Sub-lethal fungicide stress induced polymorphism in *Sclerotinia sclerotiorum* genomic DNA

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ABSTRACT

Introduction

Most of the fungicide efficacy studies have been carried out to ascertain the effect of lethal doses driving pathogen resistance by selecting for preexisting rare mutants resistant to fungicidal action. Only little work has been done to decipher contribution of sublethal fungicide doses acting as a driver of mutations. Sublethal doses of antimicrobials have been documented to damage or alter DNA in ~~pathogenic~~ microorganisms such as bacteria and fungi (Andersson DI, Hughes D (2014|| Rebecca S. Shapiro. (2015)Ref) leading to an increase in mutagenesis. There is ample chance of fungal pathogens being exposed to sublethal doses of fungicides in an agricultural setting. Farmers may spray sublethal doses intentionally or accidentally. In some cases, rainfall or dew on plants can dilute the application rate to create sublethal doses. Environmental conditions prevailing in a field can degrade chemicals resulting in sublethal rates.

Most published research work had focused on studying effects of sublethal doses of antimicrobials on bacterial pathogens either in humans or livestock animals (Andersson DI, Hughes D (2014)||Rebecca S. Shapiro. (2015)Ref). The evidence suggests that there is an increase in the rates of various genetic processes such as frequency of horizontal gene transfer, recombination and mutagenesis due to exposure of bacteria to sublethal levels of antibiotics (Andersson DI, Hughes D (2014)). Antimicrobial compounds even at low concentrations can damage microbial DNA directly or indirectly. For example antitumor antibiotic bleomycin is capable of directly damaging DNA by binding DNA and cleaving them (Hecht SM 2000). Some antibiotics such as β-lactams, aminoglycosides, and quinolones as well as antifungals such as amphotericin B (azole group antifungal agent) induce production of reactive oxygen species (ROS) downstream of their cellular targets. ROS such as hydroxyl radicals damage DNA by formation of DNA strand breaks and modification of guanine bases in the pathogen genome (Mesa-Arango AC, 2014 || Foti JJ 2012 || Zhao X, Drlica K (2014) || Gutierrez A et al. ref). As a survival mechanism microorganisms react to damaged DNA with activating DNA damage repair pathways. However, this process can introduce mutations into the genome. In bacteria, SOS response and RpoS regulation is activated in response to antibiotics mediated DNA damage. SOS is the global response to DNA damage by bacteria in which high activity of DNA repair takes place. Derepression of SOS genes and activation of low fidelity polymerases leads to DNA repair and survival of bacteria. However, there may be a compromise on the fidelity of synthesized DNA leading to mutagenesis by introducing substitute bases or single nucleotide polymorphisms (SNP). SOS response can also activate movement of mobile elements such as integrative conjugative elements (ICEs) and transposons (Andersson DI, Hughes D (2014)). On the other hand the RpoS is a general stress response sigma factor which regulates the levels of translation and protein stability (Gutierrez A et al). Down regulation of MutS protein, a mismatch repair protein synthesized by increase RpoS expression promotes mutations in bacterial genome. RpoS induction also up regulate error prone DNA polymerase IV which may also contribute to mutations when DNA is replicated. Studies on yeast has revealed homologous DNA repair mechanisms in eukaryotes in response to DNA damage by chronic environmental stresses (Boiteux S, Jinks-Robertson S (2013)|| Shor E, Fox CA, Broach JR (2013)). This is mediated by synthesis of translesion DNA polymerase Rev1 which increases rate of mutations in fungal genome (Rebecca S. Shapiro. (2015).

Although, antifungal-induced SNP mutagenesis has not been well documented in fungal pathogens (Rebecca S. Shapiro 2015), major chromosomal changes have been reported in human fungal pathogens such as *Candida albicans* (Selmecki A, Forche A, Berman J (2010)) and *Cryptococcus neoformans* (Sionov E, Lee H, Chang YC, Kwon-Chung KJ (2010)). It has been observed that fungal pathogens exposed to stress promotes loss of heterozygocity (LOH) and aneuploidy. (Selmecki A, Forche A, Berman J (2010),|| Selmecki AM, Dulmage K, Cowen LE, Anderson JB, Berman J (2009) ). Both chromosomal changes and SNP mutagenesis may have consequences of acquiring antimicorbial resistance determinants.

Studies with isolates of fungal plant pathogens subjected sublethal doses of fungicides had shown phenotypic changes such as stimulatory growth on media (i.e. higher rate of growth than none-amended media) or either increase or decrease in aggressiveness on hosts (Sakhr Ajouz, Veronique Decognet, Philippe C. Nicot, Marc Bardin 2010|| Feng Zhou, Hong-Jie Liang, Ya-Li Di, Hong You, and Fu-Xing Zhu. 2014. Ref). However, these phenotypic modifications were not associated with genotypic changes or molecular basis was not investigated. There is a great possibility of plant pathogenic fungi exposed to environmental stresses such as sublethal fungicide doses driving above normal mutations. However, this field lacks published data.

In order to investigate how long term exposure of sublethal doses of fungicide affect mutagenesis in fungal genome, we experimented with model plant pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary. *Sclerotinia sclerotiorum* is a destructive necrotrophic fungus, [Bolton MD, Thomma BPHJ, Nelson BD (2006)] with a host range of more than 400 species of plants belonging to 75 families [Boland GJ, Hall R (1994)]. It reproduces sexually in the field under right environmental conditions, but rarely produces any spores in vitro, and largely considered as a clonally disseminated fungus. Some of the cash crops that can be devastated by this fungal pathogen include sunflower, soybean, oilseed rape, dry bean, chickpea, peanut, dry pea, lentils and various vegetables [Bolton MD, Thomma BPHJ, Nelson BD (2006)]. Since most crops are either susceptible or only partially resistant against this pathogen, farmers rely on fungicide applications for disease management during epidemics (Bolton MD, Thomma BPHJ, Nelson BD (2006)||Steadmen 1984]. Five commercial fungicide formulations namely, iprodione, thiophanate methyl, boscalid, azoxystrobin, and pyraclostrobin belonging to different modes of actions were tested for long term sublethal effect on genomic changes of *S. sclerotiorum*. Nine microsatellite markers developed for *S. sclerotiorum* (Sirjusing and Kohn, 2001) and three amplified fragment length polymorphic (AFLP) markers were used to characterize molecular changes of the fungal isolates subjected to sublethal treatments. Microsatellites (simple sequence repeats or SSR) are codominant, polymorphic markers with short tandem repeats consisting of 2-6 base pairs and have been used extensively for population genetic studies due to their high level of genotypic variation [Sweet MJ1, Scriven LA, Singleton I., 2012,|| Sirjusing and Kohn, 2001,|| Attalh 2004]. A microsatellite marker is able to detect multiple alleles in a single locus. Unlike microsatellites, AFLP is a multilocus marker with the ability to screen relatively large areas of the pathogen genome [Mueller UG, Wolfenbarger LL (1999)]. AFLP is a dominant marker which has the capacity to generate numerous polymorphic DNA fragments ~~(loci)~~ per isolate that can be used to decipher cryptic relationships between closely related species, subspecies, and isolates [(Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, et al. (1995)].

The objectives of this study were to (i) assess genomic variations of *S. sclerotiorum* isolates exposed to long term sublethal doses of fungicides in vitro using microsatellite and AFLP markers, (ii) ascertain suitability of the invoked molecular markers in assessing fungicide stress induced mutations based on results of the first objective (iii) determine in vitro trends of effective concentration of fungicides required for 50% growth inhibition (EC50) overtime in *S. sclerotiorum* isolates exposed to sublethal doses of fungicides.

Materials and Methods

Spiral plater method for amending media plates with fungicides

Nine *Sclerotinia sclerotiorum* isolates (Table 1) were exposed to sublethal doses of ~~commercial~~ formulated fungicides having different modes of action over 12 generations using the spiral gradient dilution method (Förster, H., Kanetis, L., and Adaskaveg, J. E. 2004). The fungicides used were respiration inhibitor boscalid (Emerald), iprodione (26GT) with unclear mode of action, microtubulin synthesis inhibitor thiophanate methyl (LESCO T-STORM), and quinone outside inhibitors (QoI) azoxystrobin (Heritage) and pyraclostrobin (Insignia). In spiral gradient dilution method, the fungicide is deposited in a spiral pattern on a rotating media plate with a decreasing concentration from center to periphery across the plate. We used the spiral plater Autoplate 5000 (Advanced Instruments, Inc. Norwood, MA) for our experiment. All fungicides were deposited on 150-mm-diameter petri plates using the exponential mode of deposition except for iprodione. Since isolates showed a short growth to no growth interphase for iprodione, proportional mode of deposition was employed. In exponential mode of deposition the fungicide gradient from center to periphery is approximately to 300:1 whereas the gradient of proportional mode is 3:1. The fungicide concentrations used to feed the dispensing stylus and actual deposited concentration at the center and periphery of plates are depicted in Table 2. Each fungicide concentration was optimized to avoid either too little growth or overlapping of adjacent replicates due to high growth. According to the manufacturer’s instructions, media plates were prepared by adding 50 ml of PDA and stored at least 48 hours at room temperature to obtain a dry surface prior to use. The PDA media used for QoI fungicides were amended with salicylhydroxamic acid (SHAM) dissolved in acetone to have 70 ppm concentration. SHAM was added to prevent the pathogen from taking alternative respiration pathways and thereby avoiding fungicide toxicity in vitro (Wise, K.A., C.A. Bradley, J.S. Pasche, N.C. Gudmestad, F.M. Dugan, and W. Chen. 2008). Fungicide deposited plates were kept under a sterile hood for 2-4 hours to uniformly absorb the chemicals before inoculating with *Sclerotinia* isolates.

