Reassessment of Vegetative Compatibility of *Sclerotinia homoeocarpa* Using Nitrate-Nonutilizing Mutants

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ABSTRACT

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Nitrate-nonutilizing (*nit*) mutants were recovered for the first time from 21 isolates of *Sclerotinia homoeocarpa* collected in the United States. Mutants were selected from shredded mycelium of each isolate when cultured on water agar medium amended with 4% (wt/vol) potassium chlorate. The mutants could be classified into three phenotypes: *nit1*, *nit3*, and *NitM*, based on their growth on minimal medium (Czapek solution agar) supplemented with NaNO_2 or hypoxanthine. Complementary heterokaryons were observed in pairings between different phenotypes of *nit* mutants derived from compatible isolates, but not in

self-fusions or pairings between incompatible isolates. The vigor of prototrophic growth varied with isolates and mutant phenotypes. Strong and continuous heterokaryons, as well as weak and spontaneous ones, formed depending on pairings of *nit* mutants. Stable heterokaryons between compatible isolates, but apoptotic reactions between incompatible isolates, were observed immediately after hyphal fusion under the epifluorescence microscope. The 21 isolates used in this study, which were previously assigned into 11 different vegetative compatibility groups (VCGs) based on the formation of a barrage zone at the contact site of paired isolates on complete medium (potato dextrose agar), were regrouped into five VCGs based on heterokaryon formation between *nit* mutants on minimal medium.

Dollar spot, caused by *Sclerotinia homoeocarpa* F. T. Bennett, is the most economically important disease on cultivated turfgrass worldwide (13,32,35). Golf course turfgrass, in particular, is the most susceptible to dollar spot because of low-mowing heights. Turfgrass managers spend more money on controlling dollar spot than any other disease in North America (17). Dollar spot is hard to eliminate once it infects turfgrass and risks both aesthetic quality and playability of turfgrass for the entire growing season every year. More often than not, multiple applications of fungicides are required to meet the low-tolerance level of this disease due to the high expectation of turfgrass quality. As a consequence of the intensive use of fungicides, there is great concern over the development of fungicide insensitivity. Despite the significance of dollar spot to the turfgrass industry, limited research on the biology and genetics of *S. homoeocarpa* has been conducted (19,31,36). For the development of proper cultural and chemical management strategies for dollar spot, it is necessary to have a better understanding of the biological processes that affect pathogenicity, local distribution of the disease, and genetic variation of the fungal population.

Identification of vegetative compatibility has been widely used to study genetic relationships in anamorphic populations of fungi (11,12,16,23,24), including several important plant-pathogenic fungi in the genera *Fusarium* (22,28,30) and *Verticillium* (20,29). Valuable information about populations within the same species or individuals within a population can be provided by means of intraspecific subunits of vegetative compatibility groups (VCGs). The VCGs can be further correlated with genetic affinity, important phenotypes such as virulence or host specificity, geographical distribution, and temporal or spatial dynamics of the population.

Various phenotypic markers such as macroscopic or microscopic evidence (4,12,21), and auxotrophic (15) or morphologic (29) mutants, have been developed for vegetative compatibility tests. Use of auxotrophic, nitrate-nonutilizing (*nit*) mutants is the single most effective method to determine vegetative compatibility and has contributed significantly to an understanding of fungal population biology. It is based on heterokaryosis, which is identified by distinctive aerial tufts of complementary hyphae between paired, compatible homokaryons on minimal media (8,20,28,30). A primary advantage of using *nit* mutants over other types of phenotypic markers for vegetative compatibility tests is that *nit* mutants can be readily recovered because this mutation arises spontaneously at a relatively high frequency without chemical mutagens or radiation, and can be selected on chlorate-amended selective media. Once the *nit* mutant system was established, it greatly enhanced subsequent genetic studies based on vegetative compatibility. However, difficulties in recovering *nit* mutants from certain fungi, including *Sclerotinia* species, limit their use in genetic studies (7,34).

