

# Antagonistic Actinomycete XN-1 from Phyllosphere Microorganisms of Cucumber to Control *Corynespora cassiicola*

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Strain XN-1 isolated from the cucumber phyllosphere was tested for potential application as an antagonistic actinomycete against *Corynespora cassiicola* (Berk. & Curt.) Wei. The fungistatic activity of this strain was determined by inoculation of *C. cassiicola* on PDA plates, spore germination inhibition in culture filtrate and control tests on cucumber leaves. The spore germination and hyphal growth inhibition rate of the XN-1 fermentation filtrate against *C. cassiicola* can reach 96.50% and 51.17%, respectively, as well as the 63.54% of control effect on cucumber leaves. The stability of XN-1 fermentation filtrates was studied under the treatment of pH gradient, temperature gradient, and exposure to UV. The results showed the filtrates remained relatively stable in the range of those gradients. This experiment indicates that this exogenous actinomycete XN-1 has the potential to act as an antagonistic agent in controlling the occurrence and development of cucumber target leaf spot in the greenhouse. This also confirms that phyllosphere microorganisms play an important role in combating the infection of pathogens and have a promising future in developing the biocontrol products and methods.

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*Corynespora cassiicola* is regarded as a widespread pathogen, associated with more than 70 different host plants in tropical and subtropical countries (Pollack and Stevenson 1973; Onesirosan et al. 1974). In recent years, this fungus has resulted in a great loss on cucumber cultivated in plastic greenhouses in spite of treatment with various fungicides. Target leaf spot caused by *C. cassiicola* has been an important disease of cucumber since the end of the 1970s in Japan (Hasama 1990). Recently, the occurrence of this disease was also found in Korea (Kwon et al. 2003). While, in China, cucumber target leaf spot was reported as a new plant disease (Zou et al. 2002). It is well known that cucumber target leaf spot is difficult to manage in the greenhouse because of the optimum environmental conditions that are often conducive to disease development (Menzies and Belanger 1996). Moreover, the pathogen has become readily resistant to fungicides because of prolonged and continuous use. Therefore, the search for alternatives to chemical control of plant disease, especially biological control, has gained momentum in recent years (Compant et al. 2005). The control of pathogens by biological agents has not been practiced on a large scale, but many experiments have shown the potential for further development as a promising approach in responding to disease control and consumers' health concerns. In recent years, some special phyllosphere microbes have been found to have great effects on the location of pathogens (Zhao et al. 2000).

Among microbes, the actinomycetes are important

producers of bioactive compounds and represent a high proportion of the microbial biomass. Some actinomycetes secrete herbicidal compounds (Tanaka and Omura 1993) or fix atmospheric nitrogen; others can protect plants against fungal infections. The antagonistic impact of actinomycetes on pathogenic fungi is known and several species have been used as biological control agents (Jones and Samac 1996; Bressan 2003). Most species which are isolated from the soil cannot be used as a biological agent directly. In this experiment, we sought to isolate microbes from the leaves so that some new antagonistic actinomycete isolates that exhibit a highly inhibitory effect against plant pathogens can be found.

## Materials and methods

### Sampling

The cucumber plants were collected using Zhao xinhua and Chen weiliang method (2000) from Gardenspot of Northwest University of Agriculture and Forest (Yangling, ShaanXi province, China). The leaf samples picked were placed in sterile polyethylene bags, closed tightly and stored in the refrigerator at 4°C until use. The pathogen fungi were supplied by the Plant Pathology laboratory.

