Variability of Three Isolates of *Botrytis cinerea* Affects the Inhibitory Effects of Calcium on this Fungus

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ABSTRACT

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Botrytis cinerea is an economically important pathogen. Epidemiological studies are difficult because of the genetic variability within this species. The objectives of this work were to study the variability and to compare the inhibitory effects of Ca on three isolates of *B. cinerea* from decayed apple (B) and grape (C and C77:4). Among these isolates, B had the least radial growth but had a sporulation rate 40% higher than that of both C77:4 and C. In situ, isolate C incited the largest decay area in the fruit of two of four apple cultivars examined and had the highest polygalacturonase activity in vitro. Maximum mycelial

growth was reached with CaCl_2 at 1 g liter⁻¹ for isolates B and C77:4 and at 4 g liter⁻¹ for isolate C. Calcium (CaCl_2) inhibited polygalacturonase activity at 1 g liter⁻¹ for C and C77:4 and at 16 g liter⁻¹ for B. Calcium infiltration reduced decay caused by all three isolates by three to five times. Mycelial DNA analysis showed that 42% of the character loci scored were polymorphic and the greatest similarities were found between B and C77:4. These results support the evidence that the biological and statistical variability in research can be affected by the *B. cinerea* isolate selected. Despite this variation, Ca treatment of apples reduced decay caused by all three *Botrytis cinerea* isolates.

Additional keyword: gray mold.

The genus *Botrytis* contains 22 species and a large number of host-specific pathogens, such as *B. fabae* Sardina on Leguminosae and *B. aclada* Fresenius on *Allium* spp. (7). *B. cinerea* Pers.:Fr., however, is an important pathogen that can attack more than 200 species in the field (7), greenhouse (14,35), and storage (28,29,33). Under some circumstances, *Botrytis*-infected grapes can be beneficial for wine production (7). This fungus can infect the plant at every stage of its development and has been found in every part of the plant. Its detection at early stages is difficult because *Botrytis* spp. can be found in a latent state on the host plant (31), making epidemiological studies difficult.

In vivo, only asexual multinucleate conidia are produced, and field isolates of *B. cinerea* grown on synthetic media exhibit considerable variation (7). Variations can be phenotypic or can result in resistance to fungicides or a change in pathogenicity. These latter variations can make control with fungicides difficult. Mutations and heterokaryosis are the main causes of variations. Nevertheless, the development of resistant strains has provided genetic markers and recognition that distinct genotypic populations of *B. cinerea* exist. No relationship has been found, however, between the existence of different populations and their origins or sources. Because of the emergence of resistance to fungicides, new strategies in the control of *B. cinerea* have been investigated. One alternative involves the use of biocontrol agents including bacteria (21), yeasts (13), or antagonistic fungi (14,40). Calcium improves the efficiency of biological control (45). Several studies have reported that Ca treatment of plant tissue

induces an increase in tissue Ca content (11) by cross-linking the polygalacturonate polymers (9,10), resulting in reduced decay caused by *B. cinerea* (5,10). In addition to the effect of Ca in strengthening plant cell walls, fungal polygalacturonase (PG) (EC 3.2.1.15), thought to be a key enzyme in the infection process, is inhibited by Ca (3). All the studies on the effect of Ca on *Botrytis* showed that it has an inhibitory effect on growth of this fungus at high concentrations (3,20,45).

The objectives of our work were to characterize the variability among three isolates of *B. cinerea* and their relative responses to Ca both in vitro and in situ.

MATERIALS AND METHODS

Fungal isolates. The three isolates of *B. cinerea* used were isolated from decayed 'Golden Delicious' apples (isolate B), [ATCC 90870], from American Type Culture Collection, Manassas, VA) and grapes (isolate C from Cornell University, Ithaca, NY, and isolate C77:4 from the College of Enology, Bordeaux, France). The isolates were grown on potato carrot agar (PCA) slants prepared as follows: 20 g of diced potatoes and 20 g of diced carrots were boiled in H_2O for 30 min and filtered through cheesecloth. The final volume was adjusted to 1 liter with H_2O , and 15 g of agar was added. The resulting conidia were transferred from the PCA slants to potato dextrose agar (PDA) (Sigma Chemical Co., St. Louis) plates to produce the necessary inoculum. All incubations were performed at 20°C under constant fluorescent light. Ultrapure H_2O (quality >18M Ω) was used in all experiments.

