An Analysis of Postharvest Fungal Pathogens Reveals Temporal–Spatial and Host–Pathogen Associations with Fungicide Resistance-Related Mutations

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ABSTRACT

Fungicides are the primary tools to control a wide range of postharvest fungal pathogens. Fungicide resistance is a widespread problem that has reduced the efficacy of fungicides. Resistance to FRAC-1 (Fungicide Resistance Action Committee-1) chemistries is associated with mutations in amino acid position 198 in the β -tubulin gene. In our study, we conducted a meta-analysis of β -tubulin sequences to infer temporal, spatial, plant host, and pathogen genus patterns of fungicide resistance in postharvest fungal pathogens. In total, data were acquired from 2,647 specimens from 12 genera of fungal phytopathogens residing in 53 countries on >200 hosts collected between 1926 and 2020. The specimens containing a position 198 mutation were globally distributed in a variety of pathosystems. Analyses showed that there are associations among the mutation and the year an isolate was collected, the pathogen genus, the pathogen host, and the collection region. Interestingly, fungicide-resistant β -tubulin

genotypes have been in a decline since their peak between 2005 and 2009. FRAC-1 fungicide usage data followed a similar pattern in that applications have been in a decline since their peak between 1997 and 2003. The data show that, with the reduction of selection pressure, FRAC-1 fungicide resistance in fungal populations will decline within 5 to 10 years. Based on this line of evidence, we contend that a β -tubulin position 198 mutation has uncharacterized fitness cost(s) on fungi in nature. The compiled dataset can inform end users on the regions and hosts that are most prone to contain resistant pathogens and assist decisions concerning fungicide resistance management strategies.

Keywords: β -tubulin, FRAC 1 group, fungicide resistance, 198 mutation, postharvest fungal pathogens

The use of fungicides to control phytopathogens was first noted >150 years ago in France where a mixture of lime and copper sulfate, known as “Bordeaux mixture,” was found to control fungal diseases in vineyards (Schumann 1991). Fungicides are an intricate part of agricultural systems and are a valuable asset that increases yield and agricultural efficiency. In 2012, an estimated 65 million pounds of fungicide active ingredients (costing \$1.784 billion) were applied in the United States (Atwood and Paisley-Jones 2017). The quantity of fungicides used is dependent on a variety of factors including the pathogen, the region, the crops grown, and the climate (Agrios 2005). Fungicide usage has been a valuable tool for the agriculture industry, but during the past 60 years, decreased fungicide efficacy stemming from fungicide resistance has been reported (Smith 1988).

Fungicide resistance was first reported in the *Penicillium digitatum*–lemon system by Harding (1959). However, unlike insecticides and bactericides, fungicide resistance was not initially considered a problem, with only a few field cases reported in the 1960s (Georgopoulos 1969). The lag from the first usage of fungicides to the development of resistant isolates can most likely be attributed to the broad-spectrum nature of the fungicides originally available in the market. However, in the late 1960s, specific, single-site mode-of-action (MOA) chemistries began to be widely used to control a range of fungal phytopathogens (Georgopoulos 1969). For example, in 1968 a selective benzimidazole fungicide was introduced by Dupont with the trade

name Benlate. Multiple authors reported that Fungicide Resistance Action Committee-1 (FRAC-1) fungicides (Table 1), provided systemic control for a variety of fungal diseases (Delp and Klopping 1968; Erwin et al. 1968; Freeman and Pepin 1967). Soon after the release of benomyl, a FRAC-1 fungicide, Schroeder and Provvidenti (1969) reported resistant strains of powdery mildew to FRAC-1 materials. Multiple authors followed suit with reports of resistance in other systems (Smith 1988). Fungicide resistance is a prevalent problem in the agriculture industry, predominately with single-site MOA chemistries. Resistance to FRAC-1 fungicides can occur within only a few years of fungicide applications (Jones and Walker 1976), and is especially prominent with postharvest pathogens where applications of FRAC-1 fungicides are used at high frequencies as a result of consumers’ desire for produce with limited damage or blemishes from diseases.

FRAC-1 fungicides are valuable in that they have low toxicity to humans and have high efficacy on multiple postharvest pathogens on a range of crops (Table 2). Resistance to FRAC-1 fungicides has been reported in multiple postharvest fungal pathogens including those in the genera *Botrytis*, *Colletotrichum*, *Phlyctema*, *Penicillium*, *Molinia*, and *Sclerotinia* (Baraldi et al. 2003; Bolay et al. 1974; Bryk 1997; Chen et al. 2013; Chung et al. 2010; Koenraadt et al. 1992; Martin et al. 2021; Sholberg et al. 2005). Resistance in fungi within these genera has been associated with the β -tubulin region, where mutations inhibit a fungicide’s ability to bind to tubulin, thus reducing its efficacy (Davidse 1973; Hastie and Georgopoulos 1971; Jung et al. 1992). Single-nucleotide mutations in multiple locations of the 198- and/or 200-position codon in the β -tubulin gene in multiple genera of postharvest pathogens have been found to be associated with resistance, with the E198A amino acid mutation being the most commonly reported (Baraldi et al. 2003; Buhr and Dickman 1994; Chen et al. 2009, 2013; Cosseboom and Hu 2021; Cosseboom et al. 2019; Koenraadt et al. 1992; Martin et al. 2021; Sholberg et al. 2005; Yarden and Katan 1993; Zhonghua et al. 2003). Advances in

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genomic technology allow screening of this region using PCR to predict fungicide resistance quickly and accurately (Banno et al. 2008; Luck and Gillings 1995; Luo et al. 2009; Ziogas et al. 2009).

