pathogenic fungus had been inactivated (Fig. 2B, D, and E).

Biological characteristics of KW2-1-F. When the pathogen isolate KW2-1 was treated with dazomet, its biological characteristics changed drastically. Chemical fumigation reduced the sporulation level of *F. oxysporum* f. sp. *cucumerinum* by 94.4%, and a comparison of the pigment yields between the original isolate and KW2-1-F were significantly different ($P < 0.01$). The activity of β -D-glucosaccharase in KW2-1-F decreased by 54.2% compared with KW2-1, which indicated that virulence of *F. oxysporum* might be negatively affected as well (Table 2).

Effect of KW2-1-F on germination of cucumber seed and growth of shoots. After soaking in spore suspensions of *F. oxy-*

sporum f. sp. *cucumerinum* KW2-1 or KW2-1-F, the germination rate of cucumber seed decreased slightly compared with the seed soaked in water. However, there was no significant difference among the three treatments. The growth of cucumber shoots infected by KW2-1 was greatly inhibited compared with both the water control and the fumigated isolate ($P < 0.01$) (Table 3). The seedlings treated with KW2-1 were coated with white mycelia, and the plants withered and died in 120 h. There were few mycelia observed on the plants treated with KW2-1-F, and the growth of seedlings was healthy.

Pathogenicity of KW2-1-F on cucumber. Of the cucumber seedlings infected with *F. oxysporum*, the development of symptoms of Fusarium wilt were significantly different between inoculations with spore suspensions of KW2-1 and KW2-1-F (10^6 spores/ml) on young roots. Fourteen days after transplanting, >70% of seedlings treated with the original strain showed typical wilt symptoms: the basal part of the stem turned brown accompanied by occasional constriction, and leaves were droopy and yellowed to the extent that 36% of the plants withered. On the other hand, only slight spots were observed on the seedlings soaked in the KW2-1-F spore suspension (Table 4).

Effect of soil fumigation on fungistasis to *C. rosea* 67-1. Fungistasis is a key factor that affects the activity and colonization of BCAs. Fungistasis to *C. rosea* 67-1 was greatly decreased after dazomet fumigation ($P < 0.01$). The germination rate of 67-1 spores reached $68.0 \pm 3.0\%$ in fumigated soil whereas, in natural soil, the rate was only $10.3 \pm 1.2\%$, indicating that the soil environment improved for colonization of the biocontrol fungus; thus, the BCA was able to play a more active role in the control of soilborne diseases.

Mycoparasitism of *C. rosea* 67-1 on *F. oxysporum* f. sp. *cucumerinum* KW2-1-F. Our results demonstrate that the endurance of fumigated pathogen to *C. rosea* decreased compared with the unfumigated isolate. After side-by-side cultivation for 3 days, the isolates of *C. rosea* 67-1 and *F. oxysporum* KW2-1-F grew together on the agar slide. Soon afterward, the colonies connected and extended into one another. It was obvious that the colony of *C. rosea* 67-1 was dominant in the contact zone. Under a microscope, it could be seen that the hyphae of 67-1 attached and spread along the hyphae of the pathogenic fungus in parallel or in wave form. In some cases, the hyphae of both species bonded with each other closely. The rate of *C. rosea* 67-1 hyphae contacting and binding with *F. oxysporum* f. sp. *cucumerinum* KW2-1-F hyphae was much greater than with KW2-1 (Table 5).

TABLE 1. Control of cucumber Fusarium wilt by dazomet and *Clonostachys rosea* in the greenhouse^z

Treatment	<i>Fusarium oxysporum</i> (CFU/g soil)	Disease index (%)	Control efficiency (%)
Dazomet	526 $\pm$ 5 A	7.5 $\pm$ 1.5 A	88.1 $\pm$ 2.2 A
67-1	1,018 $\pm$ 87 B	19.0 $\pm$ 1.4 B	69.8 $\pm$ 1.8 B
Dazomet + 67-1	53 $\pm$ 12 C	0.0 $\pm$ 0.0 C	100.0 $\pm$ 0.0 C
Control	4,401 $\pm$ 56 D	63.0 $\pm$ 2.8 D	...

