Variation in Cultural Morphology and Virulence Among Protoplast-Regenerated Isolates of *Sclerotinia sclerotiorum*

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ABSTRACT

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The influences of source isolates and exposure to polyethylene glycol (PEG) on variations in cultural morphology and virulence among protoplast-regenerated isolates of *Sclerotinia sclerotiorum* were determined. More than 50 germinated protoplasts were isolated from each of three source isolates. One-half of the regenerants were exposed to a PEG treatment, and one-half were unexposed. Source and regenerate isolates were compared for variations in cultural morphology on potato-dextrose agar and in lesion diameters produced on celery petioles. Qualitative variations in cultural morphology, color, and sclerotial production occurred in most treatments. Quantitative variations in lesion diameters also occurred in most treatments, but interactions among isolates and PEG treatments were not significant. The stability of variation in selected regenerants was evaluated by serial transfer and evaluation of colony morphology and virulence for five subcultures after regeneration. Depending on the source isolate used, variation in regenerants tended to be unstable and to revert back to the characteristics of the source isolate. However, several virulent regenerants that did not produce sclerotia in culture were obtained. The results indicate that protoplast methods can be used for the production and isolation of isolates with unique characteristics.

Protoplasts obtained from fungi are being used increasingly in physiological, biochemical, and genetic studies of fungi (16). The production of protoplasts of several fungi has allowed for their genetic modification through the production of mutations, intra- and interspecific fusions, and the uptake of nucleic acids (17). Protoplasts also have been used to cure diseased fungi of viruslike particles (3). The production of protoplasts from fungi that do not sporulate or do not have a sexual cycle, in association with mutagenic agents, has enabled the production of mutant isolates with unique genetic characteristics (9,11).

Protoplast techniques have been used for genetic studies of plant pathogenic fungi (6–8,22). Of particular interest to the present study is that increased variation was reported from nonmutagenized protoplast-regenerated isolates of *Pyricularia oryzae* and *Claviceps purpurea* (5,11). Several protoplast-regenerated isolates from these fungi possessed altered cultural characteristics, reduced alkaloid production, and variations in virulence. Techniques for protoplast production and regeneration may have potential for generating new sources of variation in filamentous fungi.

Sclerotinia sclerotiorum (Lib.) de Bary is an important fungal pathogen of many plant species and is responsible for substantial losses in crop production in North America each year (18). Disease symptoms on individual plants are often severe and, under suitable environmental conditions, can result in an extensive watery soft rot of infected leaf and stem tissues. The destructive nature of these symptoms has stimulated investigations of the potential for *S. sclerotiorum* as a biological control agent for selected plant weeds (13–15,19). However, the extensive host range of this pathogen and the production of persistant sclerotia that produce apothecia restrict the potential of this pathogen as a mycoherbicide because of the potential for spread to susceptible, nontarget plants. Pathogenic isolates of *S. sclerotiorum* with a reduced host range and isolates that do not produce sclerotia were obtained by mutagenization of ascospores (13–15).

In preliminary experiments to incorporate foreign nucleic acids into protoplasts of *S. sclerotiorum*, considerable morphological variation among protoplast-regenerated isolates from both experimental and control treatments was observed (G. J. Boland, *unpublished*). Many regenerants differed in colony color, growth rate, virulence, and the production of sclerotia in comparison with the source isolates from which the protoplasts were produced. In addition, all treatments in the preliminary experiments were exposed to polyethylene glycol (PEG) to enhance the incorporation of nucleic acids. The importance of PEG treatments on the observed variation among regenerants was not determined; however, we felt that variation among protoplast-regenerated isolates had potential for the production of isolates of *S. sclerotiorum* with unique genetic characteristics.

The objectives of this study were to evaluate variation in cultural morphology and virulence among protoplast-regenerated isolates of three source isolates of *S. sclerotiorum*; to determine the influence of PEG treatment of protoplasts on variation among regenerants; to evaluate the stability of variation among regenerants during serial subcultures of growth; and to isolate virulent, nonsclerotial-forming isolates of *S. sclerotiorum*.