Growth on PDA. Plugs (0.6 cm diameter) of fully developed hyphae were placed in the center of a PDA plate, and the mycelial area was recorded over 4 days. Three replicates of each isolate were analyzed. After 5 days, four mycelial plugs (2 cm diameter)

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were removed from each plate and mixed with 10 ml of H₂O for 1 min. The conidial suspension was filtered through two layers of cheesecloth, and the conidial concentration was determined with a hemacytometer. Three replicates of each isolate were analyzed.

Growth in modified Richard's solution. Conidial suspensions (1×10^6 conidia per ml) were prepared from the PDA plates after 2 weeks of growth. Flasks (500-ml) containing 100 ml of Richard's solution, using sucrose at 50 g liter⁻¹ (15) as the carbon source, were inoculated with 2 ml of the conidial suspension. Four Ca (CaCl₂) concentrations at 0, 1, 4, and 16 g liter⁻¹ were added to the media. The pH of all media was adjusted to 4.2 (pH of the medium prior to the addition of Ca). The cultures were incubated at 22°C on a rotary shaker (50 rpm) under constant light. Mycelia were collected by centrifugation ($8,000 \times g$, 20 min, 4°C) after 3, 5, 7, and 10 days and washed three times with H₂O. Hyphae were dried at 70°C until dry weight was constant. Four replicates per treatment were analyzed.

Protein and PG activity determination. The three isolates were grown in Richard's solution with CaCl₂ added at 0, 1, or 16 g liter⁻¹. After 5 days, 3-ml aliquots of each culture medium were collected and dialyzed against H₂O for 16 h at 4°C. The protein content (24) and PG activity (17) were determined. Five replicates for each treatment were analyzed.

Fig. 1. Effect of calcium on the growth of three isolates of *Botrytis cinerea* in modified Richard's solution. Data bars are means ($\pm$ standard error) of four replicates.

Pathogenicity test. Four apple cultivars (Arkansas Black, Golden Delicious, Mutzu, and Rome) stored at 0°C for 6 months were used. Fruit from each cultivar were randomized and wounded with the tip of a disinfected 10-penny nail (2.5 mm diameter, 3 mm deep) on two opposite sides of each fruit. Fruit were dipped for 30 s in a conidial suspension (5×10^5 conidia per ml) and incubated at 22°C. Severity of decay was recorded 5, 6, and 7 days after inoculation by measuring the diameter of the decayed area as the mean of its width and length and computing the total area of decay. Twenty fruit pieces per cultivar were analyzed.

In another experiment, 'Red Delicious' apples were randomized, pressure-infiltrated (68.95 KPa) for 2 min with 6% CaCl₂ (99% pure), and stored at 0°C for 6 months. Fruit were wounded (2.5 mm diameter, 3 mm deep) and inoculated (1×10^5 conidia per ml) as described above. The decayed area was measured 5, 6, and 7 days after inoculation. Eighty fruits per isolate were tested.

Statistical analysis. Effects of Ca treatment, isolate, and time on all variables studied were evaluated by analysis of variance and Duncan's multiple range test was used to compare means. To compare variability among the three isolates, restricted maximum likelihood estimates of residual variances were calculated with the MIXED procedure (SAS Institute, Cary, NC). The model removed all effects of Ca treatment, isolate, and time when applicable, leaving a pooled residual variance among the replicates. Within this one model, the pooled residual was estimated separately for each isolate (i.e., variability), and those estimates were compared with pairwise *F* tests (37) between isolates (i.e., significance). The more variable isolate was arbitrarily designated as *** and the least variable was designated as *. Significant ($P < 0.01$) differences between variances were calculated and indicated by a, b, and c. Each experiment was repeated at least twice.

Nuclear staining. Conidia were collected from 2-week-old cultures on PDA and stained with mythramycin (36). Preparations were examined with a fluorescence microscope equipped with a BV-2A filter, and the number of nuclei per conidium was determined. One hundred observations were made for each isolate.