In our study, we conducted a meta-analysis of the β -tubulin sequences available in GenBank, and throughout the literature, of common postharvest pathogens, to infer temporal-, spatial-, host-, and genus-level patterns of resistance. A meta-analysis is a study that synthesizes previously acquired data to gain a better understanding of the peer-reviewed literature and infer conclusions on a wide dataset collected in different settings. Meta-analyses focusing on common genetic variants increases the power to detect associations within a population compared with single studies (Lee 2015; Munafò and Flint 2004; Oliveira et al. 2018; Panagiotou et al. 2013). We decided to focus on mutations in the β -tubulin amino acid 198 position as this is the most consistent marker associated with resistance to FRAC-1 fungicides (Chung et al. 2010; Cosseboom and Hu 2021; Cosseboom et al. 2019; Sholberg et al. 2005). With the globalization of agricultural commodities, these data can inform farmers on what regions and hosts are most prone to contain resistant phytopathogens and can assist with regional dependent Integrated Pest Management (IPM) and fungicide resistance management strategies used in a variety of systems.

MATERIALS AND METHODS

Data collection. The meta-analysis included GenBank (NCBI), an annotated collection of all publicly available DNA sequences, as well as information from 203 peer-reviewed studies taken from throughout the world. We decided to focus strictly on postharvest pathogens and thus genera were chosen that consisted of species that caused considerable postharvest diseases to common agricultural crops. Examples of the genera chosen, and their corresponding postharvest disease and host plants, are presented in Table 2. In total, 12 fungal genera were evaluated: *Alternaria*, *Aspergillus*, *Botrytis*, *Coleophoma*, *Colletotrichum*, *Fusarium*, *Monilinia*, *Neofabraea*, *Penicillium*, *Pezizula*, *Phylectema*, and *Sclerotinia*. We evaluated all available species within each genus. The amino acid 198 position from the β -tubulin region was evaluated as a genetic marker for fungicide resistance because of the high affinity of mutations in this sequence to be correlated to resistance phenotypes in multiple systems (Buhr and Dickman 1994; Chen et al. 2009, 2013; Koenraad et al. 1992; Ma et al. 2003; Sholberg et al. 2005; Yarden and Katan 1993). We decided to exclude the codon 200 position, as for many organisms this either was not reported to be associated with resistance, or was reported to be at lower association levels than that of the 198 position (Chung et al. 2010; Sholberg et al. 2005).

TABLE 1. List of commonly used Fungicide Resistance Action Committee-1 (FRAC-1) fungicides and their trade names for the control of phytopathogens, as well as their mode of action, active ingredients, and year the fungicide was introduced

Commercial products ^a	FRAC-1 active ingredient	Chemical group	Mode of action	Year of introduction
Agrocit, Benex, Benlate, Benosan, Fundazol, Fungidice 1991, Tersan 1991	Benomyl	Benzimidazole	β -tubulin assembly interference (mitosis)	1967
Delsene, Bavistin, Corbel, Konker, Bedazim, Derosal, Kombat, Kemdazin, Carbendor, Hoe 017411, Cekudazim, Equitdazin, Aimcozim	Carbendazim	Benzimidazole	β -tubulin assembly interference (mitosis)	1968
Baycor, Bayer 33172, Baytan, Baytan FS, ICI Baytan, Neovoronit, Sibutol, Sibutrol, Voronit, Voronite, W VII/117	Fuberidazole	Benzimidazole	β -tubulin assembly interference (mitosis)	1968
Arbotect, Mertect, Bioguard, Bovizole, Eprofil, Equizole, Lombristop, Metasol TK 100, Mintesole, Mycozol, Nemapan, Omnizole, Polival, Tebuzate, Tecto, Thibenzole 200, Thiprazole, Top Form Wormer, Agrosol	Thiabendazole	Benzimidazole	β -tubulin assembly interference (mitosis)	1968
Topsin M, Mertect, Decco Salt No. 19, Shield-Brite TBZ, Cleary 3336, Fungo, SysTec 1998, Cavalier, Scotts Systemic Fungicide, TM 4.5F, TM 85WDG	Thiophanate-methyl	Thiophanate	β -tubulin assembly interference (mitosis)	1969

^a Some commercial products presented here are a combination of fungicides. This is not a fully comprehensive list. These commercial products are from company sources and are trademarked.

TABLE 2. Postharvest pathogens evaluated in this study and the diseases associated with them

Genera	Common agricultural host plants	Postharvest disease
<i>Alternaria</i>	Apple, mandarin, date, tomato, sweet cherries, peach, mango, cucumber, grapefruit, melon, tangerine, fig, lychee, pear, pistachio, walnut	Black rot, black spot, Alternaria rot, stem-end rot, brown spot, moldy core, core rot
<i>Botrytis</i>	Onion, tomato, cucumber, tulip, strawberry, carrot	Bitter rot, gray mold
<i>Colletotrichum</i>	Annual bluegrass, creeping bentgrass, cranberry, orchid, olive, pear, pepper, soybean, statice, strawberry	Anthraxnose
<i>Fusarium</i>	Wheat, rice, onion, banana, potato, cucurbit fruits	Crown rot, Fusarium fruit rot
<i>Monilinia</i>	Peach, apple, plum, pear, cherry, sour cherry, apricot, quince, nectarine, blueberry, blackberry	Brown rot
<i>Neofabraea</i>	Apple, pear	Bull's eye rot
<i>Penicillium</i>	Apple, pear, peach, cherry, quince, barley, citrus	Blue mold rot, green mold
<i>Phylectema</i>	Apple	Bull's eye rot
<i>Sclerotinia</i>	Canola, German turnip (kohlrabi), blueberry, carrot, squash, French bean, pea	Sclerotinia stem rot, soft rot, nesting