The concept of vegetative compatibility has been applied to *S. homoeocarpa* (3,14,25,27,36). Microscopic or macroscopic manifestation of apoptotic barriers between paired wild-type isolates has been used in the previous vegetative compatibility studies. A major limitation associated with the macroscopic evidence is that the morphological features of apoptotic barriers are variable (14), or often not clear enough to define incompatibility. Somatic incompatibility defined by a barrage zone may not be synonymous with heterokaryon instability as described in some fungi (15,18). Vegetative compatibility studies in *S. homoeocarpa* would be significantly augmented if the system of *nit* mutants were implemented.

The objectives of this study are to recover *nit* mutants of *S. homoeocarpa* using modified protocols previously developed in *Verticillium* and *Fusarium* and to use these mutants to re-evaluate VCGs of 21 *S. homoeocarpa* isolates previously assigned to 11 different VCGs (3,27) based on macroscopic apoptotic barrier formation.

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MATERIALS AND METHODS

Recovery of *nit* mutants. Twenty-one *S. homoeocarpa* isolates, originally recovered from diseased turfgrass in Illinois, Michigan, Arkansas, Florida, and Minnesota (Table 1) between 1995 and 2001, were used in the current study. These isolates were previously characterized and grouped into 11 VCGs based on barrage formation (3,27), and further characterized for genetic diversity and pathogenicity on bentgrasses (*Agrostis* spp.) (6).

Nit mutants of each *S. homoeocarpa* isolate were screened with a modified technique described for *Verticillium* (20) and *Fusarium* spp. (28,30). Spontaneous, chlorate-resistant *nit* mutants were selected on media amended with chlorate, a toxic analog of nitrate. Initially, 21 wild-type isolates were grown on potato dextrose agar (PDA; Becton, Dickson, and Company, Sparks, MD) for 2 weeks. An agar plug (5 mm in diameter) of each *S. homoeocarpa* isolate was transferred to the center of media amended with chlorate. Two types of chlorate-amended media were prepared for selecting *nit* mutants in 9-cm-diameter petri dishes: (i) PDAC: PDA amended with 4% (wt/vol) potassium chlorate (Fluka, Sigma-Aldrich Chemie GmbH, Switzerland) and (ii) WAC: water agar (2%) amended with 0.2% (wt/vol) glucose (Mallinckrodt Baker Inc., Phillipsburg, NJ) and 4% potassium chlorate. Four PDAC and four WAC plates were prepared per isolate.

A new method of spreading shredded mycelium instead of placing agar plugs on chlorate-amended media was tried. The amount of initial inoculum could be increased in this method in order to increase the chance of selecting spontaneously occurring *nit* mutants. One milliliter of sterile water was added to each culture plate containing 2-week-old mycelium on PDA, and the mycelium was harvested by scraping with a sterilized rubber policeman. Mycelial scrapings were diluted to 10 ml with sterile water and shredded with a sterile tissue homogenizer (Brinkmann Instruments Co, Switzerland) at 15,000 rpm for 30 s. The length of shredded hyphal pieces ranged from 100 to 400 μ m when examined under the compound microscope. An aliquot of 800 μ l shredded mycelium was spread on four plates of PDAC or WAC. Plates were air-dried in a laminar flow hood for 30 min and then incubated at 23°C for 14 days.

When mycelial growth on chlorate media (PDAC or WAC) became noticeable, individual colonies were selected. To confirm

their inability to use chlorate and to minimize the false selection of the wild type, selected colonies were subcultured once more on WAC. A hyphal tip of the chlorate-resistant colony growing on WAC was cut and transferred to a minimal medium, Czapek solution agar (CDA; Becton, Dickson, and Company, Sparks, MD), which contains nitrate as the sole nitrogen source. Colonies that grew thin and expansive with no aerial mycelium were considered a *nit* mutant. Each *nit* mutant was cultured on WAC and stored at 4°C.

