Control of Cucumber Grey Mold by Endophytic Bacteria

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Abstract: Endophytic bacterial strains B12 and B13 isolated from cucumber leaves effectively controlled cucumber grey mold, *Botrytis cinerea*. Both culture broth and culture filtrates were effective in inhibiting spore germination and germtube elongation of *B. cinerea*. Preliminary studies on the effects of pH and temperature on stability of the culture filtrates in inhibition of the pathogen were made.

Introduction: Grey mold, *Botrytis cinerea*, is a widespread pathogen of economic importance in greenhouse and open-field cucumber cultivation. As chemical control is not always efficient, benign alternative methods of protection are becoming more attractive. The term endophyte refers to interior colonization of plants by microorganisms that do not have pathogenic effects on their hosts. Some bacterial endophytic species have been implicated in the promotion of plant growth and protection against pathogens (1-4, 12). Wilhelm et al. (13) demonstrated that *Bacillus subtilis* strains isolated from the xylem sap of healthy chestnut trees exhibited antifungal effects against *Cryphonectria parasitica* causing chestnut blight. *Neotyphodium* elevates host protection against pathogens, and improves survival of grasses under competition (5). Endophytic isolates of *Pezicula* were shown to produce fungicidally active metabolites that are toxic to pathogens of their hosts (9).

B12 and B13 are two bacterial strains that have been isolated from the leaves of cucumber. On PDA plates, they were observed to inhibit the growth of *Botrytis cinerea.* In the present study, the effects of culture broth and culture filtrates of these two endophytic strains against *Botrytis cinerea* on cucumber leaves and the effects of pH and temperature on stability of the culture filtrates in inhibition of the pathogen were studied.

Materials and Methods: *Endophytes, pathogen , and plant.* B12 and B13 are two endophytic bacterial strains that were isolated from the leaves of cucumber in Yangling, Shaanxi Province, China. The cucumber plants examined in this study were supplied by the Horticulture Department, Northwest A&F University. The pathogen, *Botrytis cinerea*, was obtained from Plant Pathology laboratory. The cultures were maintained on potato-dextrose agar (PDA) medium at 4°C; and fresh cultures were grown on PDA plates at 25°C before experimentation. Spore suspensions were prepared from ten-day-old PDA cultures by dislodging spores from the surface of the cultures with a sterile bacteriological loop in sterile distilled water. The concentration was adjusted to $5x10^6$ spores ml^{-1}.

Antagonist. Liquid cultures were grown in 250 ml flasks containing 100 ml of nutrient broth (NB) which had previously been added using four sterile bacteriological loops of the cultures. Flasks were then incubated on a rotary shaker (170rpm) at 28°C for 48 h. The concentration was adjusted to $5x10^9$ CFU/ml. Culture filtrates were subsequently prepared by filtering the supernatant of centrifuged cultures of antagonist through a 0.22 μm polycarbonate membrane filter.

Antagonism in vitro. To evaluate the effects of culture filtrates on control of grey mold on leaves of cucumber, 6-mm-diameter callus from three-day-old cultures of *Botrytis cinerea* grown on PDA plates were cut off and then placed on cucumber leaves that had been previously sprayed with 2ml of the culture filtrates under serial dilutions of 1x, 10x, 20x, 50x, and 100x, with sterile distilled water as control. Then the leaves were placed in Petri dishes at 25°C. There were three replicate trials of ten leaves arranged in a completely randomizated design. The lesion diameter was recorded after four days. This experiment was repeated three times.

The effects of liquid cultures on spore germination and germtube elongation of the pathogen were assessed in potato dextrose broth (PDB). One-hundred-microliter (100 µl) spores suspension of $5x10^6$ spores ml^{-1} culture broth containing bacteria at $5x10^7$, $5x10^8$, $5x10^9$ CFU/ml concentrations, and culture filtrates, plus sterile distilled water were added into 10 ml glass tubes containing 5 ml PDB. Then they were placed on a rotary shaker (140rpm) at 25°C for 20h. No fewer than 100 spores were observed microscopically per replicate to record germination and germtube length. This experiment was repeated twice (6).