Inoculum preparation and inoculation of fungicide treated plates

Each isolate of *Sclerotinia* was grown on a 10-cm-diameter petri plate. Just before the mycelial mat reached the edge of the plate, mycelial scrapings were transferred to 500 µl of sterile water in a 2 ml microtube with two glass beads and homogenized using a Fastprep homogenizer (MP Biomedical, Solon, OH). Filter strips made of Whatman grade1 paper (GE Healthcare Bio-Sciences, Pittsburg, PA) and having a dimension of 50 mm x 4 mm were placed on PDA plates and 45 µl of mycelial solution per filter strip was spread on them. The inoculated strips were incubated for 40 hours at 25° C to let mycelia grow thoroughly on paper and transferred to fungicide deposited plates. Four strips were placed per plate on two perpendicular diameters with mycelia facing downwards. Each inoculated filter strip is considered a technical replicate. Non fungicide amended 10cm-diameter PDA plates were used as positive controls by placing a single strip closer to the edge of the plate. Three control plates were used per isolate. Both control and fungicide treated plates were incubated for 38-42 hours at 25° C and the point of 50% growth inhibition on the each filter strip was determined relative to the growth of control isolates (Fig. 1). The 100% growth of an isolate was the height of mycelial mat on control plate measured perpendicularly from top of the strip to the edge of the mat. Therefore, 50% inhibition is equated to one half growth of the control plate. The radius to the point of 50% growth on the inoculated filter strip was recorded to calculate EC50 using the SGE software provided with AP 5000 spiral plater.

Long term exposure of Sclerotinia isolates to sublethal fungicide doses

The mycelia within the zone of 50% inhibition point to 100% inhibition (tail-ending point) (Fig. 2) was scraped and transferred to microtubes with sterile water as described above to prepare the inocula of the next generation. The inocula were homogenized and transferred to new PDA plates with filter strips and considered as generation 1 (G1). After approximated 40 H of incubation at 25° C, these inoculated strips were again transferred to fungicide deposited 15-cm-diameter plates. Inocula prepared for control plates were never exposed fungicides. All isolates were taken through twelve generations by repeating this process and at the end of each generation PDA plates used for inoculated filter strips were kept at room temperature for 3 weeks for sclerotia formation. The sclerotia were collected in microtubes and both plates and sclerotia were preserved at 4° C. This experiment was repeated once. Isolate 587 was removed from the study since fungicide exposed replicates died half way during the experiment. A few other isolates showed unusually low growth and had to be removed from the experiment. They were namely, thiophanate methyl exposed isolate 467 of the first experiment (TM\_467\_ Exp1); G12 control isolate 594 (Con\_594\_Exp1\_G12), TM\_594\_Exp1 and TM\_594\_Exp2. We used the following acronyms to describe control and fungicide challenges isolates. The first and repeated experiment was denoted by Exp1 and Exp2, respectively. G0 and G12 were used to describe isolates at the beginning of the experiment and at the end, respectively. All isolates challenged with fungicides were from G12 and therefore generation was not mentioned. However control isolates were from both G0 and G12 generations. We abbreviated the five fungicides as follows: TM (thiophanate-methyl), Bos (boscalid), Ip (iprodione), Az (azoxystrobin), and Py (pyraclostrobin). Control isolates were denoted as Con.

DNA extraction and molecular analysis

Each isolate before exposed to sublethal fungicide doses (G0) and after challenging with fungicides for 12 generations (G12) was used to extract DNA. A DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) was employed for the DNA extraction according to the manufacturer’s instructions and stored at -20° C for future use. The G12 DNA included isolates that were continuously challenged with sublethal doses of fungicides as well as their control isolates which were only transferred 12 times but never exposed to fungicides.

Microsatellite analysis

In a previous study we characterized more than 350 isolates of *S. sclerotiorum* including the isolates in this study using 16 microsatellite markers identified by Sirjusingh and Kohn 2001). The results of the analysis were used to select 6 microsatellite markers that showed polymorphism with unambiguous repeat lengths. The selected markers were namely, 6-2, 17-3, 20-3, 55-4, 110-4, 114-4 (Sirjusingh and Kohn 2001). Polymerase chain reaction (PCR) was carried out as described by Sirjusingh and Kohn (2001) using primers labeled with FAM fluoropore. The PCR products were first resolved in a 1.5% agarose gel stained with ethidium bromide to ensure product was within the expected size range. Capillary fragment analysis of PCR products added with size standard GeneScan™ 600 LIZ® was performed using a 3730 genetic analyzer (Applied Biosystems Inc., Foster City, CA) at the Ohio State University Plant-Microbe Genomics Facility. Microsatellite analysis was carried for all isolates of both experiments except for the irregular ones described above.

AFLP analysis

Initially 12 primer pairs were tested with four representative *Sclerotinia* isolates and three promising primers which produced clear and relatively higher number of fragments were selected for the analysis. We characterized 32 isolates of *Sclerotinia* to investigate molecular differences between fungicide challenged and unchallenged control isolates. These isolates included all survived controls (G0 and G12) from Exp1 and ten TM exposed isolates from both Exp1 and Exp2: TM\_152\_ Exp1, TM\_462\_ Exp1, TM\_555\_ Exp1, TM\_588\_ Exp1, TM\_646\_ Exp1, TM\_152\_ Exp2, TM\_467\_ Exp2, and TM\_555\_ Exp2, TM\_646\_ Exp2, and TM\_655\_ Exp2. Another seven isolates exposed to rest of the four fungicides were also included in the analysis. They were Az\_646\_Exp1, Bos\_555\_Exp1, Bos\_646\_Exp1, Ip\_467\_Exp1, Ip\_594\_Exp1, Py\_588\_Exp1, and Py\_594\_Exp1. Most of these seven isolates showed at least one mutation for the microsatellite loci characterized.

AFLP was carried out based on the method described by Vos et al (Vos and others 1995) with the following minor modifications. AFLP® Core Reagent Kit (Invitrogen™, Carlsbad, California) was used for restriction digestion and ligation steps according to the given instructions. Preamplification and selective amplification primers were ordered from Life Technologies Corporation (Grand Island, NY). Approximately 250-400 ng of genomic DNA was digested with *Eco*RI and *Mse*I. Thereafter, digested products were ligated with *Eco*RI and *Mse*I double stranded (ds) adapters provided with the AFLP Core Reagent Kit. After the ligation step was completed, the reaction mixture was diluted ten‐fold by adding sterile Tris‐EDTA (TE) buffer and used for preamplification step. The preamplification step was carried out with *Eco*RI (5′-GTAGACTGCGTACCAATTC-3′) and *Mse*I (5′-GACGATGAGTCCTGAGTAA-3′) primers compatible with respective oligonucleotide adapters used in the ligation step. The preamplification mixture of 50 µl included 5µl of the diluted restriction-ligation reaction, 0.1125 µM each of *Eco*RI and *Mse*I primers, 1× Taq polymerase reaction buffer, 0.2 mM of each dNTP and 1 U of Taq polymerase (Invitrogen™). The PCR was performed in a Mastercycler®Pro thermocycler (Eppendorf, Hamburg, Germany) with first cycle at 72° C for 2 min, then initial denaturation at 94° C for 2 min followed by 30 cycles of 30 s at 94° C, 1 min at 60° C and 2 min at 72° C. The PCR products were diluted fifteen‐fold with TE buffer and used as template DNA for the selective amplification step. For selective amplification, primer pairs consisting of *Eco*RI and *Mse*I adapter sequences with 2 - 3 selective nucleotides each were used (*Eco*RI + *AAC* and *Mse*I + *CA*, *Eco*RI + *AAC* and *Mse*I + *CC*, *Eco*RI + TGand *Mse*I + *CA*). All *Eco*RI selective primers were labeled with fluorescent dye 6‐FAM at the 5′ end for automated sequencing on a 3730 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Each selective PCR mixture of 20 µl included 5 µl of diluted preamplification product, 0.25 µM each of *Eco*RI and *Mse*I primers, 1× standard Taq polymerase reaction buffer, 0.2 mM each dNTP and 1 U of Taq polymerase (Invitrogen™). The PCR reaction was performed for 36 cycles with the following cycle profile. The first twelve cycles consisted of 30 s DNA denaturation at 94° C, 30 s annealing at 65° C (-0.7 C/cycle), and 1 min extension step at 72 C. The remaining cycles consisted of 30 s at 94° C, 30 s at 56° C, and 1 min at 72° C. A final extension step of 5 min at 72° C completed the reaction.

