1. Introduction

Sclerotinia homoeocarpa F.T. Bennett is the causal organism of dollar spot in turfgrasses and is a multinucleate fungus with a history of resistance to multiple fungicide classes. Heterokaryosis gives rise to the coexistence of genetically distinct nuclei within a cell, which contributes to genotypic and phenotypic plasticity in multinucleate fungi. We demonstrate that field isolates, resistant to either a demethylation inhibitor or methyl benzimidazole carbamate fungicide, can form heterokaryons with resistance to each fungicide and adaptability to serial combinations of different fungicide concentrations. Field isolates and putative heterokaryons were assayed on fungicide-amended media for in vitro sensitivity. Shifts in fungicide sensitivity and microsatellite genotypes indicated that heterokaryons could adapt to changes in fungicide pressure. Presence of both nuclei in heterokaryons was confirmed by detection of a single nucleotide polymorphism in the β-tubulin gene, the presence of microsatellite alleles of both field isolates, and the live-cell imaging of two different fluorescently tagged nuclei using laser scanning confocal microscopy. Nucleic adaptability of heterokaryons to fungicides was strongly supported by the visualization of changes in fluorescently labeled nuclei to fungicide pressure. Results from this study suggest that heterokaryosis is a mechanism by which the pathogen adapts to multiple fungicide pressures in the field.

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study from Canada using random amplified polymorphic DNA markers found evidence for sexual or asexual genetic recombination (Hsiang and Mahuku, 1999). There is also evidence that individual isolates within populations of this fungus undergo heterokaryon formation, and thus, genetic diversity in S. homoeocarpa might be explained by heterokaryotic isolates and/or parasexuality through vegetative compatibility (Jo et al., 2008; Liberti et al., 2012). Vegetative compatibility refers to the ability of different fungal individuals to form stable heterokaryotic mycelium. Hyphal strands fuse regularly and freely between individuals in filamentous fungi and lead to cytoplasmic mixing, allowing the exchange of nuclei between hyphal cells. When genetically similar fungal strains exchange nuclei, a heterokaryon is formed. Control over whether these nuclei are compatible resides with the heterokaryon (het) and/or vegetative compatibility (vic) loci (Glass et al., 2000; Leslie, 1993). In some fungi, nuclei in heterokaryons fuse (karyogamy) following by haploidization or recombination, which may result in parasexual exchange of genes (Franco et al., 2011).

Previous reports on responses of heterokaryons to fungicide pressure indicate that heterokaryosis can result in enhanced fungicide tolerance (Esuruoso and Wood, 1971; Meyer and Parmeter, 1968; Ogden and Grindle, 1983; Webster et al., 1968). In addition, these works showed that heterokaryons displayed phenotypic plasticity in response to presence or absence of fungicides. However, none of the aforementioned studies analyzed the adaptive ability of heterokaryons when subjected to sequential, disparate fungicide pressures, a situation potentially simulating fungicide application programs in the field.

Fungicide resistance in dollar spot is prevalent on golf courses, however, little is known about the evolution of resistance development. Therefore, we sought to determine if fungicide sensitivity alleles could be shared in heterokaryotic isolates of S. homoeocarpa, potentially contributing to the spread of resistance in field populations of this pathogen. Furthermore, the study of nucleic adaptability of heterokaryons to stresses, fungicides in this study, adds to the body of knowledge on the biology of multinucleate fungi. Our objectives were the following: (1) determine if heterokaryons display greater adaptability to fungicides than homokaryons based on growth assays, (2) determine if there are genotypic shifts corresponding to heterokaryon adaptability observed in growth assays, and (3) to visualize heterokaryons’ adaptability to fungicides using fluorescently labeled nuclei and live-cell imaging.

2. Materials and methods

2.1. Sclerotinia homoeocarpa isolates

Two vegetatively compatible pairs of field isolates with different fungicide resistance profiles were selected for this study (Table S1). Field isolates B1 and R1, collected from MA, were sensitive to the demethylation inhibitor (DMI) fungicide propiconazole (PP) (as determined by no mycelial growth after 3 days of incubation on potato dextrose agar (PDA, Difco Laboratories, Inc.) amended with 1 \( \mu \)g ml\(^{-1}\) of PP), and resistant to the methyl benzimidazole carbamate (MBC) fungicide thiophanate-methyl (TM) (as determined by growth after 3 days of incubation on PDA amended with 1000 \( \mu \)g ml\(^{-1}\) of TM) (Jo et al., 2006; Popko et al., 2013). Field isolate 7 was collected from OH and field isolate 46 was collected from WI, and both were known to be resistant to PP and sensitive to TM (Jo et al., 2006; Koch et al., 2009). These isolate pairs are referred to as the TM resistant (TMR) and PP resistant (PPR) parents. In addition, two field isolates HRI11 and JTS30, which were resistant or sensitive to both PP and TM fungicides, respectively (Sang et al., 2015), were used as growth controls in all growth assays.

All isolates were maintained in long-term storage using the method described in Chakraborty et al. (2006). Briefly, Kentucky bluegrass (Poa pratensis L.) seeds autoclaved twice in potato dextrose broth (PDB) (Difco Laboratories, Inc.) in 1.5 mL screw-cap microcentrifuge tubes were inoculated with mycelial agar plugs from each isolate. Once abundant mycelia were observed on the infected seeds, tubes were desiccated and kept at 4 °C.

