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Full Length Research Paper

Potent antagonistic activity of newly isolated biological control *Bacillus subtilis* and novel antibiotic against *Erysiphe graminis* f.sp *tritici*

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A newly isolated *Bacillus subtilis*, as well as its antibiotic, had potent antagonistic activity against *Erysiphe graminis* f.sp *tritici.* Minimum inhibitory concentration (MIC) of the antibiotic for *E. graminis* f.sp *tritici* was determined. We arrived at an opinion: for some bacteria, unless the ammonium sulfate-precipitated substance had been filtered with a membrane, even if it had bio-activity in agar disc diffusion test, it is not necessarily true that such activity does come from the ammonium sulfate-precipitated substance.

Key word: *Bacillus subtilis*, *Erysiphe graminis* f.sp *tritici*, antibiotic.

INTRODUICTION

The indiscriminate use of synthetic pesticides has brought undesired problems to human health, agriculture, and the environment. Increasing concern for pesticide risks by governments and the public is seen as the main impetus for change in "traditional" crop protection practices and for investment in other more ecological products like biological control agent or biopesticide (Saenz-de-Cabezon et al., 2010). Biopesticide encompasses a broad array of microbial pesticides, biochemicals derived from micro-organisms and other natural sources (Sudakin and Trevathan, 2003). Microbial pesticides are being introduced in crop protection and currently several beneficial microorganisms are the active ingredients of a new generation of microbial pesticides (Dayan et al., 2009). We have isolated a strain of *Bacillus subtilis* that had shown potent antagonistic activity against *Erysiphe graminis* f.sp tritici, the pathogen of powdery mild of wheat. We are very interested in this bacterium's potential as a biopesticide against *E. graminis* f.sp tritici.

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MATERIALS AND METHODS

Culture medium and microorganism

KMB medium

It consists of BBI company peptone 20 g, glycerol 15 ml, K_2HPO_4 1.5 g, $MgSO_4$ 0.75 g, volume was adjusted to 1000 ml by distilled water, sterilized at 121℃ for 20 min. PDA medium consists of: potato extract, 230 ml, glucose, 20 g, distilled water, 770 ml, potato extraction was prepared by adding 100 g potato (peeled and sliced in a minicer) to 300 ml distilled water, it was left overnight at 4℃, and filled through cloth.

Quartz sand

Quartz Sand was immersed in acidic potassium dichromate solution for 24 h to oxidize organic substance (the acidic potassium dichromate solution: $K_2Cr_2O_7$ 37 g plus 300 ml water was heated and stirred until potassium dichromate was dissolved. After it was cooled, 300 ml of 98% sulphuric acid was gradually added). The quartz sand was eluted with distilled water for 10 h to remove metallic ion and oxidizing substances. Then, it was sterilized at180℃ for 2 h. Microorganism: *B. subtitle*, as well as *E. graminis* f.sp *tritici* was kindly given by the college of agriculture and biotechnology of Zhejiang University, P.R.C. *B. subtitle* was isolated from the leaf of egg plant derived from Hangzhou suburbs,

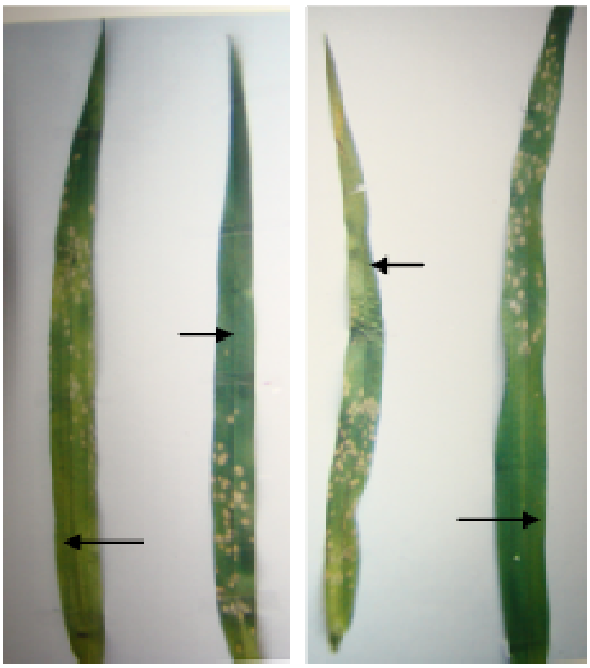

Figure 1. The B. subtilis could inhibit the germination of spores of *E. graminis* f.sp *tritici*. On the leaf that denoted by the arrow and that were spraying with water (as negative control), the spores of *E. graminis* f.sp *tritici* could germinate. On the other half leafs that was without arrow and that were spraying with the culture of the B. *subtilis*, such fungus could germinate.

Zhejiang Province.

Antagonistic effect of *B. subtilis* (the antibiotic-producing bacteria)

Because *E. graminis* f.sp *tritici* could only grown on living wheat leaf (that is, it could not be cultured in any *in vitro* culture medium), the activity of *B. subtilis* against this pathogen had to be carried out by scattering the spores of the pathogen on living wheat leaf. Before scattering these spores, one half of the leaf was spraying with the culture of *B. subtilis,* while the other half of the same leaf was spraying with water (as negative control). The antagonistic activity was evaluated by inhibited effect of the bacterium towards fungal growth.