DNA extraction and analysis. DNA amplification fingerprinting (DAF) was used to assess the genetic relationships between the three *B. cinerea* isolates (4). Mycelia were harvested from 3-day-old potato dextrose broth (Sigma) cultures by filtering through Whatman No. 1 filter paper (Whatman International, Maidstone, England) with a gentle vacuum. The samples were stored at -75°C until needed. Samples consisting of 20 mg of mycelia were ground in liquid N₂, and the DNA was extracted according to Yoon et al. (46) as modified by Trigiano et al. (39). Genomic DNA was resuspended in sterile H₂O and adjusted to 5 ng ml⁻¹. DNA templates were amplified according to Trigiano et al. (38). Primers (Integrated DNA Technologies Inc., Coralville, IA) used had the following 5' to 3' sequences: 8.6C: AACG-GGTG; 8.6D: GACGTAGG; 8.6F: GATCGCAG; 8.6G: CTAA-CGCC; 8.6H: GAAACGCC; and 8.7D: CCGAGCTG. DNA was amplified in a thermocycler (Ericomp TwinBlock, San Diego, CA) for 35 cycles (each $\approx$ 5.5 min) of denaturing for 10 s at 96°C and followed by annealing for 10 s at 30°C (38). All experiments were repeated twice. Amplified DNA products were used either

TABLE 1. Comparison of the mycelial growth areas (square centimeters) of three isolates of *Botrytis cinerea* on potato dextrose agar (PDA)

Day	Isolate C	Isolate B	Isolate C77:4
1	1.03 a ^y	0.46 b	1.08 a
2	13.21 a	5.58 c	10.20 b
3	39.95 a	30.60 a	29.51 a
Spores ^z ($\times 10^{-4}$ /ml)	2.43 b	4.00 a	2.63 b

^y Means within a row followed by the same letter are not significantly ($P < 0.05$) different based on Duncan's multiple range test.

^z Spore concentration was determined after 5 days on PDA.

directly from the reaction mixture or diluted 1:1 with sterile distilled H₂O. Samples were prepared for electrophoresis by mixing 3 µl of product with an equal volume of loading buffer (4) and separated on 0.45-mm-thick, 10% polyacrylamide 7 M urea gels backed on Gelbond polyacrylamide gel electrophoresis with a constant 180 V. DNA was visualized with a fast, sensitive silver-staining procedure that detects picogram quantities of DNA (2). Gels were examined with a light box, and bands of 700 bp or fewer were recorded as either present (1) or absent (0); missing or uncertain data was designated as 9. All binary data were entered as unordered, nondirected, and unweighted characters by primer and isolate, and Jaccard similarity coefficients were calculated using the Numerical Taxonomy and Multivariate Analysis System, version 1.8 (Exeter Software, Setauket, NY).

RESULTS

Growth in vitro. The growth of the three isolates of *B. cinerea* on PDA is shown in Table 1. At 1 and 2 days after inoculation, isolate B had the smallest mycelial growth area. At day 3, no significant ($P < 0.05$) difference in growth area was found between the isolates. After 4 days, the mycelia had covered the 8.3-cm-diameter agar plate in all samples, with a total growth area of 50 cm². The conidial concentration was determined after 5 days of growth on PDA. Isolate B produced more conidia than the other isolates, and the sporulation rate of isolate B was 40% higher than that of isolates C and C77:4. No visible morphological differences were detected among the three isolates when grown on PDA and microscopically examined. Nuclear staining of the conidia revealed an average of two nuclei per conidium for each isolate.

When grown in liquid culture with modified Richard's solution, isolate C grew well in the control media (no Ca added), and mycelial dry weight continued to increase up to 10 days after inoculation (Fig. 1A). Mycelial growth of isolate B followed the same pattern, and the growth rate was twofold higher compared with isolate C (Fig. 1B). Isolate C77:4 had the lowest growth rate in this medium and growth stopped 7 days after inoculation (Fig. 1C). Dry weight of isolate C increased and growth was stimulated by Ca (CaCl₂) concentrations up to 4 g liter⁻¹. No further stimulation was observed when grown at a concentration of 16 g liter⁻¹ (Fig. 1A). Growth of isolate B was particularly stimulated at the lowest CaCl₂ concentration (1 g liter⁻¹) (Fig. 1B). Maximum growth rate was reached when isolate C77:4 was grown at a concentration of CaCl₂ of 1 g liter⁻¹ (Fig. 1C).

