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Population Structure of the Sour Rot Pathogens *Galactomyces citri-aurantii* and *G. geotrichum* and Evaluation of Sterol Demethylation Inhibitors for Postharvest Management of Citrus Decays

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Pathology

by

Alistair Hartley McKay

March 2011

Dissertation Committee:
Dr. James E. Adaskaveg, Chairperson
Dr. Michael D. Coffey
Dr. Michael E. Stanghellini

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The Dissertation of Alistair McKay is approved:

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Committee Chairperson

University of California, Riverside

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Alistair McKay

March 2nd 2011.

DEDICATION

I dedicate this dissertation with gratitude and love for my wife, Tonya and my children Duncan and Madison who have supported me unconditionally, and my parents Roderick and Margaret for their love and support.

ABSTRACT OF THE DISSERTATION

Population Structure of the Sour Rot Pathogens *Galactomyces citri-aurantii* and *G. geotrichum* and Evaluation of Sterol Demethylation Inhibitors for Postharvest Management of Citrus Decays

by

Alistair Hartley McKay

Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, March 2011
Dr. James E. Adaskaveg, Chairperson

Sour rot of citrus caused by *Galactomyces citri-aurantii* (anamorph: *Geotrichum citri-aurantii* (Ferraris) Butler) is an important postharvest disease that affects all varieties of citrus fruit grown in California and is particularly prevalent in the lemon industry since the fruit is stored at approximately 12°C and 95% relative humidity. The postharvest fungicides currently registered to manage green mold caused by *Penicillium digitatum* including imazalil, thiabendazole, as well as azoxystrobin, fludioxonil, and pyrimethanil are not effective against *G. citri-aurantii*. Using a fungicide gradient dilution method, sensitivities were evaluated against selected demethylation-inhibiting triazole (DMI-triazole) fungicides including propiconazole that recently received emergency registration on stone fruit to manage sour rot caused by *G. geotrichum*. Propiconazole effectively reduced mycelial growth in vitro of *G. citri-aurantii, G. geotrichum* and imazalil-sensitive strains of *P. digitatum* with mean EC_{50} values of 0.34 µg/ml and 0.14 µg/ml and 0.008 µg/ml for the three species respectively. Species-specific PCR primers were developed from genes encoding β-tubulin and endopolygalacturonase proteins to differentiate the two *Galactomyces* species. To evaluate fungicide resistance potential, the population genetic structure and genetic diversity of the two *Galactomyces* species was studied using amplified-fragment-

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CHAPTER I. GENERAL INTRODUCTION

Sour rot is an important postharvest disease of citrus fruit that is caused by the fungal pathogen *Galactomyces citri-aurantii* Butler (anamorph: *Geotrichum citri-aurantii* (Ferraris) Butler). The disease has been reported from most areas of the world where citrus is grown and infects all varieties of citrus including tangerines (*Citrus reticulata* Blanco), oranges (*C. sinensis* (L.) Osbeck), grapefruit (*C. paradisi* Macfadyen), and lemons (*C. limon* (L.) N.L. Burm.) (Eckert, 1959; Butler et al., 1965, 1988; Hershenhorn et al., 1992; Suprapta et al., 1995). Senescent and aging fruit are the most susceptible. The pathogen enters through wounds (Baudoin and Eckert, 1982; Brown, 1979). The presence of free water increases decay incidence (Cohen et al., 1991). Disease development is also temperature-dependent requiring temperatures >10ºC (Eckert, 1959; Brown, 1979; Baudoin and Eckert, 1982). Sour rot is particularly prevalent in California's lemon industry because for marketing considerations, lemons are stored for long periods (up to 6 months) at high relative humidity and at relatively high temperatures (12 to 15ºC) to avoid chilling injury (Butler et al., 1965; Eckert, 1959). Although crop losses due to sour rot are less severe world-wide than those resulting from blue and green mold decays caused by *Penicillium* spp., in years with high rainfall the disease can be potentially devastating to California's estimated $1.7 billion (annual 2009) citrus industry (Eckert, 1959; California Agricultural Resource Directory 2010–2011). Sour rot is not effectively controlled by fungicides currently registered for use on citrus that include imazalil, thiabendazole (Brown, 1986), as well as the recently introduced fungicides azoxystrobin, fludioxonil, and pyrimethanil (Smilanick et al., 2008). The broad-spectrum fungicide sodium *o*-phenylphenate (SOPP) has limited efficacy against the disease (Eckert and Eaks, 1989), but this compound is no longer in common use due to concerns for public health, along with environmental and disposal considerations. For this reason, the primary goals of this dissertation were to explore strategies for the effective

management of postharvest sour rot of citrus. This included i) investigating the population genetic structure of *G. citri-aurantii* and *G. geotrichum*, ii) developing baseline sensitivities of the pathogens to sterol demethylation inhibitor (DMI) -triazole fungicides, particularly propiconazole, and iii) exploring effective postharvest application strategies for DMI-triazole fungicides with emphasis on propiconazole.

Establishing baseline sensitivities and the resistance potential of target pathogen populations to particular fungicides first requires accurate identification of the population under evaluation. For the two *Galactomyces* spp., there have been mixed reports in the literature if they are races or types of a single species or if they are two distinct species. Infection of citrus by *G. citri-aurantii* results in symptoms that are virtually identical to sour rot of various other fruits and vegetables such as tomatoes, stone fruit, and melons caused by *G. geotrichum* (Butler & Petersen) Redhead & Malloch (anamorph: *Geotrichum candidum* Link) (Butler, 1960; Butler and Petersen, 1972; Brown, 1979). Also, morphological and physiological similarities of the two species make differentiation difficult. For example, reports indicate differences in endo-polygalacturonase (PG) activity (Nakamura et al., 2001) and carbohydrate metabolism (Suprapta et al., 1996c) , differential growth in citrus juices (Butler et al., 1988; Suprapta et al.,1996b; Nakamura et al., 2008), differential pathogenicity to citrus fruits (Suprapta et al., 1996a; Nakamura et al., 2008), as well as sequence variations in the internal transcribed spacer region (ITS) of ribosomal DNA (Nakamura et al., 2008). In Japan, however, researchers were unable to detect the sexual phase of *G. citri-aurantii* and thus, maintained the contention that the two species were in fact races or types of the same species, *G. geotrichum*. In contrast, Butler et al. (1972, 1988), described the sexual stage of both pathogens and classified each as separate species. For this reason, another goal of this dissertation was to establish PCR-based species-specific molecular identification techniques for *G. citri-aurantii* and *G. geotrichum*.

The population genetic structure and reproductive strategy of *G. citri-aurantii* and *G. geotrichum* have not been established. This information is important for disease management since fungal pathogens that have a sexual stage and display random mating, generally have increased genotypic diversity as a result of genetic recombination compared to species that primarily reproduce asexually. This may allow the pathogen to better respond to selection pressures such as the introduction of new fungicide treatments (Milgroom, 1996; McDonald and Linde, 2002). Many plant pathogenic fungi use both a sexual and an asexual reproductive stage in an "epidemic" style life strategy that is described by Taylor et al. (1999) as the most common fungal lifestyle. A pathogen with such a mixed reproduction strategy has a high probability of developing resistance to fungicides because genetic diversity due to sexual recombination can result in variants able to survive selection from a fungicide treatment and the asexual phase results in the widespread dispersal of variants with the greatest fitness. Also, pathogens that exhibit a high degree of gene (or genotype) flow and migration within and between populations are expected to have higher genetic diversity than pathogens with low degrees of gene flow (McDonald and Linde, 2002). Individuals from pathogen populations that share a mixed mode of reproduction, random mating or outcrossing, and a high rate of gene flow pose the greatest risk of developing resistance to fungicides. For example, powdery mildew of barley caused by *Blumeria graminis* f. sp. *hordei* developed resistance to several DMI fungicides (Blatter et al., 1998).

One method of evaluating the extent of sexual reproduction in the life history of plant pathogenic fungi is by determining mating-type segregation ratios of natural populations. The teleomorphs of both *Galactomyces* pathogens have been described as heterothallic with A_1 and A_2 mating types (Butler and Petersen, 1972; Butler et al., 1988). Thus, we endeavored to quantify segregation ratios of diverse populations of the two species collected from California, other locations within the United States, and twelve countries worldwide. Another method is to

examine genetic variability in natural populations through multilocus genotypic analysis using DNA-based amplified fragment length polymorphisms (AFLPs). Sexually reproducing populations, consistent with random mating, show little association between alleles at one locus compared to those of another locus. Therefore, multilocus genotypes will be highly variable when comparing individuals of the same species (Anderson and Kohn, 1995; Milgroom, 1996). In asexually or clonally reproducing fungi, alleles at different loci are strongly associated with one another, and the same multilocus genotype is commonly recovered from different individuals within a population (Anderson and Kohn, 1995). Pathogens with a mixed reproductive strategy share attributes of both. Another aim of this dissertation was to determine the intrinsic resistance potential of the two sour rot causing pathogens *G. citri-aurantii* and *G. geotrichum* using population-based analysis of genetic variability, the gene/genotype flow within each species, the population genetic structure, and mating type segregation ratios.

Limited screening of a variety of fungicides demonstrated that some of the DMI-triazole fungicides including etaconazole, propiconazole, and flutriafol were highly effective in reducing in vitro mycelial growth of *G. citri-aurantii* and in preventing sour rot development of oranges (Brown, 1986; Cohen et al., 1988; McKay et al., 2007). Two compounds, etaconazole and the closely related propiconazole were particularly effective, however, etaconazole showed high levels of phytotoxicity following application to citrus (Brown, 1986). Propiconazole, although slightly less effective, does not cause phytotoxicity and currently holds a registration in California for management of sour rot of stone fruit caused by *G. geotrichum*. For this reason, one of our goals was to establish baselines for sensitivity of a large and diverse population of *G. citri-aurantii* and *G. geotrichum* to selected DMI-triazole fungicides with an emphasis on propiconazole using the spiral gradient dilution method (Förster et al., 2004). Simultaneously, sensitivity data were collected for *P. digitatum* that causes green mold of citrus. Such knowledge

on the range in sensitivities of target pathogens to novel fungicides is vital in developing effective methods for preventing the development of resistance to those fungicides (Georgopoulos, 1982).

The potential of plant pathogens to develop resistance to postharvest fungicide applications is largely based on i) the fecundity and reproductive strategy of the pathogen, ii) the evolutionary potential of the pathogen, iii) the genetic control of resistance, whether it is polygenic or monogenic, qualitative or quantitative, and iv) the selection pressure exerted by the fungicide treatment (Kendall and Hollomon, 1998; Russell, 2004). For green mold of citrus caused by *P. digitatum,* the potential for resistance to new fungicides has been shown to be very high (Kanetis et al., 2010). For example, resistance to the benzimidazole fungicide thiabendazole (TBZ) occurred in lemon packinghouses within 15 months after the widespread use of the compound (Eckert, 1988). Based on current concepts, such rapid development of resistance was predictable because i) *P. digitatum* has a very high fecundity with approximately$1x10^8$ airborne spores produced after seven days per infected fruit and ii) a high evolutionary potential, possibly the result of heterokaryosis or a high rate of mutation (Kanetis et al., 2010), iii) benzimidazole resistance is due to a single-site fixed mutation in the gene encoding β-tubulin (Eckert, 1988), and iv) TBZ is highly effective at very low rates in the sensitive population, exerting a very strong selection pressure toward resistant strains (Kendall and Holloman, 1998). Kanetis et al. (2010) developed a practical field method to determine the resistance frequency of pathogen populations by exposing large populations to selection pressure by the fungicide through plating large quantities of reproductive spores onto amended media. Following the introduction of a fungicide, this strategy can be employed for resistance monitoring purposes. Prior to the introduction of the fungicide the method can be used to establish the intrinsic resistance potential of a pathogen to the fungicide. Because until now, postharvest single-site mode of action fungicides have not been used to manage sour rot, the resistance potential of *G. citri-aurantii* and *G. geotrichum* to

fungicides such as propiconazole is not known. Thus, an important aim of this research was to determine the natural resistance frequencies and the resistance potential of these pathogens to propiconazole using the procedures outlined by Kanetis et al. (2010).

Another goal of this dissertation was to determine the most effective strategy for applying propiconazole to citrus during processing at packinghouses. Depending on the pathogen, the host, and the fungicide, delays in application timing can result in a different post-infection efficacy. For example, pyrimethanil and imazalil provided 100% control of green mold decay incidence of lemons when aqueous solutions were applied 21 h after inoculation, whereas at the same rate and timing, fludioxonil and azoxystrobin provided 82 and 68% reduction of green mold incidence, respectively (Kanetis et al., 2008). The same four compounds were 96 to 100% effective when applied only 9 h after inoculation. The reduced efficacy of fungicides due to delayed application is largely due to i) the rate of development of the fungus in the host tissue and ii) the apoplastic and symplastic mobility of the fungicide in the host tissues (Hewitt, 1998). In some cases, the reduced efficacy due to delayed application timing can be overcome by increasing the application rate, in a classic dose-response manner. For example, the imazalil concentration required to inhibit growth of *P. digitatum* was found to be three times higher when applied after 24 h compared to 12 h (Holmes and Eckert, 1999). Thus, an important objective of this research was to establish dose-response curves for the effective use of propiconazole in managing sour rot of citrus fruit.

In California, citrus packers use various strategies to optimize the performance of fungicide treatments. For example, the three newly registered fungicides fludioxonil, pyrimethanil, and azoxystrobin demonstrated improved control of green mold of infected lemons when applied as high-volume drench applications (Kanetis et al., 2008). This application method improves coverage and allows the fungicide to enter the wound sites where infections develop

(Kanetis et al., 2007). Also, the practice of heating high-volume re-circulating aqueous imazalil drench solutions that are applied to fruit over commercial brush beds at a temperature of ca. 50°C has been commonly adopted by the California citrus industry (Smilanick et al., 2008). Heating the solution presumably improves apoplastic and symplastic mobility of the fungicide enabling better post-infection decay control (Smilanick et al., 2008). Mixing certain fungicides with inorganic salts such as sodium bicarbonate ($NaHCO_3$) or with sanitizers such as hydrogen peroxide/peroxyacetic acid (HPPA) (Kanetis et al., 2008) or calcium hypochlorite $Ca(OCl)_2$ (Smilanick et al., 2005) has also provided improved decay control efficacy of green mold and other postharvest diseases of citrus. Finally, imazalil and TBZ are frequently applied to citrus fruit as a component of the fruit coatings that are used commercially to improve fruit appearance and prevent desiccation after harvest. This practice results in excellent control of sporulation from diseased fruit infected with fungicide-sensitive strains of *Penicillium* spp. and prevents contamination of adjacent fruit lots or packing equipment (Holmes and Eckert, 1999; Kanetis et al., 2008). For propiconazole, the most effective packinghouse application strategy has not been determined. Thus, another objective of this dissertation was to develop optimal application strategies to manage postharvest sour rot of citrus fruit with propiconazole.

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CHAPTER II. DISTINGUISHING *GALACTOMYES CITRI-AURANTII* FROM *G. GEOTRICHUM* AND CHARACTERIZING POPULATION STRUCTURE OF THE TWO POSTHARVEST SOUR ROT PATHOGENS OF FRUIT CROPS IN CALIFORNIA

ABSTRACT

A growth assay in lemon juice and PCR amplifications using newly designed species-specific primers from endo-polygalacturonase and beta-tubulin genes rapidly differentiated isolates of the morphologically similar fruit sour rot pathogens *Galactomyces citri-aurantii* and *G. geotrichum*. Isolates of both species were collected from agricultural soils and decaying fruit at locations in California and outside California, including world-wide locations, and were used in population genetic studies based on amplified fragment length polymorphic (AFLP) DNA markers. For all four geographically defined sub-populations (three counties of California and five locations outside California) among 97 isolates of *G. citri-aurantii* and for the two sub-populations (origin within or outside California) among 35 isolates of *G. geotrichum,* the proportion of polymorphic loci and haplotypic diversity were high. A total of 82 unique haplotypes was identified for *G. citri-aurantii* for the four sub-populations, and of these, 80 haplotypes were unique among sub-populations. For *G. geotrichum,* 25 unique haplotypes were identified among the two sub-populations and no haplotype was shared. Indices of genetic differences (F_{ST}) between sub-populations within each species were all low (0.03835 to 0.2263) indicating a low level of genetic differentiation. The effective migration rate *Nm* was calculated as 1.709 to 2.862 migrations per generation for *G. citri-aurantii* and as 12.53 migrations per generation for *G. geotrichum*. Following clone correction, mating type segregation ratios for *G. citri-aurantii* did not significantly ($P > 0.1$) deviate from a 1:1 ratio for all four sub-populations or the entire population, indicating a random mating structure for this species. Tests of the index of association

I_A and parsimony tree-length permutation tests (PTLPT) also supported a random mating structure for clone-corrected data for the Kern, Tulare, and Ventura sub-populations and the null hypothesis of random mating could not be rejected. Additionally, PTLPT also supported random mating for the Outside of California population. For *G. geotrichum,* random mating was only tested using I_A and PTLPT and the null hypothesis of random mating was not rejected ($P > 0.05$) using clone-corrected data. Further evidence that sexual recombination likely occurs in both species of *Galactomyces* was the lack of phylogenetic consistency in the nearest neighbor joining tree analyses of AFLP data. A high confidence based on bootstrap values was only obtained for few of the nodes in each of the two trees. A mixed reproduction system with an out-crossing sexual mating system and a prolific asexual phase is proposed for both species.

INTRODUCTION

Galactomyces citri-aurantii Butler the causal agent of sour rot of citrus fruit is a haploid, heterothallic ascomycete fungus. The anamorph *Geotrichum citri-aurantii* (Ferr) Butler was first described in the genus *Oidium* in1899 but was re-named *Oospora citri-aurantii* (Ferr.) Sacc. & Syd. in 1902. It was first reported on lemon fruit in California in 1917 (Smith 1917). The fungus is morphologically very similar to *Galactomyces geotrichum* (Butler & Petersen) Redhead & Malloch (anamorph *Geotrichum candidum* Link.), the pathogen that causes sour rot of stone fruits, melons, tomatoes, and other fruits (Adaskaveg et al., 2007, Suprapta et al., 1996b). The citrus pathogen has also been recognized as *Geotrichum candidum* Link var. *citri-aurantii* R. Cif. & F. Cif., given a variety name due to the ability to infect citrus fruit and grow at a low pH below 2.7 (Butler, 1960; Butler et al., 1965). In 1972, the sexual stage of *G. candidum* Link. was observed for the first time in crosses between isolates from Puerto Rico, and the teleomorph

Endomyces geotrichum Butler & Peterson was described (Butler and Peterson, 1972). In 1988, Butler et al. also described the teleomorph of the citrus pathogen and separated the two pathogens as species naming the citrus pathogen *Galactomyces citri-aurantii* Butler (Butler et al., 1988). Both species of *Galactomyces* were described as heterothallic with two mating types present (Butler and Peterson, 1972; Butler et al., 1988). Due to sexual incompatibility of the citrus pathogen with isolates of *G. geotrichum* from non-citrus hosts, the two pathogens were placed into separate biological species. Researchers in Japan, however, were unable to detect the sexual phase of *G. citri-aurantii*, and thus have contended that the two species are races of the same species, *G. candidum* (Suprapta et al., 1995). *G. citri-aurantii* and *G. geotrichum* are widely distributed in agricultural soils worldwide, but the citrus pathogen is thought to be geographically and biologically confined to the citrus environment (Butler et al., 1988).

