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Biocontrol Activity of *Pseudomonas cepacia* AF2001 and Anthelmintic Activity of Its Novel Metabolite, Cepacidine A

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Abstract Cepacidine A was previously isolated as a novel antifungal antibiotic from the culture broth of *Pseudomonas cepacia* AF2001. It exhibits a potent *in vitro* antifungal activity against various plant pathogenic fungi, such as *Plasmopora veticola* on grapes, *Septoria nodorum* and *Fusarium culmorum* on wheat, as well as *Colletotrichum lagenarium* on cucumbers. Accordingly, this study was conducted to evaluate the potential crop protection activity of strain *P. cepacia* AF2001. The strain was tested in semi-greenhouse biocontrol assays, and showed an excellent biological activity against *Pythium ultimum* in cotton and cucumbers; however, only a minor activity against *Rhizoctonia solani* in cotton was observed. Furthermore, the anthelmintic activity of cepacidine A against the gastrointestinal nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* was also evaluated. However, the compound only exhibited a moderate activity in the *in vitro* larval development assay with no activity in the *in vivo* animal model.

Key words: Biocontrol activity, cepacidine A, anthelmintic, *Pseudomonas*

Plant diseases, caused primarily by fungal and bacterial pathogens, result in severe losses of agricultural and horticultural crops every year. These losses can result in reduced food supplies, poor quality of agricultural products, economic hardship for growers and producers, and ultimately, higher prices. Traditionally, applied chemical control methods are not always economical or effective, and may bring unwanted health safety and environmental risks.

Biological control of plants involves the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens which cause diseases. Such biocontrol offers an environmentally facile approach for the management of plant disease, and can be incorporated in cultural and physical controls with limited chemical usage to produce an effective integrated disease management system. Accordingly, biological control is an important component in the development of more sustainable agricultural systems.

As a result, the biological control of soil-borne plant diseases by strains of *Pseudomonas* has been studied intensively in recent years [3, 4, 5, 9]. Many of the most promising biocontrol strains produce the antimicrobial metabolites that are relevant for the biocontrol activity [3, 7], and also are toxic to a range of different soil microorganisms *in vitro* [8, 10]. These antibiotics have been implicated for the control of fungal pathogens for the rhizosphere. An important factor in biological control is the ability of an organism to compete in a given environment. Thus, it is desirable to obtain strains of biocontrol agents which are effective to control the growth of plant pathogenic fungi, and also able to aggressively compete with the indigenous bacteria and microflora that exist in the rhizosphere of the plant.

In the course of screening for novel antifungal substances of microbial origin, cepacidine A, a novel glycopeptide, was found in the fermentation broth of a strain of *Pseudomonas cepacia* AF2001 [11, 12, 13]. This strain was deposited in the Korean Federation of Culture Collections, Seoul, Korea under the registration number KFCC 10773. Cepacidine A is a mixture of two closely related compounds, cepacidine A_1 and A_2 (Fig. 1). This report mainly focuses on the biocontrol activity of *P. cepacia* AF2001 against *Pythium* and *Rhizoctonia*, pathogens that cause damping off in cotton and cucumbers.

In addition, the anthelmintic activity of the purified cepacidine A against gastrointestinal nematodes, such as *Haemonchus contortus* and *Trichostrongylus colubriformis*, was also examined in order to evaluate the potential for the application of cepacidine A in the field of animal healthcare.

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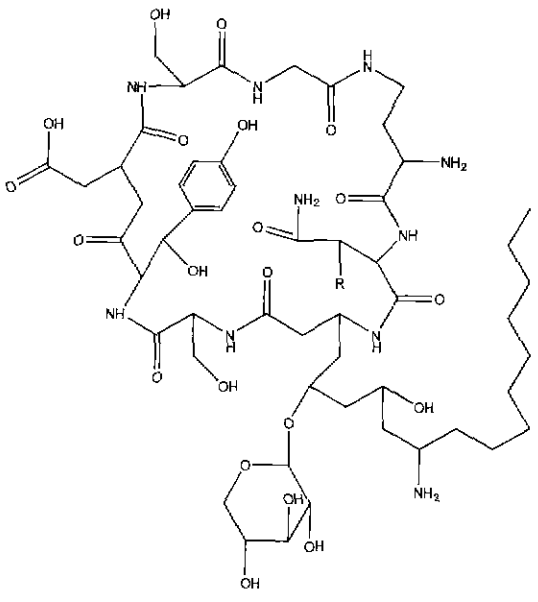

Fig. 1. Structure of cepacidine A.