### Isolation of phyllosphere actinomycetes

The picked leaves were placed into 200 ml sterile distilled water, and then shaken for 30min at room temperature. The suspension obtained was diluted by a

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factor of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ; 0.1 ml of each dilution was placed in sterile petri dishes containing Actinomycetes Isolation Agar (AIA, Olson 1968) (5% glycerol, 0.2% sodium caseinate, 0.01% L-asparagine, 0.4% sodium propionate, 0.05%  $K_2HPO_4$ , 0.0001%  $FeSO_4$  and 1.5% agar Difco). which was supplemented with 25% lactic acid to inhibit the development of bacteria without affecting the growth of fungi and actinomycetes (Naureen et al. 2009). Petri dishes were incubated for 3 days at 28°C. The isolates were purified and transferred to AIA media and stored at 4°C.

### *Antifungal assays*

The antifungal activity of the isolates was determined by the plate diffusion method (Barakate et al. 2002) against *C. cassiicola*. Isolates were grown on Bennett medium for 14 days and 5-mm diameter agar disks containing actinomycete colonial mass were prepared using sterile borers. Disks were then transferred aseptically to PDA plates in which fungal mycelial disk (5 mm diameter) was placed in the centre. Plates were incubated at 25°C. Inhibition zones were determined after 3 days of incubation. The isolates that showed an inhibition zone greater than 8 mm were considered as active ones, which were selected to be further studied.

### *Culture filtrate preparation of actinomycetes*

Liquid cultures were grown in 250 ml flasks containing 100 ml of nutrient broth (NB) which had previously been added two sterile bacteriological loops of the culture. Flasks were incubated on a shaker at 180rpm at 28°C for 72 h. The culture was transferred to 50 ml centrifugation tubes, and centrifuged at 10000 rpm/min for 20 minutes, and then the supernatant of centrifuged cultures of antagonist was filtered through a 0.22 mm polycarbonate membrane filter to be culture filtrates.

### *Antagonism in vitro*

In order to test the antagonism of culture filtrates against *C. cassiicola*, 5-mm diameter disks of agar from three-day-old *C. cassiicola* grown on PDA were transferred to the centre of plates containing 1 ml filtrates mixed into 10 ml molten PDA. Three days later, the diameter of pathogen colony was recorded. Results were expressed as mean % inhibition of the growth of *C. cassiicola* in the absence of the actinomycete. Percent inhibition was calculated using the following formula: % inhibition =  $(1 - [\text{fungal growth} / \text{control growth}]) \times 100$ .

The effect of liquid cultures on spore germination was also estimated in this experiment. The primary filtrate and the *C. cassiicola* spores were made into spores suspension of  $5 \times 10^4$  spores  $ml^{-1}$ . One-hundred-microliter (100ml) of the suspension was placed on a hollow-

groundslide kept in a sterile Petri dish of which the bottom was covered by a filter paper saturated with SDW. The Petri dishes were then stored at 25°C and observations were made to check the percent of spore germination and inhibition after 12h. Each treatment had three replicates. The percent of germination was calculated using the formula: % germination =  $(\text{number of germinated spores} / \text{number of checked spores}) \times 100$ . % inhibition =  $(\text{percent of germination of control} - \text{percent of germination of treatment} / \text{percent of germination of control}) \times 100$ . The experiment was repeated twice.

### *Inoculation plant assays*

Cucumber plantlets grown for 6 weeks were used for the inoculation. Uniform plantlets (n=24) were selected for each treatment in this experiment (Ait Barka et al. 2000). The pathogen *C. cassiicola* was incubated under 0.2 mmol/m<sup>2</sup>/s white fluorescent light for 5d. The spore suspension was made and adjusted to  $10^4$  spores/ml by plate counting, and then evenly sprayed on the surface of the cucumber leaves after the treatment of XN-1 filtrate. Control plants were treated only with sterile distilled water. Both treatments were kept moist in the next two days before the plants were transferred out from moisturizing device. Another three days later, the target leaf spot symptoms were evaluated.

