The Role of Phyllosphere Bacteria in Pathogenesis by Botrytis squamosa and B. cinerea on Onion Leaves

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ABSTRACT

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Numbers of lesions formed by *Botrytis squamosa* on onion leaves were reduced only slightly by two of thirteen onion leaf surface bacteria. Ten of the isolates stimulated greater frequency of lesion formation. Conidial germination of *B. squamosa* and *B. cinerea* in water in vitro was inhibited by several of the bacteria. Most of the isolates did not inhibit germination in nutrients. Two bacterial isolates were selected as potential nutrient competitors based on their ability to inhibit germination of the two *Botrytis* species in water, but not in nutrients. They grew equally as well on leaves inoculated with *B. squamosa* or *B. cinerea* as on noninoculated leaves. The same isolates grew moderately well in vitro in cell-free diffusates of conidia of both pathogens, but poorly in suspensions of conidia. Treatment of leaves with antibiotics (streptomycin, penicillin, chloramphenicol, or penicillin plus chloramphenicol) reduced natural bacterial populations. None of the antibiotics stimulated lesion formation on onion leaves following inoculation by *B. cinerea* or *B. squamosa* conidia in water suspension.

Additional key words: Allium cepa.

Conidia of *Botrytis cinerea* Pers. are dependent on exogenous nutrients for in vitro germination. Conidia of *Botrytis squamosa* Walker are nutrient-independent. On onion leaves, *B. cinerea* fails to produce expanding lesions without exogenous nutrients. *Botrytis squamosa* produces expanding lesions when sprayed in water onto leaves, but produces greater numbers of lesions when sprayed in water containing nutrients (10).

Numbers of naturally occurring bacterial cells on leaves of onion plants grown outdoors ranged from 10 to 5,000 per square centimeter of leaf surface; propagules of yeasts and filamentous fungi ranged from 10 to 500 propagules per square centimeter (8). The fungal flora of the onion leaf surface is weakly antagonistic to *B. cinerea* and *B. squamosa* (11).

Bacteria normally present on the surface of beet and chrysanthemum leaves inhibit development of *B. cinerea* on the leaf surface (2, 3, 4). This appears due to nutrient competition between *Botrytis* conidia and bacteria (3). Leaching conidia with water inhibits germination (15). Bacteria take up ^{14}C initially present in conidia (7). Age of the leaf, antibiotic treatment, and germicidal light treatment, all of which alter bacterial populations on leaf surfaces, also increase development of *B. cinerea* on the leaf surface (2, 5, 16). Such treatments also increase nutrient leakage from the leaf. The presence of conidia of *B. cinerea* on cabbage, or of *Colletotrichum lagenarium* on cucumber, does not increase numbers of bacterial cells on leaves, and bacteria alone appear not to prevent pathogenesis (13, 17).

This study was undertaken to evaluate the role of onion leaf surface bacteria in pathogenesis of *B. cinerea* and *B. squamosa* on onion leaves.

MATERIALS AND METHODS

Cultures of *B. squamosa* (isolate 64a) and *B. cinerea* (isolate 61-34) were maintained by monoconidial transfers to slants of a complete medium (Difco Czapek-Dox broth, 35 g; agar, 15 g; Difco yeast extract, 2.5 g; Difco malt extract, 7.5 g; sodium nucleate, 10 mg; hydrolyzed casein, 250 mg; trace element stock solution [$Fe(NO_3)_3 \cdot 9H_2O$, 723.5 mg; $ZnSO_4 \cdot 4H_2O$, 203.0 mg; H_3BO_3, 2.0 mg; H_3MoO_3, 2.0 mg; $CuSO_4$, 2.0 mg; distilled water, 1,000 ml), 1 ml; distilled water, 1,000 ml] (1). Slants were incubated at 21 C under fluorescent light (12-hour photoperiod). Conidia used for incubation in vitro and onion leaf inoculations were produced on onion leaf-straw agar. This was prepared by placing cut onion leaf-straw in a 9-cm diameter petri dish with 20-30 ml water agar and autoclaving. To collect conidia, the complete medium slants were rinsed with 10 ml of distilled water. One milliliter of the suspension of conidia was transferred to each plate of onion leaf-straw agar. The plates were incubated at 21 C under fluorescent light (Sylvania F20 T12-CW, 12-hour photoperiod). *Botrytis squamosa* conidia were harvested after 6-7 days of incubation and *B. cinerea* after 5-6 days by aspirating the conidia into 250-ml Erlenmeyer flasks containing 30 ml of sterile glass-distilled water.

