Production and Transformation of Conidia of *Venturia inaequalis*

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ABSTRACT

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High yields of conidia of *Venturia inaequalis* were produced from either mycelial fragments or conidia on cellophane-covered surfaces of agar media incubated under continuous near-ultraviolet light. Optimal conditions required a cellophane-covered surface of potato-dextrose agar in combination with light and incubation for 1 wk. The procedure represents a convenient techique for the mass-production of clonal and sterile *V. inaequalis* conidia. Conidia were biolistically transformed to hygromycin B resistance with a plasmid (pOHT) containing a bacterial phosphotransferase gene spliced between regulatory elements from *Aspergillus nidulans*. Southern hybridization analysis showed that the plasmid was incorporated into heterologous regions of the genome. Hygromycin B–resistant transformants were mitotically stable during nonselective propagation. The heterologous gene conferring hygromycin B resistance was expressed during early stages of conidia germination and during vegetative growth of mycelium.

Venturia inaequalis (Cooke) G. Wint., the causal agent of apple scab, is an attractive fungal pathogen for the refined analysis of host-pathogen interactions. Mycelial and conidial cells are ordinarily uninucleate, the fungus is strictly haploid throughout its parasitic stages, and ascospores suited for tetrad analysis can be produced in vitro (1). The pathogen has been employed in recent studies on the role of cell-wall-degrading enzymes and cutinase in pathogenicity (15,16,30,31) and the sequence analysis of tubulin genes cloned from benzimidazole-sensitive and -resistant genotypes (14). One of the current shortcomings of the fungus is the lack of a transformation technique as an important tool for advanced molecular studies (18). Mycelial growth is slow and, thus, restricts the availability of large quantities of protoplasts, the most widely used material for established transformation systems (7,8).

Biolistic transformation of fungal conidia has been employed more recently as an alternative to more established transformation procedures (10,19,23). Unfortunately, conidia formation of *V. inaequalis* is sparse on readily available agar media (2,21,28,33,34). Poor sporulation is caused by slow mycelial expansion and, more importantly, the adverse effect of a film of free liquid on the surface of agar medium (2). Many of the reported alternatives for producing clonal and sterile conidia require host components such as apple twigs (34) or apple-leaf decoctions (5,29), which are not always accessible. The most widely used "wick" culture technique developed by Nusbaum and Keitt (21) and modified by others (13,33) is labor intensive and requires two steps for mass production of conidia.

Because the film of free liquid on agar surfaces is a major obstacle to sporulation of *V. inaequalis*, the growing mycelium

must be separated from the liquid phase on agar plates. This can be achieved with a permeable cellophane membrane as a boundary between the agar surface and growing mycelium, a method first employed by Fleming and Smith (9) for several fungi and briefly described for the production of conidia of *V. inaequalis* (31). The objective of the present study was to optimize the latter procedure of conidial production on solid media, and to biolistically transform conidia with pOHT, a plasmid carrying the bacterial phosphotransferase gene conferring resistance to hygromycin B as the selectable marker. This marker gene has been used in the transformation of numerous other fungi, including plant pathogens (8,18).

MATERIALS AND METHODS

Materials and fungal cultures. Cellophane membranes (215-PUT80) used in this study were obtained from Flexel Corporation (Atlanta, GA). Potato-dextrose agar (PDA), malt-extract agar (MEA), Bacto-agar (Difco) and malt extract were from Difco Laboratories (Detroit, MI). FISHER*brand* disposable petri dishes (100 × 15 mm) were from Fisher Scientific (Pittsburg, PA) and V8 juice from Campbell Soup Co. (Camden, NJ). If not specified otherwise, all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Isolate Maine 8 (15,27) was maintained by successive culturing on PDA and reisolation of single conidia from infected apple seedlings once per year. Additional monoconidial isolates used in the study were collected from orchards in New York State in 1992. Single conidia were isolated according to the procedure described by Smith et al (26).