2.2. Co-inoculation of parents and culturing of heterokaryons

Each pair of TM resistant (TMr) and PP resistant (PPr) parents in all four crosses (B1 × 7, B1 × 46, R1 × 7, and R1 × 46). Each co-inoculation was set up by placing of parent agar plugs 10 mm apart from each other on a 90 × 15 mm Petri plate (Krackler Scientific, Inc.) on full-strength PDA. After four days of incubation (all plate incubations throughout this experiment were performed at 25 °C), ample white aerial mycelium developed at the zone of interaction, where the mycelium of parent isolates converged. Mycelium from the center of each zone of interaction between parents, and the edges of the parent sides of each cross plate was subcultured onto PDA plates. Subcultures from the parent sides of the co-inoculations were used as controls for SSR genotyping within each cross. In addition, two replications of each parent and of the growth control isolates HRI11 and JTS30 were set up by subculture from long-term storage onto PDA.

After two days of incubation, plugs from all isolates were taken and plated under water agar to facilitate isolation of individual hyphal tips. Hyphal tip isolation has been previously used as a method to isolate pure cultures of heterokaryons (James et al., 2011; Meyer and Parmeter, 1968). Two percent water agar (WA) in 90 × 15 mm Petri plates was prepared. Plugs were taken from the periphery of each parent, parent control, growth control or heterokaryon plate and were arranged radially in empty 90 × 15 mm Petri plates. Rectangles of WA measuring about 9 cm\(^2\) by 5 mm thick were cut out of the WA plates and centered over each piece of inoculum, and were arranged so that they did not touch adjacent pieces. Plates were incubated until individual hyphal tips penetrated the top surface of the WA, within 96 h. Single hyphal tips were aseptically removed from the agar using forceps with the aid of a dissecting microscope at 50 × magnification as they penetrated the surface, after 24, 48 or 96 h.

Individual hyphal tips were subcultured onto PDA plates and incubated until viable tips yielded sufficient mycelium for subsequent subculture. Of the surviving hyphal tip subcultures, a subset of isolates for each cross was randomly selected from the most robust cultures. Among isolate types ten expected heterokaryons (EHs) and four parent controls (two of each parent) were isolated per cross, as well as one of each parent, and one of each growth control isolate. All isolates were subcultured onto 90 × 15 mm PDA plates to provide sufficient area for in vitro fungicide mycelial growth assays. Media were prepared by amending full-strength PDA with three concentrations of each fungicide. In brief, bottles of PDA were cooled to ~55 °C before adding fungicide amendments. PP amended media (abbreviated as PP) were prepared by performing serial dilutions in sterile Milli-Q water using commercial grade Banner MAXX 1.3EC (Syngenta Crop Protection) to achieve final concentrations of 0.01, 0.1, and 1 \( \mu \)g ml\(^{-1}\) in PDA. TM amended media (abbreviated as TM) were prepared by addition of commercial grade Cleary’s 3336 4F (Cleary Chemical Corporation) to achieve final concentrations of 0.1, 1, and 1000 \( \mu \)g ml\(^{-1}\) in PDA. In the following text concentrations are abbreviated by omitting the units e.g. “0.1 PP”, “1 TM”.

2.3. Fungicide mycelial growth assays

In an initial fungicide mycelial growth assay, hyphal tip cultures were subcultured onto fungicide amendments in duplicate. Mycelial growth (MG) of each isolate was quantified by measuring two approximately perpendicular diameters of S. homoeocarpa colonies using digital calipers (Mahr16EX) and subtracting the plug diameter. Diameter measurements were averaged to yield mean diameter. Mycelial growth of each isolate was measured using this method three, six, and thirteen days after inoculation (3-DAI, 6-DAI, 13-DAI).
Maximum MG was 48 mm due to Petri plate size. Mood’s median test was used to determine significance.

In an effort to characterize the adaptive ability of putative heterokaryons a “swap” fungicide MG assay was performed. Seven days after the start of the initial fungicide MG assay, inocula from one biological replicate of each isolate on every PP amendment was swapped to plates of every TM amendment in a fully factorial experimental design, as well as to non-amended PDA. Fourteen days after the start of the initial fungicide MG assay, inocula from one biological replicate of all single TM amendments were swapped to plates of every PP amendment in a fully factorial experimental design, as well as to non-amended PDA. Mycelial growth of each isolate was quantified in the same way as in the initial fungicide mycelial growth assay and measured at 2-DAI, 5-DAI, and 12-DAI. Mood’s median test was used to determine significance.

2.4. DNA extraction, PCR, SSR genotyping and β-tubulin sequencing

DNA was extracted from one biological replicate of every isolate that displayed over ~10 mm of healthy mycelial growth. The DNA extraction protocol described in Hulvey et al. (2012) was used. Forty primers were screened and gradient PCR was performed to yield a primer that gave the clearest possible polymorphism between TMR and PPr parents (Table S2). Both PPr parents yielded a fragment of ~ 245 bp (PPr genotype), while both TMR parents yielded a fragment of ~ 255 bp (TMR genotype). The microsatellite primers were designed and microsatellites were amplified using methods of Hulvey et al. (2012). PCR in 10 μl volume reactions was performed in 96-well plates using a MJ PTC-200 thermocycler (MJ Research). In order to separate amplicons, gel electrophoresis was performed by loading five μl of PCR product into 3% (wt/vol) Metaphor agarose (Cambrex Bio Science Rockland, Inc.) and run in 1 x TBE buffer at 130 V for 4 h. Gels were stained with ethidium bromide at 0.5 μl/ml for 30 min, followed by 15 min of destaining in deionized water. Gel bands were visualized under UV light and photographed, and gel pictures were captured as TIFF files.

Genotypes in a subset of EHs were further characterized by amplifying and sequencing a 250 bp segment of β-tubulin gene using a primer set (Table S2), which was known to contain a single nucleotide polymorphism (SNP) conferring TM resistance in S. homoeocarpa (Koenraadt et al., 1992). The same PCR profile was used as described above, and amplicons were purified prior to sequencing by using ExoSAP-IT PCR cleanup reagents (Affymetrix). For sequencing, the β-tubulin amplicons were submitted to the Genomics Resource Laboratory at the University of Massachusetts, Amherst, MA, USA. Amplicons were visualized using MEGA5 software (Tamura et al., 2011).