Activities of Cepacidine A against 14 Plant Pathogens in Initial Screening Assays

To test the antifungal activity of cepacidine A for crop protection, preliminary *in vitro* screening assays were carried out against *Phytophthora infestans* on tomatoes, *Phytophthora infestans* on potatoes, *Plasmopora viticola* on grapes, *Puccinia recondita* on wheat, *Gaemannomyces graminis* on barley, *Botrytis cinerea* on apples, *Botrytis cinerea* on grapes, *Pythium oryzae* on rice, *Venturia inaequalis* on apples, *Septoria nodorum* on wheat, *Pyrenophora teres* on barley, *Colletotrichum lagenarium* on cucumbers, *Rhizoctonia solani* on rice, and *Fusarium culmorum* on wheat.

As shown in Table 1, at a concentration of 200 μg/ml, cepacidine A showed a potent activity (90–99% inhibition) against *Plasmopora veticola* on grapes and *Septoria nodorum* on wheat, and a moderate activity (50–79% inhibition) against *Fusarium culmorum* on wheat, *Colletotrichum lagenarium* on cucumbers, *R. solani* on rice, and *Puccinia recondita* on wheat.

Furthermore, the antifungal activity of the strain *P. cepacia* AF2001 against *Py. ultimum* and *R. solani* isolated from cotton was evaluated on an agar plate as follows: The strain AF2001 was streaked onto one side of a PDA plate. The plate was then incubated on a laboratory bench at room temperature for 14 days. A 7-mm-diameter plug taken from the margin of an actively growing colony of the pathogens was placed opposite to the AF2001 colony (1 cm distance). Thereafter, the plates were incubated at room temperature, and the radial growth of the pathogens was measured daily for three days.

As shown in Table 1, the *in vitro* activity (inhibition zone in mm) of strain *P. cepacia* AF2001 against *Py. ultimum* and *R. solani* on cotton was 6 mm and 14 mm, respectively. Accordingly, since this strain would appear to be a good candidate for a biocontrol fungicide, a semi-greenhouse test using strain *P. cepacia* AF2001 as a candidate strain for biocontrol against *Py. ultimum* and *R. solani* was carried out.

Table 1. Activities of cepacidine A and *P. cepacia* AF2001 against various plant pathogens in preliminary screening assays.

Pathogens	Activity of Cepacidine A (% inhibition)		
	200 ppm	60 ppm	20 ppm
Phytophthora infestans on tomatoes	0	0	0
Phytophthora infestans on potatoes	0	0	0
Plasmopora viticola on grapes	90–99	90–99	90–99
Puccinia recondita on wheat	60–69	30–39	0
Gaemannomyces graminis on barley	0	0	0
Botrytis cinerea on apples	0	0	0
Botrytis cinerea on grapes	0	0	0
Pythium oryzae on rice	0	0	0
Venturia inaequalis on apples	0	0	0
Septoria nodorum on wheat	90–99	50–59	30–39
Pyrenophora teres on barley	0	0	0
Colletotrichum lagenarium on cucumbers	70–79	60–69	60–69
Rhizoctonia solani on rice	50–59	0	0
Fusarium culmorum on wheat	70–79	60–69	40–49
Activity of *P. cepacia* AF2001 (mm inhibition zone)			
Pythium ultimum on cotton	6		
Rhizoctonia solani on cotton	14		

Biocontrol Activity of Strain *P. cepacia* AF2001 against *Py. ultimum* in Cotton and Cucumbers, and *R. solani* in Cotton

Biocontrol strains should be able to aggressively compete in the plant rhizospere as well as produce some antifungal substances that are effective against a broad spectrum of plant pathogenic fungi, particularly *Pythium* and *Rhizoctonia*. Currently, there are no environmentally safe and effective treatments of fungicide available for the protection of crops against *Pythium* and *Rhizoctonia*. Therefore, the use of biocontrol strains to control or prevent *Pythium* and *Rhizoctonia* infections in crop plants would provide an environmentally safe and effective method to control these pathogens.

The strain *P. cepacia* AF2001, which demonstrated the desired antifungal activity, was thus further characterized in semi-greenhouse biocontrol assays on cotton and cucumbers with *Py. ultimum* and on cotton with *R. solani*. Pencycuron (Novartis, Switzerland) was used as the chemical standard. Strain AF2001 was applied as a suspension of vegetable cells in a formulation-buffer at a concentration of 2×10^8 cfu/ml. Twenty-milliliters of this suspension were drenched per

Table 2. Biocontrol activity of *P. cepacia* AF2001 in a semi-greenhouse test.