Following AFLP reaction, 1.5 µl of each of PCR sample was mixed with 11 µl Hi‐Di formamide (Applied Biosystems, Warrington, UK) and 0.2 µl of GeneScan™ 600LIZ® internal marker. The mixtures were denatured at 95° C for 5 min and placed on ice. Thereafter, samples were sent to the Ohio State University Plant-Microbe Genomics Facility for fragment analysis by 3730 Genetic Analyzer (Applied Biosystems).

Data analysis

The GeneMapper software version 4.1 (Applied Biosystems) was used to extract and analyze data files obtained from the 3730 fragment analyzer. Individual AFLP bands were scored as either absent (zero) or present (one) by this software. The binary table produced by the GeneMapper was used to generate a neighbor joining (NJ) tree with Rogers index (Rogers, J.S. 1972) since it gave best separation between control and fungicide challenged isolates. The *aboot* function of Poppr package version 2.0.2 (Kamvar ZN, Tabima JF, Grünwald NJ) in R program was used for tree construction. The bootstrap values of groups were determined by resampling data 1000 times.

We also explored similarities and dissimilarities of AFLP data for control and treated isolates by principal coordinate analysis (PCoA) (Zuur AF, Leno EN, Smith GM). This was performed using Adegenet package version 2.0.0 in R (Jombart, T. 2008). PCoA uses eiganvalues derived from a distance matrix (or any measure of association) and produces a graphical figure in a low-dimensional Euclidian space. Analysis was conducted with several similarity coefficients available with the Adegenet package and Simple matching index (R. R. Sokal, C. D. Michener) was selected since it gave the best resolution between control and fungicide exposed isolates. The significance of the clusters formed were tested with analysis of molecular variance (AMOVA) using GENALEX version 6.41 (Peakall and Smouse 2006). PhiPT which is an analogue of Wright’s *F*ST was used to ascertain significance of populations. An FST value of 0.05 or less is generally considered as reasonably low and may be interpreted to mean that structuring between populations is weak [33,34].

To see congruence of results, discriminant analysis of principal components (DAPC) was also performed on AFLP data. DAPC is powerful multivariate method designed to identify and describe clusters of genetically related individuals. Discriminant analysis maximizes the separation between groups while minimizing variation within group (Jombart, T, Devillard S, and Balloux F. 2010). This method is also capable of deciphering how clear-cut or admixed individuals are since it can calculate the membership probabilities of isolates included in a predefined group (ex. control and fungicide challenged isolates). ~~DAPC can calculate the membership probability of clusters other than the assigned one for these admixed isolates.~~ First we used the *find.clusters* function in Adegenet to identify the optimal number of clusters of the analyzed samples. This is achieved by running *k*-means clustering algorithm for increasing number of groups and identifying the group number which maximizes the variation between groups. The *assignplot* was used to visualize the membership probabilities after successful reassignment of individuals based on discriminant functions. Large probability values indicate clear-cut clusters, while low values suggest admixed groups.

Censored data

AFLP data of control isolates of G0 and G12 showed polymorphic alleles for some loci probably due to random mutations and background noise. We removed these polymorphic alleles in order to arrive with same multilocus genotype for each control isolate of GO and G12 (i.e. converted them to clones). The corresponding alleles of fungicide challenged isolates were also removed. This censored AFLP binary table was analyzed ~~after removing clones~~ to construct a neighbor joining tree using Rogers ~~index based~~ distance matrix using Poppr. DAPC was performed on censored AFLP alleles after clone correction in order identify optimal number of clusters and probability of membership of individuals falling into each cluster.

Results

Microsatellite satellite

The microsatellite alleles of control isolates did not show any difference between G0 and G12. A few alleles of the fungicide challenged isolates expressed mutations (Table 3). Most mutations were insertions except for iprodione, which mainly consisted of deletions. All deletions and insertions were represented by addition or removal of repeat units corresponding to the microsatellite marker except for the loci 6-2, which according to Sirjusingh and Kohn (2001) is polymorphic locus rather than a microsatellite. Insertions and deletions ranged from a few base pairs to 80 bp while most mutations were less than 20 bp in length. Large mutations were observed only on 114-4 locus (data not shown).

AFLP analysis

The three AFLP primers resulted in 602 polymorphic alleles for the 32 isolates analyzed. There were no clonal genotypes. However, large number of alleles of control GO and G12 isolates were common. The NJ tree largely separated fungicide challenged isolates from control isolates (Fig 2A). A cluster with high boot strap value of 60.7% included 12 of 17 fungicide exposed isolates. However, this cluster also included two control isolates Con\_462\_G12 and Con\_467\_G0 (Fig 2A). Nine of 15 control isolates grouped in a single cluster with bootstrap value of 50.7%. The NJ tree for censored data is depicted in Fig 2B. Majority of isolates exposed to thiophanate methyl clustered together away from control isolates. Most clonal control isolates grouped in separate clusters from rest of the fungicide exposed isolates showing high diversity. The PCoA corroborated the results of NJ tree of Fig 2A by clearly separating fungicide exposed and control isolates into two groups (Fig 3). The distance matrix calculated using the Rogers index gave the best separation for isolates for eiganvectors one and three (Fig. 3). The fungicide challenged and unchallenged groups were significantly different (*P* = 0.001) according to the AMOVA with 15% of between group genetic variation as indicated by PhiPT.

The summary statistics of *find.clusters* in discriminant analysis indicated two as the optimal number of clusters for the analyzed samples (data not shown). The graph derived by *assignplot* function resulted in higher membership probabilities (posterior values provided by discriminant analysis) for most individuals in their preassigned clusters, i.e. fungicide challenged or unchallenged (Fig 4A). There were three isolates with less than 90% probability of membership in the preassigned groups. They were Az\_646\_Exp1, Ip\_467\_Exp1, and Con\_467\_G0 (Fig 4A). The censored, clone corrected data consisted of eight controls and 17 fungicide challenged isolates. DAPC analysis by *find.clusters* couldn’t decipher a clear solution for optimum number of clusters. Therefore, we grouped isolates into increasing number of clusters and checked for high membership probabilities (clear cut groups) to arrive with an appropriate number of clusters. We found four clusters as a good fit for isolates since membership probabilities of all isolates were 100% (Fig 4B) at that level. The largest cluster (cluster one in Fig 4B) consisted of all control isolates and nine fungicide exposed ones. The isolates grouped outside of control samples were mostly thiophanate methyl exposed isolates (Fig 4B) which largely agreed with the NJ tree of Fig 2B which also grouped the above isolates together. The only non thiophanate methyl exposed isolate to group outside of control isolates was the Py\_588\_Exp1, which fell in cluster 4 with TM\_467\_Exp2 and TM\_555\_Exp2 (Fig 4B).

Number of alleles scored for each isolate before and after censoring are depicted in Fig 5. The isolates with alleles less than Py\_588\_Exp1 in Fig 5A clustered with control isolates as shown in Fig 4B. It is apparent that majority of the thiophanate methyl (TM) exposed isolates (n = 7/10) survived with more alleles after the censoring process compared to other isolates. For example average survived alleles of TM isolates after censoring was 16 while other fungicide exposed isolates only had an average of five alleles. According to results of TM exposed isolates of exp1 and exp2, it is apparent that mutations are largely independent of the isolates. For example, TM exposed isolate 646 of Exp1 grouped outside of control isolates while same isolate of Exp2 grouped with control isolates. The TM exposed isolates 152 and 555 of Exp1 and 2 clustered in different groups.

The EC50 values of isolates exposed to different fungicides did not show any trend (results not shown). Some isolates exposed same fungicide showed an increase of EC50 while other decrease at the end of G12. The EC50 values of Exp1 and Exp2 also did not show any correlation.

**Discussion**

The fungicides tested had different modes of action. Thiophanate methyl (TM) is a benzimidazole fungicide which binds to tubulins coded by β-tubulin gene. This inhibits assembly of microtubules preventing nuclear division of fungal cells (Leen C. Davidse 1986). Both azoxystrobin and pyraclostrobin have a quinone outside inhibitor (QoI) mode of action. These fungicides inhibit mitochondrial respiration by binding to cytochrome bc1 enzyme complex at the Qo site (Bartlett, D.W., Clough, J.M, at el. 2002). Iprodione belongs to dicarboximide fungicide group having an unknown modes of action (Zhonghua Ma, Themis J. Michailides, 2005). Succinate dehydrogenase inhibitor (SDHI) fungicide boscalid targets succinate dehydrogenase complex in the respiration chain and inhibit fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II (Avenot HF, Michailides TJ 2010).

