Prescreening Slash Pine and *Cronartium* Pedigrees for Evaluation of Complementary Gene Action in Fusiform Rust Disease

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ABSTRACT

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Single-urediniospore cultures of the fusiform rust fungus were used to inoculate seedlings from 10 full-sib families of a five-parent slash pine diallel at two different times in 1994. The presence or absence of fusiform rust galls was recorded for each inoculated seedling at 9 months postinoculation, and percent infection levels for each family-inoculum-time combination were used for detecting differences among host families and fungal cultures and for identifying differential interactions. The existence of differential interactions between two or more fungal cultures and two or more host families verifies that complementary gene action does exist in this pathosystem. Some host families may be excluded from more detailed interaction studies on the basis of their redundancy and lack of participation in differential interactions.

Additional keyword: *Pinus elliottii*

Despite 40 years of phenotypic selection and breeding in both loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm. var. *elliottii*) against the fusiform rust fungus (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow), the genetic basis of this host-pathogen interaction is not clearly understood (4,24). Recent efforts to characterize the genetic interactions involved in the development of this disease on loblolly and slash pines have resulted in the hypothesis that the interaction may conform to a complementary genetic system (16,17,21,30,32).

A principal argument against the complementary genetic system is that family and race specificity are artifacts of plant breeding and thus are very unlikely to occur in a natural pathosystem such as that of *P. elliottii* var. *elliottii* and *C. quercuum* f. sp. *fusiforme* (2). Such specificity, however, has been demonstrated in wild populations of *Glycine canescens* and *G. argyrea* and the rust pathogen *Phakopsora pachyrhizi* (3). Furthermore, it has been argued that much of what is called "general" or "quantitative" resistance is the result of specificity and can be explained by the interactions among complementary gene pairs (19).

The complementary genetics model for fusiform rust disease has three basic tenets (15,30). First, low levels of reaction (i.e., resistance) in the host and of pathogenicity (i.e., avirulence) in the fungus are considered dominant traits. Second, if the genotype of the complementary gene from either the host or pathogen is homozygous recessive, then a high infection type (e.g., a gall) will occur. Finally, when two or more pairs of complementary genes are present, the gene pair that imparts the lowest infection type (e.g., no gall) is epistatic to all other gene pairs.

The fusiform rust pathosystem, however, presents some challenges in verifying the model and identifying complementary gene pairs. First, the nonrepeating spermatial-aecial stage of the fusiform rust fungus is the one that causes the economic damage we wish to control. An in vitro inoculation technique in which single-genotype haploid cultures of the fungus were used has been reported for infecting loblolly pine hypocotyls (13,31). However, in vitro efforts to infect epicotyls or micropropagated shoots were not successful (31). Recent attempts to inoculate slash pine epicotyls by using agar plugs containing mixed- or single-genotype hyphal cultures of the fungus have been highly variable, thus restricting at present the potential usefulness of the technique for research (R. Schmidt, *personal communication*). Single-aeciospore and single-urediniospore cultures exhibiting varying degrees of pathogenicity have been developed for use in complementary genetics research of this pathosystem (6,17,21,30,32). The basidiospores that infect pine, however, have been shown to be haploid products of meiosis and hence represent a segregating population (5,7).

Significant problems with loblolly and slash pine include the long time it takes to generate F_1 and F_2 populations (compared with agronomic species) and the inability to clonally propagate large numbers of selected host genotypes. Rooted cuttings are currently the most efficient means of vegetative propagation. Rooting success, however, is still dependent upon the genotype of the individual. In addition, there are reports of rooted cuttings exhibiting an apparent increased resistance to the rust fungus (9,10).

Genomic mapping and bulk segregant analysis have been used recently to identify regions of the loblolly host genome that confer a low infection type when single-aeciospore cultures of the rust fungus that possess the corresponding complementary gene are used for inoculation (17,32). These techniques, in conjunction with the basic hypotheses of the complementary genetics model, are being used to identify reaction genes in the host (H. Amerson, *personal communication*). This work has utilized a megagametophyte DNA approach that can discern only genes of a heterozygous nature in the maternal host parent.