Sequences were filtered using GenBank based on genera and genomic region (β -tubulin). All sequences were aligned with the tool Geneious v.11.0.2 (<https://www.geneious.com>) and the codon 198 position was visually analyzed to infer the genotype of codon position 198. Any codon 198 genotype that resulted in an amino acid change from glutamic acid was considered a “mutation” in analysis. The GenBank accession number of the obtained sequences were analyzed with the tool BLAST (NCBI) to attain additional information provided by GenBank (i.e., isolate number and occasionally host, region, reference, etc.). The published reference corresponding to each accession number was analyzed to obtain essential information, with email correspondences sent to the publishing author as needed. Additional isolate information from the peer reviewed literature was added to our database for instances where sequences had not been uploaded to GenBank.

The main factors gathered as potential predictive variables for the response variable (amino acid mutation) included the year an isolate was collected, the pathogen genus, the plant host, and the collection region. Data were not included if the authors submitted biased information to GenBank. For example, Sholberg et al. (2005) only reported sequences on isolates that grew on fungicide-selective media. Additionally, often-times, authors only sequenced representative phenotypes to evaluate if the β -tubulin region was associated with resistance (Cosseboom et al. 2019; Yarden and Katan 1993). Isolates were removed if they contained identical isolate numbers and/or we deemed the sequences to be duplicates. We also did not include the majority of full genome sequences and assumed no sequencing errors had occurred.

In the analysis evaluating the amino acid mutation rate over time, time was binned into 5-year periods. In the data where the collection years were reported within a range, we binned the data into the earliest year of the range (if ranges were >5 years, we omitted the data).

We also evaluated data based on the different genotypes of codon 198. In this analysis we did not include specimens from the genus *Monilinia* or the species *Fusarium graminearum* as their wild-type sequence was glutamic acid (GAA) and we wanted to ascertain the probability of a silent/synonymous mutation from glutamic acid (GAG) to glutamic acid (GAA).

Fungicide usage data collection. FRAC-1 fungicide usage data were collected from California and Arizona, as these are the only localities noted that require users to report their fungicide data. The data included the total active ingredients used and total acreage on which FRAC-1 fungicides were applied. As California and Arizona consist of such a large fraction of agriculture in the United States (USDA 2021), grow a variety of different crops, and have different climates, data from these areas were considered as a “proxy” for worldwide FRAC-1 fungicide usage over time. In California, data quality before 1990 is less certain, as error-checking procedures that are currently in place were not then in effect. Additionally, full-use reporting did not become a requirement until 1990 (Crump et al. 2018; Farrar et al. 2016). In Arizona, growers are not required to report 100% of all pesticide uses, but they must report all for-hire applications, which tend to represent a significant portion, if not a majority (Fournier et al. 2007), of all pesticide applications in the state. Data on the total amount of acreage on which FRAC-1 fungicides were applied were obtained, as this could be a better indication of fungicide usage over time with active ingredient-recommended rates changing as products become more efficient.

Statistical analysis. As the response variable (amino acid mutation) was binary (yes/no), statistical analyses were done using a logistic regression in the software R v.3.31 (R Core Team 2017) to evaluate the association between an amino acid mutation with the year an isolate was collected, the pathogen genus, the host, and the collection region. The predictor variables were evaluated individually and in a model that accounted for interaction effects. Multicollinear predictor variables were not used in the same model. For example, host and pathogen were not used in the same model as certain hosts are often correlated with certain pathogen genera. Models were followed by a χ^2 test to evaluate predictor variable significance.

In total, data were acquired from 2,647 specimens from GenBank and from 203 references from around the world (Supplementary Table S1). Data were obtained for the pathogen species, plant host, year the specimen was isolated, and the locality of the collection. The specimens consisted of data from 53 countries, on >200 hosts collected between the years 1926 and 2020. Results were nearly identical between the two models, as well as when the variables were analyzed individually. All predictor variables (host, locality, pathogen genus, and time) were significantly ($P < 0.05$) associated with the mutation in all models and when they were analyzed individually.

Genotype frequency. In total, 15.34% of the specimens evaluated contained an amino acid mutation differing from the GAG wild-type codon in position 198 of the β -tubulin region (Table 3). The genotype frequencies of codon position 198 differed substantially, with GAG (84.88%; glutamic acid) being the most common genotype. GCG, coding for alanine, was the second most common, with a frequency of 12.24%. All other genotypes, including the silent/synonymous mutation GAA, occurred at frequencies <1%.