Some technical replicates in both fungicide exposed and control groups exhibited slow growth and even death during the continuous transfer process. For example, we had to remove isolate 587 from the experiment since all treated and control isolates either died or performed very poorly by midway of the experiment. The common morphological change observed was the growth rate differences of mycelia in fungicide exposed isolates. ~~Some replicates of control isolates exhibited below normal growth rate while fungicide exposed being~~ Some isolates during the chronic exposure to sub-lethal doses of fungicides showed irregular growth by sectoring. Especially isolates exposed to iprodione showed a higher frequency of sectoring with a few pockets of rapid growth compared to other isolates. Also, the mycelial mat of these isolates were thinner than the control isolates. Isolates exposed to boscalid produced sclerotia faster than the control isolates with no other morphological changes. The isolates exposed to azoxystrobin and pyraclostrobin showed more aerial mycelia than the control isolates.

Why large number of polymorphisms in TM.

PCoA shows random mutations in Ss subjected to chronic exposure

Isolates are from different geographies.different selection pressures.

the microsatellites in genomic DNA of *Alternaria* were modified as an adaptative response to the ITC toxic effect.

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Sublethal doses can increase or decrease growth rate of the fungus.

Genomic plasticity

Resistance development to fungicides has been a major problem and an ongoing research topic since the introduction of single-site, target specific fungicides to the market in the 1970s (Z. Ma, T.J. Michailides / Crop Protection 24 (2005) 853–863| John A. Lucas1, Nichola J. Hawkins and Bart A. Fraaije, Advances in Applied Microbiology, Volume 90). Most newer single site fungicides are highly efficacious because of their systemic nature and curative effect. This inhibits most of the sensitive isolates of a pathogen population and creates an environment to select even rare preexisting mutants with ability to withstand the fungicide pressure. Since most fungal pathogens have a rapid reproduction rate, resistance isolates will dominate the population provided their ecological fitness was not compromised by different genetic makeup.

When the majority of isolates of a pathogen population become less sensitive to a fungicide year after year, and this effect is stable and transferred genetically or epigenetically to its progeny, that population is considered to be resistant to the fungicide applied at the recommended rate.

It is well-documented that the resistance emergence is due to natural mutations occurring in the pathogen genome (Dekker, J. (1976).| Avenot, H. F., & Michailides, T. J. (2007)| Miles, T. D., Miles, L. A., Fairchild, K. L., & Wharton, P. S. (2014)| Ma et al. 2003, | De Waard, M. (1994)| Chin, K. M. (2001) ref). Mutations conferring resistance to a fungicide have been identified in a single or multiple genes (Sierotzki et al., 2007, ||Lim TP, 2015). Though resistant isolates may increase their frequency under selection pressure, sensitive isolates will revert back to former levels when selection pressure is removed provided resistance is associated with low ecological fitness. Resistant isolates without a fitness cost (Baraldi et al 2003, Gisi et al. 2002) may remain for a long period of time even in the absence of fungicide pressure and remerge to dominance with the resumption of fungicide application.

In the past decades, research and development cost of new fungicide chemistries have increased dramatically. Due to adverse effect of chemical residues on the environment and potential risk for agricultural workers, pesticides are being increasingly regulated world over. Today, many studies on pesticide toxicity, persistence, and environmental impact are needed before registration is approved. It is estimated that from synthesis of candidate chemicals and biological screening to bringing a new fungicide to market cost more than US$250 million (Russel 2006). Therefore, it is important to place more emphasis on preventing or delaying pathogen resistance to existing fungicides than research and development of new chemistries. The strategies to delay selection and dominance of fungal pathogens by lethal doses include mixing two fungicides with different modes of action; avoid continuous use of the same fungicide by alternating sprays; avoidance of low dose rate applications; and combining non chemical cultural practices such as crop rotation, biological control and fallowing.

These two markers were selected for the present study since Kohn et al. (2008) found them to be stable over time through serial transfer and growth of laboratory strains of *S. sclerotiorum*. Conversely, a study on *Saccharomyces cerevisiae* revealed increased rate of microsatellite instability with the increasing length of the repetitive tract (Wierdl M, Dominska M, Petes TD, 1997).

**Figure 5** Histogram of number of AFLP alleles detected in *Sclerotinia sclerotiorum* isolates exposed to sublethal doses of fungicides as well as non-exposed controls. The isolates were exposed to fungicides for 12 generations before molecular characterization. Control isolates were characterized at the beginning (G0) of the experiment as well as after transferring for 12 generations (G12). Fungicides included boscalid (Bos), iprodione (Ip), thiophanate methyl (TM), azoxystrobin (Az), and pyraclostrobin (Py). Each fungicide exposed isolate is described by the fungicide used, isolate name, and experiment number. The experiment was repeated once. All control isolates were from the first experiment and depicted as Con followed by isolate name and generation (G0 or G12) **4A**) Original number of alleles detected for each isolate. **4B**) Number of alleles present after censoring AFLP data by removing polymorphic loci from G0 and G12 control isolates to arrive with same multilocus genotypes for controls.

**Figure 4** Heat map of membership probabilities of *Sclerotinia sclerotiorum* isolates belonging to fungicide challenged and unchallenged clusters as depicted in Figure 3. The 100% probability is denoted by red while white is for 0% probability. The blue crosses represent the prior cluster of isolates provided to DAPC. **4A**) The membership probabilities of isolates preassigned to fungicide challenged isolates and unchallenged controls. For example, isolates Az\_646\_Exp1, Con\_467\_G0, and Ip\_467 had membership probability >90% to a single cluster. **4B**) The membership probabilities of isolates after censoring AFLP data by removing polymorphic loci from G0 and G12 control isolates to arrive with same multilocus genotypes for controls.

**Figure 3** Principal coordinate analysis (PCoA) AFLP fragments of *Sclerotinia sclerotiorum* isolates before and after exposed to sublethal doses of fungicides. The isolates were exposed to fungicides for 12 generations before molecular characterization. Control isolates were characterized at the beginning (G0) of the experiment as well as after transferring for 12 generations (G12). The group of control isolates (both G0 and G12) are shown with a C and fungicide exposed group of isolates are depicted as T at the center of the respective populations in the diagram. The group of isolates of Ip\_467\_Exp1, Az\_646\_Exp1, and Con\_467\_G0 were not clear cut representing less than 90% probability to the preassigned group as shown in Fig 4.

**Figure 2** Neighbor joining (NJ) tree constructed from AFLP data of *Sclerotinia sclerotiorum* isolates exposed to sublethal doses of fungicides and non-fungicide exposed controls. Rogers similarity coefficient was used for calculating pairwise distances between isolates. The isolates at the beginning (G0) of the experiment and after fungicide exposure for 12 generations (G12) were characterizing with AFLP markers. The unchallenged control isolates were also taken through 12 generations and included in the analysis. The experiment was repeated once. Fungicides included boscalid (Bos), iprodione (Ip), thiophanate methyl (TM), azoxystrobin (Az), and pyraclostrobin (Py). The fungicide used, isolate name, and experiment number are mentioned for each taxon of the tree. The control isolates are depicted as Con followed by isolates name and their generation (either G0 or G12). All control isolates were used from first experiment. The bootstrap values above 50% are shown at the beginning of clusters. **A**) NJ tree for uncensored data. **B**) NJ tree constructed from censored AFLP data. Data were censored by removing polymorphic loci from G0 and G12 control isolates to arrive with same multilocus genotypes for controls. The corresponding loci of fungicide challenged isolates were also removed.

**Figure 1** Determination of 50%-100% growth inhibition zone of *Sclerotinia sclerotiorum* isolates for inocula preparation. The mycelial growth on control plates (A) were considered as 100% growth. The mycelial growth corresponding to 50% inhibition was determined on fungicide challenged plate by measuring A/2 distance perpendicular to inoculated strip. The radius to this point was used to calculate EC50 of the isolate using SGE software.

Table 1 *Sclerotinia sclerotiorum* isolates selected for the experiment\*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolates name | origin | Year of collection | Virulence\*\* | MCG\*\*\* |
| 152 | Nebraska | 2003 | 3.9 | 4 |
| 462 | Washington | 2003 | 4.6 | 57 |
| 467 | Colorado | 2003 | 4.6 | 45 |
| 555 | Minnesota | 2004 | 6.4 | 44 |
| 587 | Oregon | 2004 | 5.5 | 5 |
| 588 | Oregon | 2004 | 5.3 | 4 |
| 594 | California | 2004 | 4.6 | 21 |
| 646 | Washington | 2005 | 5.4 | 60 |
| 655 | North Dakota | 2005 | 4.0 | 46 |

\*All were isolated from different cultivars of dry beans. These isolates were a subset from a pool of 366 characterized isolates.

\*\*Virulence was rated on a scale of 1-9 using the straw test method (Ref) with increasing numbers representing higher virulence.

\*\*\*MCG: Mycelial compatibility group.