Protein secretion and PG activity. Seven days after growth on modified Richard's solution containing CaCl₂ at 0, 1, or 16 g liter⁻¹, the total amounts of exocellular protein and PG specific activity were determined (Table 2). Total protein secreted by isolates C and C77:4 was not significantly ($P < 0.05$) affected by CaCl₂,

while both isolates exhibited a significant ($P < 0.05$) 30% decrease in PG specific activity when grown on media containing Ca. When grown with a high Ca concentration, protein production and secretion of isolate B doubled. PG-specific activity, however, was significantly ($P < 0.05$) reduced by 60% compared with the control.

Pathogenicity. The pathogenicity of each isolate, expressed as the area of decay, was tested on four apple cultivars (Fig. 2). Isolate B was more virulent than isolates C and C77:4 on 'Golden Delicious' apples 7 days after inoculation (Fig. 2A). However, isolate C was more virulent on 'Mutsu' and 'Arkansas' (Fig. 2B and C). Virulence levels of isolates B and C on 'Rome' were similar to each other (Fig. 2D) and greater than that of isolate C77:4.

To study the effect of Ca on disease development, Ca-infiltrated 'Red Delicious' apples were inoculated with each isolate (Fig. 3).

Fig. 2. Pathogenicity of three isolates of *Botrytis cinerea* on fruits of four apple cultivars. Data bars are means ($\pm$ standard error) of 20 replicates.

TABLE 2. Calcium effect on exocellular protein production and polygalacturonase (PG) activity of three *Botrytis cinerea* isolates after 7 days of growth in Richard's solution

Isolate	CaCl ₂ (g liter ⁻¹)	Protein secreted (µg/mg mycelial dry weight)	PG specific activity (units/µg of protein)
C	0	14.22 a ^z	2.82 a
	1	13.40 a	1.89 b
	16	13.79 a	1.93 b
B	0	10.14 b	1.78 a
	1	7.58 b	2.41 a
	16	21.21 a	0.72 b
C77:4	0	20.44 a	1.94 a
	1	13.20 a	1.37 b
	16	12.29 a	1.15 b

^z Means in the same column within each isolate followed by the same letter are not significantly ($P < 0.05$) different based on Duncan's multiple range test.

Calcium infiltration reduced decay caused by each isolate. The effect of Ca was particularly evident for isolates C and C77:4 (Fig. 3A and B), resulting in a decayed area that was five times less severe than that of control fruit. Decay incited by isolate B was reduced threefold (Fig. 3C).

Statistical analysis. In vitro, the variability in growth among replicates was significantly ($P < 0.01$) different among all three isolates (Table 3). On PDA, isolate B had the most and isolate C had the least variability. In Richard's solution, isolate B still had the most but isolate C77:4 had the least variability. Isolate C77:4 exhibited the most variability in total protein content. However, isolate C77:4 had the least variability in PG specific activity among replicates. There was no significant ($P < 0.01$) difference in variability among replicates for pathogenicity among the three isolates. Variability in pathogenicity among replicates of the three isolates was not significant across cultivars or Ca treatments.

Genetic variation. DNA analysis of each isolate was performed, and DNA profiles generated by each of the six primers were reproducible. A total of 146 character loci were scored and 61 ($\approx 42\%$) were polymorphic. Figure 4 shows the profiles gene-

rated by primers 8.7D and 8.6H. The calculated Jaccard similarity coefficient indicated that isolates B and C77:4 were more similar to each other than to isolate C (data not shown).