Due to the cultural and morphological similarities of *G. citri-aurantii* and *G. geotrichum*, differentiation of the two species can be difficult. Various biochemical and physiological characteristics have been used by researchers to facilitate species identification. For example, differential pathogenicity to citrus fruits has been used in some studies (Suprapta et al., 1996a). In addition, secretion of extracellular endo-polygalacturonases that aid in the rapid breakdown of infected tissues (Davis and Baudoin, 1986; Suprapta et al., 1996) was found to be higher in the citrus pathogen (Nakamura et al., 2001). The two species also exhibit a different growth pattern in citrus juices (Butler et al. 1988; Suprapta et al., 1996b). Additionally, genetic variability was detected in the internal transcribed spacer region (ITS) of ribosomal DNA (Nakamura et al., 2008). Based on these reports, it is apparent that more specific methods are needed to separate the two species. Thus, one objective of our research was to develop identification methods based on growth characteristics and molecular specificity that would enable species distinction when

analyzing large populations of the two *Galactomyces* spp. collected from soils or diverse environments

Although sour rot causes substantial economic losses in agriculture worldwide (Eckert and Eaks, 1988; Snowdon, 1990) and both fungal pathogens are commonly found in soils, little is known about the population genetic structure of *G. citri-aurantii* and *G. geotrichum* or the extent that sexual reproduction contributes to the epidemiology and diversity of these fungi. This information could have important implications when developing strategies to manage these diseases. For example, pathogenic fungi that have a sexual stage and display out-crossing, generally have increased genetic diversity as a result of recombination, and this may allow pathogen populations to better respond to selection pressures such as the introduction of new fungicide treatments (Milgroom, 1996; McDonald and Linde, 2002). As for many ascomycete fungi, the sexual stage of *G. citri-aurantii* is not commonly observed in soils or in infected fruit because the abundantly produced asexual arthroconidia are considered the main inoculum (Butler et al., 1965) and the single-spored asci are small (8 to 12 x 7 to 9 μm in diameter) and difficult to detect (Butler and Peterson.1972; Butler et al., 1988). Thus, the extent of sexual reproduction in the fungal population may not be evident due to the predominance of asexual spores. In recent years, population genetic methods have been used increasingly to deduce information on population structure, sexual reproduction and gene flow from mating type markers, as well as molecular data such as random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and microsatellite markers (Dale et al., 2011; Peever et al., 1994; Taylor et al., 1999 b; Zhan and McDonald, 2005).

One method of determining the reproductive lifestyle of a plant pathogenic fungal species is by determining the distribution of mating types within a population. Thus, for a randomly mating species the distribution will be in roughly equal proportions (Milgroom, 1996). Another

method is to examine genetic variability in natural populations through multilocus genotypic analysis and develop deductions on the life history of a fungal species. For example, in a sexually reproducing population with random mating (panmixis), there will be little association between alleles at one locus with those of another locus, and multilocus genotypes will be highly variable when comparing individuals of the same species (Milgroom, 1996). In contrast, in an asexually, or clonally reproducing fungus, alleles at different loci are strongly associated and the same multilocus genotype is commonly recovered from different individuals within a population (Anderson and Kohn, 1995). A combination strategy of heterothallic mating with a definite asexual phase has been described as the most common fungal lifestyle (Taylor et al., 1999 b). Thus, another goal of our research was to determine the reproductive strategy of *G. citri-aurantii* and *G. geotrichum* using multi-locus genetic analysis to determine if sexual reproduction that has been observed in the laboratory is occurring in the field.

MATERIALS AND METHODS

Isolates of isolates of *G. citri-aurantii* and *G. geotrichum*. A total of 97 isolate of *G. citri-aurantii* and 35 isolates of *G. geotrichum* were using in the study. Isolates of *G. citri-aurantii* were recovered from symptomatic citrus fruit collected from packinghouses as well as from soils in commercial citrus groves and some non-citrus locations in Kern, Tulare, and Ventura counties, CA, between 2006 and 2009 (Table 1). Isolates of *G. geotrichum* were obtained from soil in citrus or nectarine orchards or from peach fruit in packinghouses in California. For all isolates collected, information on the exact geographic origin was recorded. Six isolates of *G. citri-aurantii* and fourteen isolates of *G. geotrichum* of worldwide origin were obtained from the collection of Dr. E. Butler, University of California, Davis.

Isolations from symptomatic fruit were made by excising a small section of fruit tissue from a sub-epidermal area at the margin of a sour rot lesion that was placed onto potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 1.0 ug/ml fludioxonil (Scholar 50WP, Syngenta Crop Protection) and 0.5 ug/ml novobiocin (Sigma-Aldrich, St. Louis, MO). Soil samples were collected 5 to 10 cm below the soil surface. Each composite sample consisted of 10 soil cores that were collected around the drip line from each of 5 to 10 trees per orchard. An aliquot of 3 g soil was placed into 100-ml Erlenmeyer flasks containing 25 ml of sterile water and was shaken at 150 rpm on an orbital shaker for 2 h. Aliquots of 1 ml were added to sterile tubes containing 25 ml of autoclaved lemon juice and incubated on a shaker at 200 rpm for 48 h at 24°C. From this mixture, 50 µl were spread onto PDA amended with fludioxonil and novobiocin using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA) set at the exponential deposition mode. After 48 to 72 h, colonies resembling *Galactomyces* spp. were excised and transferred to PDA for single-spore isolation. Species identification of each isolate was done using a lemon juice incubation test and by PCR with newly designed species-specific primers as described below.

Growth characteristics of *Galactomyces* spp. in lemon juice. The assay was done using a modified protocol that was previously published (Suprapta et al. 1996b). Conidial suspensions ($1x10^4$ conidia/ml) of single-spore isolates were prepared in autoclaved fresh lemon juice (pH 2.2; undiluted or diluted 1:1 with sterile water) and 1 ml of each suspension was pipetted into a 2-ml microcentrifuge tube and incubated on an orbital shaker at 150 rpm for 48 h. Aliquots of the cultures were then examined microscopically (40 x magnification). Cultures with elongated cells that fragmented into numerous conidia and with no mycelial clusters present were putatively considered *G. citri-aurantii* pending further evaluation by species- specific PCR (see below). Cultures with clusters of mycelial colonies present and with only few individual conidia were

putatively considered to be *G. geotrichum*. Each isolate was re-evaluated in two separate experiments.

DNA extraction. DNA was extracted using a modified protocol for the FastDNA® Spin kit for soil (MP Biomedicals, Solon OH) as follows. Approximately 50 mm^3 of hyphal and conidial tissue were scraped from PDA cultures and placed into a 1.5-ml screw-cap microcentrifuge tube containing six stainless steel beads 2.3 mm in diameter (BioSpec Products, Inc., Bartlesville, OK) and 100 µl of 10 mM Tris-HCl, pH 8. Samples were homogenized twice for 20 sec each using a FastPrep-24 tissue homogenizer (MP Biomedicals) at 6.0 m/s. An additional 500 µl of 10 mM Tris-HCl, pH 8, and 85 µl of MT buffer (MP Biomedicals) were added. The tubes were vortexed for 5 sec and then centrifuged at 13,000 rpm for 30 sec. The supernatant was transferred to 1.5-ml snap-cap microcentrifuge tubes and 110 µl of 7.5 M ammonium acetate was added. Tubes were inverted 10 times and centrifuged at 13,000 rpm for 5 min. The supernatant was removed and placed into a new snap-cap microcentrifuge tube containing 300 µl Binding Matrix (MP Biomedicals). Tubes were placed on a platform shaker for 5 min at 150 rpm, centrifuged at 13,000 rpm for 30 sec, and the pellet was washed twice with 300 µl SEWS-M (MP Biomedicals). The pellet was dried for 30 min in a horizontal flow hood. DNA was resuspended in 50 µl of sterile distilled water, transferred to a fresh microcentrifuge tube, and was stored at -20°C.

Design of species-specific primers for *G. citri-aurantii* and *G. geotrichum* based on endo-polygalacturonase and β-tubulin gene sequences and PCR conditions used. Primers targeting the endo-polygalacturase gene were designed from sequences for *G. citri-aurantii* and *G. geotrichum* deposited in Genbank (accession numbers AB062510.1 and AB062511.1, respectively). Sequences were aligned using Clustal-W (version 2.0; Chenna et al., 2003). From the variable region of the sequence, primers Gca F2 (5′-AGTTCTTTGCCGCTCACAAGC-3′)

and Gca R2 (5′-GGAACTTGACACCGCCTGATA-3′) for *G. citri-aurantii* and primers Gc F1 (5′-GGCACCACTGTTATCTTTGACG-3′) and Gc R3 (5′-CGGTTACCAGAGTTGACAGC-3′) for *G. geotrichum* were designed using Primer-3 (ver. 4.0; Rozen and Skaletsky, 2000). To design species-specific primers from the beta-tubulin gene, partial beta-tubulin sequences were amplified from four isolates of *G. citri-aurantii* (originating from California, Mexico, and China) and five isolates of *G. geotrichum* (originating from California, Brazil, Egypt, India, and Japan) using primers Bt2a and Bt2b (Glass and Donaldson, 1995). PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland. OH), and sequenced at the Core Instrumentation Facility of the University of California Riverside Institute for Integrative Genome Biology. Sequences were aligned using Clustal-W, and species-specific primers were designed using Primer-3 from a sequence region that was consistently variable between the two species, but homogeneous among isolates of each species. Primers were as follows: Gca F3 (5′-GAAACCATTTCTGGCGAAC-3′) and Gca R3 (5′-AGAACAGCTCTGGGGACGTA-3′) for *G. citri-aurantii* and primers Gc F5 (5′-CAAACTCCCTAGGCCTTATTC-3′) and Gc R6 (5′-ACACCAGGCTCCAGATCAAC-3′) for *G. geotrichum*.

PCR amplifications were performed in a total volume of 25 µl containing 1 µl DNA, 2 µl dNTPs (2.5 mM each dNTP), 2.5 µl PCR buffer (New England Biolabs, Inc., Ipswich, MA), 0.25 µl bovine serum albumine (10 mg/ml; Sigma-Aldrich Inc., St. Louis, MO), 1 µl of each primer (10 µM), and 0.25 µl of *Taq* polymerase (New England Biolabs, Inc.). Amplifications were performed in a PCT-100 Programmable Thermal Controller (MJ Research Inc, Watertown, MA) programmed as follows: 2 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C, and 3 min at 68°C, and a final extension at 68°C for 10 min. PCR products were visualized in 1.5% (primers based on endo-polygalacturase) or 2% (primers based on beta-tubulin) agarose gels after ethidium bromide staining.

Determination of mating type for isolates of *Galactomyces* spp. Mating type testers strains used were *G. citri-aurantii* A_1 (isolate Gca 130 = Butler 1671) and A_2 (isolate Gca 126 = butler 1655) and *G. geotrichum* A_2 (isolates Gc 109 = Butler 1619 and Gc 121 = Butler 1648; no A_1 tester strain was available for this species) (Butler et al. 1972, 1988). Loops of conidia from single-spore cultures of these cultures were directly streaked from PDA plates onto fresh PDA in 60-mm petri dishes. Loops of conidia from single-spore isolates of unknown mating type were then streaked perpendicularly over the first streak. After 5 to 10 days of incubation at 25ºC in the dark, vertical sections of the agar (approximately 1 mm thick) were mounted onto glass slides for microscopic observation at 400 x magnification. For *G. citri-aurantii*, a mating type of A_1 was assigned to an isolate when asci were present in pairings with the A_2 tester strain, and a mating type of A_2 was assigned when asci were present in pairings with the A_1 tester strain. For *G. geotrichum*, a mating type of A_1 was assigned to an isolate when asci were present in pairings with each of the A_2 tester strains. To test for monogenic segregation of the mating type locus within *G. citri-aurantii*, twelve randomly selected A_1 and A_2 isolates each were crossed against each other.

AFLP procedures. AFLP reactions were performed using a modified protocol by Vos et al. (1995). The restriction-ligation reaction contained approximately 50 ng of genomic DNA, 5 U *Eco*RI, 1 U *Mse*I, 20 U of T4 DNA ligase (all enzymes from New England BioLabs), 5 µM EcoR1 adapter, 50 µM MseI adapter, 1 x ligase buffer, 5 µg bovine serum albumine, and 50 mM NaCl in a final volume of 11 µl. After incubation at 37ºC for 2 to 2.5 h, 90 µl of $TE_{0.1}$ (10 mM Tris, 0.1 mM EDTA, pH 8) was added and the reaction was stored at -20ºC. Amplifications were done in a final volume of 12.5 µl using 1.3 µl of the diluted digestion-ligation reaction, 200 µM each dNTP, 0.8 µM Mse primer, 0.16 µM 6-carboxyhexachlorofluorescein (HEX)- or 6-carboxyfluorescein (FAM)-labeled EcoRI primer, 1 µg bovine serum albumine, 1 x *Taq*

polymerase buffer, and 0.75 U *Taq* DNA polymerase (New England BioLabs). Sixteen primer pairs were initially screened for amplification. Four primer pairs were then chosen for analysis of *G. citri-aurantii* and six primer pairs for *G. geotrichum* based on repeatability and easily distinguishable polymorphic bands. These Eco (5'- GACTGCGTACCAATTC+2 -3') and MseI (5'-GACGATGAGTCCTGAGTA+2 -3') primers are listed in Table 2.

PCR conditions comprised an initial touchdown phase of 13 cycles of 94ºC for 30 sec, 65ºC for 30 sec and 72ºC for 60 sec, lowering the annealing temperature by 0.7ºC at each successive cycle, followed by 23 cycles of 94ºC for 30 sec, 56ºC for 30 sec, and 72ºC for 60 sec, and a final extension step of 72ºC for 5 min. Aliquots of 0.5 µl PCR reaction were mixed with 0.125 µl GeneScan-500 LIZ size standard (Applied Biosystems, Carlsbad, CA) and denatured with 9.875 µl Hi-Dye formamide (Applied Biosystems) in a total volume of 11 µl for 3 min at 94ºC. AFLP fragments were separated with an ABI 3100 genetic analyzer (Applied Biosystems) and analyzed using the GeneScan software (Applied Biosystems). To validate reproducibility of the procedure, each AFLP reaction run included one or several "standard" isolates and the analysis was done at least twice for each isolate.

Neighbor-joining cluster analysis and population genetic analysis of AFLP data. GeneScan AFLP data files were imported into Genographer software (version 2.1, http://hordeum.oscs.montana.edu/genographer) for visual analysis. AFLP profiles were analyzed in the 80- to 350-bp size range on a virtual gel, and consistent fragments with bands intensities of greater than 85 units were scored for their presence or absence among isolates. Fragments were considered individual loci , that are either present (a scoring of 1) or absent (a scoring of 0), and a binary data matrix was created. AFLP.dat (Ehrich, 2006) was used to generate input files and linkage disequilibrium was evaluated for the complete data sets in Arlequin 3.1. (Excoffier, Laval, and Schneider, University of Berne, Switzerland).Any highly linked loci ($\alpha = 0.05$) were

combined into a single representative locus because inclusion of highly linked loci has the effect of reducing the power of multilocus genetic analysis to accurately determine the degree of genetic diversity within a population (Agapow and Burt, 2001; Milgroom, 1996).

The binary data matrix was subjected to phenetic analyses using the neighbor-joining method of PAUP version 4.0 (Swofford, 2002) and the tree was viewed by using TreeView (Page, 1996). Statistical support for the calculated topology was conducted in a bootstrap analysis with 1000 replications.

For population genetic analyses, two datasets were generated for each species, one using all isolates and one that was clone corrected. For this, unique haplotypes for both species were identified using Arlequin 3.1., and clones were removed from the database. The number of unique haplotypes (those that occurred only once in each population) and the proportion of polymorphic loci in each sub-population (i.e., a geographically defined sub-sample of the total population) were calculated. Polymorphic loci were defined as those where the most common allele is present at a frequency of less than 0.95 (Zhan and McDonald, 2005). Haplotypic (genotypic) diversity was defined as the probability that two individuals from within a population sampled at random have distinct genotypes (Agapow and Burt, 2001). It was calculated with the full data sets for each sub-population as $(n/n - 1)(1 - \Sigma_{Pi}^2)$ where n is the number of individuals sampled and $_{Pi}$ is the frequency of the ith genotype of both species using MultiLocus 1.3 (Agapow and Burt, 2001; http://www.agapow.net/software/ multilocus/1.3b,). Haplotypic diversity has a value of 1 when each individual is different and a value of 0 when each individual in a population is the same.

Clone corrected AFLP data for sub-populations of *G. citri-aurantii* and *G. geotrichum* were subjected to an analysis of molecular variance (AMOVA) using Arlequin 3.1. Sub-populations for *G. citri-aurantii* were based on three sampling counties in California (Kern, Tulare, and Ventura) and locations outside California (i.e., sub-population 'Outside'). For *G.*

geotrichum, isolates were divided into a California and an 'Outside' sub-population. The index of genetic difference (fixation index; F_{ST}) between populations by pair-wise difference (Weir and Cockerham, 1984), the effective migration rate *Nm,* Nei's gene diversity (average gene diversity over all loci for each population), as well as allele frequencies for each locus and sub-population were calculated using Arlequin 3.1.

Random mating was evaluated using three approaches. First, a χ^2 analysis of mating-type segregation was conducted (α=0.05) for *G. citri-aurantii* using an expected ratio of 1:1 for randomly mating haploid fungal populations (Milgroom, 1996). Two additional tests were done for both species of *Galactomyces* using the full linkage-adjusted as well as the clone-corrected data sets to account for the effects of reduced sample size due to removal of clone-mates on the probability of rejecting the null hypothesis of random mating in statistical tests (Milgroom, 1996). The index of association (I_A) that tests the random association among loci was calculated from AFLP data using MultiLocus 1.3. For a randomly mating population without linked loci the theoretical value for I_A is zero. The significance of I_A was tested by comparing the value for the observed data with randomized data (500 times; α=0.05) to assess significant deviations from gametic equilibrium expected with panmixis (Agapaw and Burt, 2001). The third test to determine the reproductive mode was the parsimony tree-length permutation test (PTLPT; Burt et al., 1996). PTLPT compares the length of the observed tree to trees produced with randomized data (500 times) and is a phylogenetic approach based on the concept that a clonal population should produce a single well-resolved tree with little homoplasy and shorter tree branches than a sexually reproducing population due to the re-assortment of alleles during meiosis (Taylor et al., 1999a). Output data files from MultiLocus 1.3 were analyzed using PAUP version 4.0.

RESULTS

Growth characteristics of isolates of *Geotrichum* spp. in lemon juice. Most isolates evaluated were assigned to either of two growth patterns when incubated in undiluted, autoclaved lemon juice. Cultures identified previously as *G. geotrichum* by others (Butler and Peterson, 1972), several cultures originating from non-citrus environments, and five isolates from soil in citrus groves in California formed clusters of mycelial colonies with irregular branching, and few individual conidia were present (Fig. 1A). In contrast, growth of isolates assigned to *G. citri-aurantii* based on their origin from citrus fruit or from soil in citrus groves occurred as elongated cells that fragmented into numerous individual conidia, and no mycelial clusters were present (Fig. 1B). Several isolates did not grow in undiluted lemon juice, but exhibited characteristic structures when incubated in lemon juice diluted 50% with sterile water and then could be placed into either of the two growth pattern groups.

PCR amplifications using species-specific primers for *G. citri-aurantii* and *G. geotrichum*. After PCR amplification, primers based on endo-polygalacturonase gene sequences yielded DNA fragments 441 bp in size for all isolates assigned to *G. citri-aurantii* (Fig. 2A) and fragments 434 bp in size for isolates assigned to *G. geotrichum* (Fig. 2B). Faint background bands were frequently observed, however, for each species using primers designed for the other species (Fig. 2A,B) even when annealing temperatures were increased above 62°C. Primers designed from beta-tubulin gene sequences yielded specific bands for *G. citri-aurantii* (Fig. 2C) and *G. geotrichum* (Fig. 2D) 208 bp and 152 bp in size, respectively, and no background bands were observed.

Determination of mating type for isolates of *Galactomyces* spp. For *G. citri-aurantii*, asci were easily observed microscopically in most pairings of mating type tester strains with

isolates of unknown mating type. Asci typically developed in clusters mainly between 1 mm and 3.5 mm below the agar surface (Fig 1). Asci were spherical and 7 to 12 µm in diameter. Of the total 87 isolates evaluated, 52 isolates were determined to be of A_1 and 35 isolates of A_2 mating type. In 10 of the pairings, ascospores failed to develop in repeated evaluations. In tests for the monogenic segregation of the mating type locus in *G. citri-aurantii*, asci were observed for all A_1 x A_2 pairings. In pairings among A_1 or A_2 isolates, ascospores were observed in two and one pairing, respectively.