Table 2 Fungicide stock solutions used for the spiral plater and concentrations resulted on PDA pales \*

|  |  |  |  |
| --- | --- | --- | --- |
| Fungicide | Sample intake concentration (ppm) | Concentration (ppm) at center (20 mm radius) | Concentration (ppm) at periphery (64 mm radius) |
| Boscalid | 750 | 9.07 | 0.05 |
| Iprodione | 400 | 2.58 | 0.807 |
| Thiophanate methyl | 9000 | 108.79 | 0.57 |
| Azoxystrobin | 250 | 2.87 | 0.01 |
| Pyraclostrobin | 200 | 2.34 | 0.01 |

\*Exponential mode was used for all fungicide depositions except for iprodione for which proportional mode was employed.

Table 3 Number of mutations at six microsatellite loci observed in eight *Sclerotinia* isolates exposed to sublethal doses of fungicide. Six microsatellite loci were characterized prior to fungicide exposure (G0) and after continuously challenging with fungicides for 12 generations (G12). The total mutations resulted from two studies as well as the nature of mutations in terms of deletion or insertion are given. Isolates not challenged with fungicides did not show any mutations for G12 compared to G0.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Fungicide | Exp. 1 | Exp. 2 | Total mutations | Insertions | Deletions |
| Boscalid | 1 | 0 | 1 | 0 | 1 |
| Iprodione | 3 | 2 | 5 | 1 | 4 |
| Thiophanate methyl | 4 | 2 | 6 | 5 | 1 |
| Azoxystrobin | 1 | 2 | 3 | 2 | 1 |
| Pyraclostrobin | 2 | 0 | 2 | 2 | 0 |

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1A)Troncoso-Rojas- Inter simple sequence repeat polymorphism in Alternaria genomic DNA exposed to lethal concentrations of isothiocyanates

Microsatellites or SSR are composed of tandemly repeated, simple DNA sequence motifs of as many as six nucleotides in length. These *loci* are commonly found in both prokaryotic and eukaryotic genomes and typically are highly polymorphic within species and populations (Dettman and Taylor, 2004).

2)Zeh, Zeh, and Yoichi Ishida :Transposable elements and an epigenetic basis for punctuated equilibria

Evolution is frequently concentrated in bursts of rapid morphological change and speciation followed by long term stasis. We propose that this pattern of punctuated equilibria results from an evolutionary tug-of-war between host genomes and transposable elements(TEs) mediated through the epigenome. According to this hypothesis, epigenetic regulatory mechanisms (RNA interference, DNA methylation and histone modifications) maintain stasis by suppressing TE mobilization. However, physiological stress, induced by climate change or invasion of new habitats, disrupts epigenetic regulation and unleashes TEs. With their capacity to drive non-adaptive host evolution, mobilized TEs can restructure the genome and displace populations from adaptive peaks, thus providing an escape from stasis and generating genetic innovations required for rapid diversification.

**DEFINITION: DNA methylation** is an epigenetic mechanism used by cells to control gene expression. A number of mechanisms exist to control gene expression in eukaryotes, but **DNA methylation** is a commonly used epigenetic signaling tool that can fix genes in the “off” position.

Furthermore, ecological communities exhibit synchrony in stasis and diversification, implicating environmental change as a critical factor generating punctuation.(8–10)

Growing evidence indicates that epigenetic regulation evolved to suppress transposable elements(TEs),(12,13) a diverse array of parasitic sequences that comprise a large fraction of eukaryotic genomes.(14)

Mobilized TEs rapidly restructure the genome and alter gene expression patterns by inserting into promoters and enhancers, and by causing chromosomal breakage, exon shuffling, sequence expansion, gene duplication, ectopic recombination, novel gene formation and expansion and re-wiring of genetic regulatory networks.(16–18)

Stress-induced breakdown in epigenetic suppression enhances the spread of TEs, with potentially deleterious effects on host fitness, and increases genetically and epigenetically based phenotypic variation.(19)

As the host genome and parasitic sequences coevolve, epigenetic silencing mechanisms regain control, heritable variation declines, and stasis is re-established.

3)Ma, and Michailides: Advances in understanding molecular mechanisms of fungicide resistance and molecular detection

However, since the introduction of the site-specific fungicides in the late 1960s, fungicide resistance in phytopathogenic fungi has become a major problem in crop protection (Brent, 1995).

Fungicide resistance is a stable, inheritable adjustment by a fungus to a fungicide, resulting in reduced sensitivity of the fungus to the fungicide. Resistance may result from single or multiple gene mutations. Resistant isolates typically arise from a very low natural rate of genetic mutation, and these isolates are less affected or not inhibited at all by a labeled application rate of a fungicide. Since the fungicide can effectively control sensitive isolates, resistant isolates may become dominant in pathogen populations under selection pressure of fungicide use over time, therefore disease control failures may eventually occur.

The ecological fitness of fungicide-resistant fungal isolates will determine the persistence of resistant genotypes once they are selected. In many instances, since resistant isolates may have lower fitness than sensitive isolates, they cannot survive well in the absence of fungicide selection pressure. In this case, the frequencies of resistant isolates in pathogen populations will decrease once the fungicide applications cease. Alternatively, resistant isolates can be as fit as sensitive isolates and persist for a long time even without any use of the fungicides. This has exemplified

by the resistance of several phytopathogenic fungi to

benzimidazole or strobilurin fungicides (Koenraadt et

al., 1992; Baraldi et al., 2003).

Fungicide resistance can be conferred by various

mechanisms (Gisi et al., 2000; Gullino et al., 2000; Fluit

et al., 2001; MgGrath, 2001), including: (I) an altered

target site, which reduces the binding of the fungicide;

(II) the synthesis of an alternative enzyme capable of

substituting the target enzyme; (III) the overproduction

of the fungicide target; (IV) an active efflux or reduced

uptake of the fungicide; and (V) a metabolic breakdown

of the fungicide.

2. Molecular mechanisms of fungicide resistance

2.1. Benzimidazoles In most cases, resistance was correlated with point mutations in the b-tubulin gene, which result in altered amino acid sequences at the benzimidazole-binding site.

4)Rebecca S. Shapiro: Antimicrobial-Induced DNA Damage and Genomic Instability in Microbial Pathogens

GOOD for DIScusson

Whereas lethal doses of antimicrobials may select for preexisting resistant microbes, there is increasing interest in uncovering the cellular consequences of sublethal antimicrobial exposure on the development of antimicrobial resistance. Sublethal antimicrobial exposure can trigger DNA damage and genomic instability across the diversity of microbial pathogens, including bacterial and fungal species.

Overall, this review aims to explore genomic pressure exerted on bacterial and fungal pathogens by antimicrobial treatment, and implications for antimicrobial resistance.

Even low doses of antimicrobials can directly or indirectly induce DNA damage and alterations

In fungi, treatment with antifungals can lead to DNA damage, resulting in homologous recombination and loss of heterozygosity (LOH), or other cellular stress responses, leading to unequal chromosomal segregation during mitosis and aneuploidy

First, several antimicrobial agents cause direct chemical damage to DNA. An example of this is the

Finally, numerous antimicrobials result in metabolic perturbations, downstream of the interaction with their respective cellular targets. A commonly observed example of this is the production of reactive oxygen species (ROS) in response to antibiotics (including β-lactams, aminoglycosides, and quinolones) [7–9], antifungals (including polyenes and azoles) [10,11], and antiparasitics [12]. Antimicrobial-induced ROS, such as hydroxyl radicals, damage DNA through the formation of DNA strand breaks, and the incorporation of oxidized guanine residues into the genome [13,14].

Repair of damaged DNA is critical for microbial survival, yet certain DNA damage repair pathways may introduce mutations into the genome.

For bacteria, the SOS response is the global response to DNA damage. Triggered

**Description of Gene Ontology Term:** error-prone translesion synthesis

The conversion of DNA-damage induced single-stranded gaps into large molecular weight DNA after replication by using a specialized DNA polymerase or replication complex to insert a defined nucleotide across the lesion. This process does not remove the replication-blocking lesions and causes an increase in the endogenous mutation level. For example, in E. coli, a low fidelity DNA polymerase, pol V, copies lesions that block replication fork progress. This produces mutations specifically targeted to DNA template damage sites, but it can also produce mutations at undamaged sites.

Nucleotide excision repair (NER) is a particularly important excision mechanism that removes DNA damage induced by ultraviolet light (UV). UV DNA damage results in bulky DNA adducts - these adducts are mostly thymine dimers and 6,4-photoproducts.

Eukaryotic microbes have homologous strategies to repair or tolerate DNA damage, with a global response involving the expression of genes involved in nucleotide excision repair, and error-prone translesion synthesis polymerases such as DNA polymerase zeta, and Rev1 [18]. In both bacteria and fungi, repair of DNA double-strand breaks may occur through non-homologous end joining, where cut ends are re-ligated in a manner that may be mutagenic, or through homologous recombination, using a homologous sequence as a template for repair [18,19].

As a result of antimicrobial-induced DNA damage and repair discussed above, as well as additional stress-response pathways, microbial species may experience genomic instability. One example of this is an increase in the number of single nucleotide polymorphisms (SNPs) in response to antimicrobial treatment (Fig. 1). The mechanisms by which this occurs can broadly be categorized into DNA damage response pathways, and other stress response signaling pathways.