DISCUSSION

The three *B. cinerea* isolates used were isolated from different sources (decayed grape and apple), and origins (United States and France), and had each been subcultured numerous times. When grown on PDA, isolate B showed the slowest growth rate during the first 2 days. This slow growth rate might be correlated with the high sporulation rate we observed for this isolate after 5 days on PDA. Variations in growth and sporulation rates have already been shown in different strains and isolates of *B. cinerea* (16), but the determining factor has not yet been elucidated. Isolate B also had the highest growth rate in modified Richard's solution. Variations in fungi are caused mainly by mutation and heterokaryosis (7). Therefore, the more frequently an isolate is subcultured, the higher the possibility of variation. The variation between the three isolates was more apparent in vitro. In situ, all three isolates exhibited the same variability. Each isolate used contained an average of 2 nuclei per conidium, but some *Botrytis* strains have up to 20 nuclei per conidium (27). This implies that heterokaryosis was limited in the three isolates used in our study. DAF analysis of the three isolates showed that 42% of the character loci were polymorphic, and the Jaccard similarity coefficient showed that isolates C77:4 and B, not related in origin or source, were closer to each other than to isolate C. This agreed with previous work showing that *Botrytis* strains isolated from the same host were not genetically alike (43).

Fungal mutation is one of the main sources for the emergence of isolates or strains resistant to fungicides (7). Isolate B produced twice as much biomass as isolate C in Richard's solution. This growth, however, was not related to an increase in protein secreted nor to an increase in PG-specific activity. Nevertheless, some differences observed in vitro were associated with differences observed in situ. No significant difference between isolates was observed regarding the secretion of fungal protein in vitro, but isolate C showed the highest PG-specific activity and exhibited the highest virulence on two of four apple cultivars. Enzyme activities of *Botrytis* spp. depend on the isolate or strain (44), particularly pectic enzymes, such as PG, which are involved in the infection process. PG has been studied extensively (23,42) and is composed of isoenzymes whose expression is influenced by the

Fig. 3. Effect of calcium infiltration of 'Red Delicious' apples on the pathogenicity of three isolates of *Botrytis cinerea*. Data bars are means ($\pm$ standard error) of 80 replicates.

TABLE 3. Variability estimates from growth, protein content, and pathogenicity data from three isolates of *Botrytis cinerea*^{y,z}

Variable	Isolate C	Isolate B	Isolate C77:4
Growth			
PDA	* _a	*** _b	** _c
Richard's Solution	** _a	*** _b	* _c
Protein			
Total protein	** _a	* _a	*** _b
PG specific activity	** _a	*** _a	* _b
Pathogenicity			
'Mutzu'	** _a	*** _a	* _a
'Rome'	** _a	*** _a	* _a
'Arkansas'	*** _a	** _a	* _a
'Golden Delicious'	** _a	* _a	*** _a
Ca effect on pathogenicity	*** _a	* _a	** _a

^y Restricted maximum likelihood estimates of residual variances were calculated using the MIXED procedure. The model removed all effects of Ca treatment, isolate, and time when applicable, leaving a pooled residual variance among the replicates. Within this model, the pooled residual was estimated separately for each isolate. The more variable isolate was arbitrarily qualified as ***, the least variable as *.

^z Estimates were compared with pairwise *F* tests between isolates. Variances within a row followed by the same letter are not significantly ($P < 0.01$) different.

growth medium (41). The constitutive PG measured in this study has been identified as PG2 and is part of the pool of PG isoenzymes involved in pathogenesis (23). The correlation we found between virulence and PG activity agreed with previous work (42). We also found that isolate B, originally isolated from Golden Delicious, was more virulent on this cultivar, compared with the two other isolates. Since host-specificity has never been found in *B. cinerea*, this result might be purely coincidental. Some apple cultivars were more (Mutzu) or less (Arkansas) susceptible to infection. A number of reasons could explain this observation, such as differences in carbohydrate content (25) or the presence of phenolic compounds (22,44) and phytoalexins (18,19), as well as the quality of the cell wall after 6 months storage at 0°C (1).

Calcium is required in minute quantities for mycelial growth (12) and the control medium (Richard's solution) used in our study contained Ca at 3.74 mg liter⁻¹ (determined by inductively coupled argon plasma atomic emission spectrometry). The cation is involved in several functions, such as synthesis of fungal cell wall polymers, vesicle migration, and tip growth (34). We found that Ca stimulated growth of all three isolates at low concentration (CaCl₂ at up to 4 g liter⁻¹) and that increasing Ca concentration fourfold in the media did not increase mycelial growth to the same extent. Kaile et al. (20) reported that mycelia cultured with 100 to 200 mM Ca²⁺ exhibited a lower viability compared with mycelia grown with 10 mM Ca and Ca decreased growth rate (45) and PG activity (3) for some isolates of *Botrytis* spp. Our data showed that low Ca concentration has no effect on protein secretion but did inhibit PG activity. Polygalacturonase activity of isolates C and C77:4 was inhibited at low Ca concentrations, whereas PG activity of isolate B was inhibited at higher Ca concentrations. This difference in susceptibility to Ca inhibition, as well as the difference in PG activity in the control medium, implied that the

three isolates produced PGs that were different in their isoenzyme composition, as has been reported for some *B. cinerea* isolates (26).