It has been demonstrated in the barley–mildew (*Hordeum vulgare–Erysiphe graminis* f. sp. *hordei*) disease association that the genetics of the pathogen can be inferred from a detailed genetic study of the host (20). A similar approach could be used for studying the southern pine–fusiform rust disease association. By inoculating an array of host pedigrees from a full-diallel mating scheme with basidiospores from several single-urediniospore fungal cultures and using the tenets of the model, hypothetical genotypes in both organisms for each complementary gene pair could be derived.

Once the initial host and pathogen genetic arrays have been rogued of uninformative pedigrees, the resources required for subsequent studies involved in mapping reaction genes in the host or pathogenicity genes in the rust fungus could be substantially reduced. The objective of this study was to determine whether seedlings

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from full-sib families of slash pine could be used to prescreen host plant material and cultures of the fusiform rust fungus for their potential to exhibit complementary genetic interactions.

MATERIALS AND METHODS

Host seedlings. Five first-generation selections of *P. elliottii* var. *elliottii* were control pollinated in a full-diallel crossing design. All five selections were chosen from the U.S. Forest Service's Harrison Experimental Forest in Harrison County, MS. In previous research, in which bulk inocula of the rust fungus was used, these selections were classified as follows: parent 8-7, resistant; parents 9-2 and 18-27, moderately resistant; and parents 18-26 and 18-62, susceptible (14,27). Twenty of the 25 full-sib families were propagated from seed (8) (selfs were excluded because of insufficient seed). Seed germination was staggered so that the seedlings were 8 weeks old at the time of each inoculation series.

Pathogen cultures. Four single-urediniospore cultures of *C. quercuum* f. sp. *fusiforme* were used in this study: CCA-2.SS1.SS1, WLP-10-2.SS1.SS1, CZ.SS1, and LM-5.SS1.SS1 (5; U.S. Forest Service SRS Study Plan 2.01, *unpublished,* available upon request). All four cultures originated from aeciospore collections made in 1984: CCA-2 on planted Livingston Parish loblolly pine in Madison County, FL; WLP-10-2 on planted Livingston Parish loblolly pine in Livingston Parish, LA; CZ on native slash pine in Washington Parish, LA; and LM-5 on native slash pine in Jones County, MS. Single-urediniospore cultures were then developed from the aeciospore collections (5,23).

Artificial inoculations. All inoculations were performed at the Southern Institute of Forest Genetics in Gulfport, MS, on 22 to 25 May and 11 to 14 July 1994. For each inoculation time and rust fungus culture, an average number of 36 seedlings from each full-sib family were randomly divided into four replications. In each replication (day), families and cultures were randomly ordered prior to the start of that day's inoculations. A forced-air apparatus (29) was used to deliver 12 to 18 basidiospores/mm^2 of each fungal culture to the juncture of the stem and base of the terminal tuft of juvenile needles on each seedling. Spore density was monitored after inoculation of every tenth seedling and adjusted to ensure close adherence to the target density.

After inoculation, the seedlings were incubated in the dark at 20 to 22°C and 100% relative humidity for 24 h. After incubation, the seedlings were returned to the greenhouse and grown under an 18-h-light photoperiod provided by 1,000-W metal halide lamps. Two weeks after inoculation and every second week thereafter, the seedlings were fertilized (20-20-20 N-P-K, 200 ppm N).

Data collection. The presence or absence of fusiform rust galls on the seedlings was recorded 9 months after inoculation. The percent infection was then determined for each replication within a given full-sib family × inoculation time × inoculum treatment combination.

Given the simplest scenario of only one complementary gene pair operating in this biological array, infection levels can range from 0 to 100%, depending upon the segregation ratios of the complementary genes in each organism. If the single-urediniospore culture is homozygous for high pathogenicity (i.e., aa), then all the haploid basidiospores would be virulent and the expected infection would be 100%, regardless of segregation in the host. If the culture is homozygous for low pathogenicity (i.e., AA), then all the haploid basidiospores would be avirulent and the expected infection could be 0, 25, 50, or 100%, depending on whether the R:r segregation ratio is 1:0, 3:1, 1:1, or 0:1, respectively. Given a 1:1 segregation of avirulent and virulent basidiospores resulting from a heterozygous single-urediniospore culture and R:r host segregation ratios of 1:0, 3:1, 1:1, or 0:1, the expected infection levels could be 50, 62.5, 75, or 100%, respectively. The array of expected percent infection levels expands as more complementary gene pairs are added to the model (30).