Characterization of *nit* mutant phenotypes. *Nit* mutants were assigned to different phenotypic classes pursuant to their growth on CDA amended with different supplements such as NaNO₂ or hypoxanthine (8). An agar plug (5 mm in diameter) was cut from the edge of each *nit* mutant growing on CDA and transferred to three types of selective media: CDA; CDA amended with NaNO₂ (0.05%, wt/vol); and CDA amended with hypoxanthine (0.02%, wt/vol). An agar plug of each wild-type isolate growing on CDA was also included as a wild-type control for each experiment. The plates were incubated at 23°C for 7 days, and the colony morphology was evaluated by comparison with the wild type. This experiment was conducted twice for each *nit* mutant isolate. The genetic symbols for *nit* mutants found in this study followed the same nomenclature used in *F. oxysporum* Schlechtend.:Fr. (8). In short, the mutants that did not use nitrate but used both nitrite and hypoxanthine, were designated as *nit1* (associated with mutation on nitrate reductase); those that did not use either nitrate or nitrite but used hypoxanthine were designated as *nit3* (associated with mutation on the regulatory gene for the nitrate assimilation pathway); and those that did not use either nitrate or hypoxanthine but used nitrite were designated NitM (associated with mutation on genes controlling production of a molybdenum-containing cofactor).

Vegetative compatibility assays. Vegetative compatibility of the 21 *S. homoeocarpa* isolates was tested and compared between two different methods based on (i) heterokaryon formation in pairings between *nit* mutants on CDA and (ii) barrage-zone formation on PDA. Vegetative compatibility based on complementation of *nit* mutants was conducted by cutting an agar plug (5 mm in diameter) from the edge of each *nit* mutant colony and placing a pair of agar plugs 4 cm apart on a 9-cm-diameter plate of CDA. Pairings were made between two different *nit* mutants in all possible combinations and also between the same isolate (self-fusion), which were included for each test run on the same petri dish as a

TABLE 1. *Sclerotinia homoeocarpa* isolates and their nitrate-nonutilizing (*nit*) mutants recovered in this study

Isolate ^a	Location	Number of mutants recovered			
		<i>nit1</i>	<i>nit3</i>	NitM	ND ^b
1. FL17	Unknown golf course, FL			1	
2. Southbrook	Southbrook, MN			1	
3. ARK	Unknown golf course, AR				
4. 30B-13	Lakewood Shores, MI		1		1
5. 30B-24	Lakewood Shores, MI	2	1		
6. 30B-48	Lakewood Shores, MI	1			
7. 46-3	Forest Akers Golf Course, East Lansing, MI	1			
8. A7	Southbrook, MN	1		1	
9. TB64D	Unknown	1			
10. L36	Unknown	2			
11. 64-41	Evergreen Golf, Hudson, MI			1	
12. 64-49	Evergreen Golf, Hudson, MI	1			1
13. MNI	Unknown golf course, MN	1			
14. 48-54	North Shore County Club, Chicago, IL			2	
15. 32-47	Evergreen Golf, Hudson, MI	1			
16. 32F	Evergreen Golf, Hudson, MI				4
17. 33A-9	Hancock Turf Research Center, East Lansing, MI		2		
18. I16	Les Bolstead, St. Paul, MN	4		1	
19. I18	Les Bolstead, St. Paul, MN		1		
20. Les Bolstead	Les Bolstead, St. Paul, MN	1			
21. BRS	Unknown		1		
	Total	16	6	8	7

^a Isolates were previously collected by Powell and Vargas (27) and Bernick (3).

^b Not determined.

negative control representing no complementation of mutation. The plates were incubated at 23°C for 10 days before examination. A complementary reaction was determined by evidence of the development of dense heterokaryotic aerial mycelial growth at the zone of hyphal contact. The experiment was conducted twice.

A vegetative compatibility assay based on barrage formation was conducted using both *nit* mutant and wild-type isolates. Agar plugs of the 21 wild-type isolates growing on PDA were cut and paired in all possible combinations on PDA. Agar plugs of *nit* mutants growing on CDA were cut and paired on PDA. Pairings with the same isolate (self-fusion) were also included for each test run on the same petri dish as a negative control representing no barrage formation. The formation of a barrage zone between wild-type pairs and between mutant pairs, representing somatic incompatibility, was determined at 10 days after incubation at 23°C.