Calcium infiltration is effective in controlling decay. Externally applied Ca can accumulate in plant tissue (10), especially in the cell wall (6,9), where it cross-links the polygalacturonate polymers. Consequently, the plant cell wall is strengthened, resulting in a reduction in decay caused by *B. cinerea* (8). Previous work showed that when apple fruit were infiltrated with 4% CaCl₂, Ca content of the cell wall was increased 5 to 6 times (1,8). Therefore, we could expect the same increase in Ca content of the apple cell wall in our experiment. During the infection process, Ca²⁺ concentration in the apparent free space (AFS) increased (20) due to the hydrolysis of pectic substances by fungal enzymes. Apparently, the increase in Ca content of the cell wall after infiltration and the consecutive increase in free Ca in the AFS were sufficient enough to reduce fungal infection by all three isolates.

Resistance to organic and inorganic fungicides has been shown in *Botrytis* spp., such as resistance to copper sulfate in *B. cinerea* (30). Calcium is not fungicidal and no resistance to high Ca concentration has been shown. Biological control of *B. cinerea* has been introduced as an alternative strategy to limit fungal infection. The use of yeasts (28,32,45), bacteria (21), or antagonistic fungi (14,40) has successfully controlled infection by *Botrytis* spp. in greenhouses and the field. Calcium increases the efficacy of this biological control, presumably by directly affecting spore germination and metabolism of the pathogen and indirectly by not altering the normal metabolism of the biocontrol organism when Ca is present at high concentrations (45).

Our results indicate that biological and statistical variability can be affected by the *B. cinerea* isolate selected. Despite this variation, Ca reduces the potential of the fungus to infect the host by limiting fungal growth, inhibiting PG activity, and decreasing plant tissue susceptibility to decay caused by *B. cinerea*.

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LITERATURE CITED

- Abbott, J. A., Conway, W. S., and Sams, C. E. 1989. Postharvest calcium chloride infiltration affects textural attributes of apples. *J. Am. Soc. Hortic. Sci.* 114:932-936.
- Bassam, B. J., Caetano-Anollés, G., and Gresshoff, P. M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196:80-83.
- Biggs, A. R., El-Kholi, M. M., El-Neshawy, S., and Nickerson, R. 1997. Effects of calcium salts on growth, polygalacturonase activity, and infection of peach fruit by *Monilinia fructicola*. *Plant Dis.* 81:399-403.
- Caetano-Anollés, G., Bassam, B. J., and Gresshoff, P. M. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* 9:553-557.
- Chardonnet, C. O., and Donèche, B. 1995. Relation entre la teneur en calcium et la résistance à la digestion enzymatique du tissu pelliculaire au cours de la maturation du raisin. *Vitis* 34:95-98.
- Chardonnet, C. O., and Donèche, B. 1995. Influence of calcium pretreatment on pectic substance evolution in cucumber fruit (*Cucumis sativus*) during *Botrytis cinerea* infection. *Phytoparasitica* 23:335-344.
- Coley-Smith, J. R., Verhoeff, K., and Jarvis, W. R. 1980. *The Biology of Botrytis*. Academic Press, London.
- Conway, W. S., Gross, K. C., Boyer, C. D., and Sams, C. E. 1988. Inhibition of *Penicillium expansum* polygalacturonase activity by increased apple cell wall calcium. *Phytopathology* 78:1052-1055.
- Conway, W. S., Gross, K. C., and Sams, C. E. 1987. Relationship of bound calcium and inoculum concentration to the effect of postharvest

Fig. 4. DNA amplification fingerprinting of three isolates of *Botrytis cinerea* with primers 8.7D (CCGAGCTG) and 8.6H (GAAACGCC). Some polymorphisms are indicated by arrow heads. M = molecular weight (base pairs); CN = isolate C; BL = isolate B; and FR = isolate C77:4.