Up-regulation of general stress response pathways similarly mediate stress-induced mutations in fungal species. In the model yeast Saccharomyces cerevisiae, stress triggers an environmental stress response pathway, mediated through transcriptional regulators Msn2 and Msn4. Similar to what is observed in bacteria, these transcription factors activate downstream error-prone translesion synthesis via the Rev1 polymerase, thus increasing mutagenesis [25]. While antifungal-induced SNP mutagenesis has not been well documented in fungal pathogens, analysis of S. cerevisiae, with conserved regulatory machinery with pathogenic fungi [26], may provide novel mechanistic insight for fungal pathogens.

For both bacterial and fungal pathogens, antimicrobial induced mutagenesis has the capability to accelerate the acquisition of drug resistance and multi-drug resistance by increasing genetic and phenotypic diversity within the population [27], with important consequences for clinical use of antibiotics.

**Large-Scale Genomic Alterations Induced by Antimicrobial Treatmen**t

In addition to nucleotide mutagenesis, treatment with sublethal antimicrobial agents can also promote larger-scale genomic rearrangements in microbial pathogens. This includes movement of mobile genetic elements, chromosomal rearrangements, and whole chromosome aneuploidies.

Thus antibiotic treatment can both induce movement of antibiotic-encoding mobile elements, and stimulate cellular competence, which together can strongly promote the acquisition and spread of genetic resistance determinants within populations.

Although horizontal gene transfer is rarely observed amongst human fungal pathogens, treatment with antifungals can promote alternative forms of genomic instability, via gross chromosomal rearrangements. The antifungal fluconazole, which targets fungal membrane integrity, also leads to the up-regulation of cellular stress response pathways [29], and promotes genomic rearrangements [4]. For the diploid fungal pathogen Candida albicans, sublethal doses of fluconazole promote increased rates of loss of heterozygosity (LOH) [34], a form of gross chromosomal rearrangement in diploid organisms that results in the loss of genetic heterozygosity at a particular locus or throughout an entire chromosome (Fig. 1). Furthermore, C. albicans exposed to antifungal stress promotes the formation of isochromosomes, in which entire chromosome arms are exchanged, creating a chromosome comprised of two identical chromosome arms flanking a centromere [35]. Although the mechanism of antifungal mediated chromosomal alterations is unknown, it has been suggested that DNA double-strand breaks induced by antifungal agents [34,36] and repaired via recombination between chromosomes, may contribute to such genomic rearrangements.

Both LOH and isochromosomes play an important role in acquired resistance to antifungals in C. albicans, through homozygosis and duplication of genes encoding both the drug target of the azoles (ergosterol biosynthesis enzyme Erg11), and regulators of drug efflux [4]. Duplication and thus overexpression of Erg11 reduces the efficacy of the azole drugs and promotes resistance, while duplication of transcriptional regulators of drug efflux pumps (such as Tac1 and Mrr1), may promote multidrug resistance by increasing the efficacy by which antifungals are exported from the cell [4].

**Definition(s)**

(LOH) At a particular locus heterozygous for a deleterious mutant allele and a normal allele, a deletion or other mutational event within the normal allele renders the cell either hemizygous (one deleterious allele and one deleted allele) or homozygous for the deleterious allele

The antifungal agent fluconazole induces the formation of aneuploidies in C. albicans [35,39], and chromosome disomies in C. neoformans [40,41] (Fig. 1), both of which are linked with the development of antifungal drug resistance from increased copy numbers of key antifungal resistance determinants, including antifungal target proteins and drug transporters [40].

In Candida species, this stress induced aneuploidy occurs from aberrant mitosis due to antifungal stress, resulting in the formation of tetraploid cells, and unequal chromosomal segregation [42]. In S. cerevisiae, stress-induced aneuploidies occur under diverse stress conditions, including low-dose antifungal treatment, and are linked to protein chaperone Hsp90-mediated disruption of the kinetochore complex, leading to chromosomal instability [43].

Antimicrobial-induced genomic instability leading the chromosomal aneuploidies, including those associated with drug resistance, is a unique way in which fungal pathogens adapt to antimicrobial stress conditions.

The scale of stress-induced genomic alterations, from SNPs to whole chromosome aneuploidy, likely has varying degrees of phenotypic consequences for microbial pathogens. For many fungal pathogens, which unlike bacteria, cannot increase their genetic diversity through horizontal gene transfer, and which rarely undergo sexual reproduction for genetic recombination [44], large-scale chromosomal rearrangements and aneuploidies may provide a unique mechanism to rapidly generate genetic diversity and adapt to their environments under conditions

of stress.

As stress-induced mutation provides a mechanism for microbial pathogens to develop resistance, it is critical to understand how antimicrobial therapeutics may enhance or limit pathogen evolvability. One therapeutic strategy to limit acquired drug resistance is to target the pathogen response to antimicrobials [19]. For instance, preventing SOS induction by targeting central SOS regulators such as the protease LexA can prevent mutations and the evolution of antibiotic drug resistance in E. coli [22,46]. Similarly, quinolone antibiotics do not induce mutations in Salmonella typhimurium strains lacking the Pol V homolog [47]. Additionally, new research has identified certain antimicrobial peptides that, unlike antibiotics, do not elicit an SOS response or increase bacterial mutation rate [48]. This finding suggests promising avenues for identifying novel antimicrobial agents that do not expedite the evolution of

antimicrobial resistance.

5)Book chapter 2

The Evolution of Fungicide Resistance

John A. Lucas1, Nichola J. Hawkins and Bart A. Fraaije

Similarly, for the QoI fungicides, a review of cytochrome b mutations in yeast and other eukaryotes listed 22 mutations over 12 codons conferring resistance to QoIs (Brasseur, Saribas, & Daldal, 1996), of which 3 (G143A, F129L, and G137R) have been reported in field isolates of phytopathogenic fungi (Sierotzki et al., 2007). In Magnaporthe grisea, laboratory mutants had G143A or G143S substitutions in cytochrome b (Avila-Adame & K€oller, 2003), but only G143A has been reported in the field (Ma & Uddin, 2009). In Cercospora beticola, UV mutants were generated with G143S or F129V (Malandrakis, Markoglou, Nikou, Vontas, & Ziogas, 2006), but again, only G143A has been reported in the field (Birla, Rivera-Varas, Secor, Khan, & Bolton, 2012). Spontaneous mutants of B. cinerea with resistance to the QoI trifloxystrobin were selected in the laboratory from wild-type sensitive isolates (Angelini et al., 2012), but the G143A mutation could not be detected, unlike resistant isolates from the field that all had the mutation. The laboratory mutants were unstable in the absence of fungicide selection, and these authors suggested that the heteroplasmic state of resistant mitochondria might account for the lack of detection of G143A and rapid loss of resistance.

Recently, in vitro mutagenesis studies have been carried out to predict possible mechanisms of resistance to the new SDHI fungicides (Avenot & Michailides, 2010; Sierotzki & Scalliet, 2013). In B. cinerea, mutations in sdhB codon 225 and 272 have been generated in vitro and reported in the field (Walker, Gredt, & Leroux, 2011). The B-R54G substitution has been reported in both laboratory mutants and field isolates (Fraaije et al., 2012), but only in combination with different further changes. Eight mutations found in laboratory mutants of Z. tritici (Fraaije et al., 2012; Scalliet et al., 2012) and Aspergillus oryzae (Shima et al., 2009) have been reported in field isolates of other species, ….. but a further 30 mutations have so far only been reported in laboratory mutants of various species and 20 other mutations only in the field, although not all are associated with reduced fungicide sensitivity.

6.2 Fitness Costs

A frequently suggested reason for mutations generated in vitro not emerging in the field is that some mutations result in pleiotropic fitness costs (Avila- Adame & K€oller, 2003; Davidse & Ishii, 1995). In typical in vitro selection experiments, fungicide-resistant mutants are able to grow on agar plates as single-spore colonies unless the mutations are actually lethal, whereas in the field, mutants must be pathogenic in planta and competitive against other isolates. Fusarium moniliforme Y50R mutants (Yan & Dickman, 1996), S. cerevisiae F167Y mutants (Li, Katiyar, & Edlind, 1996), and three of the benomyl-resistant S. cerevisiae mutants generated by Reijo et al. (1994) were cold sensitive. Therefore, it seems likely that mutations conferring higher levels of resistance, with lower fitness costs under field conditions, are more likely to be selected.

8.2 Evaluating Management Strategies

Mavroeidi and Shaw (2006) tested the effect of different doses of a triazole fungicide, with or without a QoI partner, on selection for azole resistance in Zymoseptoria tritici. Selection for resistance was shown to increase in proportion to dose, while addition of the QoI at higher azole doses reduced selection. These results supported the idea that selection for resistance is positively related to fungicide dose, but that effects of mixtures on selection may be variable.

The dose rate debate: does the risk of fungicide resistance increase or decrease with dose?