Mutation frequency by year. The dataset evaluating the association between collection year and amino acid 198 mutation frequency included a total of 1,754 specimens. Fifty-six samples were sequenced that were collected before 1986 (none contained a mutation). The first sequence that contained a mutation in our dataset was from *Colletotrichum gloeosporioides* on wild strawberry (*Fragaria vulgaris*) from 1986 in the United States (GenBank accession number DQ522504). The years 2005 to 2009 had the highest frequency of an amino acid mutation (29.78%), followed by 2000 to 2004 (20.23%) and 2010 to 2014 (17.63%). Mutations were seen in all time periods evaluated after 1980 to 1984 (Fig. 1). Sample sizes of β -tubulin sequences tended to increase over time. Between 1985 and 1989, the sample size of β -tubulin sequences was 12, followed by 43 between 1990 and 1994, 83 between 1995 and 1999, 173 between 2000 and 2004, 188 between 2005 and 2009, 744 between 2010 and 2014, and 531 between 2015 and 2020. In statistical analysis with collection year as a predictor variable, collection year is significantly associated with an amino acid mutation ($P < 0.001$). The years 2005 to 2009 had the lowest P value ($P < 0.001$), followed by 2000 to 2004 and 2010 to 2014 ($P < 0.05$).

Fungicide usage over time. FRAC-1 fungicide usage in California and Arizona depicted similar patterns where FRAC-1 active ingredients' usage started to decline between 1995 and 2005 (Fig. 2). In Arizona, the pounds of FRAC-1 active ingredients used peaked in 2003 at 3,990 pounds and has been steadily decreasing since. In California, the pounds of FRAC-1 active ingredients peaked in 1998 at 435,232 pounds. The total amount of acreage treated with FRAC-1 fungicides peaked in Arizona in 1997 at 11,567 acres, and in California in 1999 at 612,609.37 acres.

Mutation frequency by pathogen genus. An amino acid mutation was significantly associated with pathogen genus ($P < 0.001$). Data using pathogen genus as a predictor variable are presented in Figure 3. Thirty-four different species were found to have a mutation (Table 4). No mutations were noted in *Aspergillus* ($n = 58$), *Coleophoma* ($n = 5$), and *Pezizula* ($n = 81$). The genera with the highest amino acid 198 mutation frequencies were *Colletotrichum* (34%)

TABLE 3. Genotype frequency of the β -tubulin 198 codon position of the specimens evaluated in this study

Genotype	Frequency (%)	Amino acid
GAG	84.88	Glutamic acid
GAA	0.45	Glutamic acid
GCG	12.24	Alanine
AAG	0.93	Lysine
GTG	0.69	Valine
CAG	0.77	Glutamine
GAT	0.04	Aspartate

and *Botrytis* (25.46%). The association between an amino acid 198 mutation and fungicide resistance differed based upon the pathogen species/genus (Table 4). For example, the mutation in *Botrytis cinerea* is close to 100% correlated with fungicide resistance (Cai et al. 2015; Cosseboom et al. 2019; Yarden and Katan 1993), whereas the mutation in *Penicillium* tends to be associated with resistance <70% of the time (Baraldi et al. 2003; Cabañas et al. 2009; Sholberg et al. 2005).

Mutation frequency by host. The dataset evaluating the frequency of the association between the host source and amino acid 198 mutation frequency included a total of 2,377 specimens. Host was significantly associated with isolates containing the mutation

($P < 0.001$) (Fig. 4). In total, >36 different hosts had fungal pathogens that contained an amino acid mutation in position 198 (Table 5). Blueberry, nectarine, pea, and raspberry were not included in Figure 4 or Table 5 because of their low sample size ($n < 10$); however, they were all significantly associated with fungi that possessed this mutation ($P < 0.05$). Peppers (95.6%) and soybeans (84.2%) had the highest frequency. Statice and peppers had the lowest P values ($P < 0.001$) in statistical analysis; however, the majority of data from these two hosts came from two studies: one in Israel evaluating *C. gloeosporioides* on Statice (Maymon et al. 2006), and one from Trinidad and Tobago evaluating *Colletotrichum truncatum* on peppers (Ramdial et al. 2016).

Mutation frequency by locality. The dataset evaluating the association between collection locality and amino acid 198 mutation frequency included a total of 2,511 specimens. Raw data from all of the countries ($n > 10$) evaluated in this study are presented in Table 6. The mutation was found in 24 countries from all continents, with the exceptions of Africa and Antarctica (Fig. 5). The sample size in Africa was small ($n = 3$), so no definite conclusions can be made on its presence/absence. The following countries were significantly associated with the mutation ($P < 0.05$): Argentina, Brazil, Canada, Chile, China, France, India, Iran, Israel, South Korea, Taiwan, and the United States. Regions that are statistically associated with the mutation tend to harbor specimens that have a higher mutation frequency. A lower P value, for a certain region, often has a higher mutation frequency in their population whereas insignificant regions tend to have lower mutation frequencies. All of the specimens from Trinidad and Tobago ($n = 87$) contained the mutation.

Fig. 1. Percent of pathogens evaluated harboring an amino acid 198 mutation over the past 40 years.

Fig. 2. Fungicide Resistance Action Committee-1 fungicide usage over time in California and Arizona presented as total pounds of active ingredients applied and acres treated.

DISCUSSION

Fungicide resistance of several postharvest fungal pathogens was evaluated from around the world on a range of hosts within the last 90 years, to infer temporal, spatial, species, and host patterns of fungicide resistance. Statistical analyses showed that fungicide resistance (measured as a proxy of a mutation in the β -tubulin amino acid 198 position) is significantly associated with time of isolate collection, the pathogen genus, the pathogen host, and the collection locality.