Cellophane and media preparation. Cellophane membranes were cut into 8.0-cm disks and soaked in 3 L of water (HPLC grade) for 12 h. Soaked disks were layered between 9.0-cm-diameter filter paper (Whatman #4 qualitative) and placed in a glass petri dish. The dish was filled with water (HPLC grade), wrapped in aluminum foil, and autoclaved for 15 min at 120 C. The sterile disks were layered onto hardened surfaces of V8-juice agar (200 ml of V8 juice, 2.0 g of $CaCO_3$, and 1.7 g of agar in 1 L of water), PDA or MEA, avoiding the formation of air spaces.

Plasmid. The plasmid pOHT was provided by M. Ward (Genencor International, San Francisco, CA). The 5.3-kb pUC18 derivative contains a 2.65 insert consisting of the bacterial phosphotransferase gene (*hph*) fused to the *oliC* promoter and *trpC* terminator (32) from *Aspergillus nidulans* (Eidam) G. Wint. Plasmid DNA was amplified and purified according to standard procedures (22).

Conidial production. Mycelium of *V. inaequalis* was derived from fungal cultures grown on PDA for 4 wk (2 cm colony diameter). Three colonies were removed from the agar surface and blended in a Waring blender equipped with a sterile stainless steel container in 20 ml of water, for 10 × 10 s with pauses (2 s).

For conidial production, a suspension of blended mycelium (0.75 ml) was transferred to the cellophane-covered agar surface. Alternatively, the surface was seeded with a suspension of conidia (0.5 ml) at various densities. The dish was tipped until the suspension was evenly distributed across the surface. Dishes were sealed with Parafilm and incubated at 20 C in the dark. Culture dishes incubated in the light were placed in a single layer, at a distance of 25 cm from a continuous light source (General Electric, black light, F15T8/BLB, 15 watt). The light intensity at 390 nm (emission maximum) was 8.3 μW cm^{-2}.

After incubation for 1 to 4 wk, the cellophane was removed from the agar surface and placed in a 150-ml beaker containing 50 ml of water. The disk was stirred slowly for approximately 5 min to remove the conidia from the cellophane. The conidial suspension was poured through glass wool in a 20-ml syringe in order to remove residual mycelium. The suspension was centrifuged at 2,500 rpm for 10 min, the pellet resuspended in water, and the number of conidia was determined with a hemacytometer. Germination rates were determined after incubation of conidia on water agar (4%) for 24 h.

Transformation. Sterile conidial suspensions (isolate JF5 18) were centrifuged at 4,000 *g* for 10 min. The pellet was resuspended in water, and the conidial density was adjusted to 2.5×10^6 ml^{-1}. PDA (7 ml) containing either no osmoticum, or sorbitol at concentrations of 0.125 M or 0.25 M, was poured over the wet surface of a sterile Whatman No. 4 filter paper (9 cm diameter). This "pagar" procedure has been described in detail by Smith et al (25). A suspension of conidia (0.2 ml) was spread over the PDA, and the surface was air dried under sterile conditions prior to bombardment.

M5 tungsten particles (Sylvania, GTE Products Corp., Towanda, PA) were used as microprojectiles. The plasmid pOHT was precipitated onto the particles according to the coating protocol of Sanford et al (23) with the following modifications: For the preparation of aliquots sufficient for 6 bombardments, 50 μl particle suspensions containing 6-mg tungsten particles in 50% (v/v) glycerol, 5 μl of plasmid DNA (1 mg ml^{-1}), 50 μl of CaCl2 (2.5 M), and 20 μl of spermidine (0.1 M) were combined. The mixture was vortexed for 10 min and gently pelleted for 10 s by pulse centrifugation. The supernatant was removed, and the pellet was washed with 100 μl of 70% (v/v) and resuspended in 24 μl of 100% ethanol. The particle suspension was mixed by sonication in an ultrasonic cleaner bath (Branson 1200), followed by gentle shaking until the particle suspension appeared homogenous. Aliquots of 4 μl (0.8 μg of DNA precipitated on 1 mg tungsten particles) were spread over the centers of Kapton flying disks. Loaded disks were dried and stored in a dessicator until use.