The stability of culture filtrates. Twenty ml glass tubes that contained 10 ml culture filtrates were placed in water bathes for 20 min each at 50, 60, 70, 80, 90 and 100°C before experimentation. The pH of other culture broths were adjusted each at 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with HCl or NaOH. Subsequently, 6-mm-diameter callus from 3-day-old cultures of *Botrytis cinerea* on PDA plates were taken and placed on PDA plates which contained 1ml of culture broth containing bacteria of $5x10^9$ CFU/ml, culture filtrates, sterile distilled water, and 20ml PDA. The lesion diameter was measured 96 h later. This experiment, containing three replications, was repeated three times.

Results: *The effect of culture filtrates against Botrytis cinerea on detached leaves of cucumber.* Culture filtrates were effective in inhibiting the development of disease on detached leaves of cucumber (Table 1). The pathogen was controlled at the high concentrations, the inhibition rate of B12 and B13 were 83.2 (± 3.2) % and 81.4(± 3.1) %, respectively.

Effect of B12 and B13 against Botrytis cinerea in vitro. Spore germination of *Botrytis cinerea* in PDB was significantly inhibited as the concentration of cultures broth increased ($P<0.05$) (Table1). Spore germination rates treated with $5x10^9$ CFU/ml of B12 and B13 culture broths had the most pronounced effects; being 5.5(±0.2) % and 8.6 (±0.4) %, respectively. The germtube length of the pathogen also decreased significantly compared to the control ($P<0.05$). The germtube lengths treated with $5x10^9$ CFU/ml cultures broth of the strains B12 and B13 were 25.9 (± 2.7) µm and 33.4 (±3.1) µm, respectively. Spore germination and germtube elongation of the pathogen were also significantly inhibited by culture filtrates, suggesting that in the culture filtrates there might be some metabolites produced toxic to the pathogen. The inhibition effect was similar with cultures broth at $5x10^9$ CFU/ml. In general, B12 had a more pronounced effect than B13 did in inhibiting *B. cinerea*.

The stability of culture filtrates. Culture filtrates of the two strains were stable at high temperatures, and although the effects on inhibition rate of the pathogen decreased slightly, the biocontrol effect was stable (Figure 1).

Culture filtrates of the two strains were stable against the pathogen at pH values between 6.0 and 9.0 (Figure 2). The effects of pH in culture filtrates on inhibition rate of pathogen decreased with the increase of alkalinity or acidity.

Discussion: Microbial endophytes are typically defined as microorganisms that are detected after surface sterilization of a plant part (7), and are assumed to originate from seed and/or the surrounding growing environment. Van Buren et al. (11) demonstrated that 32% of 192 endophytic bacterial strains isolated from potato stems exhibited biocontrol activity against the bacterial ring rot pathogen. Results of the present study showed that endophytic bacterial strains isolated from cucumber leaves can effectively control *Botrytis cinerea*, and that both of culture broth and culture filtrate types examined were effective in inhibiting the spore germination and germtube elongation of *B. cinerea*. We agree with Reiter (8) that endophytes represent a promising source of biocontrol strains, and that their use may be more successful than that of rhizosphere bacteria due to less competition with other bacteria in the apoplast.

Seghers et al. (10) demonstrated that agricultural practices significantly influence certain populations of the root endophytic community. Results of Reiter et al. (8) suggest that similar phylogenetic groups and genera, but different species and strains, were present in the different plant varieties. Our preliminary investigation indicates that differing cultivars might contain different endophytic communities. Thus, biocontrol mechanisms need further study. It is likely from such studies that the interactions among plants, pathogens, and bacterial endophytic communities will be an active field of research in the future.