Test sample	*In vivo* Activity (% control)[a]					
P. cepacia AF2001	*Rhizoctonia*/ cotton		*Pythium*/ cotton		*Pythium*/ cucumber	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
	18	25	79	94	52	30
Standard[b]	107	103	102	97	92	104

[a]The biocontrol activity was calculated in relation to a pathogen check (disease incidence defined as 0% control) and the non-inoculated check (disease incidence defined as 100% control).
[b]Pencycuron (Novartis, Switzerland) was used as the chemical standard.

pot, which contained 200 ml soil, thus the final concentration of the strain was 2×10^7 cfu/ml soil. The biocontrol activity of strain AF2001 was then calculated in relation to a pathogen check (disease incidence defined as 0%) and the non-inoculated check (disease incidence as 100%). As described in Table 2, strain AF2001 showed an excellent biocontrol activity (79–94%) against *Py. ultimum* in cotton, but under average activity (30–52%) against the same pathogen in cucumbers. However, the biocontrol activity of strain AF2001 against *R. solani* in cotton was minor (18-25%). Interestingly, there was no correlation between the *in vitro* activity of strain AF2001 against *Py. ultimum* and *R. solani* on cotton on the agar plate assays, where the strain demonstrated a more significant activity against *R. solani* (Table 1) compared to the biocontrol activity against the same pathogens (Table 2).

Many biological disease-controlling *Pseudomonas* strains produce antibiotics that inhibit the growth of fungal pathogens, such as cepacidine A. However, these antibiotics have been implicated in control of fungal pathogens in the rhizosphere rather than on an agar plate. Therefore, an important factor in biological control is the ability of a strain to compete under an unusual environment. So far, strain AF2001 would appear to be more effective in controlling the growth of *Pythium* rather than *Rhizoctonia* in the rhizosphere of cotton. Accordingly, strain *P. cepacia* AF2001 appears to be an excellent target for development of an environmentally safe and effective biocontrol agent, when the strain is understood further on how to enhance its stability in the rhizosphere. In this connection, it should be mentioned that the pathogenic potential of *P. cepacia* in the pulmonary colonization in cystic fibrosis patients has been reported recently [1, 6], although no such case was yet reported in Korea.

Anthelmintic Screening of Cepacidine A against Gastrointestinal Nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis*

To test the potential use of cepacidine A as an anthelmintic, the compound was also examined both in *in vitro* and *in vivo* model systems. The efficacy of cepacidine A was evaluated with larval development assays from egg to the L3 stage of *H. contortus* and *T. colubrifornis* as the *in vitro* assay, and also evaluated in Mongolian gerbils (*Meriones unguiculatus*) against an induced mixed infection of *H. contortus* and *T. colubriformis* as the *in vivo* screening. In general, a larval development assay is used to test the anthelmintic activity of the compound by interrupting the life cycle of a nematode. As shown in Table 3, 100% effective concentration (EC_{100}) of cepacidine A for the developmental disruption at the egg/larval stages of *H. contortus* and *T. colubriformis* was 3.2 ppm and 10 ppm, respectively. Although this potency of cepacidine A in the larval development assay is less effective than commercially available anthelmintics [14], cepacidine A has potentiality to become one of the leading compounds in this therapeutic field. Therefore, a further evaluation against a mixed infection of *H. contortus* and *T. colubriformis* in Mongolian gerbils was performed as outlined by Conder *et al.* [2]. The activity against each of the 2 nematode species was determined by a single oral

Table 3. *In vitro* & *in vivo* anthelmintic activity of cepacidine A.

Test	Discriminating concentration or dose	Cepacidine A
In vitro activity:		
Larval development assay (egg-L3)		
- *Haemonchus contortus* (*Hc*):	EC_{100}	3.2 ppm
- *Trichostrongylus colubriformis* (*Tc*);	EC_{100}	10 ppm
In vivo activity:		
In gerbils		
- *Hc*+*Tc*; single treatment p.o.[a] (L3-adults)	ED_{80}	>10 mg/kg
- *Hc*+*Tc*; medicated feed from day 0 to day 9 i.p.[b] (L3 to immature adults)	ED_{80}	>3.2 mg/kg/d
- *Hc*+*Tc*: medicated feed from day 10 to day 19 i.p. (Immature to mature adults)	ED_{80}	>3.2 mg/kg/d

[a]Per os.
[b]Intraperitoneally.

treatment (p.o.) with a maximal dose of 10 mg/kg, by intraperitoneal medications (i.p.) on day 0 to day 9 post-infection, and by daily medications (i.p.) on day 10 to day 19 post-infection with a maximal dose of 3.2 mg/kg/day. Ten days after the last treatment, all animals were sacrificed and their small intestines were removed, and subjected to microscopic examination. As shown in Table 3, unfortunately, cepacidine A showed no activity (ED_{80}) against each of the 2 nematode species examined. Although cepacidine A showed a significant anthelmintic activity against the 2 nematode species in the *in vitro* larval development assay, the compound showed no activity in the animal model, possibly due to low penetration of the compound into tissue upon i.p. treatment, or degradation of the compound in the gastrointestinal tract upon p.o. treatment. Accordingly, further studies are required for the enhancement of the tissue penetration rate and for an optimal formulation of cepacidine A to confirm the potential properties of the compound as a novel anthelmintic in animal healthcare.

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