The amino acid mutation frequency in this study (Table 3) is likely an underestimate of the actual percent of the population that is resistant to FRAC-1 fungicides. For example, in multiple systems, the pathogen can be resistant if it carries the 198 or the 200 mutation (Chen et al. 2013; Lee et al. 2011; Young et al. 2010) and resistance based on a mutation in amino acid position 200 would not have been calculated into the data presented. However, the amino acid 200 position is not highly associated with resistance (Chung et al. 2010; Sholberg et al. 2005). Additionally, low to moderately resistant strains often do not contain an amino acid mutation (Chen et al. 2009; Chung et al. 2006; Ma et al. 2003; Yarden and Katan 1993; Ziogas et al. 2009), and thus, our data may only correspond to “highly resistant” isolates that coincide with practical resistance (e.g., failure of a given product to control a specific pathogen). On the contrary, it is possible we are overestimating the true frequency, as it has been reported that sensitive isolates can also contain the mutation; indeed, Adnan et al. (2018) found that 2/10 sensitive *B. cinerea* isolates contained a 198 amino acid mutation.

Although multiple mutations (synonymous and nonsynonymous) in the 198 codon are known to be associated with FRAC-1 fungicide resistance (Koenraadt et al. 1992), there are large differences in the population frequency of the different mutations (Table 3). The

different genotypic frequencies are likely to be from discrepancies in the fitness costs of the different amino acid 198 mutations. The E198A mutation is the most prevalent mutation in the population (12.24%) and thus, fitness cost is likely less than the other mutations. Baraldi et al. (2003) found that resistant *Penicillium expansum* isolates had a higher infection severity on fruits and produced larger lesions than sensitive isolates.

There has been a decline in fungicide usage (Fig. 2), which has likely contributed to the decline in the worldwide 198 mutation frequency (Fig. 1). Fungicide resistance frequency tends to follow fungicide usage after ~5 to 10 years. We hypothesize that the quick decline in resistance in response to reduced selection pressure is indicative of the mutation having a previously uncharacterized fitness cost. Our hypothesis is contrary to previous research that noted that there was no fitness cost via the 198 mutation. However, research evaluating fitness of pathogenic fungi tends to be accomplished mainly in laboratory settings and under controlled conditions (Baraldi et al. 2003; Ziogas et al. 2009). In a laboratory setting, ecological factors that impact fitness can be overlooked. For example, there could be an effect of the mutation on its ability to withstand harsh environmental conditions, spread by wind, or on its competitive fitness to interact with the consortium of microbes that live on the viable host. In the future, if FRAC-1 fungicide applications continue to decline, we expect fungicide resistance will decline and thus result in an increase in FRAC-1 fungicide efficacy. However, as seen from California (Fig. 2), FRAC-1 usage seems to be gaining popularity within the past 5 years, and in the future an increase in FRAC-1 resistance is expected. It should also be noted that the fitness cost/benefit of an E198A mutation may be species dependent, and this has yet to be explored in natural settings.

Fig. 3. Bar graph showing the percent of each postharvest pathogen genus evaluated that contained an amino acid 198 mutation. There is a statistically significant association in a logistic regression analysis between the host and the resistant mutation. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

The first sequence containing a 198 mutation was from a *C. gloeosporioides* specimen collected from wild strawberry (*F. vulgaris*) in 1986. As resistance to FRAC-1 fungicides was reported in the late 1960s (Schroeder and Provvidenti 1969), we believe the mutation was present in higher levels in the 1970s and 1980s than reported in this study, as we were limited by a small sample size ($n = 22$ in the 1970s and $n = 20$ in the 1980s). The *C. gloeosporioides* specimen

from 1986 on wild strawberry provides evidence for this claim; fungicides are not applied on wild strawberries, thus, the pathogen must have been prevalent in the population, and have come from a nearby, heavily sprayed, strawberry field or, less likely, was a stochastic event that resulted in its presence without fungicide pressure. Therefore, it is probable that, by 1986, resistant genotypes of *C. gloeosporioides*, and possibly other pathogens, were already established and prevalent

TABLE 4. Amino acid 198 mutation frequency of different postharvest pathogen species and genera evaluated in this study