For the total of 35 isolates evaluated for *G. geotrichum,* asci in pairings with A_2 tester strains Gc 109 and Gc 121 were only observed for two isolates (i.e., 78 and 86). Asci were similar in appearance as for *G. citri-aurantii*, but were produced much less abundantly.

Neighbor-joining cluster analysis and population genetic analysis of AFLP data. Examples of virtual AFLP gels for representative isolates of *G. citri-aurantii* and *G. geotrichum* using two primer pairs are shown in Fig. 3A,B. Both species are clearly differentiated by their distinct banding patterns. For 97 isolates of *G. citri-aurantii*, 68 DNA fragments were scored initially following AFLP analysis with four primer pairs (Table 2). After analysis of genetic linkage and removal of polymorphic bands that were only present in single isolates, the final database for this species comprised 19 polymorphic fragments. For 35 isolates of *G. geotrichum*, 73 DNA fragments were scored initially following AFLP analysis with six primer pairs (Table 2) and the final database comprised 11 polymorphic fragments.

Neighbor-joining analyses generated a dendrogram with a high degree of diversity for each species, however, only few branching nodes showed strong support in the bootstrap analysis (Figs. 4,5). The shorter scale bar for character changes in the dendrogram for *G. citri-aurantii* tentatively indicated that the degree of diversity was higher among isolates of this species as compared to *G. geotrichum*. Few clonal groups were identified for each species. The largest

clonal group for *G. citri-aurantii* comprised six isolates (i.e., 143, 192, 193, 195, 195, and 199) that originated from fruit and soil at two locations in Ventura county. The remaining nine smaller clonal groups each contained isolates from the same or from different areas within California. The larger of the two clonal groups found in *G. geotrichum* contained isolates (i.e., isolates 102, 238, 242, 243, 244) from peach in California collected over a period of over 20 years, whereas the smaller clonal group contained isolates (i.e., isolates 121, 140, 144) from soil in California, including two isolates from citrus groves. The dendrogram for *G. geotrichum* includes three divergent isolates (i.e., isolates 101, 105, and 108) that originated from India, an unknown location, and from Zimbabwe, respectively (Fig. 5).

Data analysis for *G. citri-aurantii* using Arlequin 3.1 where linked loci were combined, identified a total of 82 unique haplotypes for the four sub-populations, and of these, 80 haplotypes were unique among sub-populations. Of the total number of AFLP loci scored, the Ventura county sub-population had the highest and the Kern County sub-population had the lowest proportion of polymorphic loci (Table 3). For all sub-populations of this species, haplotypic diversity was high and the Outside of California sub-population had the highest value (Table 3). Nei's gene diversity values ranged from 0.164 to 0.302. For *G. geotrichum,* 25 unique haplotypes were identified among the two sub-populations and no haplotype was shared for the two sub-populations. The proportion of polymorphic loci and haplotypic diversity were high (Table 3) and Nei's gene diversity values ranged from 0.275 to 0.369.

Indices of genetic differences (F_{ST}) between sub-populations of *G. citri-aurantii* that were calculated by pair-wise comparisons were all low and ranged from 0.0846 to 0.2263 (Table 4). In these calculations, F_{ST} values in all comparisons with the outside of California sub-population were the highest and were significant ($P < 0.002$) indicating the highest divergence of this sub-population. Based on conditions of migration and drift equilibrium, values for the effective

migration rate *Nm* indicate that the Outside sub-population would be expected to share between 1.709 to 2.862 migrations per generation with the three sub-populations collected from within California (Table 4). In contrast, the Tulare county sub-population showed the least divergence from the one from Ventura county (F_{ST} value 0.0846; $P < 0.001$) and based on the *Nm* value would be expected to share 5.412 migrations per generation. For *G. geotrichum,* the pair-wise F_{ST} value for the two geographically defined sub-populations was 0.03835 ($P = 0.094$) indicating a low level of genetic differentiation. The effective migration rate was calculated as $Nm = 12.53$ migrations per generation.

Allele frequencies that were calculated using clone corrected data were generally consistent across the four populations for individual loci, however two loci represented by fragments 82A (Fig 3), and 226D were present in only the Ventura population at low frequencies of 0.176 and 0.118 respectively. The Outside of California sub-population lacked five alleles that were present in the total population; whereas the Kern, Tulare, and Ventura sub-populations lacked four, three, and one allele, respectively (Table 5). For the California sub-population of *G. geotrichum* allele frequencies ranged from 0.176 (locus 154C) to 0.941 (loci 117A and 98F) for the 11 loci evaluated for the final analysis, and for the population collected from outside of California, allele frequencies ranged from 0.333 (locus 209C) to 1.00 (loci 130A, 151B) (*data not shown*).

Reproductive mode. Using non-clone-corrected data sets, mating type distribution for *G. citri-aurantii* did not significantly ($P > 0.1$) deviate from a 1:1 ratio in χ^2-square tests for the Kern, Tulare, or outside California sub-populations (Table 6). Following clone correction, mating type segregation ratios did not deviate from a 1:1 ratio for all four sub-populations and the entire population indicating a random mating structure for species. Tests of I_A supported a random mating structure only for clone-corrected data for the Kern, Tulare, and Ventura sub-populations

and the null hypothesis of random mating could not be rejected with values of $P > 0.1$ (Table 6). The null hypothesis was rejected for non-clone-corrected data of these sub-populations and for clone-corrected and non-corrected data for the Outside of California sub-population and the total population of this species. In the PTLPT analyses, probability values of 0.314 to 0.538 indicated that random mating could not be rejected for the clone-corrected data of the entire population, as well as of all four sub-populations. Thus, the lengths of the observed trees were not statistically different from those generated after randomization of data 500 times. For *G. geotrichum,* random mating was only tested using I_A and PTLPT. In the I_A calculations, the null hypothesis of random mating was not rejected ($P > 0.05$) except for the non-clone-corrected data for the California population (Table 6). The PTLPT analysis with *P* values between 0.386 and 0.680 indicated random mating based on clone-corrected data for the two sub-populations and for the total population. For the non-clone-corrected data, the null hypothesis of random mating was only rejected ($P = 0.014$) for data of for total population.

DISCUSSION

Isolates of the morphologically similar sour rot pathogens *G. citri-aurantii* and *G. geotrichum* could be easily differentiated based on characteristic growth in a lemon juice assay and by PCR reactions using newly designed species-specific primers from the endo-polygalacturose and beta-tubulin genes. Specific primers derived from the endo-polygalacturase gene were recently also designed by others and were used to differentiate isolates pathogenic and non-pathogenic to citrus (Nakamura et al., 2008). These latter authors do not acknowledge that two separate species cause sour rot of fruit crops and instead recognize a single species, *G. geotrichum* with two pathogenicity types in citrus fruit assays. Our studies add to the evidence

that two distinct biological and molecular species exist, and the highly diverse AFLP banding patterns that we obtained for the two species with virtually no shared fragments further supports this taxonomic separation. Growth and molecular assays on species identification in our study ensured that isolates were properly classified before analyses of population structure were done. Although *G. citri-aurantii* has been ecologically associated with the citrus environment such as citrus fruit or soil from citrus groves (Butler, et al., 1988), exceptions are known. For example, isolates of *G. geotrichum* have been recovered from citrus groves and likewise, isolates of *G. citri-aurantii* have been obtained from non-citrus environments (Suprapta et al., 1995). Similarly, in our study, five isolates of *G. geotrichum* were recovered from soil in citrus groves.

The three California sub-populations of *G. citri-aurantii* displayed a wide range of proportion of polymorphic loci. The low value for Kern county (i.e., 0.321) was probably due to the low sample size of only eight isolates and a clone-corrected size of seven. The other two sub-populations with clone-corrected sample sizes of 34 or 35 had values of 0.678 to 0.857, indicating a higher level of polymorphism. The Outside sub-population with a sample size of only six clone-corrected isolates also had a high proportion of polymorphic loci that was probably due to the worldwide origin of these isolates. Haplotypic diversity values were high for all four sub-populations of *G. citri-aurantii* indicating high genetic differences among individual haplotypes. The California sub-populations showed a moderate, whereas the Outside population showed higher average gene diversity over all loci (Nei's gene diversity values). Similar to most of the sub-populations of *G. citri-aurantii,* those of *G. geotrichum* had a high proportion of polymorphic loci and high haplotypic diversity. In contrast, average gene diversity was moderate to high in this latter species considering that the theoretical maximum value for a panmictic population is 0.5 for multilocus data based on biallelic markers such as the AFLPs reported here (Zhan and McDonald, 2005). Given that sexual reproduction is readily obtained in the laboratory, has been observed in

decaying lemon fruit (Butler et al., 1988), and is assumed to occur in citrus groves, the genetic diversity could be explained by sexual recombination within each sub-population. It has been stated that even low frequencies of recombination may produce a population structure that has all the appearances of random mating (Milgroom, 1995). Mutation is unlikely the cause for the diversity found, because random mutations would result in novel alleles not widely shared among sub-populations. In the analysis of allele frequencies for *G. citri-aurantii*, only two alleles were unique to a single sub-population.

Sub-populations within both species were defined based on the geographic origin of the isolates. F_{ST} values in pair-wise comparisons of sub-populations were all low with moderately high migration rates (e.g., *Nm* for *G. citri-aurantii* ranged from 1.709 to 5.412; *Nm* for *G. geotrichum* was 12.53). Still, for *G. citri-aurantii*, F_{ST} values were higher in comparisons between the Outside of California sub-population and the three California sub-populations than of comparisons among the California sub-populations. Thus, sub-populations were not strongly differentiated genetically indicating that gene flow and migration may be important in shaping the population genetic structure of these pathogens. Moreover, the number of shared alleles among sub-populations of *G. citri-aurantii* was relatively high for most of the loci (e.g., 17 of 25). This may reflect the difficulty of defining sub-populations in some agricultural host-pathogen systems. Although both species of *Galactomyces* are soil fungi, conidia are not air-borne, and inoculum in the field is not easily dispersed long-distance. Still, conidia are produced in extremely high quantities on decaying fruit and commodities are often shipped long-distance from the orchard to the packinghouse and subsequently to market. Furthermore, contaminated harvest bins may be taken to distant locations upon demand and decayed cull fruit in the packinghouse are sometimes taken back to orchard sites where they may be buried in the ground. Thus, individuals of the

pathogen may be disseminated over large areas during agricultural activities (thus, contributing to migration and gene flow), resulting in heterogeneous populations that are not strongly separated.

Ascospores of *Galactomyces* spp. can be obtained in pairings with isolates of the opposite mating type in the laboratory and ascospores of *G. citri-aurantii* have been observed in decaying lemon fruit in the field (Butler et al. 1988). Furthermore, isolates of the latter species with wide phenotypic diversity have been recovered from citrus grove soils worldwide (Hershenhorn et al., 1992; Suprapta et al., 1996b) and both mating types have been isolated from soil under the same citrus trees (Butler et al., 1988). The significance of sexual reproduction in generating genetic diversity in the field has remained unclear. Not all of our tests that we conducted supported evidence for random mating but there is a strong indication that sexual reproduction of both species of *Galactomyces* actually does occur in the field. This finding and the overall genetic diversity observed could have practical implications for the management of sour rot diseases. For example, pathogens with large genetic diversity and the ability to adapt through sexual means are thought to more likely to develop resistance against a fungicide (McDonald and Linde, 2002).

For *G. citri-aurantii* we found that for three sub-populations, mating type segregation did not deviate significantly from a 1:1 ratio for all sub-populations and the entire population following clone correction. Similarly, tests of I_A and PTLPT supported a random mating structure for clone-corrected, but not for non-clone-corrected data of most of the sub-populations. For *G. geotrichum,* matings with tester strains were mostly unsuccessful. Because only A_2 tester strains were available, the absence of ascospores could indicate that most isolates were of the A_2 mating type. Due to lack of data, no mating type segregation analysis was done. For *G. geotrichum*, the null hypothesis of random mating in the tests of I_A and PTLPT was not rejected, mainly using the clone-corrected data as with *G. citri-aurantii*. Contrasting results using clone- and non-clone-corrected data sets have also been observed in other ascomycete fungi and this has been

associated with a mixed mode of mating (Dale et al., 2010; Douhan et al., 2002). For example, mating type segregation of the needle blight fungus *Dothiostroma septosporum* did not deviate significantly from a 1:1 ratio in a diverse population, but I_A or PTLPT tests indicated a more clonal population structure unless clones were removed from the analysis (Dale et al., 2010). Other factors that may be contributing to the assessment of recombination in a population include linkage of loci, high mutation rates, and small sample size (Milgroom, 1996). Linkage correction was conducted for our data sets. The number of isolates of *G. candidum* evaluated may have been limiting. It has been suggested that 60 or more isolates should be used for multilocus evaluation of random mating (Zhan and McDonald, 2005). A final evidence from our study that sexual recombination likely occurs in both species of *Galactomyces* is the lack of phylogenetic consistency in the nearest neighbor joining tree analyses. A high confidence based on bootstrap values was only obtained for a few of the nodes in each of the two trees. It has been established that recombining genotypes, such as in sexually reproducing fungal species, show little or no phylogenetic consistency because different loci reflect different patterns of descent among individuals (Milgroom 1996).

Based on our data, sexual reproduction seems to have a major role the life cycle of both species of *Galactomyces*. As additional evidence, few clonal isolates were detected for each species. Still, the relative importance of sexual reproduction is likely overestimated due to the selection of isolates used. In our own collections, isolates were obtained over several years and from diverse locations. Only between one and three isolates were included in the analyses from a single soil sampling location or fruit lot. Thus, the collection may have been biased toward more diversity. Both sour rot pathogens produce abundant asexual conidia on infected fruit that can infect healthy fruit and spread the disease and thus, are important in the disease cycle. The relative importance of sexual reproduction in both species will have to be elucidated in additional

studies by using a sampling strategy where smaller sample areas (for example, soil from under a single tree) are evaluated. A mixed reproduction system with an out-crossing sexual mating system and an asexual phase with a high capacity of reproduction (McDonald and Linde, 2002) can be proposed for *G. citri-aurantii* and *G. geotrichum*. This life cycle with heterothallic meiosporic, as well as mitosporic reproduction was described to be most common for fungi (Taylor et al., 1999b). In a mixed reproduction system, new recombinants derived from sexual reproduction will be continually tested in changing environments (McDonald and Linde, 2002). Selected genotypes will be amplified asexually and may be distributed through gene flow. In an agricultural system with numerous selection pressures imposed by human activity, this may contribute to resistance development against pesticides or the development of new pathogenicity types.

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Table 2.1. Isolates of *Galactomyces citri-aurantii* and *G. geotrichum* used in this study

Species	Isolate code	No. of isolates	Population[a]	Collection date	Source	Geographic origin
G. citri-aurantii[b]	2	1	Kern	2006	Lemon	Maricopa, CA
	14	1	Kern	2006	Navel orange	Edison, CA
	24, 28, 31	3	Kern	2006	Lemon	Delano, CA
	246-248	3	Kern	2008	Mandarin	Maricopa, CA
	81	1	Tulare	2007	Citrus soil	Exeter, CA
	151-153, 155-160, 163-165, 167,168, 171, 173, 182-183, 185-186	21	Tulare	2008	Citrus soil	Exeter, CA
	30, 33, 46	3	Tulare	2006	Lemon	Ivanhoe. CA
	70, 72	2	Tulare	2007	Navel orange	Ivanhoe, CA
	23	1	Tulare	2007	Valencia orange	Lindsay, CA
	149, 150, 175	3	Tulare	2008	Citrus soil	Lindsay, CA
	3-5, 13, 84, 87, 91	7	Tulare	2006	Lemon	Porterville, CA
	89	1	Ventura	2007	Grapefruit	Fillmore, CA
	131, 133, 136-139, 141-143, 145-147	12	Ventura	2007, 2008	Citrus soil	Fillmore, CA
	188-195, 199, 200	10	Ventura	2006 - 2008	Lemon	Oxnard, CA
	32, 40, 45, 48, 73, 77, 88	7	Ventura	2006, 2007	Lemon	Santa Paula, CA
	71, 74	2	Ventura	2008	Citrus soil	Santa Paula, CA
	239, 241	2	Ventura	2009	Lemon	Somis, CA
	12, 15, 16, 18, 20, 66, 67, 90, 92-94, 134	12	Ventura	2006	Lemon	Ventura, CA
	120	1	Outside	NA[c]	Soil	FL
	115, 123	2	Outside	1984	Soil	Israel
	130	1	Outside	1963	Lemon	Trinidad
	124	1	Outside	1986	Soil	Argentina
	127	1	Outside	1986	Soil	China
		Total=97				

[a] Populations were assigned based on geographic origin: three counties of California and outside California for *G. citri-aurantii*; California and outside California for *G. geotrichum*.

[b] Positive species identification as *G. citri-aurantii* or *G. geotrichum* was based on the use of species-specific PCR primers derived from β-tubulin and endo-polygalacturonase sequences and growth characteristics in a lemon juice assay.

[c] Sample date not available.

Table 2.1. (Cont.) Isolates of *Galactomyces citri-aurantii* and *G. geotrichum* used in this study

Species	Isolate code	No. of isolates	Population[a]	Collection date	Source	Geographic origin
G. geotrichum	102, 110, 117, 118, 121	5	California	1957, 1963, 1969, 1984, 1986	Soil	CA
	231, 232	2	California	NA	Soil	Riverside Co., CA
	86, 235, 238, 242-244	6	California	2000, 2005, 2006	Peach	Fresno Co., CA
	75, 132, 135, 140, 144	5	California	2007, 2008	Citrus soil	Ventura Co., CA
	237	1	Outside	1973	Soil	PA
	106	1	Outside	1963	Soil	Brazil
	104, 234	2	Outside	1963	Soil	Egypt
	100, 111	2	Outside	1963	Soil	Israel
	99, 103, 233	3	Outside	1963	Soil	Japan
	101, 105	2	Outside	1963	Soil	Zimbabwe
	122, 236	2	Outside	1968	Soil	Puerto Rico
	107	1	Outside	1970	Soil	Turkey
	108, 109	2	Outside	1974	Soil	India
	113	1	Outside	1981	Soil	Costa Rica
		Total=35				

[a] Populations were assigned based on geographic origin: three counties of California and outside California for *G. citri-aurantii*; California and outside California for *G. geotrichum*.

[b] Positive species identification as *G. citri-aurantii* or *G. geotrichum* was based on the use of species-specific PCR primers derived from β-tubulin and endo-polygalacturonase sequences and growth characteristics in a lemon juice assay.

[c] Sample date not available.

Table 2.2. Selective primer pairs used in AFLP reactions for two species of *Galactomyces*

*Mse*I primers (adapter +2)	*Eco*RI primers (label, adapter+2)	No. of markers[a]	
		G. citri-aurantii	*G. geotrichum*
5′-TC-3′	5′-FAM-AC-3′	20	9
5′-AC-3′	5′-HEX-TA-3′	23	11
5′-TT-3′	5′-FAM-CA-3′	10	13
5′-GC-3′	5′-HEX-TA-3′	15	---
5′-GA-3′	5′-FAM-AC-3′	---	11
5′-TA-3′	5′-FAM-AC-3′	---	18
5′-TG-3′	5′-HEX-TA-3′	---	11
		Total = 68	Total = 73

[a] Consistently amplified DNA fragments scored as being potentially descriptive.

Table 2.3. Population genetic statistics for *Galactomyces citri-aurantii* and *G. geotrichum*

Species and sub-population	Total sample size	Clone-corrected sample size	Proportion of polymorphic loci[a]	No. of unique haplotypes	Diversity		
					Haplotypic[b]	*P*	Nei's gene[c]
G. citri-aurantii							
Kern	8	7	0.321	7	0.976	0.030	0.164
Tulare	37	35	0.678	35	0.994	0.016	0.188
Ventura	46	34	0.857	34	0.978	<0.002	0.193
Outside	6	6	0.607	6	1.000	1.000	0.302
G. geotrichum							
California	18	12	0.818	12	0.944	0.010	0.275
Outside	17	14	1.000	13	0.987	0.008	0.369

[a] Polymorphic loci were defined as those where the most common allele is present at a frequency of less than 0.95.

[b] Haplotypic diversity (Multilocus 1.3, based on Agapow and Burt, 2001) was calculated using complete data set.