Statistical analysis. The data were initially analyzed by the general linear model procedure (6th ed., SAS Institute, Cary, NC) to test for significant effects. Because percent infection data are based upon a binomial response and some data points lie outside the stable variance range of 30 to 70%, all data were transformed to the arcsine of the square root of the percent infection (1). Family (FAM), inoculum (INOC), and inoculation time (TIME) were all considered to be fixed effects. Reciprocal cross effects were treated as a nested effect within family (RECIP[FAM]) and considered fixed as well. The replication effect was nested within inoculation time (REP[TIME]) and regarded as random.

Differential interactions. Within a particular inoculation time, a significant family × inoculum interaction effect will suggest differential interactions between two or more fungal cultures and two or more host families. These differential interactions are characteristic of the complementary genetics system (19) and have been reported in earlier research on half-sib families of loblolly pine inoculated by using bulk inocula collected from single galls (25). For each inoculation time, a series of two-way contingency tables for individual combinations of two fungal cultures and two families were constructed, and significant (P = 0.05) differential interactions were identified by chi-square analysis (22).

RESULTS

Analysis of variance revealed no significant reciprocal cross effects. Therefore, forward and reciprocal cross data were pooled (Table 1), after which the replication effect was no longer significant (P = 0.1311). Hence, replications were pooled. Increasing the average sample size for a given family-inoculum-time combination to 72 greatly increased the power of estimating percent infection levels. Family, inoculum, and family × inoculum effects were all significant (P = 0.0001) and accounted for 39% of the observed variation. These data suggest that infection type reversals exist. While the time main effect was not significant (P = 0.8129), all higher order interactions involving time implied that time did play some role in the development of disease symptoms.

Percent infection data for all family-inoculum-time combinations are given in Table 2. For inoculations conducted in July, any host family having 8-7 as a parent differentiated the four inocula. On these families, culture CCA-2.SS1 averaged more than 50% infection, while LM-5.SS1 averaged 18%. Cultures CZ.SS1 and WLP-10-2.SS1 were indistinguishable, each scoring less than 5% infection. The May inoculations yielded no host families capable of

Table 1. Analysis of variance for the effects of replication (REP), family (FAM), cross direction (RECIP), inoculation time (TIME), and inoculum (INOC) on the arcsine of the square root of percent infection of slash pine seedlings after inoculation with basidiospores from four single-urediniospore cultures of the fusiform rust fungus[x]

	Unpooled data[y]			Pooled data[z]		
Effect	df	Mean square	P	df	Mean square	P
FAM	9	1.9251	0.0001	9	1.9069	0.0001
RECIP(FAM)	9	0.1056	0.1042	...	...	...
INOC	3	0.8579	0.0001	3	0.8727	0.0001
FAM*INOC	27	0.2553	0.0001	27	0.2529	0.0001
TIME	1	0.0036	0.8123	1	0.0036	0.8129
REP(TIME)	6	0.1469	0.0363	7	0.0946	0.1311
FAM*TIME	9	0.4087	0.0001	9	0.4228	0.0001
INOC*TIME	3	1.1029	0.0001	3	1.0783	0.0001
FAM*INOC*TIME	27	0.1038	0.0297	27	0.1089	0.0216

[x] Mean squares and P values were determined by the SAS general linear model procedure.
[y] R^2 for unpooled reciprocal cross data = 0.57.
[z] R^2 for pooled reciprocal cross data = 0.56.

differentiating the four inocula. None of the fungal cultures were able to identify any general difference patterns among the host families.

Differences in percent infection data between the May and July inoculations were observed in several families for each of the four fungal cultures (Table 2). When challenged with CZ.SS1 and WLP-10-2.SS1 inoculum, all host families having 8-7 as one of the parents exhibited a noticeable reduction in infection levels for the July inoculations compared with those in May. For CCA-2.SS1, the percent infection was greater for the May inoculation series, regardless of host pedigree.

Differential interactions. Several significant ($P = 0.05$) differential interactions among particular pairs of host families and fungal cultures were identified in both the May and July inoculation series (Table 3). The standard error of the mean percent infection data was 6%, but this error did not interfere with the detection of a differential interaction.