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Conidia for in vitro germination studies were suspended in glass-distilled water (10,000/ml) and placed in round incubation dishes (22-mm diameter by 5-mm deep) containing a final volume of 0.6 ml liquid. The incubation dishes were placed in sterile petri dishes containing filter paper moistened with 5% (v/v) glycerine. Czapek-Dox broth (50%, v/v) plus yeast extract (0.05%, w/v) was used as a nutrient solution. The conidia were incubated in darkness at 21 C for 12 hours and then killed and stained by the addition of one drop of cotton blue in lactophenol per incubation dish.

Three- to 5-month-old onion plants (cultivar Elba Globe) were placed in a mist chamber at 21 C, 12-hour photoperiod (fluorescent light, Sylvania F96 T12-WW-VHO), constant humidification, and misting 5 seconds every 10 minutes for 24 hours prior to inoculation. Inoculum was adjusted to a concentration of 10,000-30,000 conidia per milliliter and one drop of Tween-20 per 100 ml was added. Each plant was sprayed (by atomizer) with 10 ml of inoculum and returned to the mist chamber for 48 hours and then lesion counts were made.

Cultures of unidentified bacteria isolated by washing from the phyllosphere of onions grown outdoors in New York were maintained on nutrient agar. Cell suspensions were prepared from nutrient broth cultures incubated at 30 C for 24 hours. The broth cultures were centrifuged at 20,000 *g* for 10 minutes, the supernatant liquid was discarded, the cells were resuspended in sterile glass-distilled water, recentrifuged at 20,000 *g* for 10 minutes, and the pellet was resuspended in 10 ml of sterile glass-distilled water. The cell suspension (0.1 ml) was added to each incubation dish. The cell suspensions were diluted 1:5 and 10 ml was sprayed (by atomizer) on each plant.

Streptomycin sulfate, penicillin G (K salt), and chloramphenicol (all from Sigma Chemical Co., St. Louis, Mo.) were added individually to the inocula at the rate of 50 μg/ml to test their effects on lesion formation by *B. squamosa* and *B. cinerea*. The 50 μg/ml dose was selected based on preliminary work and another report (5) which indicated some antibacterial activity, but little effect on germination of *Botrytis* spp. conidia (with the exception of streptomycin). The same antibiotics were assayed at 50 μg/ml for in vitro effect on germination of conidia of *B. squamosa* and *B. cinerea*.

Conidial diffusates were prepared from suspensions of conidia in water. The suspended conidia were incubated at room temperature (20-24 C) on a platform shaker for 4 hours and then were removed by filtration through a Millipore filter (0.22-μm pore size).

RESULTS

Six bacterial isolates (numbered 5, 10, 11, 16, 20, and 2c) from onion phyllospheres inhibited germination of *B. cinerea* conidia in water more than in nutrients (Table 1). None of the bacteria totally inhibited germination. Isolates 5, 10, 16, 34, 47, and 54 inhibited germination of *B. squamosa* conidia only in water (Table 1). Germination of conidia of both *B. cinerea* and *B. squamosa* was inhibited by isolates 2 and T-3 in water and nutrient solution. *Botrytis cinerea* also was inhibited by isolate 23. Isolates 34 and 51 stimulated germination of *B. cinerea* conidia in water (Table 1). Isolates 10 and 16 were studied further for nutrient competition with conidia. Since *B. cinerea* normally does not produce expanding lesions in the absence of nutrients, the effect of the bacteria on lesion formation was not determined. The effect of onion phyllosphere bacteria on lesion formation by *B. squamosa* (inoculated in water) was inconsistent. Only two isolates (1 and T-3) had a consistent inhibitory effect (Table 1). With the exception of isolate 16 (no effect), the remaining isolates increased lesion formation.