MATERIALS AND METHODS

Fungal isolates. Three wild-type source isolates were used to generate protoplasts of *S. sclerotiorum*. Isolate 101 was derived from a single sclerotium, isolate 137 from a single ascospore, and isolate 220 from a mass ascospore transfer. Isolates were maintained on potato-dextrose agar (PDA) at 4 C and cultured on PDA at 22–25 C, unless otherwise described.

Production of protoplasts. Mycelium was grown by transferring five agar disks (5 mm diameter) cut from the actively growing colony margins of individual isolates to 50 ml of potato-dextrose broth (PDB) in 125-ml flasks. Cultures were incubated at 100 rpm on a rotary shaker at 22–25 C for 72 h. Protoplasts of *S. sclerotiorum* were prepared from vegetative hyphae by the methods of Yelton et al (24).

Treatment of protoplasts with PEG. To determine the influence

of PEG on variation in regenerated protoplasts, the protoplast sample was divided into two subsamples of 100 μl each. The first subsample was used for the direct regeneration of protoplasts. To the second subsample, a PEG solution (60% PEG 4000, [w/v; Fisher Scientific Co., Fair Lawn, NJ], 10 mM Tris hydrochloride [pH 7.5], 10 mM $CaCl_2$) was added in steps of 200, 200, and 850 μl, with mixing after each addition (24). The suspension was incubated for 20 min at 22–25 C. Protoplasts were pelleted by centrifugation at 3,000 *g* for 15 min at 4 C, resuspended in 2.5 ml of yeast extract-glucose-sorbitol medium (YGS; 0.5% yeast extract, 2% glucose, 1.2 M sorbitol), and incubated at 37 C for 2 h. Cells were pelleted at 3,000 *g* for 5 min at 4 C and resuspended in STC buffer (1.2 M sorbitol, 10 mM Tris hydrochloride [pH 7.5], 10 mM $CaCl_2$).

Regeneration of protoplasts. Each subsample (three source isolates × two PEG treatments) was serially diluted in STC, and 100-μl aliquots were spread on regeneration medium (RM; 1.5% agar, 1.2 M sorbitol). After incubation for 48 h at 22–25 C, 26–30 regenerated protoplasts were subcultured individually from each subsample to PDA. Each isolate subsequently was subcultured once for evaluations of cultural morphology and virulence.

Evaluation of protoplast-regenerated isolates. Protoplast-regenerated isolates were compared with the source isolates and categorized for atypical colony morphology and the absence of sclerotia after 14 days of growth. These comparisons were made during the second subculture on PDA after regeneration. Atypical colony morphology included variations in color and texture. Cultures not producing sclerotia were reevaluated 7 days later. Quantitative variations in virulence were determined by removing 7-mm-diameter agar disks from the actively growing colony margins of individual isolates, and inoculating the plugs on detached celery petioles. Celery petioles were chosen as a commercially available, susceptible host that provided a consistent disease response. Each petiole was inoculated with three plugs, and petioles were arranged in a completely randomized design with three replicates per isolate (20). Inoculated petioles were incubated at 18 C and 100% relative humidity for 72 h when lesion diameters were measured parallel to the length of the celery petioles. All experiments were repeated once.