[c] Average gene diversity over all loci calculated from clone-corrected data using Arlequin 3.1.

Table 2.4. Pair-wise population F_{ST} (below the diagonal) and *Nm* (above the diagonal) values for four sub-populations of *Galactomyces citri-aurantii* [a]

Sub-population	Sub-population			
	Outside	Kern	Tulare	Ventura
Outside	---	2.072	2.862	1.709
Kern	0.1944	---	3.099	3.689
Tulare	0.1487	0.1389	---	5.412
Ventura	0.2263	0.1193	0.0846	---

[a] Calculated between distinct sub-populations using clone corrected data.

Table 2.5. Population allele frequencies for clone corrected amplified fragment length polymorphism data for *Galactomyces citri-aurantii*

	Population allele frequency			
Locus	Ventura	Tulare	Kern	Outside
210A	0.029	0.171	***0.000*** [a]	***0.000***
171A	0.824	0.829	0.800	0.333
161A	0.882	1.000	1.000	1.000
143A	0.147	0.143	***0.000***	0.333
138A	0.941	0.914	1.000	0.333
83A	0.824	1.000	1.000	1.000
82A	0.176	***0.000***	***0.000***	***0.000***
78A	0.353	0.257	0.200	0.333
198B	0.353	0.257	1.000	0.500
186B	0.882	0.829	1.000	0.500
177B	0.118	0.743	1.000	0.333
169B	0.882	0.657	0.400	0.333
156B	0.029	0.200	0.400	***0.000***
142B	0.971	0.800	1.000	0.833
136B	0.971	0.914	1.000	0.500
110B	0.588	0.200	1.000	0.667
98B	0.647	0.171	0.400	0.883
198C	0.941	0.971	1.000	1.000
195C	0.118	0.057	0.200	***0.000***
161C	0.824	0.714	1.000	0.833
105C	0.912	0.886	1.000	0.667
228D	0.029	***0.000***	0.600	0.333
227D	0.853	0.971	0.400	0.667
226D	0.118	***0.000***	***0.000***	***0.000***
148D	***0.000***	0.114	0.200	0.117

[a] Allele frequencies of ***0.000*** indicate an absence of the allele at the indicated locus for the specified sub-population.

Table 2.6. Random mating tests for *Galactomyces citri-aurantii* and *G. geotrichum* using the full and clone-corrected (CC) data sets

		Mating type segregation			Index of association (I_A)[a]			PTLPT[b]		
Species	Sub-population[c]	A_1:A_2[d]	χ^2	*P*	N	I_A	*P*	L	L^*	*P*
G. citri-aurantii	Kern	4:2	1.0	>0.1	8	0.885	0.014	17 (3)	16	0.018
	Tulare	19:16	0.35	>0.5	37	0.199	0.068	60 (2)	67	0.002
	Ventura	28:15	3.86	<0.05	46	0.268	0.036	70 (2)	74	0.002
	Outside	2:3	0.33	>0.5	6	0.785	0.028	25 (1)	22	0.328
	Total	52:35	4.01	<0.05	97	0.373	<0.002	156 (1)	156	0.001
	Kern CC	3:1	1.0	>0.1	5	0.037	0.446	11 (1)	10	0.514
	Tulare CC	17:15	0.13	>0.5	34	0.184	0.118	11 (1)	10	0.538
	Ventura CC	18:15	0.27	>0.5	35	0.036	0.400	70 (1)	63	0.412
	Outside CC	2:3	0.33	>0.5	6	0.785	0.028	25 (1)	23	0.314
	Total CC	40:34	0.63	>0.5	80	0.277	0.002	161 (1)	155	0.426
G. geotrichum	California	ND[e]	ND	ND	18	0.440	0.010	18 (5)	15	0.238
	Outside	ND	ND	ND	17	0.155	0.154	24 (1)	21	0.094
	Total	ND	ND	ND	35	0.120	0.082	35 (3)	33	0.014
	California CC	ND	ND	ND	12	0.052	0.350	17 (5)	14	0.576
	Outside CC	ND	ND	ND	14	0.025	0.408	24 (1)	20	0.680
	Total CC	ND	ND	ND	26	0.024	0.382	34 (1)	30	0.386

[a] Index of association values obtained by MultiLocus version 1.3, with 500 randomizations of data.

[b] Parsimony tree-length permutation test; L = observed length of tree generated in parsimony analysis (numbers in parentheses reflect the number of most parsimonious trees found). L^* = length of the shortest tree for randomized data (500 replicates).

[c] Isolates originating from national and international locations outside California.

[d] Sample size for mating type (A_1 or A_2) is less than *N* in some populations due to missing data.

[e] Not determined.

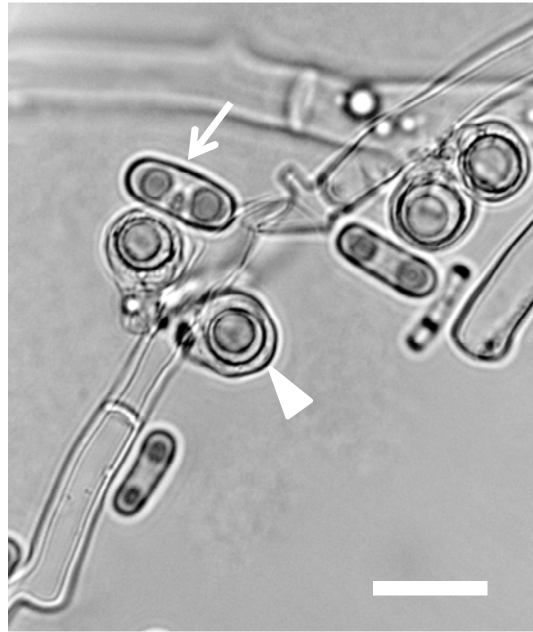

Figure 2.1. Single-spored asci with thickened wall (arrowhead at one ascus) and conidia (arrow at one conidium) in a pairing of A_1 and A_2 mating types of *Galactomyces citri-aurantii*. Scale bar equals 10 µm.

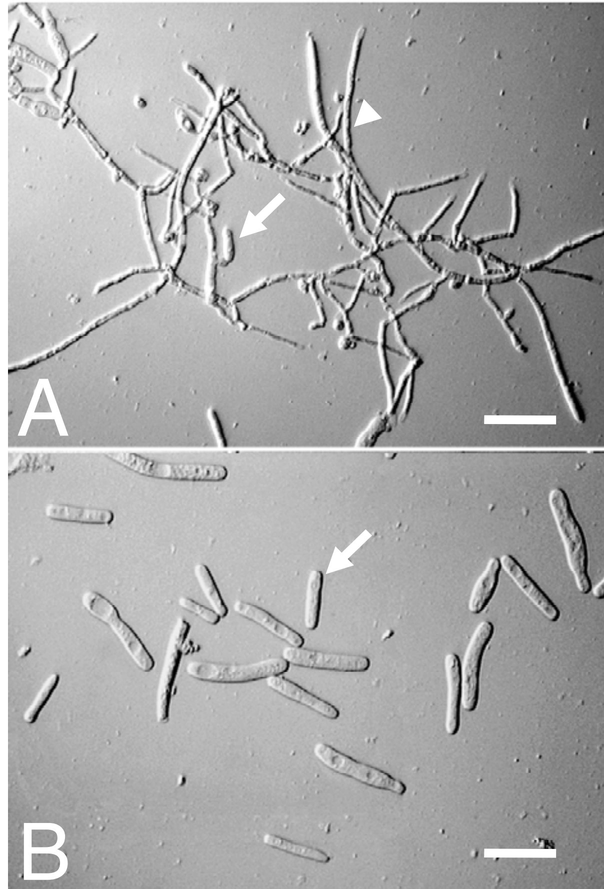

Figure 2.2. Growth characteristics of **A,** *Galactomyces geotrichum* and **B,** *G. citri-aurantii* in autoclaved lemon juice at pH 2.2. For *G. geotrichum*, growth occurred as small filamentous colonies (arrowhead) with irregular branching and with only few individual conidia (arrow). For *G. citri-aurantii*, growth occurred as elongated cells that fragmented into numerous individual conidia (arrow). Scale bar equals **A,** 50 µm and **B,** 15 µm.

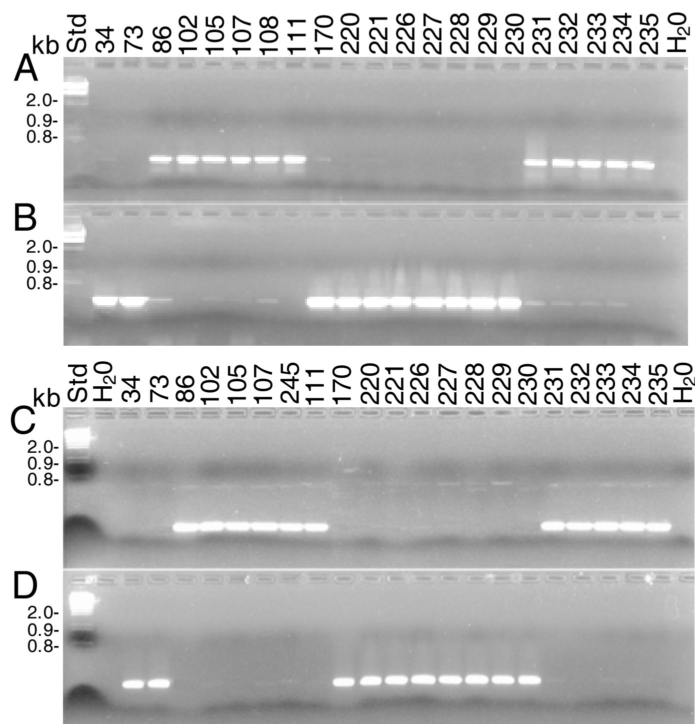

Figure 2.3. PCR amplifications using species-specific primers developed from DNA sequences of **A and B,** endo-polygalacturonase or **C and D,** beta-tubulin genes for identification of **A and C,** *Galactomyces geotrichum* and **B and D,** *G. citri-aurantii*. Primer pairs used for the identification of *G. geotrichum* were Gc F1/Gc R3 and Cand F5/Cand R6 and of *G. citri-aurantii* were Gca F2/Gca R2 and Citri F3/Citri R3 for the endo-polygalacturonase and beta-tubulin genes, respectively (*see* Table 2 for details).

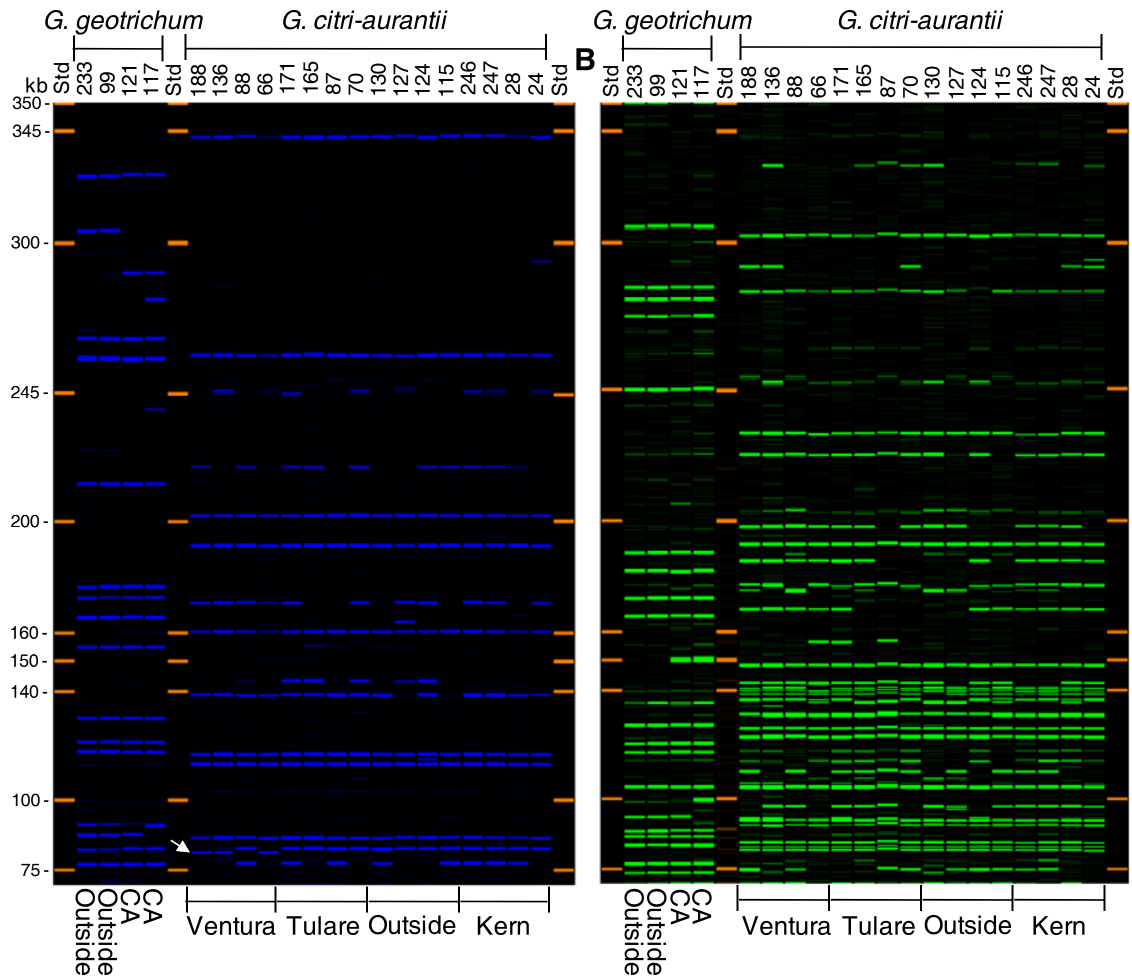

Figure 2.4. Re-constructed image of an AFLP DNA gel using Genographer (Ver. 2.1) for *Galactomyces citri-aurantii* and *G. geotrichum*. Primer pairs **A,** *Eco*R1-AC-FAM + *Mse*I-TC and **B,** *Eco*R1-TA-HEX + *Mse*I-AC were used. Four isolates of *G. geotrichum* representing two sub-populations (CA and outside CA) and sixteen isolates of *G. citri-aurantii* representing four sub-populations (Kern, Tulare, Ventura, and outside of CA) are depicted for each set of primers. The arrow indicates locus 82A that is only present in the Ventura sub-population of *G. citri-aurantii*. The GS-500-LIZ standard was used and sizes of each fragment are shown.

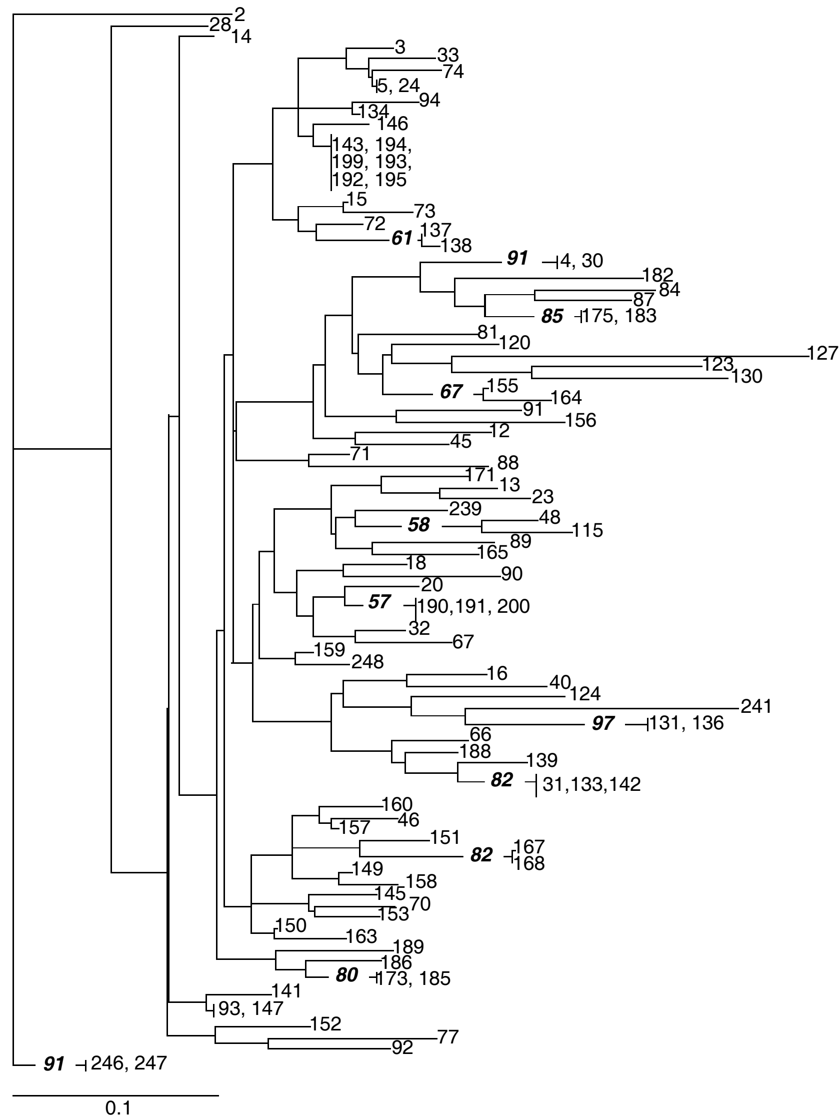

Figure 2.5. Neighbor-joining dendrogram for 97 isolates of *Galactomyces citri-aurantii* using 52 AFLP fragments (19 polymorphic fragments) generated by PAUP (Ver 4.0). Isolate numbers are shown on the right side of the tree at the branch ends. Clones are shown by a vertical line at the end of the branch followed by the isolate numbers. Numbers in bold italic at the nodes are bootstrap values based on 1000 replications and only values over 50% are shown. The scale bar is equivalent to 0.1 changes per character.

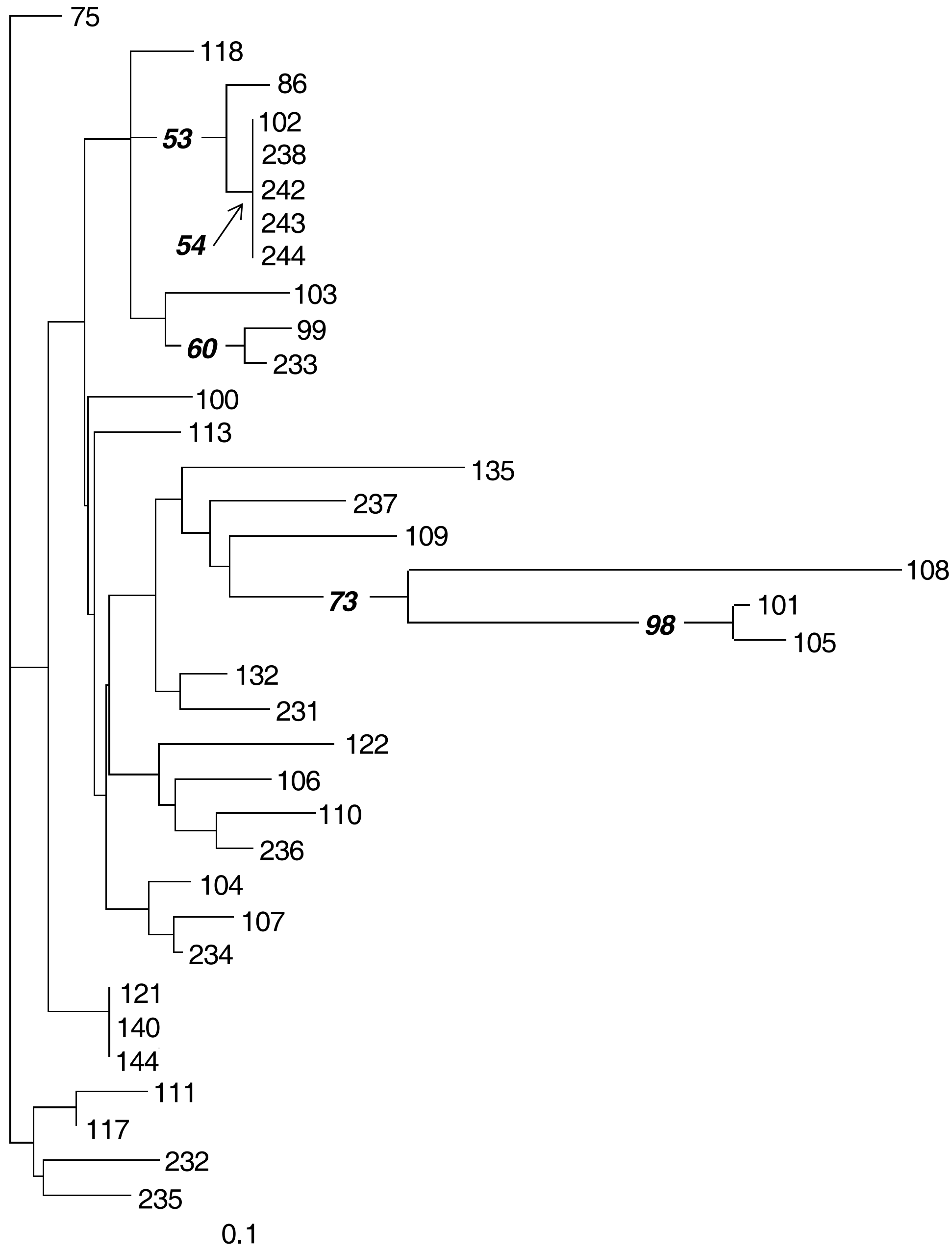

Figure 2.6. Neighbor-joining dendrogram for 36 isolates of *Galactomyces geotrichum* using 73 AFLP fragments (11 polymorphic fragments) generated by PAUP (Ver 4.0). Isolate numbers are shown on the right side of the tree at the branch ends. Clones are shown by a vertical line at the end of the branch followed by the isolate numbers. Numbers in bold italic at the nodes are bootstrap values based on 1000 replications and only values over 50% are shown. The scale bar is equivalent to 0.1 changes per character.