A helium-driven particle accelerater device with flying disks for particle delivery was used for biolistic transformation (23,24). The distance between the helium source and the flying disk was 1 cm, and the distance between the particle launch site and conidia was 6 cm. Prior to drawing the vacuum (736 mm of Hg), the vacuum chamber was flushed with helium for 5 s. Helium pressures used to accelerate the flying disk were 1,000, 1,250 or 1,500 psi.

Selection of transformants. The bombarded "pagars" (filter papers overlayered with PDA) were transferred, either immediately after bombardment or with a 17 h delay, to the surface of 21 ml of PDA (10-cm-diameter petri dishes) containing 0.133 mg ml^{-1} hygromycin B. The final hygromycin B concentration after full equilibration of the inhibitor between both agar layers was 0.1 mg ml^{-1}. Two controls in at least three replicates were included in each experiment: Conidia not bombarded but transferred to the selection medium and conidia bombarded with particles not coated with DNA were included as controls.

DNA analysis. Mycelium of fungal cultures (1 cm colony diameter) grown on PDA was removed and blended in 100 ml of potato-dextrose broth (Difco Laboratories, Detroit, MI) and incubated for 18 days at 18 C. All media involving transformants contained 0.1 mg ml^{-1} hygromycin B. Mycelium was harvested by filtration through cheesecloth, frozen in liquid nitrogen in a mortar, and ground to a powder. Mycelial powder (0.75 ml approximate volume) was suspended in 0.75 ml of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% mercaptoethanol, pH 8). The suspension was heated to 65 C for 2 h, mixed with 0.75 ml of chloroform/phenol (1:1) and centrifuged for 5 min at 4,000 *g*. The aqueous phase was removed and mixed with 0.7 ml of chloroform. After brief centrifugation, 20 μl of 3 M sodium acetate (pH 5.2) and 1 ml of isopropanol were added to the aqueous phase. After incubation for 10 min at room temperature and centrifugation for 2 min at 10,000 *g*, the pellet was suspended in 0.3 ml of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and 50 μl of 3 M sodium acetate (pH 5.2). Cold (−20 C) 100% ethanol (1 ml) was added, and DNA was recovered by centrifugation (2 min at 10,000 *g*). The DNA pellet was washed with 70% (v/v) ethanol, dried under vacuum, resuspended in 100 μl of TE, and incubated for 15 h at 4 C.

DNA was digested with *Xho*I (Promega, Madison, WI) according to the instruction of the manufacturer. Electrophoresis of DNA fragments was in 0.7% agarose gels (22), and transfer to Hybond-N membranes (Amersham, Arlington Height, IL) was done by vacuum blotting (10 × SSC) according to the procedure

of the manufacturer of the Model 785 vacuum blotter (Bio-Rad, Richmond, CA). Hybridization was performed according to standard procedures (22). Plasmid DNA (pOHT) was digested with *Hind*III (one restriction site) and labeled with α^{-32}P-dCTP (Du Pont, Boston, MA) according to the random prime labeling method (6).

Mitotic stability. Mycelium from the edges of transformant colonies was recultured twice for 4 wk at 20 C on PDA, or on PDA containing hygromycin B (0.1 mg ml^{-1}). Conidia were produced from mycelial colonies obtained from the second round of culturing. Conidial suspension (20 μl of 10^5 conidia ml^{-1}) were transferred to disks (4 mm diameter) of PDA containing no inhibitor or hygromycin B at concentrations of 0.1, 0.2, 0.5, or 1.0 mg ml^{-1}. Germination rates of 50 conidia each from three replicates were determined after 17 h incubation at 18 C. The sensitivity of mycelial growth to hygromycin B was determined by transfer (upside down) of hygromycin B–free agar disks containing germinated conidia to PDA plates amended with the above range of hygromycin B concentrations. Colony diameters were determined after incubation at 20 C for 21 days.