2.5. Generation of histone H1::eYFP or eCFP fusion mutants in S. Homoeocarpa

The histone H1 gene (hH1) mined from previously published transcriptomic data of S. homoeocarpa (NCBI Accession no. PRJNA167377) (Hulvey et al., 2012) was fused with enhanced yellow fluorescent protein (eYFP) or enhanced cyan fluorescent protein (eCFP) by two rounds of PCR (Yu et al., 2004). An upstream flanking sequence (500 bp) and full length sequence (786 bp) without stop codon (TAA) of hH1 was amplified from genomic DNA (gDNA) of isolate 7 or B1 using the primer pair F_KpnI_uphH1 and R_hH1down. The full length (717 bp) without start codon (ATG) of eYFP or eCFP was amplified from pYHN3 or pCHN3 (Rech et al., 2007) using the primer pair F_YCFP and R_SpeI_YCFP. The two amplicons (upstream and hH1 region from isolate 7 and eYFP) or two amplicons (upstream and hH1 region from isolate B1 and eCFP) were mixed and used as templates for the second round of PCR using the primer pair F_KpnI_uphH1 and R_SpeI_YCFP. The final products, hH1::eYFP or hH1::eCFP (2003 bp), were inserted into KpnI and SpeI sites of the plasmid Topo-hph containing the hygromycin resistance cassette (Ptcp-hph) (Sang et al., 2017) to generate the plasmid Topo-hph-hH1::eYFP or Topo-hph-hH1::eCFP.

Subsequently, a downstream flanking sequence (1235 bp) of hH1 amplified from gDNA of isolate 7 or B1 using the primers F_NotI_hH1down and R_Apal_hH1down was inserted into NotI and Apal sites of the plasmid Topo-hph-hH1::eYFP or Topo-hph-hH1::eCFP to generate the plasmid Topo-hH1::eYFP-7 or Topo-hH1::eCFP-B1. Two constructs were amplified from the Topo-hH1::eYFP-7 or Topo-hH1::eCFP-B1 with primers, F_uphH1/R_YG and F_HY/R_hH1down, respectively. The Zymoclean Gel DNA Recovery Kit (Zymo Research) was used for the purification of the two constructs. The polyethylene glycol-mediated protoplast transformation protocol described previously (Sang et al., 2017) was used for the generation of hH1::eYFP fusion mutant from the isolate 7 (the mutant named in 7-hH1::eYFP) and hH1::eCFP fusion mutants from the isolate B1 (the mutant named in B1-hH1::eCFP). Heterokaryons were generated from co-inoculation of two histone-tagged fluorescent protein mutants (7-hH1::eYFP and B1-hH1::eCFP) using the method described above. Primers for hH1::eYFP or hH1::eCFP fusion mutants are listed in Table S2.

2.6. Preparation of fungi for live-cell imaging and confocal microscopic analysis

Two mutants (7-hH1::eYFP and B1-hH1::eCFP) and a heterokaryon fused from the two mutants were grown on half strength PDA media, plus PP (0.01, 0.1, and 1 μg ml-1) and TM (0.1, 1, and 1000 μg ml-1) amended media for 2-4 days. Subsequently, the heterokaryon grown on 1 PP for 4 days was transferred to untreated media and TM media (0.1, 1, and 1000 μg ml-1), and the heterokaryon grown on 1000 PP for 3 days was transferred to untreated media and PP media (0.01, 0.1, and 1 μg ml-1). The heterokaryon was grown for additional 3–4 days after transfer.

All the samples were prepared by using the modified inverted agar block method described previously (Hickey et al., 2002) and live-cell images were obtained using a Nikon A1 * scanning confocal microscope (Nikon Instruments Inc.) at the Central Microscopy facility, University of Massachusetts, Amherst. YFP images were taken by excitation at 488 nm with emission collected at 540 nm. CFP images were taken by excitation at 405 nm and emission at 482 nm. The camera resolution was typically 512 x 512 pixels. To take two-color images, the sample was scanned sequentially line by line. Images were obtained using Nikon Imaging System (NIS)-Elements and analyzed with the Nikon NIS and Fiji software from imageJ (http://fiji.sc/Fiji). Number of eCFP and eYFP tagged nuclear foci and heterotypic nuclear foci containing both hH1::eYFP and hH1::eCFP labels were manually counted from each compartment (septa) in the mycelial images. The proportion of eCFP and eYFP tagged nuclei and heterotypic nuclei in a total number of nuclei was calculated.

For preparation of Videos, time-lapse microscopy (Axio Observer.Z1, Carl Zeiss; Axiosivision Version 4.8 software, Carl Zeiss) in Field Science Center for Northern Biosphere, Hokkaido University was used with filter set 46HE and 47HE (Carl Zeiss) for detecting YFP and CFP, respectively.

3. Results

3.1. Initial fungicide mycelial growth assays

All 40 EHs displayed growth phenotypes not significantly different from PPr parents on PDA, 0.1 TM, and all concentrations of PP at all DAI, with the exception of 0.01 PP at 3-DAI (p = 0.0341). EHs displayed different phenotypes than either parent on 1 TM or 1000 TM (Fig. 1). A subset of 11 EHs grew comparably to TMR parents on 1000 TM and comparable to the PPr parents on 1 PP at 13-DAI, while a subset of 24 EHs grew comparably to PPr parents on both 1 PP and 1000 TM (Table 1 and Fig. 1). Five EHs showed different MG phenotypes between biological replicates, and are not included in the results. Dividing EHs based on growth on 1000 TM separated the population into isolates
that appeared heterokaryotic for fungicide sensitivity, or putative heterokaryons (PHETs), and isolates that appeared homokaryotic for fungicide sensitivity (sensitive to TM, resistant to PP), or putative homokaryons (PHOMs). PHOMs were mostly excluded from following results and analyses, with the exception of noting that the tendency of PHOMs for PP resistant phenotype, and the mention of the overall SSR genotype ratio below.