CHAPTER III. TOXICITY OF SELECTED DEMETHYLATION INHIBITORS TO THE POSTHARVEST PATHOGENS *GALACTOMYCES CITRI-AURANTII, G. GEOTRICHUM,* AND *PENICILLIUM* DIGITATUM AND EVALUATION OF THE RESISTANCE POTENTIAL TO PROPICONAZOLE

ABSTRACT

A diverse population of *Galactomyces citri-aurantii*, the causal pathogen of sour rot of citrus fruit, was evaluated for sensitivity to demethylation inhibiting (DMI) fungicides of the triazole group. Propiconazole was found to be highly effective in reducing mycelial growth in vitro with a mean EC_{50} value of 0.34 µg/ml for 139 isolates evaluated. For *G. geotrichum,* that causes sour rot of different fruits including stone fruits, a mean EC_{50} value of 0.14 µg/ml was determined for 33 isolates. In a comparison using additional DMI fungicides, mean EC_{50} values for 60 isolates of *G. citri-aurantii* and 20 isolates of *G. geotrichum*, were 0.27 and 0.17 µg/ml for cyproconazole, 0.25 and 0.14 µg/ml for metconazole and 1.16 and 0.73 µg/ml for tebuconazole, respectively. Propiconazole was also highly active against mycelial growth of imazalil-sensitive isolates of *Penicillium digitatum,* the pathogen that causes green mold of citrus, with a mean EC_{50} value of 0.008 µg/ml using 63 isolates. Imazalil-resistant isolates of this fungus were cross-resistant to propiconazole. When *G. citri-aurantii* and *P. digitatum* were grown at selected pH values between 3 and 9 inhibition by propiconazole occurred over the entire range. The fungicide was shown to be most effective at pH 5 when compared to the non-fungicide-amended control grown at the same pH range. In laboratory mass platings of single-spore isolates sensitive to propiconazole onto selective media, isolates with an up to 81.6-fold decrease in sensitivity to the fungicide were recovered for *P. digitatum.* For *G. geotrichum,* isolates with an approximately two-fold decrease in sensitivity were obtained. No isolates with reduced sensitivity were recovered for *G. citri-aurantii*. Propiconazole is currently being registered for postharvest use on

citrus and other crops, and the information provided will be valuable in monitoring of fungicide resistance and in designing effective fungicide application strategies.

INTRODUCTION

The sterol biosynthesis or demethylation inhibitor (DMI) propiconazole is a potential new postharvest fungicide in the United States that is planned for registration on citrus, stone fruit, and other fruit crops such as tomato. It is highly effective against several important decays including those caused by species of *Penicillium, Rhizopus,* and *Monilinia*. Most importantly, however, it is effective against sour rot caused by *Galactomyces citri-aurantii* Butler (anamorph: *Geotrichum citri-aurantii* (Ferraris) Butler) on citrus fruits and by *G. geotrichum* Butler & Petersen (anamorph: *Geotrichum candidum* Link) on stone fruits, tomato, and other crops (Butler and Petersen 1972; Butler et al., 1988; Adaskaveg et al., 2007; Cochran et al., 2009). Sour rot is not effectively managed by any other postharvest fungicide currently registered in the United States.

The two sour rot pathogens are yeast-like fungi that commonly occur in soils. Conidia are disseminated by water splash from irrigation or rain or by wind in dust particles to the surface of fruit where they can cause infections at sites of injury (Eckert, 1959; Brown, 1979; Hershenhorn et al., 1992). Although sour rot can occur on fruit in the field, it is most economically important as a postharvest decay. On citrus in California, the disease is particularly destructive on lemon, grapefruit, and mandarin that are often stored for prolonged periods, depending on market demands, at relatively high temperatures (12 to 15°C) and at high relative humidity (92 to 98%) (Eckert, 1959; Brown, 1979; Baudoin and Eckert, 1982; Baudoin and Eckert, 1985; Suprapta et al., 1996). Endo-polygalacturonases excreted by the pathogen rapidly macerate the fruit tissue, and juices dripping onto healthy fruit can rapidly spread the decay in storage (Davis and Baudoin,

1986). Outbreaks of sour rot on stone fruit have caused economic losses to the stone fruit industry in the United States. In California, propiconazole received successive emergency registrations for managing sour rot of peaches and nectarines from 2005 to 2011.

Decay of citrus fruit by species of *Penicillium* is an on-going major problem for the citrus industries. Green mold caused by *P. digitatum* (Pers.: Fr.) Sacc. is the most important postharvest disease of citrus produced in semi-arid climates like California (Eckert and Eaks, 1989). The pathogen produces very large numbers of aerially dispersed conidia that contaminate virtually every fruit in citrus orchards and packinghouses (Kanetis et al., 2007). As is the case for *Galactomyces* spp., fruit injuries are required for infection by *P. digitatum* and these occur commonly during harvest and postharvest handling (Eckert, 1959; Eckert and Eaks, 1989). Thus, handling practices that minimize fruit injuries are important to decrease the incidence of decay. Additionally, sanitation treatments that reduce the level of inoculum and postharvest fungicides are essential components in the integrated management of these decays. On citrus, several compounds, each with a different mode of action, are currently registered for postharvest use in the United States. These include the DMI-imidazole imazalil and the methyl benzimidazole carbamate thiabendazole that have been used for more than 30 years, Resistance in populations of *Penicillium* spp. against these compounds has become widespread. The anilinopyrimidine pyrimethanil, the quinone outside inhibitor (QoI) azoxystrobin, and the phenylpyrrole fludioxonil are recently registered postharvest fungicides. All these compounds are primarily used to manage citrus green mold. Only sodium ortho-phenylphenate (SOPP), a phenolic based fungicide, has some efficacy against sour rot, but concentrations required to effectively manage the decay can be phytotoxic (Brown, 1979). Furthermore, use of SOPP has been in decline over recent years due to disposal and human safety concerns (Smilanick and Sorenson, 2001). Guazatine, a guanidine fungicide is used in other parts of the world, has very good efficacy against sour rot (Wild, 1992),

but will not be registered in the United States due to its multiple active ingredients and difficulty in measuring chemical residues on fruit. Thus, no effective postharvest fungicide is currently available to the United States citrus industry for the management of sour rot.

Preliminary screening of several DMI-triazole fungicidesfor toxicity to *G. citri-aurantii* identified propiconazole and cyproconazole as highly toxic to the pathogen, whereas metconazole and tebuconazole were less effective (McKay et al., 2007). Due to the stereochemical nature of DMI compounds and site-specific binding to the cytochrome P450 14α demethylase enzyme ($P450_{14DM}$) this class of fungicides can have different specificities within and among different organisms (Köller, 1988). Their activity against *G. geotrichum* is not known and cross-resistance to the DMI-imidazole imazalil has not been evaluated.

Fungicide efficacy in the postharvest environment is dependent on a variety of factors including application method, use of fruit coatings and additives, as well as pH of treatment solutions. For example, to maintain decay control efficacy and minimize phytotoxicity on treated citrus fruit, SOPP should be applied within at a pH range between 11.7 and 12.0 (Eckert and Eaks, 1989). For imazalil a pH of 7.5 was found to be more effective in reducing green mold incidence of inoculated lemon fruit than a pH of 4.0 (Smilanick et al., 2005). This information needs to be obtained for any fungicide including propiconazole before large-scale commercial use.

The prolonged use of single chemistries to control diseases has resulted in the development of resistance in many fungicide-pathogen interactions (Georgopoulos, 1982; Smilanick et al., 2006; de Waard and Fuchs, 1982; de Waard and Nistelrooy, 1990). For example, the California citrus industry has experienced significant economic losses due to reduced efficacy of imazalil and thiabendazole against *Penicillium* decays (Harding, 1962, 1972; Eckert, 1988; Eckert and Eaks, 1989). A practical tool in estimating the risk of pathogen

populations for developing resistance is knowledge on the intrinsic resistance potential or resistance frequency to a specific fungicide. For example, resistance frequencies have been established for *P. digitatum* against pyrimethanil, azoxystrobin and fludioxonil and these data helped in the design of best management strategies for maintaining the long-term utility of these fungicides (Kanetis et al., 2010). Information on natural resistance frequencies has not been obtained for propiconazole.

Objectives of this research focused on the use of propiconazole as a new tool for management of postharvest decays with a focus on the specific needs of the citrus industry and included: i) the evaluation of sensitivities against propiconazole as compared to selected other DMI-triazole fungicides (metconazole, cyproconazole, tebuconazole) against *G. citri-aurantii* and *G. geotrichum*; ii) evaluation of the effect of pH on the activity of propiconazole against *P. digitatum* and *G. citri-aurantii;* iii) evaluation of cross-resistance in imazalil-resistant isolates of *P. digitatum* to propiconazole; and iv) estimation of natural resistance frequencies in populations of *P. digitatum*, *G. citri-aurantii*, and *G. geotrichum* to propiconazole, as well as characterization of resistant isolates that may be recovered in these studies.

MATERIALS AND METHODS

Fungicides. Fungicides used include the DMI-triazoles cyproconazole (Alto 100SL; Syngenta Crop Protection, Greensboro, NC), difenoconazole (Inspire; Syngenta Crop Protection), fenbuconazole (Indar 75WSP; Dow AgroSciences, Indianapolis, IN), metconazole (Quash 50WDG; Valent USA, Walnut Creek, CA), myclobutanil (Laredo EC; Dow AgroSciences), propiconazole (Mentor 45WP; Syngenta Crop Protection), tebuconazole (Elite 45WP, Bayer CropScience, Research Triangle Park, NC), and tetraconazole (Eminent 125SL,

Sipcam Agro USA, Durham, NC), as well as several fungicides registered for postharvest use in the United States: the DMI-imidazole imazalil (Freshgard 700; JBT FoodTech, Chicago, IL), the QoI azoxystrobin (Abound 2F, Syngenta Crop Protection), the phenylpyrrole fludioxonil (Scholar 50WP, Syngenta Crop Protection), the anilinopyrimidine pyrimethanil (Penbotec 400SC; Janssen Pharmaceutica, Titusville, NJ), and the methyl benzimidazole carbamate thiabendazole (Decco Salt No. 19; Decco US Post-Harvest, Inc., Monrovia, CA).

Fungal isolates. A total of 139 isolates of *G. citri-aurantii* were recovered from soil in citrus groves or from symptomatic citrus fruit in packinghouses throughout the citrus growing areas of California (Table 1). Twelve isolates of *G. geotrichum* were obtained from soil in citrus or nectarine orchards or from peach fruit in packinghouses in California (Table 2). An additional 14 isolates of *G. citri-aurantii* and 26 isolates of *G. geotrichum* of worldwide origin came from the collection of Dr. E. Butler, University of California, Davis. Isolates of *P. digitatum* were chosen randomly from a previous study (Table 3) (Kanetis et al. 2008).

Isolations for *Galactomyces* spp. from symptomatic fruit were made by excising a small tissue section below the epidermal area at the margin of a decay lesion and plating onto potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with 1 μg/ml fludioxonil and 500 μg/ml novobiocin (Sigma Aldrich, St. Louis, MO). Soil samples were collected 5 to 10 cm below the soil surface in commercial orchards in Central and Southern California and in the Southwestern Arizona. Each composite sample consisted of 10 soil cores that were collected around the drip line from each of 5 to 10 trees per orchard. Isolations from soil were done using a procedure by Eckert and Butler (1962) modified as follows. An aliquot of 3 g soil was placed into 100-ml Erlenmeyer flasks containing 25 ml of sterile water and was shaken at 150 rpm on an orbital shaker for 2 h to loosen conidia and mycelium from the soil particles. Aliquots of 1 ml were added to sterile tubes containing 25 ml of autoclaved lemon juice and incubated on a shaker

at 200 rpm for 48 h at 24°C. Aliquots of 50 µl of this mixture were spread onto PDA amended with 1 µg/ml fludioxonil and 500 µg/ml novobiocin using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA) set at the exponential deposition mode. After 48 to 72 h, colonies resembling *Galactomyces* spp. were excised and spread onto PDA for single spore isolation. Identification of each isolate was confirmed using a lemon juice incubation test (Suprapta el al., 1996) and by PCR with newly designed species-specific primers (McKay et al., manuscript in preparation). Between 1 (low) and 3 (high) isolates per orchard site were used for determining fungicide sensitivities.

All fungal isolates were maintained on PDA, and conidia used for fungicide sensitivity assays were obtained from 4- to 7-day old cultures. Stock cultures were stored for up to one year in sterile water at 4°C. For storage a conidial suspension (ca. 50 µl of $1x10^6$ conidia/ml) of each isolate was spread onto 6-cm PDA plates, incubated for 48 to 72 h, and 4 to 6 mycelial plugs (5 mm in diameter) were transferred into 2-ml microcentrifuge tubes containing 1 ml of sterile water.

Determination of EC_{50} values for inhibition of mycelial growth and conidial germination using the spiral gradient dilution (SGD) method. The SGD method was performed as described previously and for measurement of mycelial growth plates were inoculated with mycelium-covered cellophane strips (Förster et al., 2004). Sixteen randomly selected isolates of each *G. citri-aurantii* and *G. geotrichum* were used to determine EC_{50} values for the eight DMI-triazole fungicides listed above to inhibit mycelial growth and conidial germination. The most effective fungicides were then tested against an expanded set of isolates: 139 and 33 isolates were tested against propiconazole, and 60 and 20 isolates were tested against cyproconazole, metconazole, tebuconazole for *G. citri-aurantii* and *G. geotrichum*, respectively. For *P. digitatum,* 63 and 37 isolates were evaluated for mycelial growth inhibition by

propiconazole and imazalil, respectively. Fungicide dilutions were prepared in sterile water. Concentrations applied to 15-cm agar plates by the spiral plater for the *Galactomyces* species were 500 µg/ml, 1000 µg/ml, 1250 µg/ml and 1500 µg/ml for cyproconazole, propiconazole, metconazole, and tebuconazole respectively, and was 5000 µg/ml for the remaining fungicides. For *P. digitatum*, 20 µg/ml and 1000 µg/ml were used for propiconazole and 30 µg/ml and 1000 µg/ml were used for imazalil for DMI- sensitive and -resistant isolates, respectively.

For determining EC_{50} values for conidial germination, conidial suspensions (10^6 conidia/ml) were streaked radially along the concentration gradient. Conidial germination was evaluated after 20 h under a microscope by establishing the point along the radial streak of conidia at which 50% of the conidia within the field of view had germinated. A conidium was considered germinated when the germ tube exceeded twice the diameter of the conidium. Two or three replicate plates were prepared for each fungicide and fungal isolate per experiment and each fungicide-isolate combination was evaluated twice.

Effect of pH on activity of propiconazole against *G. citri-aurantii* and *P. digitatum*. Conidia of *G. citri-aurantii* (isolate Gc67) or *P. digitatum* (isolate 3180) at a final concentration of 1 x 10^6 conidia/ml were added to potato dextrose broth (PDB, Difco Laboratories) that was buffered by adding 2 ml of sterilized citrate buffer/35 ml of broth (Lucas 1955). Propiconazole concentrations used (0.458 µg/ml for *G. citri-aurantii* and 0.008 µg/ml for *P. digitatum*) inhibited mycelial growth by approximately 75%. The pH was adjusted to 3, 5, 7, or 9 using 1 N HCl or NaOH and was measured with a pH meter (Orion 5 Star; Thermo Fisher Scientific Inc., Waltham, MA). Aliquots of 0.2 ml of the suspensions were pipetted into a sterile 96-well microtiter plate and the plate was placed on an orbital shaker at 180 rpm at 25°C. The absorbance at 490 nm was measured at the beginning of the experiment and after 18 h (*G. citri-aurantii*) or 24 h (*P. digitatum*) using a plate reader (Wallac Victor[2] 1420 Multilabel Counter, PerkinElmer Life

Sciences, Boston, MA). The initial optical density reading was subtracted from the final reading as an estimate of growth at each pH value. Each treatment was replicated four times and the experiment was done three times.

Estimation of resistance frequencies against propiconazole in populations of *G. citri-aurantii, G. geotrichum,* and *P. digitatum* in laboratory selection studies. Conidial suspensions were prepared from randomly selected single-spore isolates that were all determined to be sensitive to propiconazole. Eleven isolates were used for *G. citri-aurantii* (5×10^8 conidia/ml), 12 isolates for *G. geotrichum* (5×10^7 conidia/ml), and 12 isolates of *P. digitatum* (1×10^8 conidia/ml). Conidial suspensions of each species were prepared from individual isolates. For *G. citri-aurantii,* mixtures with equal amounts of conidia from each of the 11 isolates were also prepared. PDA plates 15 cm in diameter were amended with propiconazole using the spiral plater set for the exponential deposition mode. Concentrations were chosen to locate the mean EC_{95} for each species approximately 10 to 15 mm from the margin of each plate and these were 3000, 1500, and 100 µg/ml propiconazole for *G. citri-aurantii*, *G. geotrichum,* and *P. digitatum* respectively. After 2 to 4 h, 60 µl of the conidial suspensions were evenly spread onto 15-cm agar plates using the spiral plater in the "lawn" deposition mode (constant deposition across plate). This resulted in a total number of conidia for each of two replicate plates of 3×10^7, 3×10^6, and 6×10^7 for the three fungi, respectively. After 7 days incubation for *G. citri-aurantii* and *G. geotrichum* and 3 days for *P. digitatum* at 24ºC, white cottony colonies greater than 1 mm in diameter for the two *Galactomyces* spp. and green sporulating colonies for *P. digitatum* were quantified on each plate and the position as distance measured from the center of the plate was recorded. A subset of up to eight colonies per selection plate for each species was transferred to non-amended PDA plates using a sterile toothpick, re-cultured, and then evaluated for their sensitivity to propiconazole using the SGE method as described above. The change in fungicide

sensitivity as compared to the original isolate was expressed as the resistance factor and was calculated as EC_{50} value of the putative resistant isolate/ EC_{50} value of the original isolate. When resistance was detected, the resistance frequency was calculated as the proportion of the number of resistant colonies recovered of the total number of conidia plated.