The effect of inoculation with *B. squamosa* and *B. cinerea* on cell numbers of isolates 10 and 16 on leaves was

TABLE 1. Effect of different isolates of onion phyllosphere bacteria on lesion formation on onion leaves and in vitro germination of conidia by *Botrytis squamosa* and *B. cinerea* in water (H_2O) or 50% Czapek-Dox broth plus 0.05% yeast extract (NS)

Bacterial isolates no.	Conidial germination[y] of:				
	B. cinerea		*B. squamosa*		
	H_2O	NS	H_2O	NS	Lesions per leaf[z]
1	88 mno	76 ijk	101 jkl	100 kl	74
2	24 cd	20 bc	78 ef	74 de	. . .
5	84 klm	96 opqr	47 a	77 ef	288
10	66 h	89 mnop	69 d	95 hij	94
11	71 hi	101 r	92 gh	105 l	250
16	15 ab	96 opqr	54 b	99 ijkl	107
20	56 g	95 opqr	93 ghi	99 ijkl	. . .
23	21 bc	35 ef	99 ijkl	93 ghi	254
34	134 s	101 r	88 g	101 jkl	234
47	93 nopq	84 klm	68 d	99 ijkl	141
51	143 t	37 f	97 hijk	92 gh	118
52	79 ijkl	85 lmn	61 c	97 hijk	138
54	82 jklm	75 ij	81 f	96 hij	138
2c	28 cde	98 pqr	96 hij	100 jkl	136
T3	32 def	11 a	78 ef	42 a	78

[y]Expressed as a percent of control, in vitro (three replicates/treatment). Numbers under the same *Botrytis* spp. followed by the same letter are not significantly different ($P = 0.01$).

[z]Expressed as average percent of control (conidia in water, no bacteria) for two independent tests.

variable, and differences in populations between inoculated and noninoculated leaves were not significant.

Both bacterial isolates exhibited significantly better growth in diffusates of conidia of both *Botrytis* species than in distilled water. Isolate 16 produced more cells in suspensions of conidia (Table 2). Growth of both bacteria in diffusates of conidia was limited in comparison to growth in nutrient broth (Table 2). Following incubation, conidia-bacteria suspensions were examined microscopically for conidia germination and adhesion of bacteria to conidia. Neither phenomenon was observed.

Conidia germination (in vitro) of both species of *Botrytis* was inhibited totally by streptomycin in water, but not in nutrients (Table 3). The other antibiotic treatments did not affect germination appreciably. All four antibiotic treatments resulted in reduced bacterial populations on surfaces of leaves inoculated with *B. cinerea* (Table 3) and to a lesser extent on leaves inoculated with *B. squamosa* (Table 4). Lesion formation by *B. cinerea* conidia suspended in water was increased by penicillin although in no case was lesion formation significantly increased. Lesion formation by *B. squamosa* in water was inhibited partially by antibiotic treatment. With *B. squamosa,* streptomycin increased numbers of bacteria (Table 4). None of the antibiotics contained bacterial contaminants.