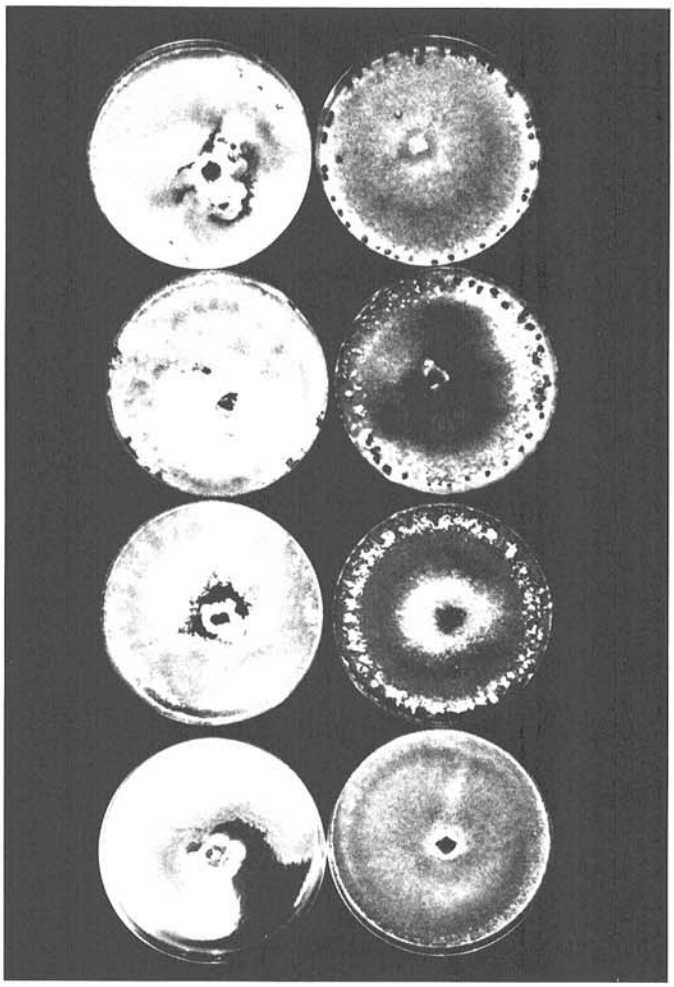

Fig. 1. Variation in cultural morphology among protoplast-regenerated isolates of *Sclerotinia sclerotiorum* after 14 days of growth on potato-dextrose agar. The source isolate (137) from which all illustrated regenerants were produced is shown in the upper, right corner.

Evaluation of protoplast-regenerated isolates during serial subculture. To determine the stability of variation among regenerants, selected isolates were serially subcultured five times and were evaluated for colony morphology and virulence during each subculture. Isolates were selected on the basis of observed variations in growth morphology and virulence from the previous experiment. Cultures were maintained, transferred, and evaluated as described previously. Agar plugs from the actively growing colony margins of individual subcultures were inoculated on celery petioles, and petioles were arranged in a completely randomized design. Lesion diameters were measured after 72 h of incubation at 18 C and 100% relative humidity.

Statistical analysis. Evaluations of virulence were conducted as factorial experiments with three (source isolates) × two (PEG treatments) sources of variation. Preliminary analysis of the data indicated a binomial response among virulent and avirulent isolates. Therefore, data were separated into two analyses. The first analysis tested the response of isolates when categorized for the presence or absence of lesions on celery petioles. These binomial data were analyzed by logistic regression to determine if experimental factors affected the probability of isolates initiating lesions (12). The second analysis tested the response among isolates that did initiate lesions on celery petioles. Measurements of lesion diameters were transformed with a natural logarithm transformation to provide normality and homogeneity of variance (20). These data were analyzed by analysis of variance as a completely randomized design with two factors (isolates and PEG treatment). Pairwise comparisons of mean lesion diameters produced by source isolates and individual regenerants were conducted with Dunnett's *t* tests.

Experiments to determine the stability of variation among regenerants were analyzed with analysis of variance as a completely randomized design. Pairwise comparisons between source isolates and individual regenerants were conducted with Student's *t* tests.

RESULTS

Variation in cultural morphology. Considerable variation in cultural morphology was observed among protoplast-regenerated isolates of *S. sclerotiorum* as compared with the source isolates (Fig. 1). The percentages of regenerants from source isolates 101, 137, and 220 that developed atypical colony morphology during the second subculture on PDA after regeneration were 23.3–50.0, 88.4, and 20.0–20.6%, respectively (Table 1). The percentages of regenerants from source isolates 101, 137, and 220 that did not develop sclerotia after 14 days of growth on PDA were 3.3–10.0, 15.3–46.1, and 0%, respectively. Many isolates initially developed sectors of discolored, slow growing hyphae (i.e., atypical colony morphology) around the agar plug used to transfer the isolate; but, as colony growth proceeded, these areas were overgrown by faster growing, typical sectors of the fungus that colonized the medium more rapidly. Such isolates often were classified as typical because of the overall appearance of the culture after 14 days of growth. Additional variation may have been obtained in some isolates if hyphal tip subcultures had been made from these atypical sectors before they were overgrown.