Estimation of natural resistance frequencies to propiconazole in populations of *G. citri-aurantii* from lemon grove soils. Soil samples from ten lemon groves in Ventura Co., CA, were obtained as described above in the winter season when wet conditions commonly occur in California. Samples from all sites within each grove were combined for a total of ten composite samples for the ten groves. Samples were processed as described above except that 1 µg/ml of fludioxonil was added to the soil-lemon juice mixture that was shaken for 24 h. Propiconazole (3000 µg/ml) was spirally applied in the exponential mode to 15-cm PDA plates amended with 1 µg/ml fludioxonil and 500 µg/ml novobiocin. Using this concentration of propiconazole, a radial concentration range from 0.2 µg/ml at the perimeter of the plate to 30 µg/ml at the center of the plate is obtained. After 2 to 4 h the soil-lemon juice mixture was applied using the spiral plater set for the "lawn" deposition mode. After 4 days of incubation at 24ºC, colonies characteristic of *G. citri-aurantii* growing in the higher propiconazole concentration range were transferred to PDA plates using sterile toothpicks and were evaluated for their sensitivity against propiconazole as described previously.

Statistical analysis of data. For repeated experiments, homogeneity of variances was tested using Bartlett's test of homogeneity. Because variances were homogeneous for each experiment, data were combined and further statistically analyzed (SAS version 9.1, SAS Institute Inc., Cary, NC). Non-transformed and transformed (log_{10}) mean EC_{50} values, as well as resistant factors were compared using analysis of variance (ANOVA) or general linear model (GLM) and least significant difference (LSD) mean separation procedures of SAS.

Mean EC_{50} values for populations of each fungal were plotted in frequency histograms. For this, EC_{50} values were first log_{10}-transformed and the bin (i.e., EC_{50} category) width h for the transformed data was determined using the method of Scott (1979) with the equation:

$$h_n = 3.49sn^{-1/3}$$

where s is an estimate of the standard deviation and n is the number of isolates used for each fungicide evaluated. Standard deviations were calculated from mean log_{10}-transformed EC_{50} values for all isolates used in each fungicide evaluation. The number of bins was then determined over the range of EC_{50} values for each fungicide. Shapiro-Wilk probability values were calculated for log_{10}-transformed values for each fungicide to determine if data were normally distributed.

To determine if cross-resistance between imazalil and propiconazole was present in *P. digitatum* populations, mean EC_{50} values for each fungicide were plotted in pair-wise comparisons. A regression analysis was then done using ANOVA and REG procedures (SAS version 9.1). Mean absorbance values from the pH study were graphed and standard errors for each pH value were determined.

RESULTS

Sensitivities of mycelial growth and conidial germination of *G. citri-aurantii* and *G. geotrichum* to selected fungicides. Mean effective concentrations to inhibit mycelial growth of *G. citri-aurantii* by 50% (EC_{50} values) for eight DMI triazole fungicides indicated that cyproconazole, metconazole, propiconazole, and tebuconazole were the most effective compounds with EC_{50} values of 0.27 µg/ml, 0.25 µg/ml, 0.34 µg/ml, and 1.16 µg/ml, respectively (Table 4). Fenbuconazole was the least effective fungicide with an EC_{50} value of >29.3 µg/ml. Higher concentrations of this fungicide were not evaluated because this concentration exceeded

its water solubility. None of the currently registered postharvest citrus fungicides including azoxystrobin, fludioxonil, imazalil, pyrimethanil, and thiabendazole was effective against growth of the citrus sour rot pathogen and EC_{50} values were all higher than 30 µg/ml. The most effective DMI triazole fungicides were also evaluated against conidial germination of *G. citri-aurantii* and mean EC_{50} values were determined to be 0.59, 0.39, 0.39, and 1.57 µg/ml for propiconazole, cyproconazole, metconazole, and tebuconazole, respectively. Values for the five registered citrus postharvest fungicides all exceeded 50 µg/ml.

The four most effective DMI triazole fungicides were used in evaluations of activity against mycelial growth for additional isolates of *G. citri-aurantii* and for isolates of *G. candidum.* For *G. citri-aurantii,* the mean EC_{50} value for propiconazole for 139 isolates was 0.34 µg/ml with a range from 0.10 µg/ml to 0.83 µg/ml.

For metconazole, cyproconazole, and tebuconazole (60 isolates evaluated each), mean EC_{50} values were 0.25 µg/ml, 0.27 µg/ml, and 1.16 µg/ml, with ranges from 0.13 to 0.55 µg/ml, 0.14 to 0.50 µg/ml, and 0.40 to 2.21 µg/ml, respectively. Log_{10}-transformed values for each fungicide were normally distributed within the population as indicated by Shapiro-Wilk probability values of P = 0.502, P = 0.579, P = 0.372, P = 0.341, respectively for propiconazole, metconazole, cyproconazole, and tebuconazole, respectively. The frequency distribution of EC_{50} categories as calculated by Scott's formula (Scott, 1979) for each fungicide is shown in the histograms of Figure 1A to D.

For isolates of *G. geotrichum*, mean EC_{50} values for propiconazole (33 isolates), metconazole, cyproconazole, and tebuconazole (20 isolates for each fungicide) were 0.14 µg/ml, 0.14 µg/ml, 0.17 µg/ml, and 0.73 µg/ml with ranges from 0.06 to 0.30 µg/ml, 0.07 to 0.22 µg/ml, 0.06 to 0.26 µg/ml, and 0.29 to 1.69 µg/ml, respectively. Shapiro-Wilk probability values were P = 0.729, P = 0.279, P = 0.129, and P = 0.982, and thus, log_{10}-transformed values for each

fungicide were normally distributed. The frequency distribution of EC_{50} categories for each fungicide is shown in the histograms of Figure 2A-D.

Sensitivity of mycelial growth of *P. digitatum* to propiconazole and imazalil, and cross-resistance between these fungicides. The frequency histograms for each of the two fungicides show the presence of two subpopulations among isolates of *P. digitatum*: one with high sensitivity and another one with reduced sensitivity (Fig. 3A,B). For the highly sensitive subpopulations, mean EC_{50} values were 0.008 µg/ml and 0.005 µg/ml with ranges from 0.003 to 0.014 µg/ml and 0.003 to 0.007 µg/ml for propiconazole (63 isolates used) and imazalil (37 isolates used), respectively. For the less sensitive subpopulations, mean EC_{50} values were 0.487 µg/ml and 0.103 µg/ml with ranges from 0.146 to 1.151 µg/ml and 0.024 to 0.329 µg/ml for propiconazole and imazalil, respectively. For *P. digitatum,* only $\log_{10}$-transformed values for the imazalil-sensitive subpopulation were normally distributed ($P = 0.294$). The imazalil-resistant, as well as the propiconazole-sensitive and -resistant subpopulations of *P. digitatum* were not normally distributed based on significant Shapiro-Wilk probability values ($P < 0.05$).

To evaluate the presence of cross-resistance between propiconazole and imazalil among 37 isolates of *P. digitatum*, $\log_{10}$-transformed EC_{50} values for propiconazole were regressed against those for imazalil. Two clusters of isolates were found in this regression (Fig. 4). The regression line for all of the data had a coefficient of determination of $r^2=0.72$ (Fig. 4). One of the clusters consisted of isolates sensitive to the two fungicides, whereas the other cluster contained isolates with reduced sensitivities.

Effect of pH on activity of propiconazole against *G. citri-aurantii* and *P. digitatum*. Growth of both fungi in non-amended PDB in microtiter plate wells that were inoculated with conidia was significantly higher at pH 5 than at pH 3, 7, or 9 (Fig. 5). Growth of *G. citri-aurantii* was equally low at pH 3, 7, and 9, whereas for *P. digitatum* the lowest amount of mycelial

production was observed at pH 9. In propiconazole-amended media, both fungi were inhibited over the entire pH range tested as compared to media not amended with the fungicide. Although for both species overall inhibition by propiconazole was highest at pH 9, inhibition relative to the non-amended control was highest at pH 5 (Fig. 5).

Estimation of resistance frequencies against propiconazole in populations of *G. citri-aurantii, G. geotrichum,* and *P. digitatum* in laboratory selection studies. Among the 11 isolates of *G. citri-aurantii* used in this study where conidial suspensions of individual isolates were subjected to propiconazole, colonies growing at propiconazole concentrations higher than the EC_{95} values were observed for two isolates (Gca22 and Gca137). These colonies grew in a propiconazole concentration range between 9.0 and 29.8 µg/ml on the selection plates (Table 5). For each of the two isolates, sub-cultures of six of these colonies grown on non-amended PDA were characterized for their fungicide sensitivity. With mean resistance factors of 0.97 and 0.98, sensitivities were not significantly different from the original isolate (Table 5). When a mixture of conidia from the 11 isolates of *G. citri-aurantii* was subjected to selection, two colonies were found growing at the higher fungicide concentration range. After sub-culturing, both of these colonies showed an EC_{50} value within the propiconazole sensitivity range of all isolates evaluated for this species (data not shown).

For *G. geotrichum,* a ten-fold lower concentration of conidia was applied to the selection plates than for *G. citri-aurantii* because numerous slow-growing colonies developed in the higher fungicide concentration range that could not be sub-cultured without cross-contamination. Among the 12 individual isolates subjected to propiconazole, putatively resistant colonies growing at propiconazole ranges between 3.0 and 9.8 µg/ml were obtained from four isolates (Table 6). Mean EC_{50} values for selected colonies from two isolates (Gc231, 6 colonies; and Gc244, 5 colonies) were not significantly different from the original isolates. Mean EC_{50} values

for selected colonies from the other two isolates (Gc75 and Gc121, 6 colonies each), however, were significantly higher than for the original isolates (i.e., 0.09 µg/ml for Gc75 and 0.07 µg/ml for isolate Gc121) with a range from 0.11 to 0.21 µg/ml for colonies recovered for isolate Gc75 and 0.13 to 0.16 µg/ml for colonies for isolate Gc121. Still, resistance factors only ranged from 1.3 to 2.3 with means of 1.71 and 1.97 for isolates Gc75 and Gc121, respectively (Table 6).

For *P. digitatum*, laboratory selections were done using conidial suspensions from isolates sensitive and less-sensitive to propiconazole. Of the total of twelve isolates used, colonies growing at concentrations of propiconazole >EC_{95} were identified for five sensitive isolates and for four less-sensitive isolates. For the selections from sensitive isolates, EC_{50} values were determined for a total of six colonies after sub-culturing on non-amended PDA. All isolates had significantly higher EC_{50} values (0.20 to 0.45 µg/ml, mean 0.35 µg/ml) than the original isolates (0.006 to 0.010 µg/ml, mean 0.007 µg/ml) (Table 7). Resistance factors ranged from 14.3 to 81.6 (mean 45.5) and resistance frequencies ranged from 8.0×10^{-8} to 1.2×10^{-7} For the selections from less-sensitive isolates, EC_{50} values for a total of six sub-cultured colonies also were significantly higher (1.29 to 2.88 µg/ml, mean 2.06 µg/ml) than the original isolates (0.33 to 0.63 µg/ml, mean 0.45 µg/ml) (Table 7). Resistance factors ranged from 2.3 to 7.9 with a mean of 5.1 and resistance frequencies ranged from 8×10^{-8} to 1.6×10^{-7}.

Estimation of natural resistance frequencies to propiconazole in populations of *G. citri-aurantii* from lemon grove soils. Propagules of *G. citri-aurantii* were enriched from mixed soil populations by shaking soil samples in lemon juice for 24 h and between 5×10^5 and 2×10^6 conidia were deposited on selection plates amended with propiconazole. In repeated experiments, a total of 6 to 10 colonies per plate were observed growing at propiconazole concentrations >EC_{95}. These colonies were very slow-growing and 4 to 6 colonies per plate were sub-cultured.

When tested for propiconazole sensitivity, EC_{50} values of none of these colonies were significantly higher than the highest value in the population (i.e., 0.83 µg/ml).

DISCUSSION

This research presents in vitro sensitivities for the DMI-triazoles propiconazole, cyproconazole, metconazole, and tebuconazole against the two sour rot pathogens *G. citri-aurantii* and *G. geotrichum*, sensitivities of additional DMI-triazoles against *G. citri-aurantii*, and a sensitivity range for propiconazole against *P. digitatum.* Our primary focus was on the sour rot pathogen of citrus fruits, *G. citri-aurantii,* and the specific needs of the citrus industry to identify and register a highly effective fungicide to manage this decay. This need was supported by our findings that none of the currently registered citrus postharvest fungicides, with the exception of SOPP, is effective against this pathogen. Thus, a larger number of isolates was evaluated for this pathogen than for the other two fungal species. Additionally, an emphasis was put on propiconazole because of its effectiveness against *Galactomyces* spp., as well as support from the registrant and regulatory agencies for introduction of this compound. The green mold pathogen of citrus, *P. digitatum*, was included due to its central role in citrus postharvest decay management, its history of fungicide resistance development (Eckert and Wild, 1983; Holmes and Eckert, 1999) and because a DMI-imidazole fungicide is already registered on citrus.

The baseline sensitivity of a fungicide is defined as the sensitivity range of a fungal population before the introduction of the specific fungicide (Russell, 2004). None of the DMI-triazole fungicides evaluated have ever been used commercially by the California citrus industry and thus, the population of *G. citri-aurantii* has not been exposed to these fungicides. Still, the sensitivity ranges we are presenting should not be called baseline sensitivity ranges. Although not

effective against this pathogen, this is because the DMI-imidazole compound imazalil has been in use for many years, specifically for control of Penicillium decays. Cross-resistance between the two DMI sub-classes is well documented (Köller, 1988) and was also demonstrated in our study (see below). For *G. geotrichum*, many of isolates included in our study were collected before the introduction of DMI fungicides and thus, for this species the sensitivity ranges presented could be described as baseline sensitivities.

A wide range of effective concentrations to inhibit growth of *G. citri-aurantii* by 50% was obtained for the eight DMI-triazole fungicides evaluated. In comparisons of the most effective fungicides (i.e., cyproconazole, propiconazole, metconazole, and tebuconazole), mean 50% inhibitory concentrations were all lower for *G. geotrichum* than for *G. citri-aurantii*. For both species, however, inhibitory concentrations were similar for cyproconazole, propiconazole, and metconazole and higher for tebuconazole. Differences in sensitivity among the DMI compounds have been observed for other plant pathogenic fungi (Karaoglanidis and Thanassoulopoulos, 2003) and can be explained by different affinities for the triazole binding site on the cytochrome P450 isoenzyme 51 (CYP51/lanosterol-14R-demethylase) (Parker et al., 2011). For *P. digitatum,* we determined that isolates were cross-resistant for propiconazole and the DMI-imidazole imazalil. Resistance to triazole and imidazole fungicides has been correlated with mutations in the gene encoding the CYP51 in several fungi, including *P. digitatum* (Hamamoto et al., 2000). For the resistant isolates in our study, however, there was no clear correlation between the degrees of insensitivity to the two DMI sub-classes. Differences in binding sites of the fungicides during fungal sterol biosynthesis again could be responsible for this lack of correlation. All DMI fungicides, however, inhibit demethylation at position 14 of lanosterol or 24-methylene dihydrolanosterol which are precursors of sterols in fungi (Scheinpflug, 1988, Köller, 1988). In a study with *Mycosphaerella fijiensis*, the same mutations

responsible for resistance to specific DMI fungicides were not correlated with propiconazole insensitivity (Cañas-Gutiérrez et al., 2009) indicating that these mutations may not impact the binding site of propiconazole to the target enzyme. It is therefore possible that propiconazole may be partially effective against decay caused by imazalil-resistant populations of *P. digitatum* that commonly occur in California packinghouses. Still, use of propiconazole after registration is recommended only as a mixture with a fungicide of a different class (i.e, azoxystrobin, fludioxonil, pyrimethanil, or possibly thiabendazole) to avoid exacerbation of the widespread DMI resistance.

For *G. citri-aurantii*, we used 139 isolates in the sensitivity tests for propiconazole. Based on the formula described by Leung et al. (1993) of $N = \log(1 - P)/\log(1 - F)$, where N is the sample size of the population and P is the probability of detecting an outlier at frequency F in the population, 97.8% of all *G. citri-aurantii* isolates will have EC_{50} values for inhibition of mycelial growth by propiconazole within the range we have described at a 95% confidence level. For *G. geotrichum* with twenty isolates evaluated, only 91.3% of all isolates will have EC_{50} values within the described range. However, because isolates were of worldwide origin and soil samples were obtained from a variety of agricultural systems, the sensitivity range that we determined is probably close to the actual range that would be based on a larger collection of isolates.

We found that growth of both citrus pathogens, *G. citri-aurantii* and *P. digitatum,* was higher at pH 5 than at pH 3, 7, or 9. Optimum growth of *P. digitatum* was previously reported in the low pH range and for *G. citri-aurantii* an optimum of pH 6 was determined (Eckert and Eaks, 1989; Suprapta et al., 1996). Thus, these fungi are adapted to the acidic environment of citrus fruit. Depending on the level of fruit maturity, the pH of lemon albedo and flavedo has been determined as between 5.1 and 5.6 (Smilanick, et al., 2003).

Inhibition of conidial germination and mycelial growth of *G. citri-aurantii* and *P. digitatum* by propiconazole was highest at pH 5 as compared to the controls grown at the same pH range without the fungicide. Growth, however, was significantly reduced in the presence of propiconazole over the entire pH range evaluated. Postharvest fungicides are applied over a wide range of pH conditions depending if the treatment is applied in water or in a fruit coating. Additionally, the fruit surface and fruit juices diffusing from wounds can modify the pH in the micro-environment. Its activity over a wide pH range is one characteristic that can make propiconazole a reliable treatment for postharvest use. In similar studies by others, pyrimethanil, another postharvest citrus fungicide, was effective against conidial germination of *P. digitatum* over a wide range of pH (Smilanick et al., 2005). In contrast, for imazalil conidial germination of *P. digitatum* was most effectively reduced at pH values above 7, whereas at values of less than 5, germination was much less affected (Smilanick et al., 2003). A higher efficacy of imazalil at more basic pH conditions was also shown for *P. italicum* and the reduced activity at lower pH values was ascribed to decreased uptake of the fungicide by the mycelium of the fungus as a result of changes in electric charge of the molecule (Siegel et al., 1977). In contrast, propiconazole has a pKa of 1.09 and is hydrolytically stable over a pH range from 1 to 13 (Tomlin, 2003), and thus, the molecule is unlikely to undergo alterations to the charge as a result of the pH of the media that would directly affect uptake by mycelium.

In laboratory selections, isolates of *P. digitatum* with reduced sensitivity against propiconazole were obtained from mass platings of conidia from several single-spored propiconazole-sensitive isolates onto selective media. Resistance factors with a maximum of 81.6 were calculated for the recovered isolates. Maximum EC_{50} values were 0.45 µg/ml and thus, only moderately resistant isolates were obtained. When isolates moderately resistant to propiconazole (EC_{50} values 0.33 to 0.63 µg/ml) were plated onto propiconazole-amended media, resistance

factors of the recovered isolates ranged from 2.3 to 7.9. We only evaluated a total of twelve colonies growing in the high-concentration range of propiconazole and thus, these data may be biased. Still, these findings could be explained by the quantitative type of resistance of DMI fungicides where mutations accumulate step-wise and multiple selection processes have to occur to reach high levels of resistance. Additionally, because of the large difference in resistance factors observed when using conidia from sensitive (mean resistant factor = 45.5) as compared to moderately resistant isolates (mean resistant factor = 5.1), there does not seem to be a linear relationship between increase in insensitivity and number of selection processes. Resistance frequencies in our selection studies ranged from 8.0×10^{-8} to 1.6×10^{-7} and thus, were similar as for fludioxonil where resistance frequencies from 9.7×10^{-8} to 1.3×10^{-7} were obtained previously in mass platings of mixed conidial suspensions from 21 to 42 isolates of *P. digitatum* to selective media (Kanetis et al., 2010). In contrast, in this latter study resistance frequencies for pyrimethanil were significantly higher and ranged from 6.9×10^{-5} to 1.1×10^{-4}.