- calcium treatment on decay of apples caused by *Penicillium expansum*. Plant Dis. 71:78-80.
10. Conway, W. S., and Sams, C. E. 1983. Calcium infiltration of Golden Delicious apples and its effect on decay. Phytopathology 73:1068-1071.
11. Conway, W. S., Sams, C. E., Abbott, J. A., and Bruton, B. D. 1991. Postharvest calcium treatment of apple fruit to provide broad-spectrum protection against postharvest pathogens. Plant Dis. 75:620-622.
12. Dhingra, O. D., and Sinclair, J. B. 1985. Basic Plant Pathology Methods. CRC Press, Boca Raton, FL.
13. Droby, S., Wisniewski, M. E., Cohen, L., Weiss, B., Toutiou, D., Eilam, Y., and Chalutz, E. 1997. Influence of CaCl_2 on *Penicillium digitatum*, grapefruit peel tissue, and biocontrol activity of *Pichia guilliermondii*. Phytopathology 87:310-315.
14. Eden, M. A., Hill, R. A., and Stewart, A. 1996. Biological control of Botrytis stem infection of greenhouse tomatoes. Plant Pathol. 45:276-284.
15. Fahmy, T. 1923. The production by *Fusarium solani* of a toxic excretory substance capable of causing wilting in plants. Phytopathology 13:543-550.
16. Grindle, M. 1979. Phenotypic differences between natural and induced variants of *Botrytis cinerea*. J. Gen. Microbiol. 111:109-120.
17. Gross, K. C. 1982. A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. HortScience 17:933-934.
18. Hrazdina, G., Borejsza-Wysocki, W., and Lester, C. 1997. Phytoalexin production in an apple cultivar resistant to *Venturia inaequalis*. Phytopathology 87:868-876.
19. Jeandet, P., Sbaghi, M., Bessis, R., Verhoeff, K., Malathrakakis, N. E., and Williamson, B. 1992. The use of phytoalexin induction and of *in vitro* methods as a tool for screening grapevines for resistance to *Botrytis cinerea*. Pages 109-112 in: Rec. Adv. in Botrytis Res., Proc. 10th Int. Symp. K. Verhoeff and N. E. Malathrakakis, eds. Pudoc Scientific Publishers, Wageningen, Netherlands.
20. Kaile, A., Pitt, D., and Khun, P. J. 1992. Calcium cytotoxicity, protoplast viability and the role of calcium in soft-rot of *Brassica napus* due to *Botrytis cinerea* Pers. Physiol. Mol. Plant Pathol. 40:49-62.
21. Ketterer, N., Fischer, B., Weltzien, H. C., Verhoeff, K., Malathrakakis, N. E., and Williamson, B. 1992. Biological control of *Botrytis cinerea* on grapevine by compost extract and their microorganisms in pure culture. Pages 179-186 in: Rec. Adv. in Botrytis Res., Proc. 10th Int. Symp. K. Verhoeff and N. E. Malathrakakis, eds. Pudoc Scientific Publishers, Wageningen, Netherlands.
22. Kritzman, G., and Chet, I. 1980. The role of phenols in the pathogenicity of *Botrytis allii*. Phytoparasitica 8:27-37.
23. Leone, G. 1990. *In vivo* and *in vitro* phosphate-dependent polygalacturonase production by different isolates of *Botrytis cinerea*. Mycol. Res. 94:1039-1045.
24. Lowry, D. H., Rosebrough, W. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.
25. Macfoy, C. A., and Smith, I. M. 1985. Interrelationship between nutrients, pathogenicity, and phytoalexin metabolism of *Botrytis cinerea* on clover leaves. Phytopathology 116:193-200.
26. Magro, P., Di Lenna, P., Marciano, P., and Pallavicini, C. 1980. Variability of polygalacturonase and protein isoelectric focusing patterns in *Botrytis cinerea* isolates. J. Gen. Microbiol. 120:105-109.
27. Menzinger, W. 1965. Karyologische untersuchungen an arten und formen der gattung *Botrytis* Mich. Arch. für Mikrobiol. 52:178-196.