Differential interference contrast and epifluorescence microscopy. Pairings between compatible *nit* mutants and pairings between incompatible mutants were selected for microscopic examination. Hyphal crossing between paired isolates was performed on glass slides (75 × 25 mm). Initially, the *nit* mutants were grown on CDA. An agar block (3 × 2 mm) was removed from the edge of active-growing mycelium on CDA. The agar block containing mycelium from one of the pairs was placed on each side of the slide. Each slide was placed in a petri dish (9 cm in diameter) with wet filter paper at the bottom and incubated at 25°C for 5 days.

When hyphae growing from each agar block were about to contact each other at the middle of the slide, mycelium was dried at room temperature for 30 min. Staining was accomplished by dropping an ethidium bromide solution (200 µg/ml) directly onto the mycelium, and then the mycelium was covered with a plastic cover slip. The slides were examined using an epifluorescence microscope (Eclipse E600 with a UV-2A cube, Nikon, Melville, NY). Each slide was first examined at 200× to locate hyphal fusions between paired isolates, and then re-examined at a higher magnification of ×400 to ×1,000. Bright-field images were captured using bright-field DIC, and images of the same spot stained with ethidium bromide were captured under fluorescent light.

Mycelium on the same slide was stained again with 100 µg/ml acridine orange solution (Difco Becton Dickinson, Sparks, MA)

without removing the coverslip. The same spots previously examined were located, and images of mycelium stained with acridine orange were captured under fluorescent light. All images were processed using Spot Diagnostic Software version 3.5.9 (Diagnostic Instruments, Sterling Heights, MI) and Photoshop 5.5 (Adobe Systems, Inc., San Jose, CA).

RESULTS

Recovery of *nit* mutants. All 21 tested isolates yielded chlorate-resistant colonies when shredded mycelium was plated on WAC but not on PDAC. Growth of wild-type colonies was greatly restricted on WAC in contrast with those on PDAC. Mycelium agar plugs transferred on WAC and PDAC failed to yield chlorate-resistant sectors or produced overgrown wild-type hyphae.

Isolates varied considerably in the frequency of developing chlorate-resistant colonies, ranging from a few colonies (e.g., isolates of FL17, Southbrook, and 30B-48) to ≈20 colonies (e.g., isolates of 32F and I16) per WAC culture plated with shredded mycelium. Two through a maximum of five chlorate-resistant colonies per isolate were selected after screening them on WAC twice. Most of the chlorate-resistant colonies (≈90%) produced thin and expansive mycelium on the minimal medium, CDA. A total of 37 colonies were considered *nit* mutants (Table 1).

Characterization of *nit* mutant phenotypes. Three different *nit* mutant phenotypes, *nit1*, *nit3*, and NitM, which were previously known in *F. oxysporum* (8), were recovered from *S. homoeocarpa*. Recovery rates of these *nit* mutant phenotypes were variable among isolates (Table 1). Out of 37 *nit* mutants that were recovered in this study, 16 (43%), 6 (16%), and 8 (22%) mutants were *nit1*, *nit3*, and NitM, respectively. The remaining seven mutants could not be clearly classified as any of the three *nit* mutant phenotypes. The undetermined mutants grew very slowly, which rendered evaluation impossible within 7 days after inoculation, and therefore, could not be used for subsequent complementation assays.

Vegetative compatibility assays. Five VCGs were determined based on the results from the complementary assay of 30 *nit* mutants generated from 21 *S. homoeocarpa* isolates (Table 2).