RESULTS

Comparison of procedures for conidia production. Initial results with the cellophane technique in combination with V8-juice agar, a medium introduced and widely used for the induction of fungal sporulation (4,29), indicated no improvement of yields over the "wick" culture procedure (13,21,33). However, additional radiation with near-ultraviolet light (17,20,29) was successful. Yields of conidia achieved with three replicate cultures seeded with identical densities of mycelial fragments and after incubation for 2 wk were 1.46×10^5 ($\pm$ 0.12×10^5) per cellophane-covered V8-juice-agar surfaces incubated in the dark, and 3.75×10^7 ($\pm$ 1.42×10^7) per identical dish exposed to continuous light. Thus, light increased conidia production by a factor of 260. The triggering effect of light on sporulation was observed in several repeated experiments.

Time course and efficacy of conidia production. A time course study revealed that a first maximum of conidia production on cellophane-covered V8-juice agar was almost reached after 1 wk (Table 1). A sharp decline was observed after 3 wk, followed by a new increase in yields after 4 wk. The dual peak cycle of conidia production is explained by the germination of the first crop of conidia, followed by a second cycle of mycelial development and renewed sporulation.

The composition of the agar medium had impact on the yield of conidia. Optimal yields were achieved after 1 wk on PDA (2.2×10^7 conidia per culture). MEA (0.5×10^7 conidia per culture) and V8-juice agar (0.8×10^7 conidia per culture) were inferior to PDA. The comparative experiment was repeated twice with similar results.

The optical density of mycelial suspensions used for transfers ranged from 0.5 to 1 at 630 nm subsequent to a fivefold dilution with water. Blended PDA pieces contained in the suspension had no impact on the optical density at this wavelength. A typical optical density of 0.8 was equivalent to 4×10^5 ml^{-1} mycelial fragments. Decreasing the density of mycelial suspensions led to proportionally decreasing yields of conidia (data not shown). Increasing the mycelial density yielded suspensions too viscous to be evenly distributed across the cellophane surface. Blending of three mycelial colonies (2 cm diameter) in 20 ml of water represents optimal conditions for efficient production of conidia.

Conidial production was not only achieved with mycelial fragments, but also with conidia as starting material. The relation between the number of conidia employed in transfers and the yield of conidia after 1 wk of incubation on PDA was linear ($r^2 = 0.97$) (Fig. 1). The number of conidia was amplified by a factor of 30–40 through this second round of conidia production. This alternative procedure has the advantage of allowing conidia production from conidia stored as a frozen suspension.

Highest yields of conidia were achieved when single cellophane membranes covered with conidiating mycelium were stirred in 50 ml of water. Sequential washing of single membranes (up to 10 membranes per 50 ml) had clear advantages over an increased number of cellophane membranes washed simultaneously in larger volumes. Under these conditions, membranes easily adhered to each other and shielded conidia-covered surfaces.

The isolate Maine 8 of *V. inaequalis* used throughout the experiments described above was maintained by successive transfers to fresh PDA. Conidia production over a period of 3 yr was consistent and reliable. However, the yield of conidia production declined toward the end of 1 yr of mycelial cultivation. It could be fully restored by reisolation of a monoconidial culture from a scab lesion on apple seedlings infected with conidia that had been prepared on cellophane-covered agar media (data not shown). This decline and recovery of sporulation capacity is in full agreement with an earlier observation made by Nusbaum and Keitt (21).