F. van den Boscha\*, N. Paveley, M. Shaw, P. Hobbelen and R. Oliver (2011)

During the ‘emergence phase’ the resistant strain has to arise through mutation and invasion. Emergence phase: no experimental publications and only one model study report on the emergence phase, and we conclude that work in this area is needed. In the mathematical models published, no evidence has been found that a lower dose could lead to a higher risk of fungicide resistance selection.

The methods suggested include (i) management of application dose, (ii) constraining the number of applications, (iii) use of fungicide mixtures, (iv) use of fungicide alternation, and (v) provision of pathogen refugia, or various combinations of the above.

We study the effect of dose in isolation and will not discuss the interaction of dose with other aspects of fungicide application (number of treatments, mixtures, alternation).

However, insects, weeds and mammals are diploid organisms and this has important consequences for the development of resistance.

In contrast to insects and weeds, many important fungal and oomycete plant pathogens are either haploid, in which case there are no heterozygotes, or diploids or dikaryons that are largely clonal, in which case selection acts on the entire clonal genotype. The high dose plus refugia mechanism is not relevant for such fungi (but see the section ‘Current research into the dose debate’).

Physiological adaptation is not resistance Selection

The implication is that applying a low dose would train the population to ‘resist’ the fungicide, rather as an animal might adapt to lower temperatures in winter. Applying a high dose on the other hand would not lead to adaptation, but would kill the pathogen. There is no evidence that such physiological adaptation is relevant in the case of resistance development. Resistance is a matter of genetic or epigenetic change in individuals in the population that is inherited by their offspring and the subsequent selection of this altered genotype causing its eventual domination in the population. The development of fungicide resistance is thus a matter of differential selection of inherited variation.

The models for fungicide resistance published so far and the new calculations with the tested model of Hobbelen et al. (2010) therefore suggest that, independent of model type or model output quantity considered, an increased dose does not decrease the risk of fungicide resistance. In the most extreme case, the risk of fungicide resistance is independent of dose, but in most cases increased dose increases the risk of fungicide resistance. However, all this work assumes that the pathogen evolves complete resistance to the fungicide.

Stress-induced fungicide resistance Mutations

Fungicide resistance generally emerges through mutations in the nuclear or mitochondrial genome. Several experiments have shown that environmental stress (caused for example by nutrient limitation, UV light, oxidative stress, antibiotic exposure or acid exposure) can increase the mutation rate in bacteria (Bjedov et al., 2003; Tenaillon et al., 2004; Kang et al., 2006; Galhardo et al., 2007). This raises the possibility that eukaryotic (for example, fungal and oomycete) mutation rates might also be increased by stress, such as exposure to fungicide. No research has been done into this area.

During the fungicide registration process each fungicide is rigorously tested for any possible mutagenic ⁄ carcinogenic effects on non target organisms, and we are not suggesting here that any registered fungicide has any such effect. However …it could be that for the target organism the fungicide presents a stress factor, which could lead to, for example, the suppression of proof-reading during copying of the genome.

Mutation limitation

Mutations occur during the replication of the genome and can occur during any cell division in a cell lineage leading to spore formation. Although there will be spatial and temporal variation in the frequency of mutant spores, the process can be summarized as a probability that a random spore contains a new mutation contributing to fungicide resistance. …Population size is thus a key component in the number of fungicide resistance mutations occurring per time unit.

In a few cases a founder population of resistant individuals will build up in sufficient numbers for selection to be as important as chance in population change

Now consider the contrasting situations of fungal populations affected by low or high doses of fungicide (Fig. 8). At low dose the density of the sensitive strain is relatively high. This implies a relatively high number of fungicide resistance mutations occurring per time unit. It also implies a low probability that a mutant will be able to build up a population and survive through the emergence phase because of the presence of a relatively high density of the sensitive strain taking up leaf area.

During the selection phase is it unlikely that further mutations occurring in the population have a quantitative

effect on the course of selection.

Refugia

The reasoning behind the high dose plus refugia strategy, that has led to resistance management in insecticide and herbicide resistance, does not apply to most fungal plant pathogens since the argument is based on the target organism reproducing sexually, being diploid (or of higher ploidy) and the heterozygote being partially sensitive to the chemical control.

It has been argued that in a situation where the majority of the fungal population lives in the refugia (=untreated) area and the resistant strain is partially sensitive to the fungicide, there may be cases where the high dose plus refugia strategy works.

Further work is needed before definitive conclusions can be drawn, but current evidence suggests that the presence of refugia does not result in a higher dose reducing the development and invasion of fungicide resistance.

Partial resistance

Published experimental evidence on the selection of fungicide resistance suggests that increased dose increases selection. This also holds for the experiments involving pathogen-fungicide combinations where the pathogen evolves partial resistance to the fungicide. The authors believe that the effect of dose on the emergence and selection of partial resistance needs further study because there are possible scenarios where partial resistance can lead to situations where increased dose can decrease the risk of fungicide resistance.

Discussion and conclusions

Resistance management strategies should be based on evidence interpreted within a sound experimental and theoretical framework. Further work is needed especially in the area of partial resistance where there are theoretical situations possible where a higher dose may lead to a lower risk of fungicide resistance.

In conclusion, a high dose may be required to obtain effective control – particularly on susceptible cultivars under high disease pressure, or where a sensitivity shift has eroded efficacy and dose has to be increased to maintain effective control (i.e. in the adjustment phase). However, current evidence and analysis suggests that, in most circumstances, a high fungicide dose will increase the speed at which fungicide resistance develops.

Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation

Peter A Smith & Floyd E Romesberg

Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies

Jonathan Gressel∗

Microsatellite stability in the plant pathogen Botrytis cinerea after exposure to different selective pressures

Sakhr AJOUZ, Ve´ronique DECOGNET, Philippe C. NICOT, Marc BARDIN\*

The stability of microsatellite markers was investigated in the spore-producing fungus Botrytis cinerea exposed to four growth conditions. This knowledge is essential in order to differentiate mutations from genetic exchanges or recombination in population genetics studies. It is also important when using strains from collections that need to be regularly propagated on medium.

The results showed that, despite the phenotypic changes observed in some generations, no changes were observed in the allele size at nine microsatellite loci whatever the selective pressure endured by the fungus.

Some of these conditions may represent a stress to which they have to adapt. For certain fungi, stresses such as nutritive deficiency could increase mutation rates in the genome (Drake et al. 1998) by modifying the activity of transposable elements and inducing systems involved in the modification or repair of DNA (Mes et al. 2000; Ikeda et al. 2001).

Botrytis cinerea Pers.:Fr (teleomorph Botryotinia fuckeliana (de Bary) Whetzel) is a haploid necrotrophic fungal pathogen of numerous plants estimated to more than 200 species (Jarvis 1980).

Microsatellite markers (simple sequence repeats) are short tandem repeated tracts of DNA composed of units that are 1-6 base pairs long, spread throughout the genome and used as genetic markers in genome mapping or population genetics studies because of their high level of polymorphism (Jarne & Lagoda 1996). Their mutation rates are thought to vary from 10\_6 to 10\_3 per locus per gamete per generation depending on organism and locus (Weber & Wong 1993; Schug et al. 1997). .. This high genetic variability is interpreted as an indication of genetic exchange between distant populations (Karchani-Balma et al. 2008; Decognet et al. 2009) and/or an indirect proof of recombination (Karchani-Balma et al. 2008; Vaczy et al. 2008). However, mutations in the microsatellite loci overtime may also reveal diversity and therefore misinterpret the conclusions of these studies. In order to properly apply these markers in population studies, one should estimate the mutation rate of the microsatellite markers used.

Lasker & Ran (2004) demonstrated the short-term stability of microsatellite profiles after the serial culture of Penicillium marneffei during 7-8 weeks. More recently, Kohn et al. (2008) reported that no mutations were detected in alleles of seven microsatellite loci after 400 d of continuous in vitro hyphal growth of 12 strains of the plant pathogenic ascomycete Sclerotinia sclerotiorum.

Materials and methods

A total of 20 successive generations were produced for each type of PDA medium. On PDA medium supplemented with pyrrolnitrin or iprodione the first ten generations were produced using a constant concentration of the compound and the ten last generations were produced with increasing doses of the compound (Table 2).

For some reasons after storage, some of the lineages produced were not able to grow anymore on PDA medium. Therefore they were not used for phenotypic and genotypic characterizations.

Microsatellite marker analyses To assess the stability of microsatellites, the microsatellite profiles of the parent isolates (G0) on pyrrolnitrin (G10P and G20P), on iprodione (G20I) and in condition of nutritive stress (G10N).

Results

Phenotypic changes

The two isolates sensitive to iprodione and dicloran (BC25 and H6) acquired resistance to these fungicides when grown on medium amended with pyrrolnitrin (G20P) or iprodione (G20I) (Table 3). Finally, no changes in the resistance of carbendazim were observed (Table 3). In parallel a modification in the level of aggressiveness on apple fruits and tomato plants was observed: the generations that acquired a resistance to pyrrolnitrin (G20P) were less aggressive compared to the corresponding control G0 isolates suggesting a fitness cost of resistance to pyrrolnitrin (Table 3).