Species ^a	N	Isolates with a mutation, %	Most prevalent host associated with a mutation	Most prevalent location associated with a mutation	Resistant isolates that contain a mutation, %	References
Total	2,647	15.34				
<i>Alternaria</i>	271	0.74				
<i>Alternaria alternata</i>	108	1.85	Date			
<i>Botrytis</i>	221	25.46				
<i>Botrytis acalada</i>	4	25	Onion	New Zealand		
<i>Botrytis cinerea</i>	158	39.87	Grape	France	100	Adnan et al. (2018) ^b ; Cai et al. (2015); Cosseboom et al. (2019); Yarden and Katan (1993) ^c
<i>Botrytis fragariae</i>	2	50	Strawberry	Germany		
<i>Botrytis squamosa</i>	27	11	Onion	Canada		
<i>Botrytis tulipiae</i>	4	25	Tulip	Canada		
<i>Colletotrichum</i>	705	34.75				
<i>Colletotrichum acutatum</i>	8	25	Strawberry and grape	Japan	0	Chung et al. (2006) ^d ;
<i>Colletotrichum cereale</i>	62	41.93	Creeping bentgrass	United States	60.87	Young et al. (2010)
<i>Colletotrichum cliviae</i>	1	100	Orchid	India		
<i>Colletotrichum cymbidiicola</i>	11	63.64	Orchid	India		
<i>Colletotrichum fruticola</i>	25	4	Strawberry			
<i>Colletotrichum gloeosporioides</i>	141	45.39	Statice	Israel	100	Chung et al. (2006) ^d ; Maymon et al. (2006)
<i>Colletotrichum musicola</i>	1	100	Soybeans	Brazil		
<i>Colletotrichum plurivorum</i>	15	100	Soybeans	Brazil		
<i>Colletotrichum siamense</i>	115	33.91	Strawberry	United States	100	Martin et al. (2021)
<i>Colletotrichum truncatum</i>	89	97.75	Peppers	Trinidad and Tobago		
<i>Fusarium</i>	707	1.56				
<i>Fusarium asiaticum</i>	13	7.69				
<i>Fusarium fujikuroi</i>	67	1.49	Rice	China		
<i>Fusarium graminearum</i>	77	9.09	Wheat	China	100	Chen et al. (2009) ^e
<i>Fusarium oxysporum</i>	473	0.21	Ctenanthe oppenheimiana	China		
<i>Fusarium proliferatum</i>	76	1.30	Onion	United Kingdom		
<i>Monilinia</i>	73	16.40				
<i>Monilinia fruticola</i>	62	19.35	Peach	United States	31.8, 100	Chen et al. (2013); Ma et al. (2003); Martini et al. (2016) ^f
<i>Neofabraea</i>	138	5.07	Apple			
<i>Neofabraea actinidiae</i>	8	37.50				
<i>Neofabraea brasiliensis</i>	3	100				
<i>Neofabraea perennans</i>	33	3.03				
<i>Penicillium</i>	212	20.75				
<i>Penicillium albocoremium</i>	1	100				
<i>Penicillium commune</i>	1	100	Apple	Canada		
<i>Penicillium cyclopium</i>	1	100	Barley	Denmark		
<i>Penicillium digitatum</i>	40	45	Orange	Taiwan	48.65	Lee et al. (2011)
<i>Penicillium expansum</i>	104	16.34	Apple/pear	Spain	23.5, 50, 66.67	Baraldi et al. (2003); Cabañas et al. (2009); Sholberg et al. (2005)
<i>Penicillium italicum</i>	1	100	Air			
<i>Penicillium solitum</i>	5	100		Canada	100	Sholberg et al. (2005)
<i>Phlyctema</i>	163	7.98				
<i>Phlyctema vagabunda</i>	162	8.02	Apple	Chile		
<i>Sclerotinia</i>	15	20				
<i>Sclerotinia sclerotiorum</i>	14	21.42	Canola	China		

^a Only species/genera that contained a 198 amino acid mutation (changed from GAG) are included in the table. The association between fungicide resistance and an amino acid 198 mutation of each species was determined from previous studies.

^b Adnan et al. (2018) reported that 2/10 sensitive isolates contained the E198A mutation. However, 100% (10/10) of the resistant isolates also contained the mutation.

^c Yarden and Katan (1993) reported that 100% of the highly resistant strains yielded an E198A mutation. However, if they were “moderately resistant” or “sensitive,” they did not contain a mutation.

^d Chung et al. (2006) found that the AAG mutant sequence was not corresponding to resistance in *C. acutatum*. Additionally, *C. gloeosporioides* that were labeled as “intermediately resistant” did not contain the E198A mutation. However, all “highly resistant” strains contained the mutation.

^e Chen et al. (2009) determined that 100% of the highly resistant isolates contained a mutation. However, none of the “moderately resistant” isolates did.

^f Ma et al. (2003) reported that 100% of the highly resistant isolates had the mutation. However, none of the “low resistant” isolates had the mutation.

throughout the United States. Since 1986, the frequency of the mutation in the population increased to a maximum of 29.78% between the years 2005 and 2009. After 2009, the mutation frequency proceeded to decline to 17.61% between 2010 and 2014 and then to 10.36% between 2015 and 2020 (Fig. 1). This may be caused by many factors, but one of the most obvious contributors could have been less usage of the FRAC-1 material, in lieu of other site-specific MOA chemistries including the demethylation inhibitors (FRAC 3), strobilurins (FRAC 11), and limited multisite materials such as Ziram and Captan (FRAC M).

The overall pattern of increased FRAC-1 fungicide resistance from the 1990s to the 2000s (Fig. 1) agrees with data from previous studies on multiple systems. For example, 25.64% of *Monilinia fructicola* collected between 1994 and 2002 on stone fruits were highly resistant to FRAC-11 fungicides (Ma et al. 2003); however, 73.02% of *M. fructicola* isolates collected in 2011 were resistant (Martini et al. 2016). Additionally, in a 3-year survey from 1991 to 1993, Sholberg and Haag (1996) found 22.9% of *Penicillium* spp. isolates to be resistant, whereas 52% of isolates collected in 2004 to 2005 were found to be resistant (Cabañas et al. 2009).

The decreasing frequency of the resistance mutation and fungicide usage (Fig. 1) can likely be attributed to the implementation of IPM strategies and fungicide resistance programs implemented by farmers. After the peak resistance frequency observed between 2005 and 2009 (29.78%), IPM strategies, such as rotating fungicides with different MOAs, could have been successful in combating or minimizing resistance. Additionally, FRAC-11 fungicides, such as quinone outside inhibitors, have gained popularity because of their high efficacy (Moparthi and Bradshaw 2020). The popularity and success of FRAC-11 fungicides, coupled with the fitness cost of a 198 mutation, is likely contributing to the decline in FRAC-1 fungicide usage and resistant fungal populations. We hypothesize that in the future, resistance will follow an oscillating pattern as fungicides continue to be rotated.

