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The Biocontrol Effect of *Paenibacillus polymyxa* Strain NBF188 on Cucumber Rhizoctonia Rot and its Growth-Promoting Effect

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Rhizoctonia solani **Kuhn is one of the soilborne pathogenic fungi have been associated with damping-off. Protection of cucumber against this pathogen is important to maximize the crop field, plant growth-promoting rhizobacteria (PGPR) have been used to suppress the growth of this pathogen as well as enhance growth of several crops. In the present study, the endophytic bacterium *Paenibacillus polymyxa* strain NBF188 was evaluated as a biological control agent for cucumber Rhizoctonia rot. *P. polymyxa* strain NBF188 showed high inhibition activity in dual culture and inhibitory halos were observed Optical microscope study revealed deformation of hyphal and enlargement of cytoplasmic vacuoles. Furthermore, the strain NBF188 showed a broad antifungal spectrum on mycelium growth of numerous important plant pathogenic fungi. In the greenhouse, seed-soaking with NBF188 (10^8 CFU·ml^{-1}) exhibited a better biological control effect on cucumber Rhizoctonia rot and plant growth promoting ability. The control effect was 76.67% and the plant height, root length and fresh weight have increased 53.48%, 49.84% and 37.57% respectively compared with the untreated control. Experiments reported here indicate that *P. polymyxa* NBF188 could be a promising agent in biocontrol cucumber Rhizoctonia rot.**

Key words: *Paenibacillus polymyxa*; Biocontrol; Rhizoctonia rot; cucumber.

Rhizoctonia solani is a destructive soil-borne plant pathogen infecting a wide range of agricultural and horticultural crops, including cucumber and rice, and causes considerable yield loss[1, 2, 3].

Currently, *Rhizoctonia* disease is managed by cultural practices, such as crop rotation with grains, and methods that minimize prolonged contact of the plant with the pathogen, such as planting in warmer, drier condition to promote rapid sprout emergence and promptly removing tubers from the field[4] . Chemical fungicides are often used when losses from *R. solani* are substantial[5] . However, current cultural and chemical controls are not completely effective, and the increasing use of chemical pesticides causes several negative effects on the environments as well as on human health[6] . Rhizoctonia disease remains a persistent problem.

Over the last decades, efforts have been directed towards developing new alternatives to chemical disease control. During these studies, the application of plant growth-promoting rhizobacteria (PGPR) to the soil as a biocontrol agent, in the greenhouse or under field conditions, not only resduced disease severity but also enhanced plant growth[7, 8, 9, 10, 11] . The object of this study was to identify the control effect of *P. polymyxa* strain NBF188 to *Rhizoctonia solani* damping-off disease and its growth-promoting effect on cucumber.

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MATERIALS AND METHODS

Strains

P.polymyxa was isolated from a cucumber rhizosphere sample collected from cucumber greenhouse of Hubei province, and identified in preliminary experiments and stored on a nutrient agar (NA) medium at 4°C.

Dual plate assays

P. polymyxa was tested for antagonism against *R. solani* by dual plate assays. The bacterial isolate of NBF188 was grown in nutrient broth on a rotary shaker at 30°C and 160rpm for 24h. The suspension was centrifuged in sterile 50-ml plastic tubes at 6000 rpm for 10 min. The pellets were re-suspended in sterile distilled water to obtain a final concentration of 10^8 cells ml^{-1} (OD = 0.5) at 600 nm, which was measured with the viable plate count and optical density methods. One 4mm disk of pure *R. solani* cultural was placed in the center of a petri dish containing potato dextrose agar (PDA), Four drops of the bacterial suspension (5¼L) were placed around the fungal inoculums at a distance of 3cm. Plates were incubated for 48h at 28°C. The plates were observed for the zone of inhibition around the bacterial strain. Additional 15 plant pathogens were also used in dual plate assay for detecting the spectrum. Each experiment was conducted in triplicate and repeated at least two times.

In dual cultures, *R. solani* mycelium on the edge of the inhibitory halo was used to observe the effects of NBF188. An optical microscope (OLYMPUS CX21, Tokyo, Japan) was used for the observations.

Biological control assay against cucumber Rhizoctonia rot

Cucumber seeds of the cultivar JinChun 4 were surfaced-srerilized by soaking in 3% NaOCl solution for 10 min, follow by five times washing with sterile distill water. Then they were germinated on 9cm plates covered with sterile wet filter paper at 25°C for 48h..

Mycelial plugs of *R. solani* was grown on PDA at 27°C for 5 days, then was added into the mixture of airdried sand and 3% ground oatmeal (w/v). Each inoculum (30g) was incubated in 20% humidity at 27°C for 3 weeks, and then was mixed thoroughly with 1000 g of natural field soil. Twenty seeds soaking with *P. polymyxa* (10^8 CFU·ml^{-1}), carbendazim (500¼g ml^{-1}) and sterile water for 6h were sown in aperture disk carrying the infested soil. The plants were kept in greenhouse and managed using standard nursery practices for the wuhan area in China. Ten days after seeding, plants were observed carefully for typical symptoms of damping-off, and the number of symptomatic plants was counted. There were 3 replications of each treatment, and each republication contained 20 plants. This experiment was repeated twice.