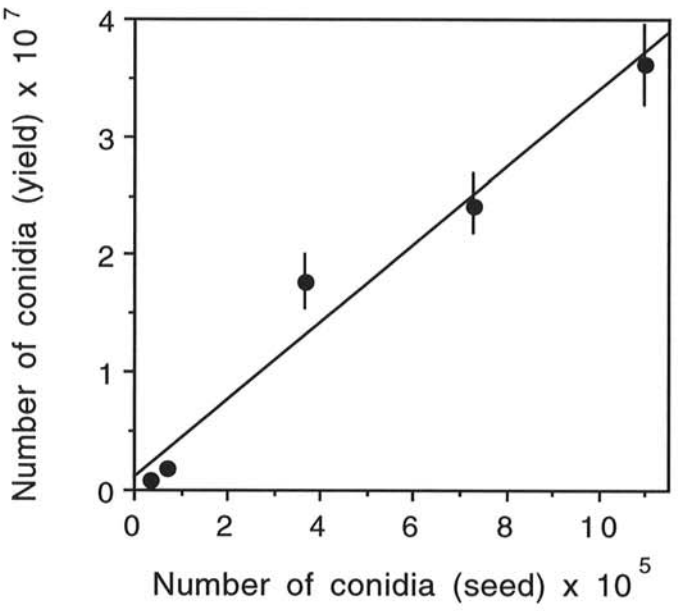

Fig. 1. Production of *Venturia inaequalis* conidia (isolate Maine-8). Cellophane-covered potato-dextrose agar plates were seeded with a conidial suspension in 0.5 ml of water and incubated for 1 wk under light. Number of conidia (seed and yield) are per plate. Values are means of three replicates; vertical bars represent standard deviations.

TABLE 1. Time course of light-induced conidia production by mycelium of *Venturia inaequalis* (isolate Maine 8) on cellophane-covered V8-juice agar

Time (wk)	Conidia per culture ($\times 10^7$)
1	0.74 (0.02)[a]
2	1.02 (0.26)
3	0.16 (0.03)
4	4.31 (0.99)

[a]Values are means of three replicates; standard deviations are in parentheses.

TABLE 2. Transformation frequencies obtained by biolistic transformation of conidia of *Venturia inaequalis*

	Sorbitol (M)		
Transformation	0	0.125	0.25
1	6.3 (6.1)[a]	9.0 (1.7)	17.0 (1.7)
2	4.4 (2.2)	6.0 (3.8)	8.6 (3.7)
3[b]	1.4 (1.3)	1.7 (1.1)	2.6 (1.4)

[a]Values are means (at least three replicates) of numbers of hygromycin B–resistant transformants per plate; each plate contained 5×10^5 conidia. Standard deviations are in parentheses.
[b]Conidia were frozen for 2 wk at −20 C prior to bombardment.

Phenotypic variation of conidia production. Preliminary results indicated that the capacity of monoconidial isolates to produce conidia was variable and dependent on the strain of *V. inaequalis*. This trait was investigated with 11 isolates collected and cultured under identical conditions and adjusted to uniform densities of mycelial suspensions prior to seeding cellophane-covered plates. The number of conidia produced per culture ranged from 0.2×10^6 to 5.3×10^6 and thus was different by a factor of 27. Similarly, conidia germination varied from 59 to 82%. The capacity of mycelium to produce conidia and the quality of conidia appear to reflect a phenotypic trait.

Transformation of conidia. Complete inhibition of germination of conidia prepared from isolate JF5 18 was achieved at 0.1 mg ml^{-1} hygromycin B, whereas the formation of mycelial colonies was fully suppressed at 0.05 mg ml^{-1}. For the selection of transformants, PDA was therefore ammended with 0.1 mg ml^{-1} hygromycin B.

After bombardment of conidia, hygromycin B–resistant colonies were visible after 10–12 days of growth at 18 C. Transformation frequencies were not significantly affected by the helium pressure employed in particle acceleration, or by a delayed transfer of bombarded "pagars" to the selective PDA agar. However, osmotic stabilization of bombarded cells with sorbitol had positive effects. In three separate experiments, highest frequencies were achieved in the presence of 0.25 M sorbitol (Table 2). Furthermore, the transformation frequency was lower with conidia that had been frozen for 2 wk at −20 C prior to bombardment (Table 2), indicating that freshly prepared conidia are the more suitable material for biolistic transformations.

No hygromycin B–resistant colonies were observed on 12 control plates not subjected to particle bombardment. Plates bombarded with particles not coated with the selectable marker gene showed a central zone of reduced growth under nonselective conditions caused by physical damage of conidia. However, the periphery of plates was completely overgrown after 10–12 days, demonstrating the viability of the conidia employed in the experiment and lack of substantial damage of cells. The locations of transformants were random over the entire surface and not restricted to the central region.