Variation in virulence. Variations in virulence were detected among protoplast-regenerated isolates from most treatments. Logistic regression analysis of binomial data (i.e., lesion versus no lesion) indicated that PEG treatments of protoplasts had no significant influence on the number of regenerants that initiated

lesions ($P = 0.20$). However, there were significant differences in the number of lesions initiated by the protoplast regenerants dependent on the source isolate from which they were derived. Regenerants derived from source isolates 101 and 220 produced significantly more lesions than regenerants from isolate 137 ($P \leq 0.001$), but isolates 101 and 220 were not significantly different ($P = 0.166$). There were no significant interactions between PEG treatments and isolates.

Analysis of variance of the sources of variation within the model (PEG and isolates) for mean lesion diameters (i.e., only regenerants that initiated lesions) indicated that PEG and isolate treatments significantly ($P \leq 0.01$) influenced the diameter of lesions produced on celery petioles. Interactions among PEG and isolate treatments were not significant, indicating that the influence of PEG treatments on the virulence of protoplast regenerants was constant across all isolates.

Dunnett's t tests were used to test for significant differences in mean lesion diameters between source isolates and regenerants. Regenerants that did not initiate lesions were excluded from the analyses. Significant differences were detected among regenerants from source isolates 101 and 220 for both PEG-treated and untreated populations, but no significant differences were detected among the populations derived from source isolate 137 (Table 2). On average, treatment of protoplasts with PEG increased the mean lesion diameters initiated by protoplast-regenerated isolates on celery petioles by 21%. Protoplast regenerants derived from source isolates 101 and 220 were not significantly different from each other ($P = 0.06$), but regenerants from these populations produced mean lesion diameters that were 327% ($P \leq 0.0001$) and 389% ($P \leq 0.0001$) larger than lesions initiated by isolate 137, respectively. Significant differences between individual regenerants and source isolates were found in 0–30% of the protoplast-regenerated isolates examined, depending on the source isolate used to produce the protoplasts. In all cases, significant differences were present for increased, but not decreased, mean lesion diameters.

Stability of variation after subculturing. Considerable variation was observed among selected protoplast-regenerated isolates that were serially subcultured five times and evaluated for colony morphology and virulence (Table 3). Several regenerate isolates that initially developed atypical colony morphology or no sclerotia developed a typical colony appearance or regained the ability to produce sclerotia after serial subculturing and evaluation (e.g., isolate 101-17). Similarly, several isolates that initially produced smaller lesion diameters on celery petioles than the source isolate became more virulent and produced lesion diameters that were equivalent to or greater than the source isolate (e.g., isolate 101-18). However, virulence among source and regenerate isolates was variable with repeated evaluation. This was particularly evident for source isolate 137, which produced variable lesion diameters throughout the five cycles of subculturing and evaluation. Source isolate 101 consistently produced lesions throughout the five subcultures but lost the ability to produce sclerotia by the end of the experiment.

The source isolates used to generate protoplasts appeared to have an influence on the stability of colony morphology and virulence during serial subcultures and evaluations. Protoplast-regenerated isolates derived from source isolate 220 (a mass ascospore culture) were similar to the source isolate in colony morphology and virulence both before and after serial evaluations (Table 3). Regenerants derived from source isolate 101 (a single sclerotium culture) that had atypical colony morphology or induced significantly different mean lesion diameters at the start of serial evaluations tended to revert to the characteristics of the source isolate by the end of the experiment. Some regenerants derived from source isolate 137 (a single ascospore culture) also reverted to typical colony morphology and virulence. However, one regenerant derived from source isolate 101 and three regenerants from source isolate 137 did not produce sclerotia in culture after five serial subcultures.