Assay for cucumber growth promotion by seed treatment

Cucumber seeds were soaked in the suspension of *P. polymyxa NBF188* and *Bacillus subtilis* QST713 (Serenade®) at the concentration of 10^8 CFU·ml^{-1} on 9cm plates covered with sterile wet filter paper at 25°C for 48h, control seeds were soaked with distilled water only, the seed germination rate was determined. Then the seeds coated with *P. polymyxa* NBF188, *B. subtilis* QST713 and distilled water were sown in a pot in greenhouse condition. There were 3 replications of each treatment, and each republication contained 30 seeds. This experiment was repeated twice. No additional nutrients were added in this experiment. After three weeks, shoot and root length, fresh weight of cucumber was measured. The seeding vigor index was calculated using the following formula:

Vigor Index=percentage of germination × (mean root length + mean shoot length)[12] .

PCR detection of antibiotic biosynthesis genes and nitrogen-fixing gene

PCR was used to amply antibiotic biosynthesis genes (gluB) and nitrogen-fixing gene (*nifH*), which are reported to involve in ²-(1,3)-(1,4)-glucanase biosynthesis and nitrogen fixation respectively in *P. polymyxa*, using primer sets previously described[13, 14] . Amplified PCR productions were purified by electrophoresis and cloned into the pMD20–T Vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) and sequenced by Shanghai Sangon Biological Engineering Technology. Sequence data were analyzed using DNAman software.

Statistical analysis

Analysis was conducted using with SPSS (Statistical Product and Service Solution), version

11.0. Multiple comparison tests (least significant difference, LSD) were used to detect differences in seed germination rate , shoot and root length, fresh weight and Vigor Index.

RESULTS

Assessment of antagonistic activity against *R. solani* in vitro and in vivo

Twenty-four hours after inoculation, inhibitory halos were observed in dual culture dishes (Fig. 1). In addition, *P. polymyxa* was shown to have broad-spectrum in vitro antibiotic activity against 14 additional plant pathogens. *Mycosphaerella melonis* was the only pathogens tested that were not inhibited (Table 1). *R.solani* hyphae from the edge of the inhibitory halo were observed under the optical microscope. Compared with the control treatment (Fig 2), 48h after inoculation, hyphal deformation and enlargement of cytoplasmic vacuoles were observed in NBF188 treatment.

Table 1. In vitro inhibition of plant pathogens with *P. polymyxa* NBF188

Plant pathogen	In vitro inhibition by *P. polymyxa* NBF188
Alternaria solani	+
Phytophthora capsici	+
Fusarium oxysporum f . sp. vasinfectum	+
Fusarium oxysporum . f . sp . niveum	+
Fusarium oxysporum . f . sp . cucumebrium	+
Fusarium graminearum	+
Phytophthora parasitica var . nicotiana	+
Verticillium dahliae	+
Phytophthora infestans	+
Cytospora mandshurica	+
Sclerotinia sclerotiorum	+
Mycosphaerella melonis	“
Corynespora cassiicola	+
Botrytis cinerea	+
Colletotrichum orbiculare	+

(+) indicates a distinguishable zone of inhibition of the plant pathogen and (“) indicates no distinguishable zone of inhibition.

The *P. polymyxa* strain NBF188 was tested for its biocontrol ability in cucumber Rhizoctonia rot, as shown in table 1, up to100% of untreated plant were damping-off after ten days of growth in soil infested with *R.solani*, whereas treatment of cucumber seed with cells of *P. polymyxa* induced a significant reduction in the percentage of diseased plants in comparison with nonbacterized plants. It was notable that the highest protection against cucumber Rhizoctonia rot. The control effect were 76.67% at the contencration of 1×10^8 CFU·ml^{-1} cell of *P. polymyxa* (Table 2).

Plant growth promotion of cucumber seedling by *P. polymyxa* strain NBF188

P. polymyxa NBF188 stimulated cucumber germination and seedling development when applied as a seed treatment containing 1×10^8 CFU·ml^{-1} cell per treatment. Germination and emergency of cucumber increased from 96.00% with control to 82.00% with *P. polymyxa* NBF188 seed treatment. The plant height, root length and fresh weight have inceased 53.48%, 49.84% and 37.57% respectively compared with the untreated control (Table 3).

PCR detection of antibiotic genes and nitrogen-fixing gene with strain NBF188

The gluB gene was detected and PCR products with expected sizes (636 bp for gluB gene), further sequence analysis showed the resultant PCR products shared 99% identity to gluB. The *nifH* gene did not be detected in strain NBF188 using specific primers.

DISCUSSION

Researchers have reported that *P. polymyxa* could secret antifungal metabolites such

Table 2. Biological control effect of strain NBF188 on Rhizoctonia rot of cucumber in pot experiment.