PHETs among the four crosses included six from B1 × 7, three from B1 × 46, two from R1 × 7, and zero from R1 × 46. Disparities in PHET recovery between crosses corroborated with data from a pilot study. This may be due to varied vegetative compatibility between parent strains. Since separation by cross would severely reduce isolate numbers within crosses, all crosses were lumped together for the analyses. Parents and parent controls showed little within group variation in MG when compared between isolate types. Therefore, all PPr parents and parent controls, as well as all TMr parents and parent controls from all crosses were combined, and referred to collectively as PPr parents and TMr parents, respectively.

3.2. In vitro sensitivity characterization of putative heterokaryons

On unamended PDA, all PHETs, PPr parents, and TMr parents reached median MG of 48 mm at 3-DAI (Fig. 2A). On PP amendments, median MG of all isolate types decreased as PP concentration increased (Fig. 2B). No significant difference in MG between PHETs, and PPr parents was observed on any PP concentration at 3-DAI, 6-DAI, or 13-DAI. On all three concentrations PHETs and PPr parents reached 48 mm median MG at 3-DAI (0.01 PP, 0.1 PP) or 13-DAI (1 PP). TMr parents grew significantly less than PHETs on all amendments and at all DAIs except 0.01 PP at 13-DAI (p < 0.0001) and did not reach 48 mm on 0.1 PP or 1 PP at 13-DAI.

On TM amendments, mycelial growth of all isolate types decreased as TM concentrations increased (Fig. 2C). On 0.1 PP all isolates reached median MG of 48 mm by 3-DAI. On 1 TM and 1000 TM PHETs had minimal growth not significantly different from PPr parents and significantly different from TMr parents (1 TM: p < 0.0001; 1000 TM: p < 0.0001) at 3-DAI. On 1 TM and 1000 TM at 6-DAI PHETs had variable, intermediate growth, and at 13-DAI MG median was 48 mm, and significantly different than PPr parents (1 TM: p < 0.0001; 1000 TM: p < 0.0001) (Table 1 and Fig. 2C).

The dual-resistant reference isolate grew comparably to the resistant parent on each amendment (Table 1). The dual-sensitive reference isolate grew comparably to the sensitive parent on each amendment, and therefore reached 48 mm MG on PDA and the lowest concentration of each fungicide only.

3.3. “Swap” fungicide mycelial growth assay

All isolates that reached ~48 mm on PP or TM media by 13-DAI were considered resistant (Table 1), and were swapped as described in the Materials and Methods. This phenotype as a criterion for fungicide resistance was corroborated by resistance phenotypes defined previously for PPr and TMr parents and dual-resistant and dual-sensitive reference isolates on 0.1 PP, 1 PP, and 1000 TM (Jo et al., 2006; Popko et al., 2013).

The data below was summarized in terms of MG, and is organized by DAI, initial fungicide amendment, and terminal fungicide amendment. Sensitive parents were swapped from low fungicide concentrations only (PPr parents from 0.1 TM, TMr parents from 0.01 PP). The

### Table 1

<table>
<thead>
<tr>
<th>Isolate type</th>
<th>Fungicide sensitivity phenotype</th>
<th>Propiconazole (μg/ml)</th>
<th>Thiophanate-methyl (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>PHET</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PHOM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PPr</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TMr</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dual-resistant</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dual-sensitive</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

**Footnotes:**

1. + + = all isolates at full growth at 13-DAI, + = isolates near full growth at 13-DAI, − = isolates much less than full growth at 13-DAI, − − = all isolates at zero growth at 13-DAI.

2. + + = all isolates at full growth at 13-DAI, + = isolates near full growth at 13-DAI, − = isolates much less than full growth at 13-DAI, − − = all isolates at zero growth at 13-DAI.

3. PHET = putative heterokaryons, PHOM = putative homokaryons, PPr = propiconazole resistant parents, TMr = thiophanate-methyl resistant parents, Dual-resistant = thiophanate-methyl and propiconazole resistant, and Dual-sensitive = thiophanate-methyl and propiconazole sensitive.
dual-resistant reference grew on all amendments in the initial growth assay, and therefore was swapped from all amendments. Data for the reference isolates is not discussed, but behaved on terminal amendments as they did in the initial growth assay. Swaps are abbreviated as follows: “→” points away from the initial fungicide amendment, and towards the terminal fungicide amendment (e.g. 1 TM → 0.1 PP indicates a swap from 1 TM to 0.1 PP). If no concentration is specified, all concentrations of that fungicide are referred to (e.g. 1 TM → PP would refer to the swapping from 1 TM to all concentrations of PP).

In PP → PDA, all isolates were fully grown at 5-DAI, and there were
no significant differences between isolate types. In PP → 0.1 TM all isolates reached 48 mm MG, there were no significant differences between isolate types, with the exception of 0.1 PP → 0.1 TM at 2-DAI between PHETs and PPr parents (p = 0.0285) (Fig. S1A–C). A subset of PHETs grew significantly more than PPr parents by 12-DAI in all PP → 1 TM concentrations (0.01 PP: p = 0.0285, 0.1 PP: p = 0.0115, 1 PP: p = 0.0005). There were no significant differences among MG of PHETs from each initial PP concentration at 2-DAI, 5-DAI, or 12-DAI. In PP → 1000 TM, PPr parents and PHETs showed 0 mm MG at 2-DAI (Fig. 3). A subset of PHETs swapped to all PP → 1000 TM concentrations were able