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Literature Cited:

1. Arnold, A. E., L. C. Mejía, D. Kyllo, E. I. Rojas, Z. Maynard, N. Robbins and E. A. Herre. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100: 15649-15654.
2. Bell, C. R., G. A. Dickie, W. L. G. Harvey and J. W. Y. F. Chan. 1995. Endophytic bacteria in grapevine. Can. J. Microbiol. 41: 46–53.
3. Chelius, M. K., and E. W. Triplett. 2001. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microb. Ecol. 41: 252–263.
4. Cho S. J., S. R. Park, M. K. Kim, W. J. Lim, S. K. Ryu, C. L. An, S. Y. Hong, Y. H. Lee, S. G. Jeong, Y. U. Cho and H. D. Yun. 2002. Endophytic *Bacillus* sp. isolated from the interior of balloon flower root. Biosci. Biotechnol. Biochem. 66: 1270-1275.
5. Crous, P. W., O.Petrini, G. F.Marais, Z. A. Pretorius and F. Rehder. 1995. Occurrence of fungal endophytes in cultivars of *Triticum aestivum* in South Africa. Mycoscience 36: 105-111.
6. Droby, S., M. E. Wisniewski, L. Cohen, B. Weiss, D. Touitou, Y. Eilam and E. Chalutz (1997). Influence of $CaCl_2$ on *Penicillium digitatum*, grapefruit peel tissue, and biocontrol activity of *Pichia guilliermondii*. *Phytopathology*, 87: 310-315.
7. Hallmann, J., A. QuadtHallmann, W. F. Mahaffee and J. W. Kloepper. 1997. Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43: 895–914.
8. Reiter B., U. Pfeifer, H. Schwab and A. Sessitsch. 2002. Response of Endophytic Bacterial Communities in Potato Plants to Infection with *Erwinia carotovora* subsp. *Atroseptica*. Applied and Environmental Microbiology, 68: 2261–2268.
9. Schulz, B., J. Sucker , H. J. Aust., K. Krohn , K. Ludewig, P. G. Jones and D. Doring. 1995. Biologically active secondary metabolites of endophytic

Pezicula species. Mycological Research, 99: 1007-1015.
10. Seghers D., L. Wittebolle, E. M. Top, W. Verstraete and S. D. Siciliano. 2004, Impact of Agricultural Practices on the *Zea mays* L. Endophytic Community. Applied and Environmental Microbiology. 70: 1475–1482.
11. Van Buren, A. M., C. Andre and C. A. Ishimaru. 1993. Biological control of the bacterial ring rot pathogen by endophytic bacteria isolated from potato. Phytopathology 83: 1406.
12. Van Wees, S. C., M. Luijendijk, I. Smoorenburg, L. C. van Loon and C. M. Pieterse. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. Plant Mol. Biol. 41: 537–549.
13. Wilhelm, E., W. Arthofer, and R. Schafleitner. 1997. *Bacillus subtilis*, an endophyte of chestnut (*Castanea sativa*), as antagonist against chestnut blight (*Cryphonectria parasitica*), p. 331–337. In A. C. Cassells (ed.), Pathogen and microbial contamination management in micropropagation. Kluwer Academic Publishers, Dortrecht, The Netherlands.

Table 1. The effect of culture endophytic bacterial filtrates (B12 and B13) against *Botrytis cinerea* on detached leaves of cucumber

Serial dilutions	Inhibition rate (%)	
	B12	B13
1x	83.2 (± 3.2) d	81.4 (± 3.1) c
10x	66.5 (± 2.9) c	62.6 (± 2.7) c
20x	46.8 (± 2.5) bc	39.4 (± 2.3) bc
50x	15.7 (± 2.1) b	13.5 (± 2.0) b
100x	2.5 (± 1.3) a	0 (± 0) a

Each value is the mean of three trials(±SE). Values followed by different letters are statistically different at P = 0.05 according to Duncan's multiple range test.

Table 2. The effects of endophytic bacterial strains on spore germination and germtube elongation of *Botrytis cinerea* spores

Treatments	Spore germination rate (%)		Germtube length (μm)	
	B12	B13	B12	B13
Cultures broth ($5x10^7$ CFU / ml)	34.0 (± 3.2) d	56.5 (± 3.5) d	39.2 (± 3.4) d	56.7 (± 3.7) d
Cultures broth ($5x10^8$ CFU / ml)	12.6 (± 2.5) c	21.5 (± 2.6) c	31.5 (± 3.1) c	44.5 (± 3.6) c
Cultures broth ($5x10^9$ CFU / ml)	5.5 (± 0.2) b	8.6 (± 0.4) b	25.9 (± 2.7) b	33.4 (± 3.1) b
Culture filtrates	9.8 (± 0) c	15.4 (± 2.3) b	28.7 (± 2.9) c	38.3 (± 3.4) b
Sterile distilled water	98.5 (± 0) a		164.5 (± 3.8) a	

Each value is the mean of three trials(±standard error). Values in each row followed by the different letter are significantly different at P = 0.05 according to Duncan's multiple range test.