TABLE 2. Number of cells of phyllosphere bacteria after 24 hours of incubation at 24 C in the presence of conidia or cell-free conidial diffusate of *Botrytis squamosa* or *B. cinerea*

	Change in cells/ml ($\times 10^3$)[w]	
Treatment[v]	Bacterial isolate 10[x,y]	Bacterial isolate 16[x,z]
B. squamosa conidia diffusate	497.4 b	+59.8 b
B. cinerea conidia diffusate	549.2 b	+14.9 a
B. squamosa conidia suspension	−1.7 a	+ 2.8 a
B. cinerea conidia suspension	−1.4 a	+ 0.5 a
Glass-distilled water	−1.4 a	+ 0.1 a

[v]Conidia were suspended in water (5,000/ml) and used as such for conidia suspensions. Conidia diffusates (cell-free) were obtained by filter-sterilizing suspensions after 4 hours of incubation at 21 C.

[w]Numbers are averages for two trials (three replicate flasks per treatment, two replicate plates per flask). Results for both trials were similar (+ = increase in numbers, − = decrease). Numbers under the same isolate followed by the same letter are not significantly different ($P = 0.01$).

[x]Isolates 10 and 16 were selected as possible nutrient competitors based on their in vitro effect on germination of conidia of *Botrytis* spp.

[y]Initial bacterial concentration = 373 cells/ml. In 10% nutrient broth there was an increase of 1.39×10^{12} cells/ml.

[z]Initial bacterial concentration = 65 cells/ml. In 10% nutrient broth there was an increase of 0.29×10^9 cells/ml.

DISCUSSION

Several isolates of bacteria from the phyllosphere of healthy onion leaves were inhibitory to in vitro germination of conidia of *B. squamosa* and *B. cinerea* in water, but not in nutrients. That *B. squamosa* conidia appeared to be less sensitive to antagonism by the bacteria may have resulted from greater nutrient independence (10) and/or the fact that *B. squamosa* conidia are larger in volume. Large size may afford a conidium greater resistance to soil fungistasis (14). Most of the bacterial isolates greatly stimulated lesion formation, whereas only two isolates inhibited lesion formation slightly.

Bacterial stimulation of pathogenesis was either direct (serving as a source of nutrients for *B. squamosa*) or indirect (altering the leaf in a manner predisposing it to infection by *B. squamosa*). In view of the results of Leben (12), who found only one antagonist of *Colletotrichum lagenarium* out of 230 bacteria isolates, the number of

TABLE 3. The effect of antibiotic treatment on formation of lesions and in vitro conidial germination by *Botrytis cinerea* and number of bacterial cells on onion leaves sprayed with water (H_2O) or 50% Czapek-Dox broth plus 0.05% yeast extract (NS)

Antibiotic[w]	Medium	Germination[x] (%)	Germ tube length (μm)[x]	Lesion counts[y]	Bacteria cells/mm^2 leaf surface[z] (cells/mm^2)
None	H_2O	83 b	14 a	3 a	478
Streptomycin	H_2O	0 a	. . .	5 a	46
Penicillin	H_2O	87 c	23 ab	17 a	59
Chloramphenicol	H_2O	82 b	16 ab	2 a	37
Penicillin and chloramphenicol	H_2O	92 d	26 b	2 a	15
None	NS	98 e	129 d	125 bc	997
Streptomycin	NS	98 e	101 c	152 c	104
Penicillin	NS	100 e	137 d	193 d	35
Chloramphenicol	NS	99 e	131 d	151 c	12
Penicillin and chloramphenicol	NS	98 e	131 d	103 b	4

[w]All antibiotics were applied at 50 μg/ml in the inoculum.

[x]Numbers followed by the same letter were not significantly different ($P = 0.01$).

[y]Lesions per leaf adjusted for differences in inoculum concentration to a standard of 30,000 conidia per milliliter. Numbers followed by the same letter were not significantly different ($P = 0.15$).

[z]Leaves were untreated prior to inoculation and had four bacteria/mm^2 of leaf surface. Differences were not significantly different ($P = 0.20$).