Selection of virulent, nonsclerotial-forming isolates. Four virulent, nonsclerotial-forming protoplast-regenerated isolates of

TABLE 1. Percentages of protoplast-regenerated isolates of *Sclerotinia sclerotiorum* with atypical colony morphology or without sclerotia after 14 days of growth on potato-dextrose agar (PDA)

Source treatment[a]	Number of regenerants	Atypical colony morphology[b] (%)	No sclerotia[c] (%)
Isolate 101	30	50.0	10.0
PEG-treated	30	23.3	3.3
Isolate 137	26	88.4	46.1
PEG treated	27	88.4	15.3
Isolate 220	30	20.0	0
PEG treated	28	20.6	0

[a] Source isolates from which protoplasts were derived. Approximately one-half of the regenerate isolates from each source isolate were treated with polyethylene glycol (PEG) before regeneration.

[b] Atypical colony morphology includes variation in the color, texture, and size of each colony in comparison with the source isolates from which protoplasts were derived.

[c] Colonies in which sclerotia had not been formed after 14 days of growth on PDA. Cultures not producing sclerotia after 14 days of growth were reevaluated 7 days later.

TABLE 2. Mean lesion diameters induced in detached celery petioles by protoplast-regenerated isolates of *Sclerotinia sclerotiorum* in comparison with source isolates after a 72-h incubation period[a]

	Mean lesion diameters of regenerants[b] (mm)					
	Isolate 101		Isolate 137		Isolate 220	
Regenerant	No PEG	PEG[c]	No PEG	PEG	No PEG	PEG
R1	27.7	13.5	8.9	0	32.0	19.7
R2	21.5	75.7*[d]	0	0	30.9	37.8
R3	22.8	55.4*	13.7	8.4	28.3	29.0
R4	13.2	77.6*	6.3	43.3	43.5	60.3
R5	51.0	63.2*	10.1	0	30.4	7.7
R6	47.2	37.8	13.7	9.6	31.9	68.0*
R7	0	74.3*	0	15.2	34.9	32.6
R8	39.9	62.7*	19.3	0	42.6	57.6
R9	39.9	28.7	23.0	10.8	31.6	42.7
R10	26.6	31.5	13.6	0	23.6	64.0*
R11	29.2	71.3*	27.7	4.5	27.6	76.4*
R12	34.0	46.3	0	···	42.2	59.2
R13	53.7	51.7	0	0	51.0	47.7
R14	37.0	43.9	3.3	26.4	57.7	58.7
R15	44.2	42.6	10.2	0	63.0*	36.5
R16	39.5	12.4	7.2	13.2	48.0	35.9
R17	14.5	0	27.3	0	25.4	51.2
R18	0	19.9	7.3	6.7	33.2	38.6
R19	18.6	24.8	6.9	19.0	39.9	43.5
R20	15.7	39.8	10.3	55.1	35.8	70.3*
R21	9.5	34.6	10.4	3.2	50.0	44.7
R22	45.3	31.1	0	37.3	40.7	75.0*
R23	35.0	23.8	15.8	3.8	37.5	39.7
R24	46.3	40.5	14.5	11.3	40.5	50.4
R25	60.4*	35.6	0	8.5	50.6	40.9
R26	42.8	35.0	16.4	28.5	39.3	54.2
R27	59.6*	42.3	···	18.0	38.3	44.7
R28	48.4	74.3*	···	···	51.7	30.4
R29	26.2	66.7*	···	···	79.3*	···
R30	10.1	0	···	···	51.3	···
Mean	32.0	41.9	10.2	12.4	41.1	46.9

[a] Agar plugs (7 mm diameter) were cut from the actively growing colony margin and inoculated on celery petioles incubated for 72 h at 18 C and 100% relative humidity.

[b] Mean lesion diameters induced by regenerants from indicated wild-type source isolates. Isolate 101 was derived from a single sclerotium, isolate 137 from a single ascospore, and isolate 220 from a mass ascospore transfer.

[c] Protoplasts in these treatments were exposed to polyethylene glycol (PEG) before regeneration.

[d] Mean lesion diameter of regenerate isolate was significantly different ($P = 0.05$) than source isolate according to pairwise comparisons with Dunnett's t test.