## **Results**

### *Evaluation of antagonistic activity of XN-1, the effect of filtrate on C. cassiicola in vitro and on cucumber leaves*

On PDA plates, XN-1 inhibited the growth of *C. cassiicola* by the presence of a transparent inhibition zone between the two organisms. There was a significant zone of inhibition around the actinomycete inoculum when the fungus was grown with the actinomycetes isolates on the same PDA plate (Fig. 1A). The hyphal growth inhibition rate was 78.34% under the treatment of XN-1 filtrate, which showed a potential for further study of this isolate (Fig. 1B). In the inoculation experiments, the cucumber leaves treated with culture filtrate of XN-1 had a few small lesions, whereas the control leaves exhibited typical symptoms of target leaf spot (Fig. 1C). The control rate of 63.54% indicated that the XN-1 markedly restricted the spread of the pathogen on cucumber leaves.

### *The inhibition of isolate XN-1 against the spore germination of C. cassiicola*

In the results of detecting the inhibitory effects of XN-1 filtrate on the spore germination, the inhibition rate

of primary filtrate could reach 96.50%. The formation of germ tubes could not successfully happen from either or both poles of the spores, and in some cases, a spherically intumescent germ tube could be observed (Fig. 2B), which might be deformed and fail to grow further.

### *The stability of culture filtrate*

The culture filtrate of the strain XN-1 was stable and relatively high for the whole range of temperatures, although the inhibition rate decreased slightly with the increase of temperature (Fig. 3).

Culture filtrate of the strain was also mainly stable against the pathogen at pH values between 3.0 and 11.0 (Fig. 4). The effects of pH on inhibition rate of pathogen decreased with the increase of alkalinity or acidity, but the rate was still above 50% except for extreme alkalinity.

The effect of culture filtrate of strain XN-1 on *C. cassiicola* decreased as the length of exposure to ultraviolet radiation was extended (Fig. 5). But the inhibition rate maintained a relatively high level at over 40%, even being exposed for 45 min.

## Discussion

In this study, XN-1 was isolated from the cucumber phyllosphere and tested in terms of spore germination, interactive reaction and inoculation assay to assess its future as a promising biocontrol agent. Being different from microorganisms selected from the soil, phyllosphere organisms can have direct effects on the target disease.

In the antifungal assays, some other actinomycetes were also found to exhibit marked antagonistic effects against *C. cassiicola*. However, the bioactive compounds of these agents did not show any antagonism against the pathogen, which might be attributed to the accumulation of the antibiotics influenced by type of the fermentation broth, primary pH value, temperature of shaker, the length of fermentation, and so on. Therefore, the fermentation conditions of these agents should be studied for a better application.

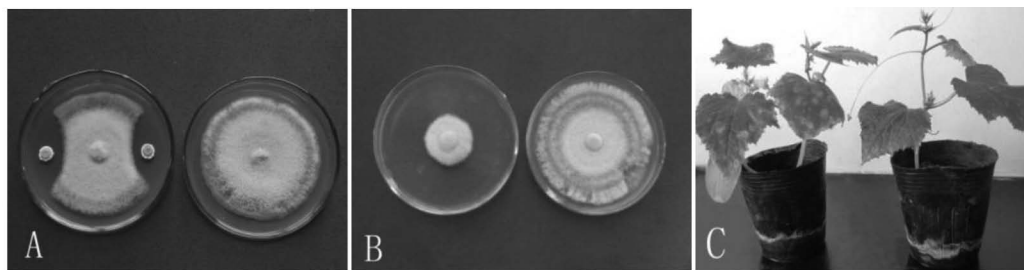
In addition, the identification of XN-1 and its colonization on the cucumber leaves deserve further studies for a better understanding of exact plant defense mechanisms, especially in the field conditions. Anyway, this study still elucidated a new path for controlling the cucumber target leaf spot.