Under optimal conditions (0.25 M sorbitol and freshly prepared conidia), the transformation frequencies of 9–17 transformants per 0.8 μg of plasmid DNA per 5×10^5 conidia (Table 2) were lower than for *Trichoderma harzianum* Rifai and *Gliocladium virens* J. H. Miller, J. E. GIddus, & A. A. Foster bombarded at higher conidial densities (600 transformants per 0.8 μg of plasmid DNA per 10^7 conidia) (19) and higher than for *Botryotinia fuckeliana* (de Bary) Whetzel (15 transformants per 0.8 μg of plasmid DNA per 3×10^7 conidia). Transformation frequencies compare favorably with frequencies normally achieved after regeneration and selection of transformed protoplasts. For example, frequencies reported for 10^7 protoplasts employed were 1–5 per 25 μg of DNA for *Botrytis squamosa* Vien. Bourg. (12), 10–50 per μg of DNA for *Ustilago* spp. (11), or 10–40 per 5 μg of DNA for several filamentous fungi (3).

Southern hybridization analysis. The incorporation of pOHT into the genome of *V. inaequalis* was investigated by Southern analysis. Genomic DNA isolated from the wild-type strain and seven randomly selected hygromycin B–resistant transformants was digested with *Xho*I, and genomic DNA fragments were hybridized to [^{32}P]-labeled pOHT plasmid DNA after electrophoresis and transfer to membranes. DNA from each of the transformants yielded fragments that hybridized to the plasmid (Fig. 2). Only one of the transformants (T1) yielded two hybridizing fragments. Because pOHT lacked a restriction site for *Xho*I, copies of the plasmid were, most likely, incorporated at two different sites. No hybridization was observed with DNA isolated from the wild-type strain.

Mitotic stability. Mitotic stability of hygromycin B–resistant transformants was tested by continuous mycelial growth in the absence and presence of selective pressure and subsequent conidiation. All transformants had retained resistance to hygromycin B in the absence of selective pressure (Table 3). Levels of resistance were identical for transformants propagated in the presence of hygromycin B (data not shown). In general, germinating conidia generated from transformants were slightly less sensitive to the inhibitor than mycelial cells (Table 3).

DISCUSSION

Most transformation techniques of fungi including plant pathogens require the preparation of large quantities of protoplasts (7,8). However, due to slow mycelial growth in liquid culture and poor germination of spores under submerged conditions, the starting material for protoplast preparation is not readily available for *V. inaequalis*.

The biolistic transformation of resting conidia has been employed as an efficient alternative to other transformation techniques (10,19,23). Adaptation of this technique to *V. inaequalis* required the development of a procedure for the production of sterile and clonal conidia. Although many variations of conidia production have been developed in the past (5,13,16,21,29,31, 33,34), none was ideally suited for conidial mass production. Optimal conidia production was accomplished by combining the technique of cellophane-covered agar surfaces (9) with the triggering effect of light (17,20,29). Conidia formation by *V. inaequalis* on malt-extract agar covered with cellophane was briefly described by Wagner et al (31), but the important effect of radiation was never reported before.

The advantages over previous procedures are manyfold. All components are readily available and not dependent on host material, the space requirements are minimal, the yields are high, and conidia can be readily produced from mycelium. Overall, the procedure should be applicable to a wide variety of studies

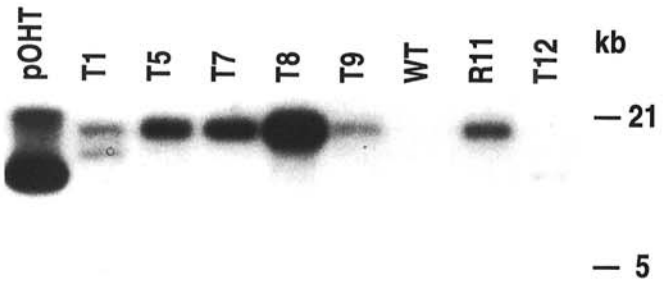

Fig. 2. Southern hybridization analysis of *Venturia inaequalis* transformants. *Xho*I-digested genomic DNA was probed with [^{32}P]-labeled pOHT following electrophoresis and transfer to membranes. pOHT, transformation vector; WT, wild-type; T1–T12, transformants.