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**Fig. 3.** In vitro mycelial growth (diameter in millimeters) at 2, 5, and 12 days after inoculation (DAI) of putative heterokaryons (PHETs), propiconazole-resistant parents (PPr), and thiophanate-methyl resistant parents (TMr) on (A–C) unamended potato dextrose agar (PDA) and (D–F) 1000 μg ml⁻¹ of TM transferred from PDA amended with 0.01, 0.1 and 1 μg ml⁻¹ of propiconazole (PP), respectively. Box and whisker plots show the median, 75th and 25th quartiles, and maximum and minimum values.
to grow fully at 12-DAI, resulting in significantly different median MGs between PHETs and PPr parents (0.01 PP: \( p < 0.0001 \); 0.1 PP: \( p = 0.0001 \), 1 PP: \( p = 0.0014 \)). There were no significant differences among MG of PHETs from each initial PP concentration at 2-DAI, 5-DAI, or 12-DAI (Fig. 3D–F). In summary of PP \( \rightarrow \) TM swaps, PP \( \rightarrow \) PDA and PP \( \rightarrow \) 0.1 TM saw no notable differences in growth phenotypes between isolates. At PP \( \rightarrow \) 1 TM and PP \( \rightarrow \) 1000 TM, a subset of PHETs were able to grow significantly more than PPr parents, and MG of these isolates was not affected by initial PP concentration (Figs. 3 and S1).

In TM \( \rightarrow \) PP swaps, increasing concentrations of TM reduced MG in PHETs (Figs. 4 and S2). In TM \( \rightarrow \) PDA, all isolates reached 48 mm MG by 5-DAI (Fig. 4A–C). In TM \( \rightarrow \) 0.01 PP, MG of PHETs deceased significantly with increasing initial TM concentration at 2-DAI (\( p = 0.0002 \)) and 5-DAI (\( p = 0.0001 \)). At TM \( \rightarrow \) 0.1 PP, PHET growth
decreased significantly with increasing initial TM concentration at 2-DAI (p = 0.0001), 5-DAI (p = 0.0004), and 12-DAI (p < 0.0001) (Fig. S2D–F). In 1 TM → 1 PP, no growth of TM parents was observed at 2-DAI and 5-DAI, with slight growth at 12-DAI (Fig. 4D–F). PHET growth decreased significantly with increasing TM initial concentration at 2-DAI (p = 0.0001), 5-DAI (p = 0.0004), and 12-DAI (p < 0.0001) (Fig. 4D and E). At 1000 TM → 1 PP, all PHETs grew in the range of the TMr parent (Fig. 4F). In summary of TM → PP swaps, increasing initial TM concentration decreased MG in PHETs. PHETs failed to reach 48 mm at 12 DAI in 1 TM → 1 PP, 1000 TM → 0.1 PP, and 1000 TM → 1 PP with a varied number of isolates growing in the range of TMr parents (Figs. 4 and S2).

3.4. Simple sequence repeat and β-tubulin single nucleotide polymorphism genotyping

Three distinct genotypes were observed in the SSR genotyping: PPr parent genotype (PPr), TMr parent genotype (TMr), and both genotypes occurring simultaneously, referred to as double (D) genotype. Both bands in D genotype were typically dimmer than the parental bands (though brightness varied between isolates), suggesting lower concentrations of DNA from each parent. In addition, a subset of isolates within each isolate type failed to display SSR or β-tub SNP genotype and are referred to as no genotype (NG). Isolates displaying NG likely yielded low quantity or quality of DNA. PCR reactions for isolates initially displaying NG were retriied with maximum possible DNA concentrations. For SSR genotyping, TMr parents yielded 0.6% PPr, 85% TMr, and 14.4% NG (n = 174), while PPr parents yielded 81.4% PPr and 18.6% NG (n = 145). Percentage distribution of SSR genotypes of PHETs among all amendments was 8.2% D, 49.5% PPr, 31.8% TMr, and 10.5% NG (n = 314). In PHOMs, genotype ratio amount all amendments was 0.1% D, 79.3% PPr, 1.1% TMr, and 19.0% NG (n = 363).

β-tubulin SNP genotyping yielded a SNP distinct to each parent in a single position (E198K) of the β-tub gene. PPr parents displayed an adenine peak resulting in a glutamic acid code (GAG) at position 198, while TMr parents displayed a cytosine peak, resulting in an alanine code (GGG) at position 198. In 0.01 PP, 0.1 PP, 0.1 TM, 1 PP, and 1000 PP amended media, the proportions of eYFP tagged nuclei (71.7, 80.2, and 76.3%) and heterokaryons (25.7, 15.9, and 19.5%) (Figs. 5C and S4A). In the hyphal tip, movements of eYFP tagged nuclei on the PP amended media were observed (Video S1).

### Table 2

Simple sequence repeat (SSR) and β-tubulin single nucleotide polymorphism (β-tub SNP) genotypes of putative heterokaryons (PHETs) on potato dextrose agar (PDA) amended with 0.01, 0.1 and 1 μg/ml -propiconazole (PP), 0.1, 1, and 1000 μg/ml thiophanate-methyl (TM), and unamended PDA.

<table>
<thead>
<tr>
<th>PHET isolate</th>
<th>SSR genotype/β-tubulin genotype</th>
<th>PDA</th>
<th>Propiconazole (μg/ml)</th>
<th>Thiophanate-methyl (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>PPr/PPr</td>
<td>0.01</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>PPr/D</td>
<td>0.1</td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>PPr/D</td>
<td>0.1</td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>8</td>
<td>PPr/TMr</td>
<td>0.1</td>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>9</td>
<td>PPr/TMr</td>
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(a single genotype indicates that SSR and β-tub SNP genotypes were concurrent. Two genotypes separated by a forward slash indicate that SSR and β-tub SNP genotypes were not concurrent, or that one was missing. SSR genotype is shown of the right side of the forward slash and β-tub SNP genotype is shown of the left side. PDA = unamended potato dextrose agar, PPr = propiconazole resistant parent genotype, TMr = thiophanate-methyl resistant parent genotype, D = double genotype, both PPr and TMr occurring simultaneously, and N = missing genotype.