In May, the host family pair 8-7 × 18-62 and 18-26 × 18-62 exhibited an infection type reversal for fungal cultures CCA-2.SS1 and CZ.SS1. Another family pair, 8-7 × 18-62 and 9-2 × 18-26, identified differences between fungal cultures CCA-2.SS1 and WLP-10-2.SS1. A third family pair, 8-7 × 18-62 and 8-7 × 18-26, differentiated fungal cultures LM-5.SS1 and WLP-10-2.SS1. A fourth differential interaction involved families 8-7 × 18-62 and 18-26 × 18-62 and fungal cultures CCA-2.SS1 and LM-5.SS1. These same two families also interacted differentially with fungal cultures CCA-2.SS1 and WLP-10-2.SS1 in the May inoculation series.

Four significant differential interactions were found among the July inoculations. All four involved the fungal culture pair CCA-2.SS1 and WLP-10-2.SS1. In addition, all four differential interactions shared the common family 18-27 × 18-62. The other host families involved were 8-7 × 9-2, 8-7 × 18-26, 8-7 × 18-62, and 9-2 × 18-26.

Within family 9-2 × 18-26, a significant differential interaction was also observed involving the two fungal cultures CCA-2.SS1 and WLP-10-2.SS1 and the two inoculation times.

DISCUSSION

Pathogenic variability was shown within *C. quercuum* f. sp. *fusiforme* when bulk aeciospore isolates from different geographic sources were tested on half-sib families of loblolly and slash pine (27,28). The observed variability was particularly striking among progeny from putative resistant parents, in which there was in some cases a complete breakdown of resistance (28). In one study with loblolly pine, the classic reversal of infection levels, indicative of specificity, was observed between two bulk aeciospore isolates and two half-sib families (25). Single-aeciospore cultures from a single gall were found to be almost as variable as bulk aeciospore collections from several galls (23). However, no infection type reversals were observed. Data from this research support existing data and show the existence of differential interactions (Table 3) and the operation of the complementary genetics model in this pathosystem.

Given the existence of differential interactions among these data, one can begin to deduce hypothetical genotypes for both organisms of this disease association. According to the July inoculation data (Tables 2 and 3), all families having 8-7 as a parent and that were inoculated with fungal culture WLP-10-2.SS1 exhibited extremely low levels of infection. Since the basidiospore is haploid and by definition of the complementary genetics model, culture WLP-10-2.SS1 must be homozygous dominant for low pathogenicity at one locus. On the host side, one of the parents must be homozygous dominant for low reaction at the same locus. Otherwise, if both parents were heterozygous, an infection level of 25% should have been observed. The odds are very low that four unique parents would all be homozygous dominant at the same locus. Hence, the common parent, 8-7, is homozygous dominant. This conclusion agrees with previous research (15).

Hypothetical genotypes have been derived for all five host parents and the two single-urediniospore cultures, CCA-2.SS1 and WLP-10-2.SS1, for the July inoculation series (30). Recognizing that heterozygosity can exist at all interaction loci in both organisms makes the generation of possible mean percent infection levels more difficult than previously reported (15). Current efforts are underway to develop computer models that will produce these phenotypic ratios and their corresponding host-pathogen genotypes.

Table 2. Percent infection levels (mean ± SD) of 10 full-sib slash pine families inoculated with basidiospores from four single-urediniospore cultures of the fusiform rust fungus on 22 to 25 May and 11 to 14 July 1994[z]