S. sclerotiorum were obtained from source isolates 101 and 137. Comparable isolates were not obtained from source isolate 220. Isolates 101-21, 137-6, 137-17, and 137-24 did not produce sclerotia on PDA throughout the five cycles of subculturing and evaluation (Table 3). On average, these isolates produced lesions on celery petioles that were not statistically different from lesions produced by the source isolate during the last subculture. However, source isolate 137 and regenerants derived from it had relatively low virulences compared with the two other source isolates.

DISCUSSION

We have shown that protoplast-regenerated isolates of *S. sclerotiorum* have increased variation in cultural morphology and virulence on celery petioles in comparison with the source isolates from which protoplasts were derived. Variations in colony morphology included reduced growth rates, discolored mycelium, reduced sclerotia size, and a lack of sclerotia in various isolates.

The heterogenous arrangement of subcelluar organelles within hyphae of filamentous fungi leads to the subdivision of the cytoplasm into a heterogenous population of protoplasts that differ in internal morphology, physiology, and biochemistry (17). Such variation has been observed for protoplasts of *Phytophthora cinnamomi* (1), *Aspergillus nidulans* (4,10), and *A. fumigatus* (21). Protoplasts initially are released from the apical or subapical regions of hyphae, normally are smaller in size, and contain fewer and smaller vacuoles than protoplasts released later from more distal regions of the hyphae.

Variations in virulence among regenerants were attributed to two sources of variation. First, agar plugs cut from the actively growing margins of fungal colonies were either virulent or avirulent, based on the ability to initiate lesions on celery petioles. The absence of lesions did not appear to be attributable to disease escape, because lesions consistently developed in control treatments. Plugs cut from slow growing or debilitated colonies often did not initiate lesions, presumably because of the genetic variation in virulence or a debilitated ability to grow and initiate disease. The relative influence of this source of variation on the experiments was removed by analyzing the binomial data separately with logistic regression analysis. From these experiments, we established that variations in the presence or absence of lesions among protoplast-regenerated isolates were significantly influenced by the source isolate used to produce the protoplasts.

A second source of variation among regenerants was measured by differences in lesion diameters that did develop on celery petioles. In these experiments, analysis of variance established that source isolates and treatment with PEG were important factors affecting variation in protoplast-regenerated isolates. It is interesting that lesion diameters produced by significantly different regenerants on celery petioles were larger than lesions produced by the source isolates. Variations in virulence (or aggressiveness) have been reported previously among protoplast-regenerated isolates of *Pyricularia oryzae* (5). Reductions in virulence in these studies were not associated with reductions of the mycelial growth rate, hyphal dry weight, sporulation, or percentage of conidial germination.

Treatment of protoplasts with PEG did not affect the number of lesions initiated by regenerants but did increase the diameter of lesions that did develop. The mechanisms of action underlying this association are not clear, but it is possible that the increased water potential caused by the addition of PEG resulted in a higher mortality rate among regenerants with reduced virulence.

The genetic natures of the three source isolates used to produce protoplasts are not well characterized. However, isolate 137 was derived from a single ascospore, isolate 101 was derived from a single sclerotium, and isolate 220 was derived from multiple ascospores. Ascospores of *S. sclerotiorum* contain two nuclei per cell that are mitotically identical, and vegetative cells are multi-

TABLE 3. Stability of variation in colony morphology and virulence on celery petioles of protoplast-regenerated isolates of *Sclerotinia sclerotiorum* during five serial subcultures[a]