No isolates with stable reduced sensitivity to propiconazole were recovered for *G. citri-aurantii* in these studies using conidial suspensions from single isolates. Resistant isolates were also not recovered in soil population enrichment assays. In these latter tests, diverse populations were sampled from citrus groves where genetic variability from various selection processes and sexual recombination could have occurred. The propiconazole sensitivity range for the 139 isolates from diverse origins evaluated was rather narrow with a factor of less than 10. Apparently, although *G. citri-aurantii* populations have been exposed to the DMI imazalil for many years, selection for DMI resistance has not occurred, possibly because the imidazole imazalil is not very active against this pathogen and the fungicide is only used in the packinghouse. For other fungi, a wide range in baseline sensitivities has been attributed with an increased risk of resistance development due to directional selection toward less sensitive

biotypes with repeated fungicide applications (Köller and Scheinpflug, 1987). For example, propiconazole sensitivity among *Colletotrichum cereale* isolates that had not been previously exposed to the fungicide ranged from 0.025 μg/ml to 0.35 μg/ml, a greater than 100-fold range in baseline sensitivity (Wong and Midland, 2007). In this study, when repeated applications of propiconazole had been made to soils, resistant isolates of the species were recovered.

For *G. geotrichum*, resistance factors for isolates obtained in selections using two isolates of *G. geotrichum* ranged between 1.3 and 2.3 and EC_{50} values were within the sensitivity range of the worldwide collection of 13 isolates. Compared to the moderately resistant isolates selected from sensitive isolates of *P. digitatum* with a mean resistance factor of 45.5, the range of resistance factors from 1.3 to 2.3 for *G. geotrichum* is relatively low in comparison. Therefore, our data indicate that species of *Galactomyces* may have an intrinsic lower risk for resistance development to DMI fungicides. Natural resistance frequencies and the risk of resistance development are dependent on the organism being exposed and the selection agent. Biological as well as genetic characteristics of a pathogen may determine the risk. Thus, for *Penicillium* species, the high reproductive potential and heterokaryosis have been attributed to the increased risk of resistance development (Kanetis et al., 2010). As indicated above, differences in resistance frequencies among fungicides have been previously reported for *P. digitatum* with higher frequencies for pyrimethanil as compared to fludioxonil, while no resistant isolates were recovered for azoxystrobin (Kanetis et al., 2010). Similarly, in laboratory studies using mass platings of single-spore isolates of the pome fruit pathogen *P. expansum*, stable resistant isolates were readily obtained for fludioxonil and pyrimethanil, but not for the DMI fungicide difenoconazole (Adaskaveg and Förster, 2010).

The establishment of sensitivity ranges in pathogen populations before the commercial introduction of a new fungicide has become an essential component in the implementation of

anti-resistance strategies (Brent, 1988, 2007). By routine monitoring for changes in sensitivity in the target populations, rotations and mixtures between different chemical classes effective against the specific pathogen can be utilized before resistant populations cause crop losses. Multiple alternatives are available for the management of Penicillium decays to apply these strategies. For sour rot, however, propiconazole will be the only highly effective treatment and it is essential that its efficacy is maintained until eventually new treatments become available. Although our studies indicate that potentially a lower risk for resistance development exists for *Galactomyces* species against DMI fungicides, microorganisms with their often immense reproductive potential have the capacity to adapt to their changing environment by different means.

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Table 3.1. Isolates of *Galactomyces citri-aurantii* used in this study

No. of isolates	Isolate No.	Collection date	Source	Sample location	Geographic origin
1	1	2007	Grapefruit fruit	Packinghouse	Ventura Co. CA
3	2-4	2006	Lemon fruit	Packinghouse	Kern Co. CA
2	5-6	2006	Lemon fruit	Packinghouse	Riverside Co. CA
4	7-10	2006-2009	Lemon fruit	Packinghouse	Tulare Co. CA
44	11-54	2006-2008	Lemon fruit	Packinghouse	Ventura Co. CA
4	55-58	2009	Mandarin fruit	Packinghouse	Kern Co. CA
1	59	2006	Navel orange fruit	Packinghouse	Riverside Co. CA
3	60-62	2007	Navel orange fruit	Packinghouse	Tulare Co. CA
1	63	2006	Valencia orange fruit	Packinghouse	Riverside Co. CA,
2	64-65	2006	Valencia orange fruit	Packinghouse	Tulare Co. CA
18	66-83	2007, 2008	Soil	Lemon grove	Ventura Co. CA
1	84	2007	Soil	Navel orange grove	Kern Co. CA
36	85-120	2008	Soil	Navel orange grove	Tulare Co. CA
4	121-124	2007, 2008	Soil	Valencia orange grove	Tulare Co. CA
1	125	2007	Soil	Valencia orange grove	Ventura Co. CA
2	126-127	NA[a]	Soil	NA[a]	Argentina
1	128	NA	Soil	NA	FL
2	129-130	NA	Soil	NA	China
2	131-132	NA	Soil	NA	Israel
1	133	NA	Soil	NA	Mexico
1	134	NA	Soil	NA	China
1	135	NA	Soil	NA	Trinidad
1	136	NA	Soil	NA	TX
1	137	NA	NA[a]	NA	NA[a]
2	138-139	NA	NA	NA	NA
Total=139					

[a] Information not available.

Table 3.2. Isolates of *Galactomyces geotrichum* used in this study

No. of isolates	Isolate No.	Collection date	Source	Sample location	Geographic origin
5	1-5	2007, 2008	Soil	Citrus orchard	Ventura Co., CA
6	6-11	1957, 1963, 1969, 1984, 1986	Soil	NA[a]	CA
1	12	1986	Soil	Orange grove	Yolo Co., CA
1	13	1973	Soil	NA	PA
1	14	1968	Soil	NA	Puerto Rico
1	15	NA	Soil	NA	Riverside Co., CA
1	16	1963	Soil	NA	Brazil
1	17	1981	Soil	Citrus grove	Costa Rica
2	18-19	1963	Soil	NA	Egypt
2	20-21	1974	Soil	Guava planting	India
1	22	1963	Soil	NA	Israel
3	23-25	1963	Soil	NA	Japan
1	26	1970	Soil	Orange grove	Turkey
1	27	1963	Soil	NA	Rhodesia
5	28-32	2000, 2005, 2006	Peach fruit	Packinghouse	Fresno Co., CA
1	33	2008	Soil	Nectarine orchard	Fresno Co., CA
Total=33					

[a] Information not available.

Table 3.3 Isolates of *Penicillium digitatum* used in this study

No. of isolates	Isolate No.	Collection date	Source (fruit)	Geographic origin
2	1-2	2003	Navel orange	Fresno Co., CA.
1	3	2004	Navel orange	Kern Co., CA
6	4-9	2003, 2004	Lemon	Kern Co., CA
6	10-15	2003, 2004	Navel orange	Riverside Co., CA
12	16-27	2003, 2004	Lemon	Riverside Co., CA
3	28-30	2003	Grapefruit	Riverside Co., CA
1	31	2003	Navel orange	San Bernardino Co., CA
5	32-36	2003	Navel orange	Tulare Co., CA
10	37-46	2003	Lemon	Tulare Co., CA
8	47-54	2003, 2004	Lemon	Ventura Co., CA
9	55-63	2003, 2004	Lemon	Yuma Co., AZ
Total=63				

Table 3.4. Effective concentrations for 50% reduction of mycelial growth (EC_{50} values) of isolates of *Galactomyces citri-aurantii* exposed to selected demethylation inhibitor (DMI)-triazole fungicides and currently registered postharvest fungicides of citrus

Fungicide	Registration status	Class	Mean EC_{50} (µg/ml)[a]
Cyproconazole	Experimental	DMI-triazole	0.27 D
Difenconazole	Experimental	DMI-triazole	2.3 C
Fenbuconazole	Experimental	DMI-triazole	29.3[c] A
Metconazole	Experimental	DMI-triazole	0.25 D
Myclobutanil	Experimental	DMI-triazole	10.3 B
Propiconazole	Experimental	DMI-triazole	0.34 D
Tebuconazole	Experimental	DMI-triazole	1.16 D
Tetraconazole	Experimental	DMI-triazole	14.9 B
Azoxystrobin	Registered	QoI	56.4[c] B
Fludioxonil	Registered	Phenylpyrrole	31.0[c] C
Imazalil	Registered	DMI-imidazole	32.7 C
Pyrimethanil	Registered	Anilinopyrimidine	≥ 60.0[c] A
Thiabendazole	Registered	Methyl benzimidazole carbamate	66.1[c] A

[a] EC_{50} values for inhibition of mycelial growth were determined using the spiral gradient dilution method. Mean values are based on 139 isolates for propiconazole, 60 isolates for cyproconazole, tebuconazole, and metconazole, and 16 isolates for all other fungicides.

[b] Statistical analyses were done separately for experimental and registered fungicides. Means followed by the same letter within a column are not significantly different ($P<0.01$) following analysis of variance and least significant difference mean separation procedures.

[c] Calculated EC_{50} value exceeds the water solubility of the fungicide active ingredient at 20°C.

Table 3.5. Characteristics of isolates of *G. citri-aurantii* recovered from propiconazole-amended selection plates

Original isolate[a]	EC_{50} value of original isolate (µg/ml)[b]	Range of propiconazole concentrations (µg/ml) used for colony selection[c]	No. of recovered colonies	Mean EC_{50} value for recovered colonies (µg/ml)[d]	Mean resistance factor[e]
Gca22	0.64 Aa[f]	16.4 - 33.6	6	0.62 Aa	0.98 A
Gca137	0.61 Aa	9.0 - 29.8	6	0.59 Aa	0.97 A
Mean	0.62 a			0.61 a	

[a] Original isolates (5 x 10^8 conidia/ml) were evenly spread onto propiconazole-amended potato dextrose agar (PDA) plates using a spiral plater (see Materials and Methods).

[b] EC_{50} values were determined using the spiral gradient dilution method.

[c] Range of propiconazole concentrations on the selection plates where putatively resistant isolates were recovered.

[d] Mean EC_{50} values of selected colonies after sub-culturing on non-amended PDA.

[e] Mean resistance factor of selected colonies based on the ratio of EC_{50} values of recovered isolates to original isolates.

[f] Values followed by the same letter are not significantly different ($P<0.01$) following an analysis of variance and least significant difference mean separation procedures. Upper case letters are for comparisons between isolates (columns), whereas lowercase letters are for comparisons within rows.

Table 3.6. Characteristics of isolates of *G. geotrichum* recovered from propiconazole-amended selection

Original isolate[a]	EC_{50} value of original isolate (µg/ml)[b]	Range of propiconazole concentrations (µg/ml) used for colony selection[c]	No. of recovered colonies	Mean EC_{50} value for recovered colonies (µg/ml)[d]	Mean resistance factor[e]
Gc75	0.09 Bb[f]	6.06 – 9.75	6	0.15 Aa	1.71 B
Gc121[a]	0.07 Bb	2.96 – 9.75	6	0.14 Aa	1.97 B
Gc244	0.21 Aa	7.68 – 9.75	5	0.21 Aa	0.98 A
Gc231	0.15 Aa	7.68 – 9.75	6	0.14 Aa	0.98 A
Mean	0.13 b			0.16 a	

[a] Original isolates (5 x 10^7 conidia/ml) were evenly spread onto propiconazole-amended potato dextrose agar (PDA) plates using a spiral plater (see Materials and Methods).

[b] EC_{50} values were determined using the spiral gradient dilution method.

[c] Range of propiconazole concentrations on the selection plates where putatively resistant isolates were recovered.

[d] Mean EC_{50} values of selected colonies after sub-culturing on non-amended PDA.

[e] Mean resistance factor of selected colonies based on the ratio of EC_{50} values of recovered isolates to original isolates.

[f] Values followed by the same letter are not significantly different (P<0.01) following an analysis of variance or general linear model and least significant difference mean separation procedures. Upper case letters are for comparisons between isolates (columns), whereas lowercase letters are for comparisons within rows.

Table 3.7. Characteristics of isolates of *P. digitatum* recovered from propiconazole-amended selection

Original Isolate[a]	EC_{50} value of original isolate (µg/ml)[b]	Range of propiconazole concentrations (µg/ml) used for colony selection[c]	No. of recovered colonies	Mean EC_{50} value of recovered colonies (µg/ml)[d]	Resistance factor[e]	Mean Resistance factor[e]
Pd 3117-S	0.008	0.48	1	0.45	64.29	45.5 a
Pd 3140-S	0.008	0.34	1	0.33	27.67	
Pd 3177-S	0.010	0.27	1	0.24	18.38	
Pd 3180-S	0.006	0.19	1	0.20	14.29	
Pd 3185-S	0.006	0.43, 0.54	2	0.44	81.64	
Mean	0.007 Bb			0.35 Ba		
Pd 3113-R	0.47	51.3	1	1.29	2.75	5.1 b
Pd 3121-R	0.36	54.4, 57.8	2	2.88	7.91	
Pd 3122-R	0.33	48.4	1	2.47	7.39	
Pd 3128-R	0.63	43.0, 45.6	2	1.41	2.25	
Mean	0.45 Ab			2.06 Aa		

[a] Original isolates (10^8 conidia/ml) were evenly spread onto propiconazole-amended potato dextrose agar (PDA) plates using a spiral plater (see Materials and Methods). Isolate designations: S = sensitive, R = reduced sensitivity to propiconazole.

[b] EC_{50} values were determined using the spiral gradient dilution method.

[c] Range of propiconazole concentrations on the selection plates where putatively resistant isolates were recovered.

[d] Mean EC_{50} values of selected colonies after sub-culturing on non-amended PDA.

[e] Mean resistance factor of selected colonies based on the ratio of EC_{50} values of recovered isolates to original isolates.

[f] Values followed by the same letter are not significantly different ($P<0.01$) following an analysis of variance or general linear model and least significant difference mean separation procedures. Upper case letters are for comparisons between isolates (columns), whereas lowercase letters are for comparisons within rows.

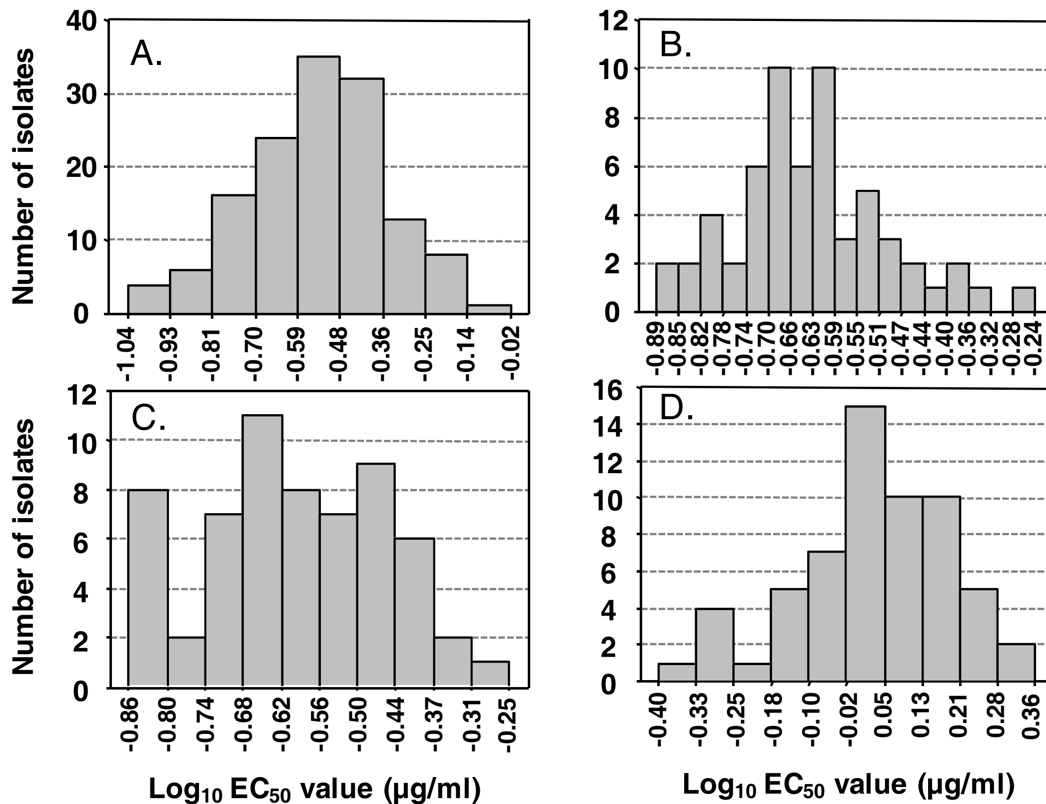

Figure 3.1. Frequency histograms of effective concentrations to inhibit mycelial_growth of *Galactomyces citri-aurantii* by 50% (EC_{50} values) for **A,** propiconazole (n=139), **B,** metconazole (n=60), **C,** cyproconazole (n=60), and **D,** tebuconazole (n=60) as determined by the spiral gradient dilution method. Bar height indicates the total number of isolates within each bin, and bin width (0.11 for propiconazole, 0.04 for metconazole 0.06 for cyproconazole, and 0.07 for tebuconazole) was based on Scott's method (Scott, 1979).

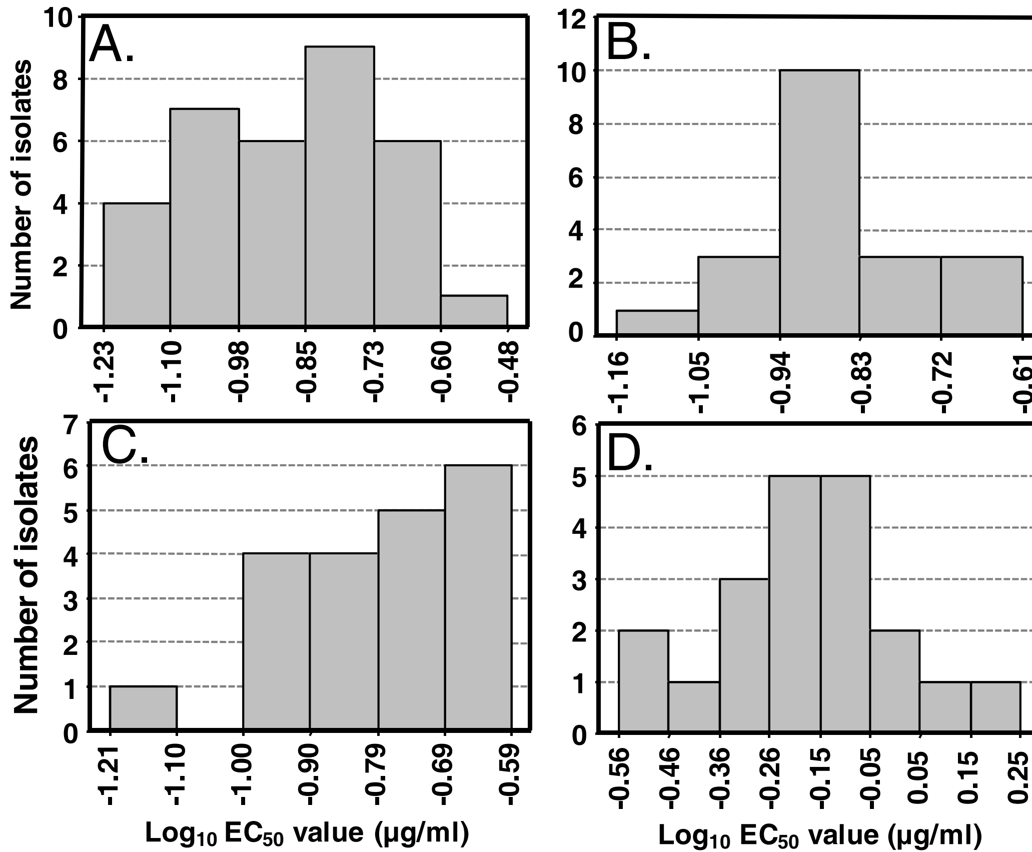

Figure 3.2. Frequency histograms of effective concentrations to inhibit mycelial growth of *Galactomyces geotrichum* by 50% (EC_{50} values) for **A,** propiconazole (n=33), **B,** metconazole (n=20), **C,** cyproconazole (n=20), and **D,** tebuconazole (n=20) as determined by the spiral gradient dilution method. Bar height indicates the total number of isolates within each bin, and bin width (0.11 for propiconazole, 0.04 for metconazole, 0.06 for cyproconazole, and 0.07 for tebuconazole) was based on Scott's method (Scott, 1979).