^z Data are means $\pm$ standard deviations of populations of *F. oxysporum* in soil, disease indices of Fusarium wilt of cucumber, and control efficiencies for different methods, including fumigation with 98% dazomet powder (1 g/1,000 g soil), *C. rosea* 67-1 (2×10^7 CFU/hole), dazomet + *C. rosea* 67-1, and the control (without fumigant and biocontrol fungus). Treatments were examined 45 days after cucumber seedlings were transplanted into 15-cm-diameter plastic pots in the greenhouse. There were three replicates of eight pots with three seedlings in each, with each treatment in a randomized complete block design. The temperatures were 26 to 30°C during the day and 15°C at night. Values within a column followed by the same letter are not significantly different ($P < 0.01$) according to Duncan's multiple range test.

Fig. 1. Regression analysis of hyphal growth of the fumigated isolate of *Fusarium oxysporum* KW2-1 versus dazomet concentration. Data points were averages of colony diameter, in centimeters, of *F. oxysporum* f. sp. *cucumerinum* grown on potato dextrose agar, exposed to different dosages of dazomet mixed with sterile field soil and 24% water. Incubation was at 26°C for 7 days with three replicates.

DISCUSSION

Biocontrol is regarded as a promising and environmentally friendly strategy for plant protection, and has been widely applied in managing various plant diseases. However, for a nonindigenous BCA, it is much harder to compete with native soil inhabitants and, therefore, unstable results are often observed in different environmental conditions in the field (28,46). Fungistasis, which hinders the germination and colonization of soil microorganisms, is a major factor for BCAs to be considered. Previous studies have demonstrated that fungistasis is related not only to nutrient deficiency but also to soil microbial flora (18,51). In protected fields where vegetables are successively planted over several seasons, the soil habitat breaks down because of the accumulation of harmful microorganisms and their toxins, and the problem of fungistasis becomes very serious under these conditions. In our research, an obvious decrease in fungistasis to the biocontrol fungus *C. rosea* 67-1 was achieved through soil pretreatment with the highly efficient, low-toxicity chemical fumigant dazomet, and the germination rate of 67-1 spores in the fumigated soil substrate approached 70%. Through soil fumigation, pests were killed and the effect of fungistasis was eliminated or weakened (34); thus, it was more likely that BCAs could colonize in the soil. Furthermore, it is very important to establish a new, healthy environment that benefits plant growth and suppresses pathogen propagation through the use of biocontrol microorganisms that can rapidly fill the temporary biological vacuum in soil caused by fumigation. In our study, the application of *C. rosea* combined with dazomet fumigation achieved essentially 100% control efficiency on cucumber *Fusarium* wilt, which was much higher than the individual control efficiencies of the two agents used alone. This

conclusion is consistent with the results of Slusarski and Pietr (46).

Bliss Independence is a statistical model to analyze the effects of multiple factors (3), and it has been used to evaluate the effects of interactions among BCAs on disease development (22,53). When several BCAs or different management practices are used together, the phenomena of no interaction (i.e., additive effect) usually occur (20,53). In some cases, antagonistic influence and incompatible mechanism among the agents and measures may be

TABLE 2. Biological characteristics of *Fusarium oxysporum* f. sp. *cucumerinum* isolates KW2-1 and KW2-1-F

Isolate	Spores ($\times 10^6$ /plate) ^x	Color value (U/ml) ^y	Glucosaccharase activity (U) ^z
KW2-1-F	6.2 $\pm$ 0.4	3.32 $\pm$ 0.09	2.07 $\pm$ 0.63
KW2-1	115.0 $\pm$ 2.6	8.15 $\pm$ 0.06	4.52 $\pm$ 0.18

^x Means and standard deviations of spore production of the original strain KW2-1 and the isolate fumigated with 98% dazomet powder (100 ppm) grown on potato dextrose agar medium at 26°C for 7 days with three replicates. The two values are significantly different ($P < 0.01$) according to the t test.