TABLE 2. Vegetative compatibility based on barrage formation of wild-type isolates on potato dextrose agar (above the diagonal) and based on complementary reaction of *nit* mutants on Czapek solution agar (below the diagonal)^a

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	VCG ^b
1. FL17	...	—	—	—	—	—	—	—	—	+	—	—	+	—	—	—	—	—	—	—	—	A
2. Southbrook	+	...	+	+/-	+/-	—	+/-	—	—	—	—	—	—	—	—	—	+/-	—	—	—	—	B
3. ARK	—	++	...	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	B
4. 30B-13	+	++	—	...	+	—	—	—	—	—	—	—	—	—	—	—	+	+/-	—	—	—	C
5. 30B-24	...	++	++	++	++	—	—	—	+/-	—	—	—	—	—	—	—	+	—	—	—	—	C
6. 30B-48	...	+	+	...	+	...	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	D
7. 46-3	+	++	—	+	++	...	...	—	+	—	—	—	—	—	+	+	+/-	—	—	—	—	F
8. A7	—	+	—	...	...	...	+	...	—	—	—	—	—	—	—	—	—	—	—	—	—	H
9. TB64D	+	...	...	...	...	...	...	+	...	—	—	—	—	—	—	—	+/-	—	—	—	—	K
10. L36	+	—	+	...	+	...	...	...	...	...	—	—	+	—	—	+/-	+/-	—	—	—	—	A
11. 64-41	...	+	++	++	++	...	+	...	+	...	...	+	—	—	—	—	+/-	—	—	—	—	E
12. 64-49	+	—	...	+	++	...	...	...	...	—	...	...	—	—	—	—	+/-	—	—	—	—	E
13. MNI	+	—	+	...	—	...	...	...	...	—	...	—	...	—	—	—	—	—	—	—	—	A
14. 48-54	...	+	+	...	...	...	+	...	...	...	...	...	...	...	—	—	—	—	—	—	—	D
15. 32-47	—	—	—	—	—	—	—	—	—	—	—	—	—	—	...	+	—	—	—	—	—	F
16. 32F	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	...	—	—	—	—	—	F
17. 33A-9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	...	—	—	—	—	C
18. I16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	...	+	+/-	—	G
19. I18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	...	—	—	G
20. Les Bolstead	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	++	...	—	J
21. BRS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	++	...	...	L

^a Results above the diagonal of the table interpret compatibility based on a barrage formation: + = no formation; — = barrage formation; and +/- = unclear because of strong accumulation of pigment. Results below the diagonal interpret compatibility based on a complementation reaction of *nit* mutants: ++ = strong heterokaryotic reaction (Fig. 2A and B); + = heterokaryotic reaction; — = no reaction (Fig. 1B); and ... = not determined.

^b Vegetative compatibility groups previously determined by barrage formation at pairings of wild-type isolates on potato dextrose agar (3,27).

Fourteen isolates (isolate numbers 1 to 14) were assigned to one VCG; four isolates (isolate numbers 18 to 21) were assigned to another VCG; and three mono-isolate VCGs. Distinct complementary heterokaryons were observed on CDA in some pairings within the same VCG (Fig. 1D). Spontaneous and discontinuous mycelium was also observed in some compatible pairings (Fig. 2C and D). No heterokaryon was formed in self-fusions of the same *nit* mutant or in pairings between *nit* mutants derived from incompatible isolates (Fig. 1B).

The vigor of heterokaryosis differed among pairings of *nit* mutant phenotypes derived from compatible isolates. Compatible pairings between NitM and *nit1* (Fig. 2B) and between NitM and *nit3* (Fig. 2A) usually developed distinct and continuous aerial

mycelium at the contact area indicating a robust complementary reaction. However, compatible pairings between *nit1* and *nit3* often showed weak or no distinct prototrophic mycelium.

In contrast, twenty-one isolates were assorted into 11 groups based on the barrage formation at pairing on PDA between wild-type isolates (Table 2) or between *nit* mutants, similar to previously determined VCGs (3,27). *Nit* mutants grew on PDA in the same way as wild types grew on PDA, producing active aerial mycelium and distinct barrage formation at certain pairings. Some paired colonies of *nit* mutants on PDA merged uniformly with no dark line, indicating a compatible reaction. In the incompatible reaction, paired *nit* mutants produced dark pigmentation (7 days after contact), and later a lytic gap along the contact zone (14

Fig. 1. Vegetative compatibility reaction using *nit* mutants of *Sclerotinia homoeocarpa*. **A**, Formation of a barrage with dark pigmentation at the contact site of the pairing between incompatible isolates on potato dextrose agar (PDA) after 10-day incubation at 23°C. **B**, No heterokaryon is formed at self-fusion or between incompatible isolates on Czapek solution agar (CDA). **C**, Obscure interference zone with dark pigmentation on PDA. **D**, Formation of vigorous heterokaryons between compatible isolates on CDA. The number on the plate indicates *nit* mutant isolates inoculated: 2 = Southbrook (NitM); 5 = 30B-24 (*nit1*); and 20 = Les Bolstead (*nit1*).