## Acknowledgments

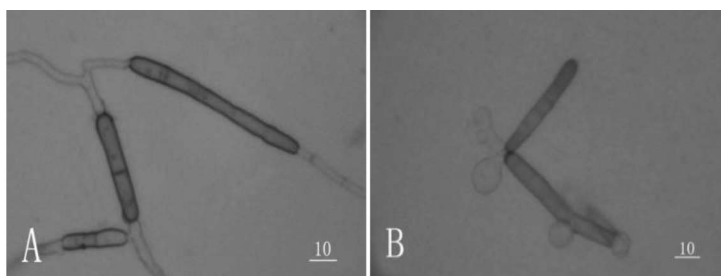
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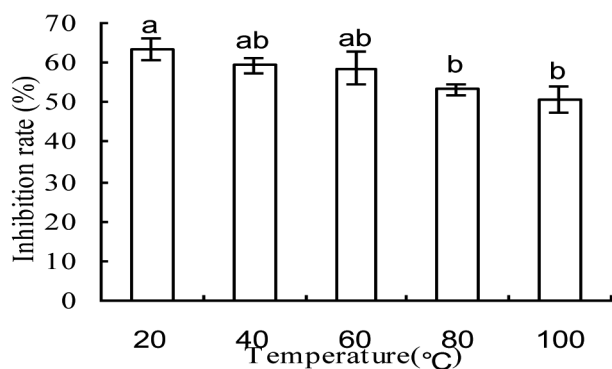
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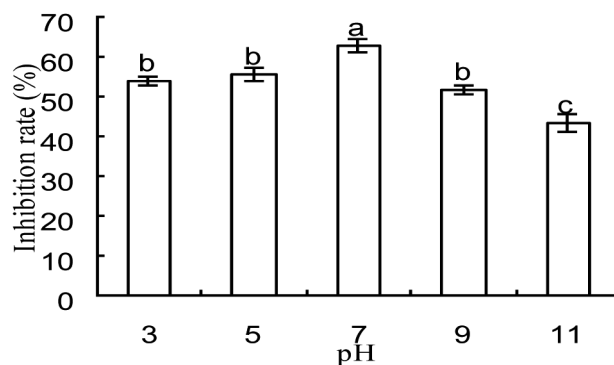
**Fig. 1** A. The antagonism between XN-1 and *C. cassiicola* B. The inhibition effects of XN-1 filtrate on the hyphal growth of *C. cassiicola* C. Cucumber leaves of treatment by XN-1 fermentation filtrates and the control.



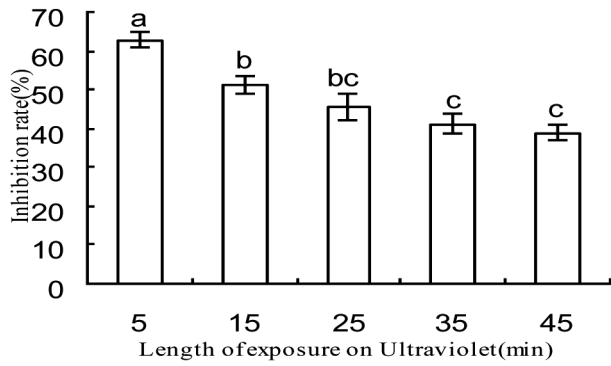
**Fig. 2** A. The normal spore germination of *C. cassiicola* within water drop B. The inhibition of XN-1 filtrate against spore germination of *C. cassiicola* Note: The bar represents 10 µm



**Fig. 3** The effects of temperature on inhibition rate of culture filtrates against *C. cassiicola*. Inhibition rates are the mean of three trials. Values followed by the different letters are statistically different at  $P = 0.05$  according to Duncan's multiple range test.



**Fig. 4** The effects of pH on inhibition rate of culture filtrates against *C. cassiicola*. Bars represent standard deviations of the means. Values followed by the same letters are not statistically different at  $P = 0.05$  according to Duncan's multiple range test.



**Fig. 5** The effects of exposure on Ultraviolet on inhibition rate of culture filtrates against *C. cassiicola*. Bars represent standard deviations of the means. Values followed by the same letters are not statistically different at  $P = 0.05$  according to Duncan's multiple range tests.