^y Means and standard deviations of pigment production in the original strain KW2-1 and the isolate fumigated with 98% dazomet powder (100 ppm) incubated in pigment-producing liquor at 28°C, 200 rpm for 5 days with three replicates. The color value was determined at 520 nm with an ultraviolet spectrophotometer. The two values are significantly different ($P < 0.01$) according to the t test.

^z Means and standard deviations of β -D-glucosaccharase activity in the original strain KW2-1 and the isolate fumigated by 98% dazomet powder (100 ppm) in potato sucrose broth at 25°C, 120 rpm for 7 days with three replicates. The two values are statistically different at $P = 0.015$ according to the t test.

Fig. 2. Microstructure of spores and mycelia of *Fusarium oxysporum* f. sp. *cucumerinum*. Isolates KW2-1 and KW2-1-F were grown on potato dextrose agar at 26°C for 7 days and observed with a scanning electron microscope (Hitachi S-570). A, Mycelium of KW2-1; B, mycelium of KW2-1-F; C, KW2-1 spore; D and E, KW2-1-F spores. Scale bars represent 1 µm.

encountered, which weaken the results compared with the individuals. In our study, both dazomet and *C. rosea* showed high control efficacy against *Fusarium* wilt under the given *F. oxysporum* f. sp. *cucumerinum* density. Furthermore, when they were applied successively the control efficacy reached 100%, revealing a synergistic effect based on the Bliss assumption. The result suggested that the combined use of dazomet and *C. rosea* was a reliable way to control cucumber *Fusarium* wilt.

Many previous studies have indicated that some chemical fumigants, such as metham sodium and dazomet, can act as substitutes for methyl bromide in plant disease control. However, some researchers disagree and consider these kinds of fumigants to be weak soil disinfectants because they are not able to kill all pests in soil, which could then lead to a bloom of disease-causing pathogens after fumigation (14). In addition, it has been demonstrated that most fumigants are subjected to accelerated microbial degradation in various geographical zones, and that the degradation can last for a long period of time. The frequent use of fumigants can accelerate the degradation and diffusion of MITC and lead to a decrease in the effective compounds in soil and also the control efficiency on soilborne diseases (1,15).

The mechanism of combined control of cucumber *Fusarium* wilt by a chemical fumigant and a BCA was twofold. First, the effectiveness depended on a reduction of the *F. oxysporum* population density; second, effectiveness was also the result of the degeneration of *F. oxysporum*. In this study, the process of field fumigation was simulated to illustrate the variation in colonization potential and activity of the pathogen. The results showed that the fumigated *F. oxysporum* f. sp. *cucumerinum* isolate had sparse hypha, slow colony extension, and a greatly reduced level of sporulation, which indicated that the physiological activities of the pathogen cells were reduced by MITC, the effective component in dazomet. Under the scanning electron microscope, it was observed that the spores and hyphal cells of

KW2-1-F were lysed to various degrees, and the structure was also damaged. Thus, it was difficult for the fumigated pathogen to invade crops and cause plant disease. In recent years, researchers have confirmed that peroxisomes in pathogenic fungi are correlated to appressorium-mediated penetration of host plants through their effect on fatty acid β -oxidation (27,50). In our study, very few fat particles were detected in the spores of KW2-1-F, whereas fat particles could be clearly observed in the original isolate. Therefore, this may suggest that the reduced number of fat particles in the fumigant-treated isolate is responsible for the observed decrease in pathogenicity of *F. oxysporum* f. sp. *cucumerinum*. After fumigation, the pathogenicity-related pigment and activity of β -D-glucosaccharase in *F. oxysporum* f. sp. *cucumerinum* also decreased significantly which, in turn, could result in a decrease in virulence of the pathogen.

In this study, we combined dazomet fumigation of soil with the biocontrol fungus *C. rosea* and achieved excellent control efficiency in disease management. After fumigation, the colonization potential and activity of *F. oxysporum* f. sp. *cucumerinum* decreased significantly, and it was much easier for the BCA to parasitize and antagonize the pathogen. As a result, it was much more likely that sustainable cucumber production would be achieved.