Fig. 2. Different prototrophic growth vigor of complementary hyphae of *Sclerotinia homoeocarpa nit* mutants. In each figure, the left plate is the culture of paired isolates grown on potato dextrose agar (PDA) and the right plate is the culture on Czapek solution agar (CDA) after 10-day incubation at 23°C. **A**, No interference zone on PDA and the formation of strong complementary heterokaryotic hyphae on CDA. **B**, No interference zone but dark pigmentation on PDA and strong heterokaryons on CDA. **C and D**, Formation of a barrage with strong pigmentation on PDA, but weak and discontinuous tuft of heterokaryons on CDA. The number on the plate indicates *nit* mutant isolates inoculated: 2 = Southbrook (NitM); 4 = 30B-13 (*nit3*); 6 = 30B-48 (*nit1*); 7 = 46-3 (*nit1*); and 14 = 48-54 (NitM).

days after contact) (Fig. 1A). Some pairings formed irregular and obscure barrages interspersed with dark pigment and overgrowth of mycelium (Fig. 1C).

Microscopic observation of heterokaryons. Stable heterokaryosis between compatible *nit* mutants and apoptosis between incompatible *nit* mutants were clearly differentiated microscopically, immediately after anastomosis (Fig. 3). Self-hyphal fusions in the same isolate occurred more often than between compatible isolates or between incompatible isolates. The fused hyphae were easily observed under DIC microscopy (Fig. 3A, D, and G). After anastomosis, heterokaryosis was visible within fused hyphae under the epifluorescence microscope after acridine orange staining (Fig. 3C and F). Instability of heterokaryosis was determined using ethidium bromide, which penetrated only dead cells and stained their nuclei (1,2,26). Ethidium bromide intensively stained nucleic acids in fused hyphae between incompatible isolates and in their adjacent hyphae (Fig. 3H), indicating that the apoptotic reaction began immediately after hyphal tips were fused. In contrast, living cells in hyphae of self-fusion or between compatible isolates were not stained by ethidium bromide, indicating no apoptosis immediately after anastomosis (Fig. 3B and E).

DISCUSSION

Nit mutants have never been identified in Sclerotiniaceae fungi up to the present. *Nit* mutants were isolated for the first time in this study from *S. homoeocarpa* using chlorate-amended minimal medium. Twenty-one isolates of *S. homoeocarpa*, originally assigned to 11 VCGs based on the barrage formation (3,27), were re-examined for vegetative compatibility using complementary, auxotrophic *nit* mutants, and regrouped into a maximum of five VCGs. The use of *nit* mutants enabled us to study vegetative compatibility of *S. homoeocarpa* based on heterokaryosis, providing a robust method for future investigation of *S. homoeocarpa* populations.

Previous vegetative compatibility assays for *S. homoeocarpa* have been performed based solely on the microscopic or macroscopic assessment of barrage formations by pairing wild-type isolates on complete medium (14,25,27,33,36). The number of VCGs determined in previous studies was highly variable, from 4 to 20, depending on the number of isolates included and the geographic range of sampling for each study. Consequently, subdivision into VCGs remains controversial, and the relationship of the VCGs with genotypes or phenotypes of *S. homoeocarpa* remains ambiguous (6,25,36). In this study, *S. homoeocarpa* isolates were conglomerated into much smaller numbers of VCGs when they were re-evaluated based on the formation of complementary heterokaryons of *nit* mutants. This result indicates that the use of *nit* mutant-based heterokaryotic reaction is a more sensitive method to determine vegetative compatibility than the macroscopic observation of barrage formation. For instance, isolates Southbrook and 30B-24 appeared incompatible when assessed macroscopically, but did yield a stable heterokaryon when assessed using pairings of *nit* mutants from these isolates (Fig. 1D). Similar cases in which different methods of analyzing vegetative compatibility produced conflicting results have been reported in *Verticillium* spp. In the study by Joaquim and Rowe (20), 22 isolates of *V. dahliae* that had been assigned to 15 VCGs by pairings of microsclerotial color mutants (29) were regrouped to only five VCGs when auxotrophic *nit* mutants were employed.