TABLE 4. The effect of antibiotic treatment on formation of lesions and in vitro conidia germination by *Botrytis squamosa* and number of bacterial cells on onion leaves sprayed with water (H_2O) or 50% Czapek-Dox broth plus 0.05% yeast extract (NS)

Antibiotic[w]	Medium	Germination[x] (%)	Germ tube length (μm)[x]	Lesion counts[y]	Bacteria cells/mm^2 leaf surface[z] (cells/mm^2)
None	H_2O	98 d	47 b	30 c	114
Streptomycin	H_2O	0 a	. . .	7 b	95
Penicillin	H_2O	99 de	46 ab	25 b	78
Chloramphenicol	H_2O	96 c	43 ab	17 abc	94
Penicillin and chloramphenicol	H_2O	94 b	38 a	4 a	21
None	NS	100 c	111 d	138 e	326
Streptomycin	NS	99 de	75 c	93 d	113
Penicillin	NS	100 c	121 e	95 d	156
Chloramphenicol	NS	99 de	127 e	118 e	18
Penicillin and chloramphenicol	NS	99 de	112 d	32 c	19

[w]All antibiotics were applied at a rate of 50 μg/ml in the inoculum.

[x]Numbers followed by the same letter are not significantly different ($P = 0.01$).

[y]Lesions per leaf adjusted for differences in inoculum concentration by multiplying lesions per leaf with NS by 10. Numbers followed by the same letter were not significantly different ($P = 0.10$).

[z]Leaves were untreated prior to inoculation and had less than nine bacteria per square millimeter of leaf surface. Differences were not statistically significant ($P = 0.20$).

isolates tested in this study was limited. Since relatively few bacteria were found on onion leaves (8) and most of these were poor antagonists, it appears that very large numbers of bacteria must be screened if useful antagonists are to be found.

Leaching conidia of both *B. cinerea* and *B. squamosa* with water but not nutrients inhibits germination (10). Such leaching has been used to qualitatively, but not quantitatively, simulate the nutrient drain on spores created by microorganisms in soil and on leaves (6, 15). Potentially, conidia of both pathogens appear to be susceptible to inhibition by nutrient competition.

That there was competition between onion leaf surface bacteria and *Botrytis* spp. conidia for nutrients (3, 7, 15) was supported by the observation that several of the bacterial isolates inhibited germination in water, but not in nutrients. When these bacteria in suspension were sprayed on onion leaves, they grew at similar rates in the presence or absence of the pathogens. Isolates 10 and 16 grew poorly in conidia suspensions and only moderately better in conidia diffusates in vitro. These observations do not support the nutrient competition hypothesis of antagonism. However, the ability of bacteria to compete for nutrients is not necessarily related to growth because various compounds can be absorbed into their extracellular polysaccharide sheaths (15).

Greater growth of bacteria in conidia suspensions rather than in conidia diffusates would be expected since the conidia diffusates contain only the nutrients present at one particular time. Conidia in suspension, however, have the potential for continued leakage of nutrients with time. The possibility that adherence of bacteria to conidia thus diminished estimates was not supported by microscopic observation. The *Botrytis* species may have been antagonistic to the bacteria.

Antibiotic treatment failed to stimulate lesion formation by *B. squamosa* (in water) and only penicillin treatment caused increased lesion formation by *B. cinerea*. However, penicillin treatment can cause increased nutrient leakage from leaves (5).

Leaf surface bacteria alone probably do not prevent lesion formation by *B. cinerea* on onion leaves. Several observations lead to that conclusion: these are (i) in a prior study bacteria were not observed in the vicinity of germinating conidia (9); (ii) bacterial populations of the onion leaf surface were very low (8); (iii) bacterial isolates from the phyllosphere of onion were only moderately antagonistic to germination of conidia in vitro; (iv) the same isolates were more stimulatory than inhibitory to lesion formation by *B. squamosa* in water; (v) inoculation of leaves with *B. cinerea* or *B. squamosa* failed to stimulate increased growth of artificially applied bacteria; (vi) antibiotic treatments which decreased bacterial populations on the leaf surface failed to stimulate increased lesion formation. Antagonism by the bacteria in vitro was more effective in water than in nutrients, suggesting that although the bacteria may not affect the ability of the *Botrytis* species to form lesions, they may compete with conidia for nutrients as suggested previously (2, 5, 7, 15). Greater antagonism in water may occur also if the conidia are more sensitive to other antagonistic factors such as antibiotics.