Pathogen genus is also significantly associated with resistance, and there is substantial variation among genera (Fig. 3). *Botrytis*, *Colletotrichum*, *Monilinia*, *Neofabraea*, *Phlyctema*, and *Penicillium* were significantly associated with β -tubulin amino acid 198 mutations, with *Colletotrichum* and *Botrytis* having the highest mutation frequency (Fig. 3). Also, there is a considerable amount of interspecies variation in the mutation frequency (Table 3). A possible contributor to this phenomenon is that the correlation between a codon 198 mutation and the resistance phenotype differs based upon species (Table 2). For example, Chung et al. (2006) and Nakaune and Nakano (2007) both reported that codon 198 mutations in *Colletotrichum acutatum* are not associated with resistance, whereas in multiple other species within this genus, there is a high association between the mutation

and resistance (Chung et al. 2006; Martin et al. 2021; Maymon et al. 2006; Young et al. 2010). Future research should evaluate the association between a 198 amino acid mutation and fungicide resistance in a variety of other systems that contain a mutation but have not yet been evaluated, such as the *Colletotrichum muscicola*–soybean and the *Colletotrichum cymbidiicola*–orchid systems.

Interestingly, no *Aspergillus* species were noted to carry the 198 mutation in GenBank (Fig. 3). This is unexpected, as multiple authors have reported that mutations in the β -tubulin region in *Aspergillus nidulans* confer resistance to FRAC-1 fungicides (Jung and Oakley 1990; Jung et al. 1992; Hastie and Georgopoulos 1971). Possible explanations for this phenomenon (as well as the low frequencies reported for other genera such as *Alternaria*) include the following:

- That fungicides are generally not used on *Aspergillus* spp., and thus there is no selecting agent for this mutation;
- That there is a deleterious effect of an amino acid 198 mutation in *Aspergillus* spp., which explains why it is nonexistent in the general population;
- That these mutations rarely occur in *Aspergillus* spp. in natural conditions; and/or
- That our sample size ($n = 58$) was too small to identify the mutation by chance.

In reference to the last, possible explanation, silent mutations in the 198 amino acid loci were found to be prevalent at $\sim 0.5\%$ frequency (Table 3; GAG-GAA), suggesting that we would not have noted the mutation in a sample size of 58. It should be noted that no definite conclusions should be derived when comparing mutations from one site to another, even in the same codon, as the mutation rate varies based on the nucleotide context (Krasovec et al. 2019).

The data reported in this study are generally consistent with the literature, especially in regard to the resistance frequency of the different genera. For example, Chung et al. (2010) found that 41.38% of *C. gloeosporioides* isolates evaluated in Taiwan were highly resistant or resistant to FRAC-1 fungicides, whereas we report 45.39% of *C. gloeosporioides* isolates contained a β -tubulin 198 amino acid mutation (Table 3). Additionally, we report a mutation frequency of 21.42% for *Sclerotinia sclerotiorum* (Table 2) whereas Wang et al. (2014) reported a 10.13% resistance frequency among *S. sclerotiorum* isolates. The differences observed in this study from previous studies can likely be attributed to the locality, host, or conditions in which isolates were collected in any single study. In addition, the possibility of sequencing error needs to be considered by the reader, especially

TABLE 5. Amino acid 198 mutation frequency of different postharvest pathogens based on host species

Host ^a	N	Mutation, %	Most prevalent pathogen with mutation
Air	17	5.89	<i>Penicillium italicum</i>
Annual bluegrass	37	32.50	<i>Colletotrichum cereale</i>
Apple	298	13.75	<i>Penicillium digitatum</i>
Creeping bentgrass	23	60.86	<i>Colletotrichum cereale</i>
Cucumber	22	18.18	<i>Botrytis cinerea</i>
Date	22	9.09	<i>Alternaria alternata</i>
Ginseng	10	10	<i>Botrytis cinerea</i>
Grape	101	24.75	<i>Botrytis cinerea</i>
Mango	82	9.76	<i>Colletotrichum gloeosporioides</i>
Onion	53	5.66	<i>Botrytis</i> spp.
Orchid	13	61.53	<i>Colletotrichum cymbidiicola</i>
Peach	31	25.81	<i>Monilinia fructicola</i>
Pear	22	13.64	<i>Colletotrichum gloeosporioides</i>
Peppers	91	95.60	<i>Colletotrichum truncatum</i>
Rice	13	7.69	<i>Fusarium fujikuroi</i>
Soybeans	19	84.20	<i>Colletotrichum plurivorum</i>
Statice	58	69	<i>Colletotrichum gloeosporioides</i>
Strawberry	82	51.22	<i>Colletotrichum siamense</i>
Tomato	26	38.46	<i>Botrytis cinerea</i>
Wheat	31	23	<i>Fusarium graminearum</i>

^a Only included if $N > 10$ and at least one host contained mutation.

Fig. 4. Bar graph showing the percent of postharvest pathogens evaluated on each host that contained an amino acid 198 mutation. There is a statistically significant association in a logistic regression analysis between the host and the presence of the resistant mutation. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

when there are outliers, as is the case with the one *Fusarium oxysporum* mutation (GenBank accession no. KX253989) out of the 473 deposited sequences.

The association among fungicide resistance, pathogen host, and geographic region (Fig. 5; Tables 5 and 6) likely stems from the variance in fungicide applications in different regions and on different

hosts. Nonetheless, an understanding of the population frequency of resistance throughout the world is important, as fungicide applications may not always be indicative of resistant pathogen populations. For example, fungal pathogens can spread hundreds of miles by wind (Schnathorst 1959), and it should be noted that countries often trade goods that are undoubtedly accompanied by their resistant pathogens.