	May				July			
Family	CCA-2	CZ	LM-5	WLP-10-2	CCA-2	CZ	LM-5	WLP-10-2
8-7 × 18-27	20 ± 5	15 ± 4	9 ± 3	11 ± 4	30 ± 5	4 ± 2	18 ± 4	1 ± 1
8-7 × 9-2	39 ± 5	18 ± 4	18 ± 4	16 ± 4	57 ± 6	3 ± 2	15 ± 4	3 ± 2
8-7 × 18-26	44 ± 7	36 ± 7	15 ± 5	40 ± 7	50 ± 6	0 ± 0	18 ± 4	4 ± 2
8-7 × 18-62	50 ± 6	35 ± 5	34 ± 5	20 ± 5	59 ± 6	5 ± 2	16 ± 4	3 ± 2
18-27 × 9-2	16 ± 5	22 ± 5	18 ± 5	18 ± 5	33 ± 6	16 ± 4	29 ± 6	21 ± 5
18-27 × 18-26	23 ± 6	29 ± 7	29 ± 7	22 ± 6	44 ± 7	39 ± 7	43 ± 7	50 ± 7
18-27 × 18-62	23 ± 5	33 ± 5	24 ± 5	29 ± 5	38 ± 5	34 ± 5	34 ± 5	55 ± 6
9-2 × 18-26	34 ± 6	41 ± 7	27 ± 6	52 ± 6	71 ± 6	34 ± 5	34 ± 5	30 ± 5
9-2 × 18-62	36 ± 5	40 ± 6	43 ± 6	36 ± 5	80 ± 5	34 ± 5	34 ± 5	44± 6
18-26 × 18-62	38 ± 5	60 ± 6	63 ± 5	65 ± 5	70 ± 6	73 ± 5	58 ± 6	70 ± 5

[z] Average sample size per family-inoculum-time combination was 72 seedlings. All four single-urediniospore cultures have the extension .SS1.

Table 3. Summary of two-way contingency analyses showing significant differential interactions ($P \leq 0.05$) among 10 full-sib slash pine families inoculated with basidiospores from four single-urediniospore cultures of the fusiform rust fungus[z]

	May				July			
Family	CCA-2	CZ	LM-5	WLP-10-2	CCA-2	CZ	LM-5	WLP-10-2
8-7 × 18-27	...	...	...	...	...	...	...	...
8-7 × 9-2	...	...	...	...	57 ± 6 B	...	...	3 ± 2 B
8-7 × 18-26	...	...	15 ± 5 c	40 ± 7 c	50 ± 6 A	...	...	4 ± 2 A
8-7 × 18-62	50 ± 6 abde	35 ± 5 a	34 ± 5 cd	20 ± 5 bce	59 ± 6 C	...	...	3 ± 2 C
18-27 × 9-2	...	...	...	...	...	...	...	...
18-27 × 18-26	...	...	...	...	...	...	...	...
18-27 × 18-62	...	...	...	...	38 ± 5 ABCD	...	...	55 ± 6 ABCD
9-2 × 18-26	34 ± 6 bI	...	...	52 ± 6 bI	71 ± 6 DI	...	...	30 ± 5 DI
9-2 × 18-62	...	...	...	...	...	...	...	...
18-26 × 18-62	38 ± 5 ade	60 ± 6 a	63 ± 5 d	65 ± 5 e	...	...	...	...

[z] Unique differential host and rust fungus pairs are identified by matching lowercase letters for the May inoculation, by uppercase letters for the July inoculation, and by Roman numerals for times between inoculations.

Some of the current data, however, appear to conflict with data from earlier studies involving some of the host pedigrees and the rust fungus culture LM-5. Virtually no galls were observed on either half-sib or full-sib progeny involving parent 8-7 when they were challenged with the bulk aeciospore inoculum LM-5 (11, 27). In addition, progeny of 18-26 × 18-62 exhibited a mean percent infection level of >90% (11). When challenged with the single-urediniospore culture LM-5.SS1, however, the mean percent infection of full-sib families with 8-7 as one of the parents ranged from 9 to 34% (Table 2). Progeny of 18-26 × 18-62, however, showed a mean infection level of only 58%. A plausible explanation lies in the way the bulk aeciospore isolates and single-urediniospore cultures were collected and subsequently handled.

The original aeciospores of LM-5 were collected from a single gall near Laurel, MS, in 1972. The isolate was soon discovered to be fairly benign, and because of its avirulent nature, LM-5 was used in subsequent studies (11,27). To supply enough inoculum for these studies, investigators either went back to the original tree and collected fresh aeciospores or they collected aeciospores from compatible interactions on slash pine seedlings of unknown genetic background inoculated with basidiospores of the LM-5 pedigree (T. Roland, *personal communication*). In regard to the first collection method, it must be noted that bulk aeciospore isolates are a complex heterogeneous mixture of dikaryotic spores resulting from the successful union of compatible mating types, and therefore the genetic composition of these mixtures can change from year to year. In the latter instance, when increasing the quantity of a putative "avirulent" isolate by collecting aeciospores from compatible interactions, one could be selecting for rare alleles in the fungal population that are capable of compromising the complementary low reaction gene or genes in the host (12).