Vegetative compatibility assays have greatly contributed to an understanding of the genetic diversity of important plant-pathogenic fungi, and have demonstrated that their VCGs have a significant correlation with formae speciales (28), strains (5,10), or races (9). Consequently, vegetative compatibility assays have been used to diagnose pathogenic strains or differentiate virulent races in various local populations, providing important information for managing diseases (34,37). Benchmarking the advanced technical approaches and accumulating knowledge about *nit* mutant-based VCGs of *Fusarium* and *Verticillium* spp. will pro-

Fig. 3. Epifluorescence microscopic images of hyphal anastomosis of *Sclerotinia homoeocarpa* on Czapek solution agar (CDA), using differential interference contrast (DIC), ethidium bromide staining and acridine orange staining. **A, B, and C**, Self-fused hyphae. **D, E, and F**, Hyphae fused between compatible isolates. **G and H**, Hyphal fusion between incompatible isolates. Acridine orange stains nuclei within both dead and living cells but ethidium bromide stains dead cells only. Scale bar = 10 μ m.

vide greater insight into the association of VCGs with genetic and phenotypic similarity of *S. homoeocarpa* in the future. Since all 21 isolates used in this study were previously examined for their genetic similarity based on random amplification of polymorphic DNA (RAPD) markers by Chakraborty et al. (6), their genetic data can be compared with our current VCGs. It is possible to find indicative evidence that *nit*-mutant-based VCGs may be associated with genetic similarity. For example, isolates belonging to the same VCG are likely to be genetically similar. However, this conclusion is arrived at with caution because the results are based on the small number of isolates. Further study is required to confirm the relationships among VCGs, genotypes, and phenotypes, such as virulence, using more isolates collected from diverse geographical locations in North America and Europe.

Determination of allelism for *nit* mutation phenotypes was not possible due to an unknown teleomorphic stage of *S. homoeocarpa* in the United States. Therefore, mutants recovered in this study could only be compared with known phenotypic characteristics and nomenclatures of alleles used in *F. oxysporum* (8) and *V. dahliae* (20). In fact, many similarities were observed in *nit* mutant phenotypes between *S. homoeocarpa* and *F. oxysporum*. Three common *nit* mutant types of *nit1*, *nit3*, and NitM were observed; *nit1* was the most prevalent in both fungi. Complementation occurred between different phenotypes, and prototrophic growth of *nit* mutants on CDA often varied among isolates and *nit* mutant phenotypes. Within a same VCG, stronger reactions occurred in certain pairings, but only weak heterokaryons formed in other pairings. Future investigation into phenotyping *nit* mutants derived from more isolates, in addition to the current 21

isolates used in this study, is necessary to clarify the similarity and uniqueness of *nit* mutants in *S. homoeocarpa*. This will help to establish reference tester isolates for further large-scaled VCG screening. Based on the current assay, NitM of isolates representing each VCG may be candidates for references because it produces strong complementary heterokaryons with *nit1* and *nit3*.

The confirmation of heterokaryosis is pivotal in terms of the evolution of *S. homoeocarpa* because heterokaryosis is one of the most important mechanisms for providing an increase of genetic variability in asexual fungi (23). Due to a lack of sexual recombination in nature as far as currently known, the genetic variation in the *S. homoeocarpa* may be caused mainly by asexual reproduction. Considering frequent in vitro heterokaryotic formation between compatible isolates confirmed in this study, heterokaryosis is also likely an important means of increasing genetic variations in *S. homoeocarpa* and may play a significant role in the epidemiology of dollar spot on turfgrass.

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