Extraction and purification of the antibiotic

From the beginning, we did not know whether the antibiotic produced by the *B. subtilis* was a protein-like substance or other kind of substance. So, we tried to isolate the antibiotic with different methods. (i) The first method: after the antibiotic-producing-bacterium (*B. subtilis*) was cultured in KMB culture medium for four days, it was centrifuged at 5000 rpm for 20 min to remove bacteria and its spores. Then the supernatant fluid was filtered through a membrane (0.22 μm aperture) to remove remaining bacteria and spores. 30% (W/V) ammonium sulfate was added into the supernatant fluid and it was left at 4℃ overnight. Then it was centrifuged at 5000 rpm for 20 min. The precipitate was dissolved in PBS and it was put in -20℃ refrigerator for late use (that is, for evaluating its bioactivity). (ii) The second method: the culture of antibiotic-producing bacterium was extracted with ethyl ether. After ethyl ether was volatilized, the remainder was put in -20℃ refrigerator for late use (tha is, for evaluating its bioactivity). Above antibiotic extract experiments, together with other experiments (not described in this paper) had shown that the antibiotic was a small compound rather than a protein-like substance and it is a volatile substance. So the following method was designed: at 37℃ for ten days, the antibiotic-producing bacterium was cultured on surface of the quartz sand that was immersed in KMB culture medium, with the surface of the quartz sand not being covered with liquid culture medium (previous work had shown that this bacteria produced more antibiotic if it was cultured on solid medium). Thereafter, the quartz sand, which had absorbed the antibiotic, was immersed in water in order for the antibiotic to be distilled. The condensed water was collected and passed through active carbon chromatographic column, which was then eluted with ether. The eluted ether was left at room temperature (25 to 30℃) overnight to evaporate ether. The remainder was chromatographed on silica gel column which was eluted with ether. The fraction with the greatest activity was further chromatographed on silica gel column and then eluted with normal pentane:ether=1:4. The normal pentane and ether were both evaporated at room temperature (25 to 30℃). In above purifying process, bio-activity was tracked by inhibition zone of *Rhizoctonia solani*, which was cultured on the surface of solid PDA (1.5% agar). About 100 mg antibiotic was produced from 100 liter KMB culture medium (about 200 kg quartz sand was consumed).

Determination of the minimum inhibitory concentration (MIC) for *E. graminis* f.sp *tritici*

The *E. graminis* f.sp tritici could only grow on living wheat leaf. So, MIC for this fungus was obtained through the following method: living wheat leaf was spraying with spores of *E. graminis* f.sp *tritici*. After the spores germinated, 2-fold serial dilutions of antibiotic (the range was from 0.02 to 0.08 μg/L) was respectively spraying onto the leaves (water was used as negative control). One week later, the antibiotic treated and the water treated leaves was respectively observed under electron microscope as described in reference (Honglan, 2004). The minimum concentration that could inhibit the fungal growth was judged as MIC.

RESULTS

Antagonistic activity of *B. subtilis* against *E. graminis* f.sp *tritici*

The spores on the half wheat leaf that were treated with water germinated well, while spores on the other half leaf that was treated with the culture of *B. subtilis* could not germinate (Figure 1). Therefore, the *E. graminis* f. sp *tritici* was very sensitive to the *B. subtilis.*

Extraction and purification of the antibiotic

Ammonium sulfate precipitation, which was prepared from the *B. subtilis's* culture and which was filtered with a membrane, had no anti-activity against *Rhizoctonia solani*. On the other hand, ethereal extract had antagonistic activity against this fungus. Even after it was treated with proteinase K, the anti-fungal activity remained

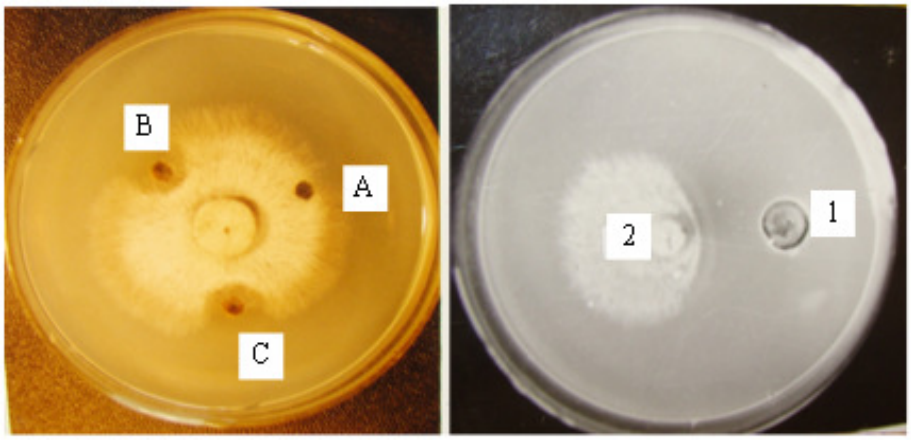

Figure 2. The ethereal extract, rather than ammonium sulfate precipitation, could inhibit the growth of *R. solani.* (A) the ammonium sulfate precipitation couldn't inhibit the growth of *R. solani*, (B) the ethereal extract was able to inhibit the growth of *R. solani*, (C) the ethereal extract, which was treated with proteinase K in order to digest anti-fungal protein that might existed, still remained its antagonistic activity against fungus. Antibiotic purified by silica gel thin-layer chromatography *R. solani.*