Isolate[b]	Colony morphology[c] (subculture 1)	Mean lesion diameter per number of subcultures (mm)[d]					Colony morphology (subculture 5)
		1	2	3	4	5	
101	Typical	22.2	19.2	23.0	18.2	15.7	No sclerotia
101-4	Typical	13.2	34.6	24.1	37.5	22.2	Typical
101-7	Typical	0*	4.0	0*	3.7*	21.0	Typical
101-13	Typical	53.7*	35.7	21.4	16.3	40.5*	Typical
101-17	No sclerotia	14.5	28.4	31.2	33.9	40.7*	Typical
101-18	Atypical	0*	28.1	17.5	19.5	27.2*	Typical
101-21	No sclerotia	9.5	8.5	9.5	3.3*	18.8	No sclerotia
137	Typical	0	2.0	9.2	23.8	14.6	Typical
137-2	No sclerotia	0	4.8	8.3	20.5	13.5	Typical
137-6	No sclerotia	13.7*	9.3	0	5.3*	9.2	No sclerotia
137-11	Typical	27.7	26.0*	25.8	30.2	44.5*	Typical
137-13	No sclerotia	0	13.2	4.3	7.2	11.1	Typical
137-17	No sclerotia	27.3*	20.8	12.8	0*	3.7	No sclerotia
137-24	Atypical	14.5	7.3	0	0	24.5	No sclerotia
220	Typical	29.1	42.6	34.6	32.5	40.7	Typical
220-10	Typical	2.6	34.3	29.0	28.0	44.3	Typical
220-15	Typical	63.0*	34.3	34.8	37.5	37.1	Typical
220-16	Typical	48.0	51.7	43.6	28.0	53.8	Typical
220-17	Typical	25.4	34.7	33.8	28.9	63.5*	Typical
220-29	Typical	79.3	25.5	29.8	30.2	40.0	Typical
220-30	Typical	51.3	47.3	44.6	22.7	46.7	Typical

[a] Agar plugs (7 m diameter) were cut from the actively growing colony margin and inoculated on detached celery petioles incubated for 72 h at 18 C and 100% relative humidity.

[b] Wild-type source isolates from which regenerants were derived. Isolate 101 was derived from a single sclerotium, isolate 137 from a single ascospore, and isolate 220 from a mass ascospore transfer. Other isolates are selected, protoplast-regenerated isolates.

[c] Typical colony morphology was represented by the source isolates. Atypical colony morphology included variations in color, texture, and size of each colony in comparison with the source isolates from which protoplasts were derived. Isolates that did not produce sclerotia on potato-dextrose agar (PDA) after 14 days of incubation were rated as producing no sclerotia. Data for colony morphology are presented only for the first and fifth subcultures.

[d] Protoplast-regenerated isolates were serially subcultured on PDA five times and evaluated for colony morphology and virulence after each subculture. * = values within each column were significantly different ($P = 0.01$) from each source isolate according to Student's t test for paired means with different variances. Each protoplast-regenerated isolate was compared with the source isolate for virulence on celery petioles 72 h after inoculation.

nucleate (23). Therefore, isolate 137 is homokaryotic, whereas isolate 220 is heterokaryotic. The nature of isolate 101 is unknown, but variations among protoplast regenerants derived from this isolate appeared to be intermediate between isolates 137 and 220. Variations in colony morphology and the production of sclerotia were most prevalent among homokaryotic isolate 137. Conversely, variations in virulence were most prevalent among heterokaryotic isolate 220. Peberdy (16) has shown that the genetic nature of the source isolate used to produce protoplasts can significantly influence the type and magnitude of variation observed in protoplast-regenerated isolates. The relatively small sample size of three source isolates used in these studies restricts any generalizations regarding the influence of isolate genotype on protoplast-induced variation. However, in subsequent experimentation with four additional isolates of *S. sclerotiorum* recovered from field-collected sclerotia, we have been unable to isolate regenerants that do not form sclerotia (*unpublished data*).

Several protoplast-regenerated isolates of *S. sclerotiorum* did not produce sclerotia in culture but were still able to initiate lesions on celery petioles. The development of effective biological control systems for the management of plant diseases and weeds often requires the genetic modification of organisms to improve selected characteristics (2). Selection of such characteristics from natural sources of variation is preferred because of deleterious effects often associated with ultraviolet- or chemically-induced mutations. In addition, regulations governing the experimental use of isolates derived through natural selection are less stringent than regulations governing organisms that have been altered through artificially induced mutations or genetic engineering. The use of protoplast-regeneration methods to generate novel sources of variation in selected isolates of fungi has potential applications in biological control.

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