For each isolate, after the generations realised on rich PDA medium (G20C), poor glucose medium (G10N), pyrrolnitrin-amended medium (G10P and G20P) or iprodione-amended medium (G20I), the amplicons obtained were all of similar sizes at the nine microsatellite loci whatever the motif and the size of the microsatellites core repeat are (Table 4). The haplotypes remained identical whatever the selective pressure endured by the fungus, revealing a good stability of the microsatellites genetic markers in Botrytis cinerea isolates (Table 4).

The main purpose of the present study was to assess if an exposure of the filamentous fungus Botrytis cinerea to different environmental stresses during successive conidial generations could have an effect on the stability of the microsatellite markers. Whatever the number of generations realised on Petri plates (G10 or G20), the duration of the consecutive transfers (280-300 d of successive cultures) and the nature of stress endured by the fungus, no mutations were observed in alleles at the nine microsatellite loci studied.

In yeasts, microsatellite instability increased as repetitive tracts became longer (Wierdl et al. 1997).

The stability in alleles at seven microsatellite loci was also observed in the non-sporulating plant pathogenic ascomycete Sclerotinia sclerotiorum after 400 d of continuous in vitro hyphal growth (Kohn et al. 2008). The short-term stability of polymorphic microsatellite marker profiles was also demonstrated in Penicillium marneffei isolates after 7-8 weeks of serial culture in liquid media (Lasker & Ran 2004).

Meanwhile, some of the selective pressures tested in this study induced phenotypic changes in B. cinerea isolates, such as a resistance to pyrrolnitrin and to some fungicides, and a reduction in aggressiveness to tomato plants and apple fruits (G20P andG20I). The stability of the microsatellite markers combined with the phenotypic modifications illustrates the fact that different phenotypes could possess the same genotype based on the use of these microsatellite markers. Moreover, the generations produced on PDA medium show that periodical transfer of isolates of B. cinerea to fresh medium, as commonly done in many laboratories, does not modify their microsatellite pattern. This stability is particularly important in population diversity studies when researchers test strains from collections that need to be regularly propagated on medium in the laboratory. To complement this study, the stability of microsatellites after successive cycles of sexual reproduction should be evaluated. Moreover, itwould be interesting to understand better at the genome level what is happening during this selection process.

Mutation as a Stress Response and the Regulation of Evolvability

Rodrigo S. Galhardo, P. J. Hastings, and Susan M. Rosenberg

Stimulatory Effects of Sublethal Doses of Dimethachlon on Sclerotinia sclerotiorum

Feng Zhou, Hong-Jie Liang, Ya-Li Di, Hong You, and Fu-Xing Zhu,

*Sclerotinia sclerotiorum* is one of the most devastating and economically important necrotrophic fungal phytopathogens, capable of infecting more than 400 species of plants worldwide. Fungicides have been a major method for control of Sclerotinia diseases due to the lack of adequate levels of host resistance (4).

In order to study stimulatory effects of sublethal doses of fungicides on *S. sclerotiorum*,55 dimethachlon-sensitive isolates and 3 dimethachlon-resistant isolates of *S. sclerotiorum* were assayed to determine effects of sublethal doses of dimethachlon on mycelial growth rate on potato dextrose agar(PDA) media and virulence on oilseed rape plants. Results showed that all 3 dimethachlon-resistant isolates and 13 of the 55 sensitive isolates exhibited stimulatory responses to sublethal doses of dimethachlon.

Dimethachlon-resistant isolates grew significantly (*P* < 0.05) faster on PDA media amended with dimethachlon at 0.5 to 4 μg/ml than on fungicide-free PDA media.

There have been some reports of plant pathogen growth and virulence stimulations by sublethal doses of fungicides or other chemicals (e.g., stimulation of Pythium damping-off of geranium by sublethal doses of mefenoxam; 18,19).

Baraldi et al. (2) reported that low concentrations of thiabendazole amended in media could increase germination percentages of some but not all thiabendazole-resistant *Penicillium expansum* isolates. Landry et al. (23) observed significantly increased radial growth of *Lyophyllum palustre* (Peck) Singer on media amended with low concentrations of propamocarb compared with the fungicide-free control.

There is a possibility that virulence stimulations maybe relate to endogenous hydrogen peroxide (H2O2) or oxalic acid in plant pathogens because these two compounds play a crucial role in fungal pathogenesis on host plants (1,4,12,15,35).

Recently, Calabrese (7) gave a comprehensive review on the mechanisms of hormesis, particularly for those mediated via receptor or cell signaling pathways. Protecting the organism from injury via adaptive and preconditioning stress responses or overcompensation for a disruption of homeostasis has been claimed to be the mechanistic basis for chemical hormesis (8). These experimental results suggested that the hormetic responses were differential for different isolates of even the same species of plant pathogen. The underlying mechanisms for hormetic responses are complex and remain to be elucidated in the future.

The reduced sensitivity of *S. sclerotiorum* to H2O2, at least in theory, could increase its virulence to host plants. Oxalic acid production has been associated with virulence of some *Sclerotinia* spp. (15,29). Oxalic acid could suppress the oxidative burst of the host plant (12), enhance activities of cell wall-degrading enzymes by reducing extracellular pH, and had other functions conducive to virulence (4).

Fungicide-Induced Mutagenesis in a Plant Pathogenic Fungus

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Stress-Induced Mutagenesis in Bacteria

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Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease

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**Fungicide-Induced Mutagenesis in a Plant Pathogenic Fungus**

Fengping Chen, Sydney E. Everhart, William C. Bridges, Chao-Xi Luo, Xili Liu and

Guido Schnabel

This is the first report of fungicide-induced, accelerated mutagenesis in a plant pathogen and suggests that prolonged exposure of field populations to azoxystrobin and maybe other QoI fungicides at sublethal doses could allow for quicker emergence of spontaneous mutations, which has important implications for fungicide resistance management.

An investigation into the nature of adaptation to SYP-Z048 and azoxystrobin revealed no evidence of resistance stability. Progeny from conidia of all mutants were as sensitive to SYP-Z048 and azoxystrobin as the parental strains indicating that the increased resistance was not a heritable trait and thus the adaptation may have been either of physiological or epigenetic nature (data not shown).

Overall, mutations in microsatellite loci appear to be common following exposure to sub-lethal levels of azoxystrobin. No indications of nucleotide changes were detected in the control or the DMI treatments.

We did not investigate the mode of action by which azoxystrobin induces mutagenesis in transposon movement or at microsatellite loci, but inhibitors of mitochondrial respiration, are known to accelerate the generation of reactive oxygen species (ROS) (25). This is supported by a recent study showing that ROS generation was enhanced in F. graminearum mycelium exposed to azoxystrobin and SHAM, compared to the azoxystrobin without SHAM treatment (7).

Transposon movement is an important mechanism in natural populations of fungi to adapt to stressful environments and is possibly more important for populations lacking sexual recombinations as a source of variation (32).

Negative effect on fitness on average

Transposable elements have been directly associated with the development of fungicide resistance in ascomycetes. For example, DMI resistance due to overexpression of the target site gene cyp51 was associated with a long interspersed nuclear element (LINE)- like retrotransposon in Blumeriella jaapii and an uncharacterized transposon of 553 bp in length in V. inaequalis (12, 36)

While there was a clear association between azoxystrobin exposure and induction of mutagenesis, the same relationship was not observed for the DMI fungicide suggesting this phenomenon is dependent on the mode of action of a fungicide.

The existence of such strains has been suggested previously to explain the observed predisposition of a pathogen population already resistant to a fungicide to accelerated resistance development to another, unrelated fungicide (10).

An alternate explanation would be that the mutagenesis induced by azoxystrobin is specific to processes that are not capable of conferring resistance to QoI or DMI fungicides.

# Sterol 14α-Demethylase Cytochrome P450 (CYP51), a P450 in all Biological Kingdoms

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The CYP51 family is an intriguing subject for fundamental P450 structure/function studies and is also an important clinical drug target. This review updates information on the variety of the CYP51 family members, including their physiological roles, natural substrates and substrate preferences, and catalytic properties *in vitro*. We present experimental support for the notion that specific conserved regions in the P450 sequences represent a CYP51 signature. Two possible roles of CYP51 in P450 evolution are discussed and the major approaches for CYP51 inhibition are summarized.

Sterol 14α-demethylation as a general part of sterol biosynthetic pathways in eukaryotes [[1](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2324071/#R1)] has been known and studied for more than 30 years [[2](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2324071/#R2)-[7](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2324071/#R7)]. The enzyme catalyzing this reaction was first purified from yeast in 1984 (*Sacharomyces cerevisiea* [[8](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2324071/#R8)]), and following determination of its primary structure [[9](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2324071/#R9)] the cytochrome P450 sterol 14α-demethylases were placed into the CYP51 family, a number reserved for fungal sequences [[10](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2324071/#R10)].