Trearment	Diseased plants (%)	Control effect (%)
Seed-soaking with NBF188	23.33b	76.67
Carbendazim 500¼g/ml	6.67a	93.33
Pathogen control	100.00d	-

as polypeptide and protein[15], and the antimicrobial protein gene of NBF188 was found. In this work, antifungal activity of *P. polymyxa* was tested against *R. solani*. In dual culture, the mycelium of *R. solani* was inhibited by *P. polymyxa* without direct contact. An antibiotic produced by NBF188 could contribute to this phenomenon. Micrographs showed that NBF188 induced hyphal deformation , enlargement of cytoplasmic vacuoles and cytoplasmic leakage in *R. solani*. (Fig. 2B). It is reported a similar observation that in vitro interaction of *R. solani* and *Bacillus pumilus* strain SQR-N43[16].

Non-chemical methods of controlling soil-borne plant pathogens are generally considered as more prospective and more environmentally friendly than chemicals ones[17] . In recent years, several research groups studying plant growth promoting rhizobacteria (PGPR) have reported advantages to use endospore-forming *Bacillus* and *Paenibacillus* spp. These bacilli have been shown to have promosing biological control in different crop system[7, 18, 19 20, 21, 22] .

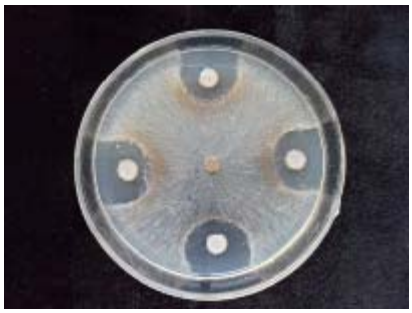

Fig. 1. Dual cultures (48h after inoculation). Inhibitory halos indicated that the growth of *R. solani* mycelium was inhibited by *P. Polymyxa* NBF188

As shown in Table 2, NBF188 was able to increased the biomass of cucumber. It has been reported that N_2-fixation of *P. polymyxa* and *p. azotofixans* plays an important role in plant-growth promotion[23, 24, 25, 26, 27] . In this study , the *nifH* gene, an indicator of N_2 fixation, could not be found from the strain NBF188 by a *nifH*-specific primers from strain NBF188, indicating no possibility of involvement of N_2 fixation in plant growth promotion.

Production of plant hormones by strain NBF188 could be a mechanism for plant growth promotion. *P. polymyxa* has been reported can synthesis the phytohormones, especially IAA and cytokinin that would have triggered the activities of specific enzymes[28] . In addition, bacterial volatile organic compounds (VOCs) have been discovered as a bacterial determinant of plant growth promotion[29, 30] . The major substance included 2,3-butanediol, which *P. polymyxa* also produced[31] .

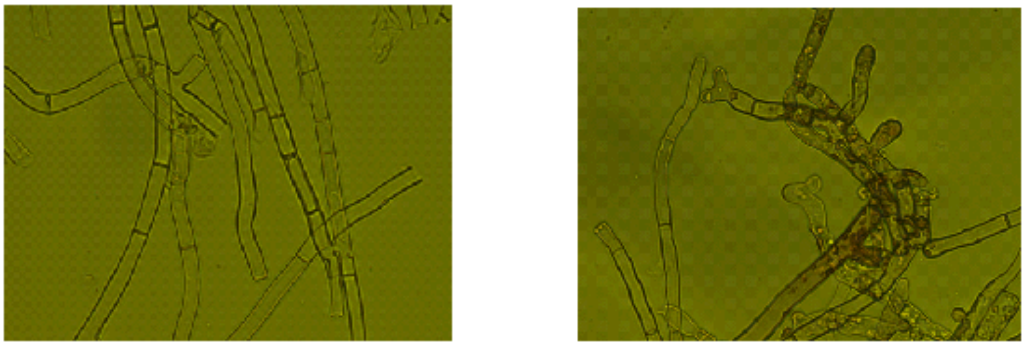

Fig. 2. Optical micrographs of the inhibition of fungal growth by NBF188 in a dual culture at 48h. (A) The control culture with only *R. Solani* . (B) *R. solani* mycelium from the edge of the inhibitoy halo.

Table 3. Effect of strain NBF188 on plant growth promoting on cucumber

Treatment	Percentage of germination (%)	Shoot length(cm)	Root length(cm)	Fresh weight (g seedling^{-1})	Vigour Index
Seed-soaking with NBF188	96.00a	25.14a	14.34a	2.49a	37.90a
Seed-soaking with QST713	91.00ab	21.63ab	13.19a	2.38a	31.69ab
control	82.00b	16.38b	9.57b	1.81ab	31.28ab

Therefore, this compound is need to identified in strain NBF188.

Root colonization was found to be an important factor in biological control of soil-borne plant pathogens[32] . Cook reported that bacteria isolated from the rhizosphere of a specific crop showed a better control of diseases than organisms isolated from other crops[33]. Therefore, in the present study, strain NBF188 was isolated from the rhizosphere of cucumber and the increased vigour indicates the ability of the isolate to colonize the roots of cucumber plants.

To conclude, based on the dual culture assay and greenhouse studies, strain NBF188 was found to exert antagonistic activity against *R. solani*. However, tests based on greenhouse studies do not always correlate with the biological control efficacy under natural conditions. Hence, biological control activities of strain NBF188 in field would be needed.

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