LITERATURE CITED

1. BERGQUIST, R. R. 1971. The biology and genetics of Botryotinia squamosa. Ph.D. Thesis, Cornell University, Ithaca, N.Y. 124 p.
2. BLAKEMAN, J. P. 1972. Effect of plant age on inhibition of Botrytis cinerea spores by bacteria on beetroot leaves. Physiol. Plant Pathol. 2:143-152.
3. BLAKEMAN, J. P. 1973. The chemical environment of leaf surfaces with special reference to spore germination of pathogenic fungi. Pestic. Sci. 4:475-488.
4. BLAKEMAN, J. P., and A. K. FRASER. 1971. Inhibition of Botrytis cinerea spores by bacteria on the surface of chrysanthemum leaves. Physiol. Plant Pathol. 1:45-54.
5. BLAKEMAN, J. P., and A. SZTEJNBERG. 1974. Germination of Botrytis cinerea spores on beetroot leaves

treated with antibiotics. Trans. Br. Mycol. Soc. 62:537-545.
6. BRISTOW, P. R., and J. L. LOCKWOOD. 1972. Soil fungistasis: a possible mechanism for the inhibition of nutrient-independent propagules. Phytopathology 62:1107 (Abstr.).
7. BRODIE, I. D. S., and J. P. BLAKEMAN. 1975. Competition for carbon compounds by a leaf surface bacterium and conidia of Botrytis cinerea. Physiol. Plant Pathol. 6:125-135.
8. CLARK, C. A. 1976. Histopathology and leaf surface relationships of Botrytis squamosa and B. cinerea on onion. Ph.D. Thesis, Cornell University, Ithaca, N.Y. 138 p.
9. CLARK, C. A., and J. W. LORBEER. 1976. Comparative histology of Botrytis squamosa and B. Cinerea on onion leaves. Phytopathology 66:1279-1289.
10. CLARK, C. A., and J. W. LORBEER. 1976. Comparative nutrient dependency of Botrytis squamosa and B. cinerea. Phytopathology 66: (In press).
11. FOKKEMA, N. J., and J. W. LORBEER. 1974. Interactions between Alternaria porri and the saprophytic mycoflora of onion leaves. Phytopathology 64:1128-1133.
12. LEBEN, C. 1964. Influence of bacteria isolated from healthy cucumber leaves on two leaf diseases of cucumber. Phytopathology 54:405-408.
13. LEBEN, C., and G. C. DAFT. 1967. Epiphytic bacteria and the germination of Colletotrichum lagenarium conidia on cucumber leaves. Phytopathology 57:527-529.
14. STEINER, G. W., and J. L. LOCKWOOD. 1969. Soil fungistasis:sensitivity of spores in relation to germination time and size. Phytopathology 59:1084-1092.
15. SZTEJNBERG, A., and J. P. BLAKEMAN. 1973. Studies on leaching of Botrytis cinerea conidia and dye absorption by bacteria in relation to competition for nutrients on leaves. J. Gen. Microbiol. 78:15-22.
16. SZTEJNBERG, A., and J. P. BLAKEMAN. 1973. Ultraviolet-induced changes in populations of epiphytic bacteria on beetroot leaves and their effect on germination of Botrytis cinerea spores. Physiol. Plant Pathol. 3:443-451.
17. YODER, O. C., and M. L. WHALEN. 1975. Factors affecting postharvest infection of stored cabbage tissue by Botrytis cinerea. Can. J. Bot. 53:691-699.