TABLE 3. Sensitivity of *Venturia inaequalis* to hygromycin B after nonselective propagation

	Inhibition (%)							
	Mycelial growth				Conidia germination			
Strain	0.1	0.2	0.5	1.0[a]	0.1	0.2	0.5	1.0[a]
WT[b]	100[c]	100	100	100	94	94	93	96
T1[d]	4	11	44	100	5	24	26	37
T5	0	0	43	100	0	0	22	50
T7	0	0	5	83	4	1	5	1
T8	0	0	47	100	3	10	10	4
T9	0	0	29	100	9	12	22	92
T11	0	15	89	100	0	4	17	58
T12	0	7	47	100	0	0	45	81

[a] Concentration of hygromycin B (mg ml^{-1}).
[b] Wild-type.
[c] Values are means of three replicates. Standard deviations did not exceed 20%.
[d] T = transformant.

involving conidia of *V. inaequalis*. Depending on the scopes of studies employing conidia, the phenotypic variability of conidia production must be considered as a potentially limiting factor.

The optimized production of sterile and clonal conidia from mycelium of *V. inaequalis* provided the basis for the biolistic transformation of the fungus.The constitutive and mitotically stable expression of the selectable marker gene in combination with high transformation frequencies provides a suitable system for studies employing genetic manipulation of the pathogen.