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TABLE 3. Effect of KW2-1-F on seed germination and shoot growth in cucumber

Treatment	Concentration ($\times 10^6$ spores/ml)	Germination rate (%) ^y	Shoot length at 72 h (cm) ^z
KW2-1	1.3	88.3 $\pm$ 7.6	5.03 $\pm$ 0.79 A
KW2-1-F	1.2	88.3 $\pm$ 2.9	8.45 $\pm$ 0.81 B
Water	...	93.3 $\pm$ 5.8	7.90 $\pm$ 0.50 B

^y Means and standard deviations of germination rates for cucumber seed coated with a suspension of *Fusarium oxysporum* f. sp. *cucumerinum* at 1×10^6 spores/ml with and without dazomet (100 ppm) fumigation and cultured at 25°C in a lighted incubator with five replicates ($n = 150$). According to Duncan's multiple range test, there is no significant difference among the treatments ($P = 0.213$).

^z Means and standard deviations of shoot lengths of cucumber seedlings grown from seed coated with *F. oxysporum* f. sp. *cucumerinum* spore suspensions (with and without dazomet fumigation) and cultured at 25°C in a lighted incubator for 72 h with five replicates ($n = 150$). Values within a column followed by the same letter are not significantly different ($P < 0.01$) according to Duncan's multiple range test.

TABLE 4. Pathogenicity of *Fusarium oxysporum* f. sp. *cucumerinum* isolates KW2-1 and KW2-1-F on cucumber seedlings

Treatment	Infected seedlings ^x					Total ^y	Disease index ^z
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4		
KW2-1	2	8	4	12	24	50	74.0 $\pm$ 6.8 A
KW2-1-F	36	11	1	0	2	50	10.5 $\pm$ 3.3 B

^x Numbers of infected cucumber seedlings grown at 26°C in a lighted incubator with 12 h of illumination in five replicates. Seedlings were treated by dipping their roots in fungal spore suspension (1×10^6 spores/ml) of KW2-1-F and KW2-1 after spouting in sterile nutrient soil and vermiculite (1:1) for 10 days, after which they were transplanted into sterile nutrient substrate in 10-cm-diameter paper cups. Incidence and severity of *Fusarium* wilt disease on the cucumber seedlings were recorded after 14 days using a five-level grading scale (from 0 to 4) based on brown areas in the root and the basal part of the stem. Grade 0 = no browning, grade 1 = <25%, grade 2 = 25 to 50%, grade 3 = 50 to 75%, and grade 4 = >75%.

^y Total number of seedlings.

^z Means and standard deviations of five replicates. Values for the different treatments are significantly different ($P < 0.01$) according to the *t* test.

TABLE 5. Mycoparasitism of hyphae of *Clonostachys rosea* 67-1 on *Fusarium oxysporum* f. sp. *cucumerinum*

Treatment	Number of <i>F. oxysporum</i> f. sp. <i>cucumerinum</i>		
	On intersections ^x	Parasitized ^y	Parasitic rate (%) ^z
KW2-1	570	336	58.9 $\pm$ 4.7
KW2-1-F	531	390	73.4 $\pm$ 3.7

^x Total number of mycelia of KW2-1 and KW2-1-F which grew on 1/4 $\times$ potato dextrose agar (PDA) on sterile glass slides at 25°C and which encountered *C. rosea* 67-1, observed on intersections of a 10-by-10 net eyepiece micrometer under a microscope at $\times 400$ magnification, in three replicates.

^y Total number of mycelia of KW2-1 and KW2-1-F which grew on 1/4 $\times$ PDA on sterile slides at 25°C, observed being parasitized by *C. rosea* 67-1 on intersections of a 10-by-10 net eyepiece micrometer under a microscope at $\times 400$ magnification, in three replicates. Mycelia of *C. rosea* 67-1 attached to or bound to the *F. oxysporum* f. sp. *cucumerinum* hyphae were regarded as parasitic.

^z Means and standard deviations of parasitic rates of the original and fumigated isolates of KW2-1 and KW2-1-F which encountered *C. rosea* 67-1 when grown on 1/4 $\times$ PDA on sterile glass slides at 25°C for 5 days, with three replicates. The two values are significantly different according to the *t* test ($P = 0.014$).

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