28. Mercier, J. and Wilson, C. L. 1995. Effect of wound moisture on the biocontrol by *Candida oleophila* of gray mold rot (*Botrytis cinerea*) of apple. Postharvest Biol. Technol. 6:9-15.
29. Michailides, T. J., and Morgan, D. P. 1996. Using incidence of *Botrytis cinerea* in kiwi fruit sepals and receptacles to predict gray mold decay in storage. Plant Dis. 80:248-254.
30. Parry, K. E., and Wood, R. K. S. 1958. The adaptation of fungi to fungicides: Adaptation to copper and mercury salts. Ann. Appl. Biol. 46:446-456.
31. Powelson, R. L. 1960. Initiation of strawberry fruit rot caused by *Botrytis cinerea*. Phytopathology 50:491-494.
32. Redmond, J. C., Marois, J. J., and MacDonald, J. C. 1987. Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. Plant Dis. 71:799-802.
33. Reyes, A. A. 1990. Pathogenicity, growth and sporulation of *Mucor mucedo* and *Botrytis cinerea* in cold or CA storage. HortScience 25:549-552.
34. Ruiz-Herrera, J. 1992. Fungal cell wall: Structure, synthesis and assembly. CRC Press, Boca Raton, FL.
35. Sirjusingh, C., and Sutton, J. C. 1996. Effects of wetness duration and temperature on infection of geranium by *Botrytis cinerea*. Plant Dis. 80:160-165.
36. Slater, M. L. 1976. Rapid nuclear staining method for *Saccharomyces cerevisiae*. J. Bacteriol. 126:1339-1341.
37. Steel, R. G. D., and Torrie, J. H. 1980. Page 111 in Comparing all means with a control: Principles and procedures of statistics. 2nd ed. McGraw-Hill, Inc.; New York.
38. Triganio, R. N., Caetano-Anollés, G., and Windham, M. T. 1995. DNA amplification fingerprinting provides evidence that *Discula destructiva*, the cause of dogwood anthracnose in North America, is an introduced pathogen. Mycologia 87:490-500.
39. Triganio, R. N., Gerharty, N. E., Windham, W. T., and Brown, D. A. 1991. A simple assay for separating fungi associated with dogwood anthracnose. Proc. of S. Nursery-men's Assoc. Res. Conf. 36:209-211.
40. Tronsmo, A., and Dennis, C. 1974. The use of *Trichoderma* species to control strawberry fruit rots. Neth. J. Plant Pathol. 83:449-455.
41. Van Den Heuvel, J., and Waterreus, L. P. 1985. Pectic enzymes associated with the phosphate-stimulated infection of French bean leaves by *Botrytis cinerea*. Neth. J. Plant Pathol. 91:253-264.
42. Van Der Cruyssen, G., Kamoen, O., Verhoeff, K., Malathrakakis, N. E., and Williamson, B. 1992. Polygalacturonases of *Botrytis cinerea*. Pages 69-75 in: Recent Advances in Botrytis Research, Proc. 10th Int. Symp. K. Verhoeff and N.E. Malathrakakis, eds. Pudoc Scientific Publishers, Wageningen, Netherlands.
43. Van Der Vlugt-Bergmans, C. J. B., Brandwagt, B. F., Vant Klooster, J. W., Wagemakers, C. A. M., and Van Kan, J. A. L. 1993. Genetic variation and segregation of DNA polymorphisms in *Botrytis cinerea*. Mycol. Res. 97:1193-1200.
44. Wasfy, E. M., Farag, S. A., Tarabieh, M. A., and Abd-Elmoety, S. M. 1978. Studies on enzymes of different strains of *Botrytis cinerea*. Phytopathology 92:168-179.
45. Wisniewski, M., Droby, S., Chalutz, E., and Eilam, Y. 1995. Effects of Ca^{2+} and Mg^{2+} on *Botrytis cinerea* and *Penicillium expansum* *in vitro* and on the biocontrol activity of *Candida oleophila*. Plant Pathol. 44:1016-1024.
46. Yoon, C. K., Glawe, D. A., and Shaw, P. D. 1991. A method for rapid small-scale preparation of fungal DNA. Mycologia 83:835-838.