LITERATURE CITED

1. Boone, D. M. 1988. *Venturia inaequalis,* cause of scab of apples. Adv. Plant Pathol. 6:249-261.
2. Bray, M. F., and Austin, W. G. 1962. Conidial suspensions of *Venturia inaequalis* for inoculation of apple rootstock. Plant Pathol. 11:106-107.
3. Daboussi, M. J., Djeballi, A., Gerlinger, C., Blaiseau, P. L., Bouvier, I., Cassan, M., Lebrun, M. H., Parisot, D., and Brygoo, Y. 1989. Transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans.* Curr. Genet. 15:453-456.
4. Diener, U. L. 1955. Sporulation in pure culture by *Stemphylium solani.* Phytopathology 45:141-145.
5. Dingra, O. D., and Sinclair, J. B. 1985. Basic Plant Pathology Methods. CRC Press, Boca Raton, FL.
6. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
7. Fincham, J. R. 1989. Transformation in fungi. Microbiol. Rev. 53:148-170.
8. Finkelstein, D. B. 1992. Transformation. Pages 113-156 in: Biotechnology of Filamentous Fungi—Technology and Products. D. B. Finkelstein and C. Ball, eds. Butterworth-Heinemann, New York.
9. Fleming, A., and Smith, G. 1944. Some methods for the study of moulds. Trans. Br. Mycol. Soc. 27:13-19.
10. Hilber, U. W., Smith, F. D., and Köller, W. 1993. Biolistic transformation of conidia of *Botryotinia fuckeliana.* Curr. Genet. 25:124-127.
11. Holden, D. W., Wang, J., and Leong, S. A. 1988. DNA-mediated transformation of *Ustilago hordei* and *Ustilago nigra.* Physiol. Mol. Plant Pathol. 33:235-239.
12. Huang, D., Bhairi, S., and Staples, R. C. 1989. A transformation procedure for *Botryotinia squamosa.* Curr. Genet. 15:411-414.
13. Kirkham, D. S. 1956. A culture technique for *Venturia* spp. and a turbidimetric method for the estimation of comparative sporulation. Nature 178:550-551.
14. Koenraadt, H., Somerville, S. C., and Jones, A. L. 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Phytopathology 82:1348-1354.
15. Köller, W., and Parker, D. M. 1989. Purification and characterization of cutinase from *Venturia inaequalis.* Phytopathology 79:278-282.
16. Köller, W., and Parker, D. M., and Becker, C. M. 1991. Role of cutinase in the penetration of apple leaves by *Venturia inaequalis.* Phytopathology 81:1375-1379.
17. Leach, C. M. 1971. A practical guide to the effects of visible and ultraviolet light on fungi. Methods Microbiol. 4:609-664.
18. Leong, S. A., and Holden, D. W. 1989. Molecular genetic approaches to the study of fungal pathogenesis. Annu. Rev. Phytopathol. 27:463-481.
19. Lorito, M., Hayes, C. K., Di-pietro, A. and Harman, G. E. 1993. Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. Curr. Genet. 24:349-356.
20. Marsh, P. B., Taylor, E. E., and Bassler, L. M. 1959. A guide to the literature on certain effects of light on fungi: Reproduction, morphology, pigmentation, and phototropic phenomena. Plant Dis. Rep. Suppl. 261:251-312.
21. Nusbaum, C. J., and Keitt, G. W. 1938. A cytological study of *Venturia inaequalis* on apple leaves. J. Agric. Res. 56:595-618.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning—A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
23. Sanford, J. C., Smith, F. D., and Russel, J. A. 1993. Optimizing the biolistic process for different biological applications. Methods Enzymol. 217:483-509.
24. Shark, K. B., Smith, F. D., Harpending, P. R., Sanford, J. C. 1991. Biolistic transformation of a prokaryote, *Bacillus megaterium.* Appl. Environ. Microbiol. 57:480-485.
25. Smith, F. D., Harpending, P. R., Sanford, J. C. 1992. Biolistic transformation of prokaryotes: Factors that affect biolistic transformation of very small cells. J. Gen. Microbiol. 138:239-248.
26. Smith, F. D., Parker, D. M., and Köller, W. 1991. Sensitivity distribution of *Venturia inaequalis* to the sterol demethylation inhibitor flusilazole: Baseline sensitivity and implications for resistance monitoring. Phytopathology 81:392-396.
27. Stanis, V. F., and Jones, A. L. 1984. Genetics of benomyl resistance in *Venturia inaequalis* from North and South America, Europe and New Zealand. Can. J. Plant Pathol. 6:283-290.
28. Szkolnik, M. 1986. Preparation of inoculum for brown rot of stone fruit and apple scab. Pages 71-72 in: Methods for Evaluating Pesticides for Control of Plant Pathogens. K. D. Hickey, ed. APS Press, St. Paul, MN.
29. Tuite, J. 1969. Plant Pathological Methods—Fungi and Bacteria. Burgess Publishing Company, Minneapolis, MN.
30. Valsangiacomo, C. and Gessler, C. 1992. Purification and characterization of an exo-polygalacturonase produced by *Venturia inaequalis.* Physiol. Mol. Plant Pathol. 40:63-77.
31. Wagner, K., Hitz-Germann, L., Seng, J. M., and Gessler, C. 1988. Cellolytic ability of the scab fungus, *Venturia inaequalis.* J. Phytopathol. (Berl.) 123:217-221.
32. Ward, M. 1991. Chymosin production in *Aspergillus.* Pages 83-106 in: Molecular Industrial Mycology—Systems and Applications for Filamentous Fungi. S. A. Leong and R. M. Berka, eds. Marcel Dekker, New York.
33. Williams, E. B. 1978. Handling the apple scab organism in laboratory and greenhouse. Pages 16-18 in: Proceedings of the Apple and Pear Workshop—APS Special Report No. 26. A. L. Jones, and J. D. Gilpatrick, eds. American Phytopathological Society, St. Paul, MN.
34. Zobrist, L., and Bohnen, K. 1958. Eine Methode zur Massenproduction einheitlicher, keimfähiger Konidien von *Venturia inaequalis* (Cke.) Wint. Phytopathol. Z. 31:367-370.