Parents 8-7 and 9-2 have been classified as resistant and moderately resistant, respectively, to bulk inocula of the pathogen (11,14,27). It would be incorrect, however, for one to assume that the progeny of 8-7 × 9-2 would also exhibit a high level of resistance. For example, in the July inoculation, family 8-7 × 9-2 was virtually uninfected by the WLP-10-2.SS1 inoculum yet exhibited 57% infection when inoculated with CCA-2.SS1. The incorrect assumption does not result from the use of the term "resistant," but rather from failing to recognize the nature of the resistance.

Distinct differences exist in the differential interaction patterns between the two inoculation times (Table 3). It could be argued that the inoculation time effect and the differential interaction within family 9-2 × 18-26 (between the May and July inoculation series and rust fungus cultures CCA-2.SS1 and WLP-10-2.SS1) are artifacts of the artificial inoculations. The forced-air system (29) and the incubation protocols, however, have yielded reliable data (5–8,11,21) and are not suspect. The effect could be caused by temperature differences. A minimum period of 18 h with temperatures fluctuating between 16 and 26°C and humidity maintained close to the moisture saturation point is needed for production of and infection by basidiospores on pines (26). Postinoculation day and night temperatures of 30 and 26°C, respectively, significantly reduced infection levels, but challenged plants growing in day-night temperature regimes ranging from 30 and 22 to 22 and 18°C did not differ in mean percent infection levels (16).

Unfortunately, postinoculation greenhouse temperature data were lost for this study. One could speculate, however, that warmer day and night temperatures during July so affected the physiology of either the host seedlings or the fungal cultures that the disease failed to develop. This hypothesis could account for the lower percent infection levels in July for pedigrees with 8-7 as a parent challenged with fungal cultures CZ.SS1 or WLP-10-2.SS1. However, this host physiological resistance does not explain the ability of these same families to exhibit higher percent infection levels when inoculated with fungal culture CCA-2.SS1 in July, nor does this hypothesis explain the ability of fungal cultures CZ.SS1 and WLP-10-2.SS1 to infect some families and not others in July. Clearly, further research is needed to discern whether this is an environmental effect on the biology of the host or the pathogen or a genetic effect such as a temperature-sensitive complementary gene pair similar to those identified in stem rust of wheat (18).

Some host families and rust fungus cultures can play important roles in advanced complementary genetic studies. Three host families with 8-7 as one parent that were inoculated with culture CCA-2.SS1 exhibited mean infection levels of approximately 50% (Table 2), and it has been hypothesized that they have at least one dominant allele for low reaction to the fungus (30). The fungal culture is thought to be heterozygous for pathogenicity at this interaction locus (30). If one were to again inoculate any of these families with CCA-2.SS1 at a higher spore density (e.g., 25 to 30 spores/mm^2) in order to ensure multiple infections and hyphal anastomosis, the resulting dikaryotic aeciospores from the compatible galls should be homozygous for high pathogenicity. If the host families are then inoculated with this genetically advanced culture at the lower inoculum density and the mean infection levels increase from 50 to 100%, it would confirm that putting selection pressure on the pathogen could break down this "resistance" in only one generation.

The value of using seedlings in the initial screening is that a large array of both plant material and fungal cultures can be economically prescreened. Identification of genotypes with the potential to exhibit a differential response can preserve large amounts of time and material, thus increasing the feasibility of developing the clonal plant material needed for more detailed analyses of complementary gene action in this pathosystem. Clonal propagation of differential host lines could also be used to selectively monitor gene frequency shifts in the pathogen population.

No differential interactions were detected with families 18-27 × 9-2, 18-27 × 18-26, and 9-2 × 18-62 (Table 3), which could be dropped from further study. An argument could be made for discontinuing the CZ.SS1 culture because its infection level pattern in this study was similar to that of WLP-10-2.SS1 (Table 2) and it was found in only one differential interaction (Table 3). By not carrying such host and pathogen pedigrees forward, the workload of future studies involving this diallel and these fungal cultures could be reduced by 50%. A workload reduction of this magnitude can either be translated into direct cost savings or allow for the reallocation of resources to other critically needed studies.

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