3.7. Visualization of homokaryons and heterokaryon in response to fungicides

To visualize different colors of nuclei from PP or TM resistant isolates, eYFP was fused to the C termini of hH1 of PP resistant isolate 7 (7-hH1::eYFP) and eCFP was fused to the C termini of hH1 of TM resistant isolate B1 (B1-hH1::eCFP). The mutant B1-hH1::eYFP was able to grow on unamended, 0.01 PP, 0.1 PP, 1 PP, and 0.1 TM amended half-strength PDA media and displayed nuclei with yellow fluorescence, however the mutant was unable to grow on 1 TM and 1000 TM for 2 days (Fig. 5A). The mutant B1-hH1::eCFP was able to grow on unamended, 0.01 PP, 0.1 PP, 1 TM, and 1000 TM amended media and displayed nuclei with cyan fluorescence, but the mutant was unable to grow on the media amended with 1 PP for 2 days (Fig. 5B).

In the heterokaryon from two mutants (7-hH1::eYFP and B1-hH1::eCFP) on the unamended media, nuclei displayed two different fluorescent colors, yellow and cyan, and also heterothetic nuclei exhibiting both colors (hH1::eYFP and hH1::eCFP labels) were observed.

In 23 septa (a total number of nuclei, n = 220), the proportion of eYFP tagged nuclei (44.1%) was higher than the proportion of eCFP tagged nuclei (24.1%) and heterothetic nuclei (31.8%) (Figs. 5C and S4A). In the heterokaryon grown on the 0.01 PP, 0.1 PP, and 1 PP amended media, the proportions of eYFP tagged nuclei (71.7, 80.2, and 76.3%, respectively) in 24 (n = 226), 27 (n = 227), and 30 (n = 215) septa were increased more than the proportion on PDA and higher than the proportions of eCFP tagged nuclei (2.7, 4.0, and 4.2%) and heterothetic nuclei (25.7, 15.9, and 19.5%) (Figs. 5C and S4A). In the hyphal tip, movements of eYFP tagged nuclei on the PP amended media were observed (Video S1).
Video S1. Live cell imaging of movements of eYFP and eCFP tagged nuclei and heterotypic nuclei containing both hH1::eYFP and hH1::eCFP labels in the heterokaryon mutant on propiconazole amended media. Each video displays the blight field and both YFP and CFP, respectively. Scale bar, 100 μm.

The eYFP tagged nuclei proportion (43.2%) in 21 septa (n = 118) from the heterokaryon at 0.1 TM was similar to the proportion on PDA but higher than the proportion of eCFP tagged nuclei (9.3%). The proportion of heterotypic nuclei (47.5%) on 0.1 TM was increased more than the proportion on PDA. In the heterokaryon on the 1 TM, the proportion of eCFP tagged nuclei (55.3%) in 21 septa (n = 94) was increased more than the proportion on PDA and higher than the proportion of eYFP tagged nuclei (23.4%) and heterotypic nuclei (21.3%). The heterokaryons at 1000 TM had mostly eCFP tagged nuclei (92.1% of proportion) in 24 septa (n = 202) and only few heterotypic nuclei (7.9% of proportion). None of eYFP tagged nuclei was detected at 1000 TM (Figs. 5C and S4A). In the hyphal tip, movements of eCFP tagged nuclei on the TM amended media were observed (Video S2).
3.8. Visualization of heterokaryon in response to “swap” fungicides

The heterokaryon grown on 1 PP was transferred to half-strength PDA and 0.1 PP, 1 TM, and 1000 TM. On 1 PP → PDA, the proportion of eYFP tagged nuclei (94.4%) in 27 septa (n = 234) was increased more than the proportion (76.3%) at 1 PP and higher than eCFP tagged nuclei (3.8%) and double-labeled nuclei (1.7%). On 1 PP → 0.1 TM, the proportion of eYFP tagged nuclei (88.5%) in 15 septa (n = 96) was increased more than the proportion at 1 PP and higher than eCFP tagged nuclei (1.0%) and double-labeled nuclei (10.4%). However, the proportion of nuclei was changed on 1 PP → 1 TM. The proportion of eCFP tagged nuclei (89.2%) in 30 septa (n = 167) was increased more than the proportion (4.2%) at 1 PP and higher than eYFP tagged nuclei (9.0%) and double-labeled nuclei (1.8%). On 1 PP → 1000 TM, only eCFP tagged nuclei (100% of proportion) were detected (Figs. 6A and S4B).

The heterokaryon from 1000 TM was transferred to half-strength PDA and 0.01 PP, 0.1 PP, and 1 PP. On 1000 TM → 0.01 PP, only eCFP tagged nuclei (100% of proportion) in 25 septa (n = 166) and in 30 septa (n = 162) were observed. However, very few double-labeled nuclei (0.6% of proportion) in 30 septa (n = 164) were detected on 1000 TM → 0.1 PP and mostly eCFP tagged nuclei (99.4% of proportion) were at the concentration. The heterokaryon from 1000 TM was unable to grow on 1 PP for 4 days (Figs. 6B and S4C).

4. Discussion

The goal of this experiment was to first determine if heterokaryons formed from single-fungicide-resistant field isolates exhibit improved adaptability to multiple disparate fungicide pressures compared with...
concentrations are abbreviated by omitting the unit (µg ml⁻¹) of propiconazole (PP) to unamended and 0.1, 1, and 1000 µg ml⁻¹ of thiofanate-methyl (TM) amended half-strength PDA.