TABLE 6. Amino acid 198 mutation frequency of different postharvest pathogens based on collection locality

Location ^a	N	Mutation rate, %	Most prevalent pathogen with mutation	Most prevalent host that contains pathogen with mutation
Argentina	12	58.33	<i>Botrytis cinerea</i>	Strawberry
Australia	127	0		
Brazil	112	19.64	<i>Colletotrichum plurivorum</i>	Soybean
Canada	107	18.6	<i>Penicillium expansum</i>	Apple
Chile	72	15.23	<i>Phlyctema cinerea</i>	Apple
China	317	11.08	<i>Botrytis cinerea</i>	Wheat and strawberry
Colombia	88	0		
Denmark	12	8.33	<i>Penicillium cyclopium</i>	Barley
France	53	43.40	<i>Botrytis cinerea</i>	Grape
Germany	22	9.09	<i>Botrytis</i> spp.	Strawberry
Hungary	14	21.42	<i>Botrytis cinerea</i>	Raspberry
India	20	40	<i>Colletotrichum cymbidicola</i>	Orchid
Iran	30	30	<i>Botrytis cinerea</i>	Grape
Israel	65	61.54	<i>Colletotrichum gloeosporioides</i>	Statice
Italy	121	2.50	<i>Monilinia fructicola</i>	Grape, nectarine, peach
Japan	78	6.41	<i>Colletotrichum gloeosporioides</i>	Pear
Malaysia	52	0		
Mexico	13	0		
The Netherlands	23	4.35		
New Zealand	33	6.06	<i>Botrytis</i> spp.	Grape and onion
Pakistan	20	5	<i>Colletotrichum gloeosporioides</i>	Strawberry
Poland	91	1.10	<i>Phlyctema vagabunda</i>	Apple
Serbia	15	0		
South Korea	10	40	<i>Colletotrichum gloeosporioides</i>	Apple
Spain	115	7	<i>Penicillium expansum</i>	Apple/pear
Taiwan	48	35.42	<i>Penicillium digitatum</i>	Orange
Trinidad and Tobago	87	100	<i>Colletotrichum truncatum</i>	Peppers
Turkey	12	0		
United Kingdom	51	1.96	<i>Fusarium proliferatum</i>	Onion
United States	591	12.52	<i>Colletotrichum siamense</i>	Strawberry

^a Only included if N > 10.

Fig. 5. Amino acid 198 mutation frequency based on the collection region and the pathogen host.

Maymon et al. (2006) found high amounts of *C. gloeosporioides* that were resistant to FRAC-1 fungicides in Israel on static even though FRAC-1 fungicides were never reported on this crop in this region, and Cosseboom et al. (2019) reported 72% of *B. cinerea* from organic strawberry fields in California were resistant to FRAC-1 fungicides. In the California strawberry industry, nurseries that grow transplants for the organic industry apply fungicides and thus, are likely spreading resistant populations to organic operations.

Similar to previous studies (Cosseboom and Hu 2021; Johnson et al. 1994), we noticed differences in resistance based on host (Fig. 4). Peppers, soybeans, static, and orchids had isolates containing the highest mutation frequency (Table 5). Of course, our data have some internal biases and are likely underrepresented/skewed in some areas/hosts and overrepresented/skewed in others. Cosseboom et al. (2019) and Johnson et al. (1994) reported that >75% of *B. cinerea* on strawberries contained the resistance mutation; however, in our study, 48.72% of pathogens collected from strawberries contained the resistant loci. The surveys by Cosseboom et al. (2019) and Johnson et al. (1994) were both accomplished in the Western United States, where high amounts of fungicides are used in the strawberry industry.

Additionally, the high amount of the resistance mutations we noted on peppers is most likely not indicative of the worldwide population; the majority of these data came from one study (i.e., Ramdial et al. 2016). However, these data should not be discounted. Ramdial et al. (2016) evaluated peppers from Trinidad and Tobago, and sequenced 87 isolates of *C. truncatum*, all of which were resistant to FRAC-1 fungicides and contained an amino acid 198 mutation.

The resistance genotype located on soybeans from Brazil (Bouffleur et al. 2020) and orchids from India (Chowdappa et al. 2014) are both worth mentioning, although neither of the studies that reported these genotypes were evaluating fungicide resistance. We hypothesize that soybeans from Brazil and orchids from India are highly resistant to FRAC-1 fungicides, and that future research should evaluate fungicide resistance on both of these crops in those localities. Countries that depend on soybeans and have large orchid-growing productions should screen incoming crops for fungicide-resistant pathogens.

As the trading of goods results in the movement of fungicide-resistant pathogens (Maymon et al. 2006), growers of certain products need to be aware of the potential harm of using FRAC-1 fungicides and consider alternative control methods. This study was conducted as a resource for countries and farmers that are reliant on FRAC-1 fungicides for the control of postharvest pathogens. Using the presented data, screenings should be implemented when crops from regions/hosts with high levels of resistance are exchanged. Future research can use the data compiled in this study (Supplementary Table S1) to make inferences on the amount of FRAC-1 fungicides used, the probability and speed at which resistance builds up, and generate models to predict the movement of resistance through space and time. Additionally, our study provides a platform to translate our methodology of analysis to other MOA chemistries that have tightly correlated resistance mutations (e.g., azoles and CYP51A1, and G143A for strobilurins) to determine similar trends, patterns, and predictive models for assessing risk of developing resistance.

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