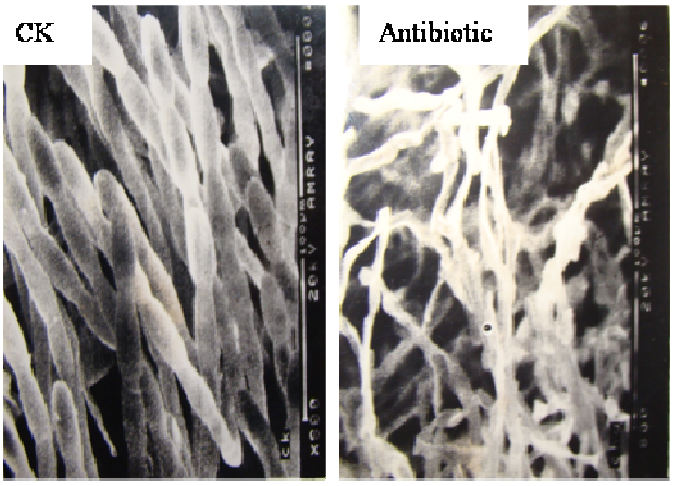

Figure 3. Antibiotic destroy the hypha of *E. graminis* f.sp tritici. CK: water treated (as control), Antibiotic: 0.08 µg/ml antibiotic could destroy hypha of *E. graminis f.sp tritici.*

(Figure 2). So, there was the indication that the antibiotic produced by the *B. subtilis* belongs to fat soluble substance. Because the antibiotic could not be precipitated by ammonium sulfate and its bio-activity could not be destroyed by proteinase K, it was supposed that the antibiotic might be a nonprotein substance.

Determination of MIC

The antibiotic's concentration of 0.08 1 µg/mL could destroy the hypha of *E. graminis* f.sp tritici shown in (Figure 3). So it was judged that the MIC for *E. graminis* f.sp tritici was 0.08 1µg/mL.

DISCUSSION

Even if the ammonium sulfate precipitation, which had been centrifuged but not filtered by a membrane, was treated with chloroform (the bactericide), it still could inhibit fungal growth (result not shown). However, after the ammonium sulfate precipitation was filtered with a membrane, it did not show any anti-fungal activity. The explanation is as follows: although the ammonium sulfate precipitation had been centrifuged and therefore the overwhelming majority of bacteria and its spores had been removed, there still remained some bacteria and spores. The chloroform could kill living bacteria but could not kill spores. So, it was not the ammonium sulfate

precipited substance that had the activity, but the spores remained in ammonium sulfate precipitation that germinated, multiplied and thus secreted antibiotic that shown anti-fungal activity. Only after the membrane filtrated the spores and the bacteria, had we excluded the false conclusion that the antibiotic produced by the *B. subtilis* might be a protein. Here, we arrived at an opinion: for some bacteria, unless the ammonium sulfate-precipitated substance had been filtered with a membrane, even if it had bio-activity in agar disc diffusion test, it is not necessarily true that such activity does come from the ammonium sulfate-precipitated substance. We think that the above mentioned experience is very valuable, because: (i) revealing the nature of a bio-activity substance is a pivotal step in determining the procedure for isolating and purifying the substance; (ii) To the best of our knowledge, such advice has not been mentioned in any documents or text-books. We have evidence that this antibiotic is rather stable (not shown in this paper). For biopesticide candidate, stability and dissolution in fatty solutions are good features because plant leaf has lipophilic character due to the fact that plant leaf has cuticle and wax coat (Yanming, 2006). Powdery mildew caused by *E. graminis* f. sp. tritici (Syn. *Blumeria graminis*), is one of the major diseases of wheat worldwide (Salari et al., 2003; Rani and Munshi, 2005). Although this wheat disease can be controlled through the use of some fungicides in agriculture, however, the pathogens often evolve that is resistant to fungicides (Fernández-Ortuño et al., 2008; Bäumler et al., 2003). So researchers are trying to search new and more effective fungicides, including such natural fungicides or biopesticide as bacterium, and many compounds derived from living organisms (Copping and Duke, 2007; Genet and Jaworska, 2009). The development of a microbial pesticide requires several steps addressed to its isolation in pure culture and screening by means of efficacy bioassays performed *in vitro, ex vivo*, *in vivo*, or in pilot trials under real conditions of application (field, greenhouse, post-harvest) (Montesinos, 2002). Our newly isolated *B. subtilis*, including its antibiotic, has shown potent *in vitro* or *ex vivo* antagonistic activity against *E. graminis* f.sp. tritici. And therefore, both merit further investigation as biopesticide candidate against powdery mildew.

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