Fig. 6. Live-cell image of eYFP and eCFP tagged nuclei and heterotypic nuclei containing both hH1::eYFP and hH1::eCFP labels in the heterokaryon mutant (7-hH1::eYFP + B1-hH1::eCFP) for the “Swap” fungicide assay. (A) eYFP and eCFP tagged nuclei and heterotypic nuclei in the heterokaryon mutant swapped from half-strength PDA amended with 1 µg ml⁻¹ of propiconazole (PP) to unamended and 0.1, 1, and 1000 µg ml⁻¹ of thiofanate-methyl (TM) amended half-strength PDA. (B) eYFP and eCFP tagged nuclei and heterotypic nuclei in the heterokaryon mutant swapped from half-strength PDA amended with 1000 µg ml⁻¹ of TM to unamended and 0.01 and 0.1 µg ml⁻¹ of PP amended half-strength PDA. Fungicide concentrations are abbreviated by omitting the unit (µg ml⁻¹) e.g. “0.1 PP”, “1 TM”. Arrows indicate heterotypic nuclei clearly shown in septa. Scale bar, 10 µm.

single-fungicide-resistant isolates, and to test the hypothesis that fungicide treatments correspond to observable changes in heterokaryon genotypes and nucleotypes. Jo et al. (2006) demonstrated the ability of S. homoeocarpa isolates to form heterokaryons based on nit-mutant compatibility, and thus at the outset of this study we showed the evidence that pairing isolates with vegetative compatibility maximized probability of heterokaryon formation and sharing of fungicide resistance alleles.

The D genotype provides strong evidence of nucleotypes from both parents in PHETs, but single allele genotypes (PPr or TMr) were prevalent throughout both fungicide MG assays. Libert et al. (2012) investigated genotypic and phenotypic diversity in S. homoeocarpa and reported that 38% of S. homoeocarpa isolates tested were heterokaryotic for mating type allele. They also found that gel electrophoresis bands were of differing intensities, suggesting unbalanced nuclear ratios between nucleotypes. Since bands were scored conservatively in the current study it is entirely plausible that the band of the alternate allele was too weak to be safely scored. The potential for such a discrepancy is supported by the data for five PHETs in which the TMr allele in the β-tubulin sequence electropherograms did not co-occur within the SSR genotype. We speculate that the alternate allele was frequently present in such low frequency in the heterokaryotic mycelium so that it was simply undetectable using the SSR or β-tubulin genotyping methods we employed, which are among the least sensitive. Inappropriate single allele genotyping could also have been reinforced through PCR bias. This occurs when one template DNA is preferentially amplified, and can occur in GC rich targets if a small amount of target template is available, has been damaged, or in the presence of contaminants in the PCR reaction (Muter and Boynton, 1995). Despite the sensitivity limits of SSR and β-tubulin genotyping, confocal images of heterokaryons clearly revealed the coexistence of both resistant nuclei with different proportions on all concentrations of TM and PP (Fig. 5C).

Interestingly, PHETs appear to be dominated by PPr nuclei when they are not subjected to significant TM fungicide pressure (Table 2). During the expected heterokaryon screening, 24 out 40 EHs (PHOMs) were considered homokaryotic from PPr nuclei based on phenotypic and genotypic data, suggesting that in the absence of fungicide pressures, the majority of EHs became homokaryotic for PPr nuclei. In the initial fungicide growth assay, there was no significant difference in MG between PHETs and PPr parents and a significant difference between PHETs and TMr parents on all PP concentrations (Fig. 2). This theory is also supported by the observation that there was no significant difference in MG between PHETs and PPr parents in 0.1 TM → PP, 0.1 TM → PDA, PP → 0.1 TM and PP → PDA swaps (Figs. 3 and 4, S1 and S2). These phenotypic data were corroborated by the SSR and β-tubulin genotyping, in which PHETs displayed a majority of PPr genotype on these amendments (Tables 2–4). Indeed, live-cell images of heterokaryons indicated that PPr (eYFP-labeled) nuclei were significantly more abundant on PP, 0.1 TM, 1 PP → PDA, and 1 PP → 0.1 TM than TMr (eCFP-labeled) nuclei (Fig. 5C and 6A). The prevalence of PPr nuclei as a result of significant PP selection pressure (0.1 and 1 PP) makes sense in light of theories on the effect of fungicide pressure on resistant nuclei in a heterokaryon. However, another mechanism must be in place that is causing PPr nuclei to dominate in the absence of any fungicide selection (in EH screen phase) or light selection pressure (when TM is below 1 µg ml⁻¹ and PP is below 0.1 µg ml⁻¹). Differential mitotic division rates between nuclei could have resulted in this discrepancy, a phenomenon that has been shown in heterokaryons previously (Clark and Anderson, 2004; James et al., 2008). Imbalanced nuclear ratios result from asynchronous division between different nucleotypes, a state common in multinucleate fungi (Roper et al., 2010), although the mechanisms underlying the coordination of nuclear division is unknown in S. homoeocarpa. Since there is evidence that nuclei in multinucleate fungi can actively regulate their ratio in the cell (Glafelter, 2006), some form of inter-nuclear communication is likely allowing for the overabundance of PPr nuclei in heterokaryotic mycelium, and the retention of the TMr at low abundance, when not under fungicide selection. Intriguingly, in some hyphae of S. homoeocarpa either the PPr (eYFP-labeled) or TMr (eCFP-labeled) nuclei were restricted to move to other septa or branches containing mixed nucleotypes (Videos S3 and S4). Previous studies have investigated the nature of inter-nuclear interactions in heterokaryotic fungi, however there were substantial differences in their findings, including whether nuclei cooperate or compete in the mycelium (James et al., 2008;
Maheshwari, 2005; Roper et al., 2010). However, in *S. homoeocarpa* the exact mechanism by which PPr nucleotypes dominate, TMr nucleotypes persist, and how certain hyphae maintain one nucleotype in heterokaryons requires future inquiry.