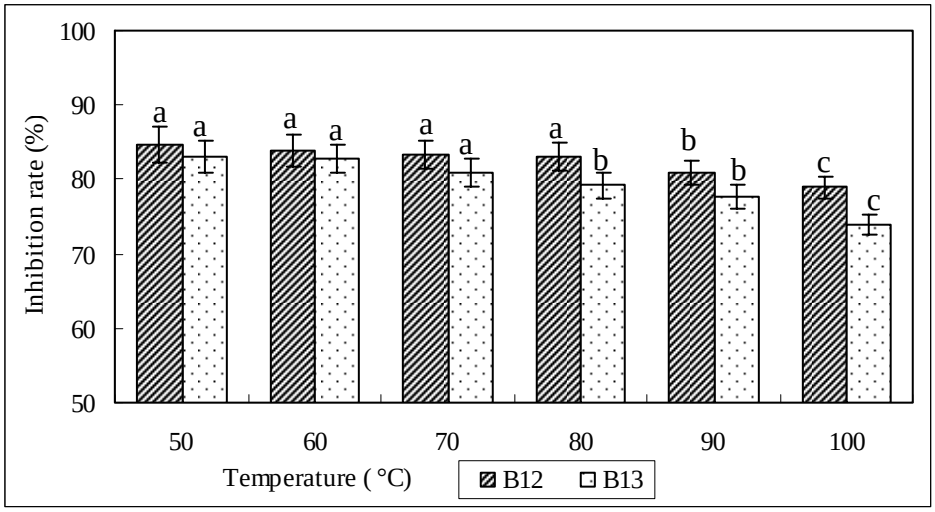

Fig. 1. The effects of temperature on inhibition rate of culture filtrates. Inhibition rates are the mean of three trials. Values followed by the different letters are statistically different at P = 0.05 according to Duncan's multiple range test.

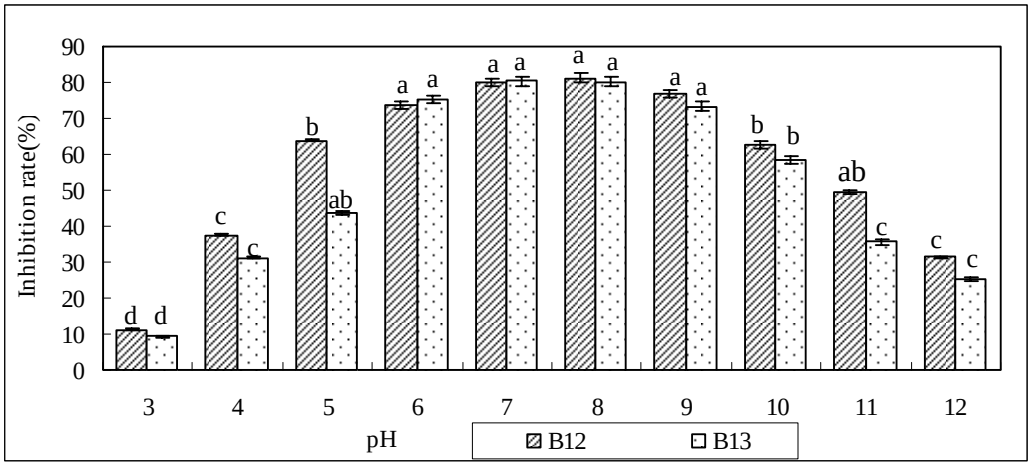

Fig. 2. The effects of pH on inhibition rate of culture filtrates. Bars represent standard deviations of the means. Values followed by the same letters are not statistically different at P =0.05 according to Duncan's multiple range test.