When PHETs were assayed on 1 TM or 1000 TM they displayed growth comparable to PPr parents at 3-DAI. PHETs showed a wide range of growth values at 6-DAI, and at least a subset displayed growth comparable to TMr parents at 13-DAI. This discrepancy is assumed to be the result of an “adaptation period” of 6 days. A similar phenomenon was postulated by Webster et al. (1968), in which *Rhizopus stolonifer* heterokaryotic sporangiophores were exposed to fungicide 2,6-dichloro-4-nitroaniline and were able to germinate after a lag time in comparison to resistant isolates, thought to be due to a small proportion of resistant nuclei present. This explanation is plausible in our experiment, since evidence indicates that although PHETs were mostly PPr genotype when not under TM selection, all PHETs had no significant difference in MG compared to TMr parents on 1 and 1000 TM and displayed the TMr or D genotype. PHETs showed a mixture of D and TMr genotypes on 1 TM, which suggests that selection with 1 TM was sufficient to allow TMr nuclei to partially or completely dominate the mycelium. This phenomenon was validated by the fact that the small proportion of TMr (eCFP-labeled) nuclei in heterokaryons on PDA were significantly increased on 1 TM and 1000 TM. Since a number of PPr (eYFP-labeled) nuclei were still survived on 1 TM, PHETs displayed D genotype on 1 TM. However, only TMr nucleotypes were detected on 1000 TM due to survival of very small proportion of PPr (eYFP-labeled) nuclei.

Overall, the fungicide swap experiments demonstrated that PHETs were able to adapt when swapped between disparate fungicide pressures. Mycelial growth and heterokaryon genotype and nucleotypes were influenced by both initial and terminal fungicide amendments and concentrations providing strong evidence for the heterokaryon adaptability to fungicide pressures. As indicated above, the genetic “baseline”

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**Video S3.** Live cell imaging of movements of eYFP and eCFP tagged nuclei and heterotypic nuclei containing both hH1::eYFP and hH1::eCFP labels in the heterokaryon mutant. The restriction of eYFP tagged nuclei movement to branched hyphae containing eCFP tagged nuclei. Each video displays both YFP and CFP, CFP and YFP, respectively. Scale bar, 100 μm.

**Video S4.** Live cell imaging of movements of eYFP and eCFP tagged nuclei and heterotypic nuclei containing both hH1::eYFP and hH1::eCFP labels in the heterokaryon mutant. The restriction of eCFP tagged nuclei movement to a hypha containing eYFP tagged nuclei. Each video displays both YFP and CFP, CFP and YFP, respectively. Scale bar, 100 μm.
heterokaryons to fungicide pressure have been made. Ogden and Grindle (1983) proposed that fungicides acting on the nuclei of a heterokaryon could be acting by nuclear or hyphal selection. In nuclear selection, unequal division rates of genetically different nuclei in hyphal tips would enable preferential proliferation of resistant nuclear types. In hyphal selection, hyphae at the growth front grow better or worse under fungicide selection pressure depending on the ratio between sensitive and resistant nuclei. As discussed above, asynchronous nuclear division is likely common in multicellular fungi, which supports possibility of nuclear selection. In addition, studies in other ascomycetes have suggested that gene products are restricted to a limited zone around different nuclei in a heterokaryons (Gerstenberger et al., 2012; Griffiths, 1976). In addition, the exact effect of a fungicide on heterokaryon nuclear ratios would depend on the specific biochemical mode of action. For instance, the fungicidal MBC fungicide TM interrupts cellular division and cytoskeletal formation by targeting β-tubulin (Koenraadt et al., 1992) preventing normal microtubule formation. This would likely only allow for division of TM resistant nuclei, while preventing division of TM sensitive nuclei. However, the DMI fungicide PP targets ergosterol synthesis, which directly influences membrane viability and integrity causing effects throughout the cell. Consequently, PP application would be more likely to select for cells with high proportions of PP resistant nuclei, indicating hyphal selection. In accordance with both the theories on nuclear behavior in fungal hyphae and the nature of biochemical modes of action of these fungicides, both nuclear and hyphal selection are plausible in S. homoeocarpa in the case of TM and PP applications.

The current study is the first empirical demonstration of nuclear selection due to stresses in multicellular fungi and also lays the groundwork for potential use of S. homoeocarpa heterokaryons as a biotechnological tool. Since the first report of shifts in nuclear ratio in heterokaryons of Penicillium in 1952 (Jinks, 1952), the question has been raised whether the shifts resulted from nuclear selection or more random processes of gain or loss in particular mycelial fractions (Strom and Bushley, 2016). The results presented in this study provide the answer that when a heterokaryon composed of nuclei with different fungicide resistance mutations is exposed to a fungicide, the resistant nuclei type is selected. The phenomenon of selection of fungicide resistant nuclei in S. homoeocarpa is of unknown importance in the field in comparison to stepwise development of multiple fungicide resistances. However, the possibility of nuclear disproportion (Pitchaimani and Maheshwari, 2003) in heterokaryons and disproportional responses to fungicide selection pressure may allow improved adaptability to fungicide pressures in this sterile filamentous fungus. Strom and Bushley (Strom and Bushley, 2016) also emphasized the biotechnological importance of heterokaryosis as a source of heterosis resultant of parasexual cycle or hyphal/protoplast fusion. Though parasexuality in S. homoeocarpa is not known, but it has been suspected (Halvey et al., 2012). Additionally, hyphal fusion was successfully performed in the current study, a procedure that could be used in the future to construct industrially useful strains. This study therefore forms the basis for the use of this fungus in industry and as a model organism for the study of heterokaryon genetics.

Conflict of interest

The authors have no competing interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/jdbg.2018.01.005.

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