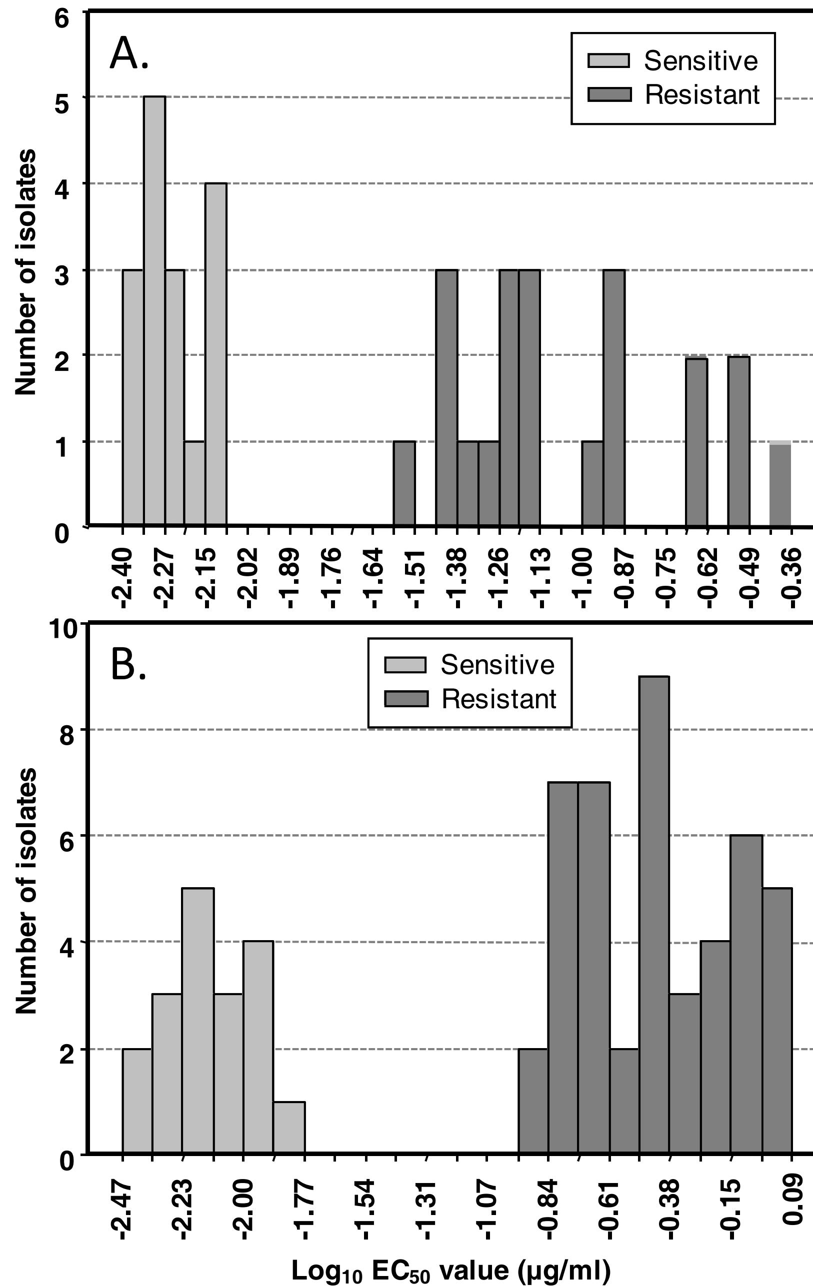

Figure 3.3. Frequency histograms of effective concentrations ($\log_{10}$ µg/ml) of **A,** imazalil (37 isolates) or **B,** propiconazole (63 isolates) to inhibit mycelial growth by 50% (EC_{50} values) for *Penicillium digitatum.* Isolates were designated as sensitive or resistant to the two fungicides based on EC_{50} values as determined by the spiral gradient dilution method. Bin height indicates the total number of isolates within each bin and bin width was based on Scott's method (Scott, 1979).

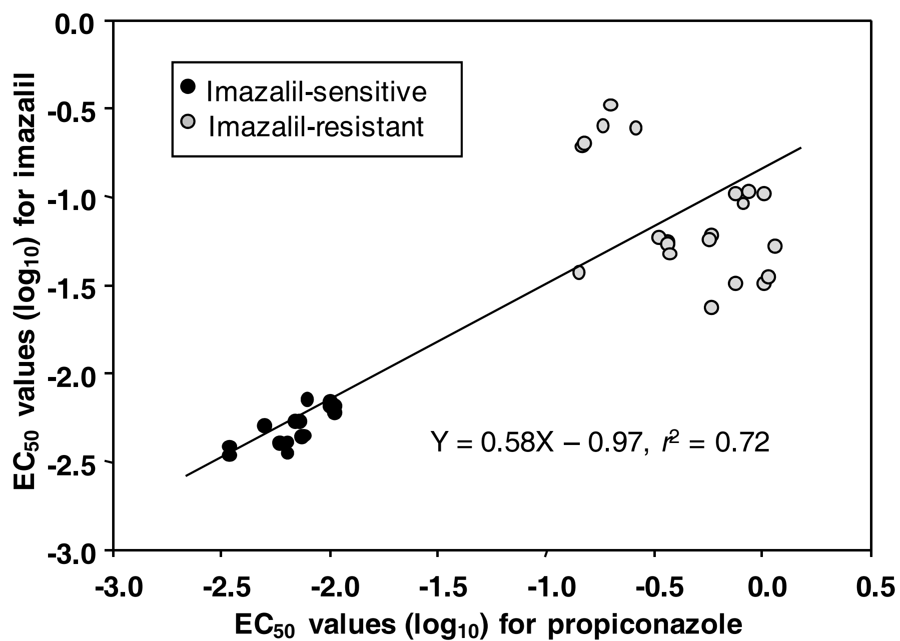

Figure 3.4. Scatter plot and linear regression of effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) for propiconazole plotted against those for imazalil. Filled and open circles designate propiconazole-sensitive and -resistant strains, respectively, among the total of 37 isolates of *Penicillium digitatum* evaluated.

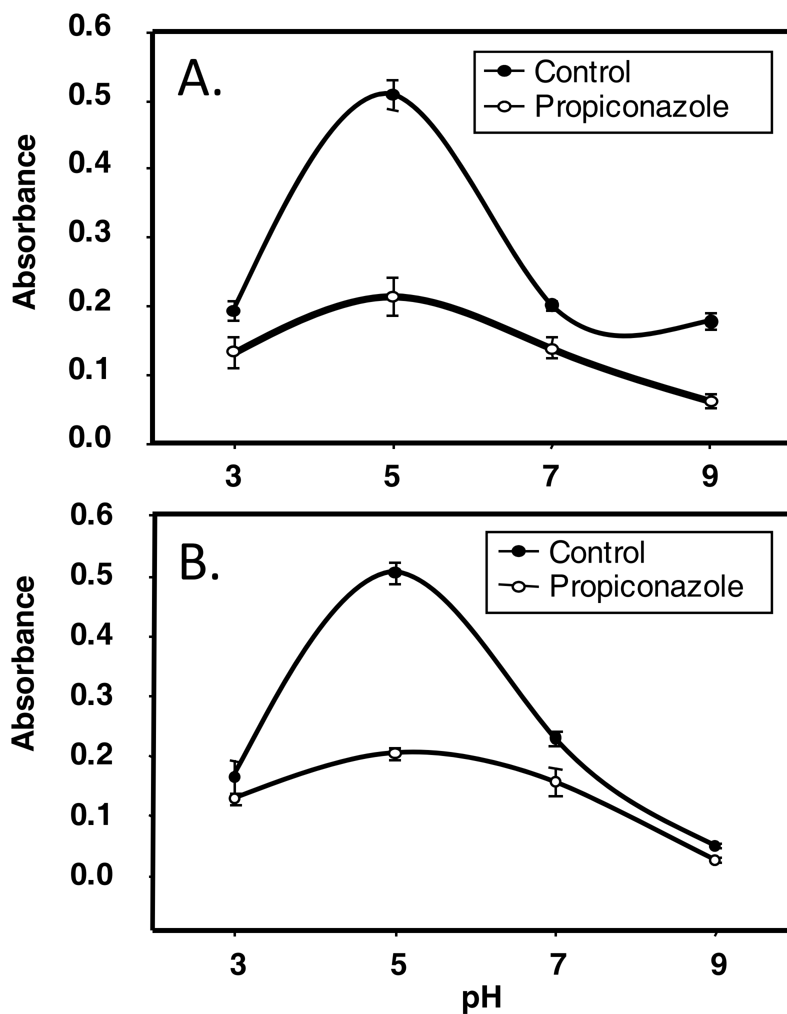

Figure 3.5. Mycelial growth of **A,** *Galactomyces citri-aurantii* after 18 h and **B,** *Penicillium digitatum* after 24 h of incubation at 25 C as measured by absorbance at 490 nm in microtiter plate assays. Potato dextrose broth was amended or not amended with propiconazole and adjusted to selected pH values.

CHAPTER IV. EFFICACY AND APPLICATION STRATEGIES FOR THE NEW POSTHARVEST FUNGICIDE PROPICONAZOLE IN MANAGING SOUR ROT AND GREEN MOLD OF CITRUS FRUIT IN STORAGE

ABSTRACT

The demethylation inhibiting (DMI)-triazole fungicides propiconazole and cyproconazole were found to be more effective than metconazole and tebuconazole in reducing postharvest sour rot of citrus caused by *Galactomyces citri-aurantii*. Additional studies were conducted with propiconazole as a postharvest treatment because it has favorable toxicology characteristics for agricultural food crop registration in the United States and the registrant supports a worldwide registration. Regression and covariance analyses were performed to determine optimal time of application after inoculation and fungicide rate. In laboratory studies decay incidence increased when propiconazole applications were delayed between from 8 to 24 h (lemons) or 18 to 42 h (grapefruit) after inoculation. Effective rates of the fungicide ranged from 64 to 512 µg/ml and were dependent on inoculum concentration of the sour rot pathogen used and on the type of citrus fruit. Propiconazole was found to be compatible with 100 µg/ml sodium hypochlorite and 1 to 3% sodium bicarbonate without loss of efficacy for decay control on lemons. The addition of 80 µg/ml hydrogen peroxide/peroxyacetic acid slightly decreased the effectiveness of propiconazole to reduce sour rot. Heated (48°C) solutions of propiconazole did not significantly improve the efficacy as compared to solutions at 22°C. In experimental packingline studies, aqueous drench applications applied alone or followed by applications of the fungicide in storage or packing fruit coatings were highly effective reducing sour rot to 0% or 1.2% as compared to 83.8% decay incidence in the control when treatments were made 14 to 16 h after inoculation. When the fungicide was applied alone in either fruit coating, decay was reduced to 49.1% to 57.1% incidence. Tank mixtures of propiconazole with the citrus postharvest fungicides fludioxonil and

azoxystrobin were highly effective in reducing green mold caused by isolates of *Penicillium digitatum* sensitive or moderately resistant to imazalil and sour rot. Propiconazole will be an important postharvest fungicide for managing sour rot of citrus and potentially can be integrated into current management practices to reduce postharvest crop losses caused by DMI-sensitive isolates of *P. digitatum*.

INTRODUCTION

Sour rot of citrus fruit caused by the soil-borne fungal pathogen *Galactomyces citri-aurantii* Butler (anamorph: *Geotrichum citri-aurantii* (Ferraris) Butler) is an economically important postharvest disease affecting California's estimated $1.7 billion annual (2008 to 2009) citrus crop (Anonymous, 2010 - 2011). Sour rot ranks second in postharvest decay losses after decays caused by *Penicillium* species. Of the latter, *P. digitatum* (Pers.: Fr.) Sacc.is the most important postharvest pathogen of citrus produced in semi-arid climates like California (Eckert and Eaks, 1989). Sour rot inoculum can contaminate fruit through contact with soil, by splash from soil during wet conditions, or by dust arising during orchard cultivation (Baudoin and Eckert, 1982; Eckert, 1959). The pathogen infects through fruit injuries that occur during harvest and subsequent postharvest handling (Brown, 1979; Eckert, 1959; Eckert and Eaks, 1989). In storage, the disease may result in complete collapse and liquefaction of infected fruit. Juices dripping from infected fruit can readily spread the pathogen to healthy fruit (Eckert and Eaks, 1989). In California, sour rot is particularly problematic on lemons (*Citrus limon* (L.) N.L. Burm.) grown in the coastal regions, as well as on mandarins (*C. reticulata* Blanco) that are often stored for extended periods at relatively high temperatures of 12° to 14°C to obtain marketable rind color (Eckert, 1959; Suprapta et al, 1996). Sour rot can also cause high postharvest losses on

grapefruit (*C. paradisi* Macf.) (Butler et al., 1965) and on oranges (*C. sinensis* L.). Overall, outbreaks of the disease are sporadic with highest incidences on fruit that are harvested during prolonged wet conditions (Baudoin and Eckert, 1982; Eckert, 1959).

Several fungicides are currently registered in the United States for postharvest management of Penicillium decays including the sterol demethylation-inhibiting (DMI) imidazole imazalil, the methyl benzimidazole carbamate thiabendazole, as well as three recently introduced compounds: the quinone outside inhibitor (QoI) azoxystrobin, the phenylpyrrole fludioxonil, and the anilinopyrimidine pyrimethanil. None of these, however, are effective against sour rot (Cohen, 1989; Smilanick et al., 2005). Sodium ortho-phenylphenate (SOPP), a broad-spectrum phenolic based fungicide, has some activity against sour rot and is registered for postharvest application to citrus, but concentrations required to effectively manage sour rot of citrus can result in phytotoxicity (Brown, 1979). Additionally, use of SOPP by the citrus industry has been in decline over recent years due to disposal and human safety concerns (Smilanick and Sorenson, 2001). Guazatine, a guanidine fungicide available in other parts of the world, has very good efficacy against sour rot (Rippon and Morris, 1981; Wild, 1992). This fungicide, however, will not be registered in the United States because it contains multiple active ingredients and there is no method to analyze fungicide residues on fruit.

The DMI-triazole propiconazole is a new postharvest fungicide in the United States that is planned for registration on citrus, stone fruit, and other fruit crops such as tomato. It is highly effective against several important decays including those caused by species of *Penicillium, Rhizopus,* and *Monilinia*. Most importantly, however, it is effective against the sour rot pathogens *G. geotrichum* (Butler & Petersen) Redhead & Malloch (anamorph: *Geotrichum candidum* Link) that affects stone fruits, tomato, and other crops (Cochran et al., 2009) and *G. citri-aurantii* (McKay et al., 2007). In comparative evaluations of several DMI-triazole fungicides against

growth of *G. citri-aurantii* in vitro, propiconazole, cyproconazole, and metconazole were the most effective compounds evaluated (McKay et al., manuscript in preparation).

The goal of this study was to evaluate the efficacy of propiconazole against citrus sour rot and also green mold. We addressed several aspects in optimizing efficacy that will be valuable for the upcoming commercial use of the fungicide. Usage rates have been determined for stone fruits and tomatoes (Adaskaveg et al., 2007; Cochran et al., 2009), but need to be established for citrus fruits. This is partly due to a lower toxicity of propiconazole against mycelial growth of *G. citri-aurantii* as compared to *G. geotrichum* (McKay et al., 2007), but also because different types of crops and handling systems are involved. Proper timing of postharvest applications is another critical aspect of postharvest treatments, and different fungicides have been shown to have different post-infection characteristics. For example, imazalil and pyrimethanil provided almost complete decay control when applied 9 h to 21 h after inoculation for green mold control of lemon fruit, whereas efficacy of azoxystrobin and fludioxonil was significantly reduced when applied more than 12 h after inoculation (Kanetis et al., 2007).

Postharvest fungicides are commonly applied in combination with a fruit coating to prevent water loss from fruit during storage (storage coatings) or to improve the appearance of fruit prior to marketing (packing coatings). When directly mixed with fruit coatings, fungicide efficacy can be affected as shown previously for fungicides used to control citrus green mold (Kanetis et al., 2007). This interaction also needs to be investigated for propiconazole. Other strategies that have been employed to maximize postharvest treatment efficacy for citrus fruit are the use of fungicide solutions heated to between 41 and 50ºC as shown for imazalil and thiabendazole (Smilanick et al., 1997). The addition of sodium bicarbonate (SBC) or potassium sorbate improved the performance of citrus postharvest fungicides for management of green mold decay (Smilanick et al., 1999, 2005, 2008). Due to their high effectiveness, re-circulating in-line

drench applications of fungicides are increasingly being used commercially for citrus fruit in California. With this practice, the build-up of microbial populations in the treatment solutions should be prevented by using a sanitizer to avoid contamination of healthy fruit and to minimize selection of fungicide-resistant propagules (Kanetis et al., 2008). Thus, the compatibility of propiconazole with SBC and sanitizers needs to be evaluated.

Thus, the specific objectives of this study were to evaluate: i) the efficacy of selected DMI-triazole fungicides in reducing sour rot of citrus; ii) the efficacy of propiconazole as affected by rate and application timing after inoculation; iii) interactions of propiconazole with the sanitizing agents sodium hypochlorite and hydrogen peroxide/peroxyacetic acid (HPPA), with SBC, as well as with citrus fruit coatings; and iv) the effect of heated propiconazole solutions on decay control. Additionally, we evaluated the effectiveness of propiconazole applied by itself or in a mixture with azoxystrobin and fludioxonil against decay caused by isolates of *P. digitatum* sensitive or resistant to imazalil.

MATERIALS AND METHODS

Fungal isolates and inoculation of fruit. Single-spore isolates of *G. citri-aurantii* (isolate Gca-67) and *P. digitatum* were obtained from infected lemon fruit in citrus packinghouse in California. Two isolates of *P. digitatum* were used: an isolate sensitive to imazalil and thiabendazole (isolate Pd; EC_{50} values for mycelial growth 0.05 and 0.08 µg/ml, respectively) and an isolate less sensitive to the two fungicides (isolate 3129; EC_{50} value for mycelial growth 0.32 µg/ml and >7.82 µg/ml, respectively). For inoculum production, isolates were grown for 5 to 14 days at 25ºC on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI). Isolates were maintained as mycelial plugs in sterile water at 4ºC for up to one year.

‘Eureka’ and ‘Lisbon’ lemon fruit, as well as ‘Oroblanco’ grapefruit used in the studies were not treated with any pre- or postharvest fungicide and were obtained from research orchards at the University of California, Riverside, and the University of California, Kearney Agricultural Center in Parlier, or from citrus packinghouses. Fruit arriving from the orchard at the packinghouses were collected following a dump tank treatment with 100 µg/ml sodium hypochlorite and a potable water rinse. Fruit from the research orchards were hand washed with 100 µg/ml sodium hypochlorite and rinsed with tap water. Fruit were placed in plastic fruit trays in cardboard fruit boxes, and allowed to air-dry at ambient temperature before inoculation. Fruit were either inoculated the day of harvest or stored at 10°C for up to 7 days before use.

For inoculation with *G. citri-aurantii,* conidial suspensions (5 x 10^5 or 1 x 10^7 conidia/ml) were prepared in sterile water or in autoclaved lemon juice with or without the addition of 2 µg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO) modified after Eckert and Brown (1986). For *P. digitatum*, conidial inoculum (1 x 10^6 conidia/ml) was prepared in sterile water with the addition of 0.01% (vol/vol) Tween 20 (Sigma-Aldrich). For each treatment and for each of the four replications used in each experiment, 12 grapefruit or 24 lemon fruit were used. When two inoculum concentrations were used in experiments with *G. citri-aurantii*, they were done on the same fruit approximately 20 mm from the stem and blossom ends of the fruit. For *P. digitatum*, each fruit received a single inoculation. Conidial suspensions of each fungus (20-µl droplets) were pipetted onto the fruit, and the flavedo (exocarp) and albedo (mesocarp) beneath each droplet were then wounded using a stainless steel probe that created a 1-by-2-mm injury that did not penetrate beyond the albedo. Trays with fruit inoculated with *G. citri-aurantii* were placed into plastic storage boxes (volume approximately 28 liter; Sterilite, Townsend, MA) and 200 ml of water was added to the bottom of each box to provide high relative humidity (fruit were not in contact with the water). For *P. digitatum*, fruit trays were placed into cardboard fruit boxes that

were covered with plastic bags. Fruit were incubated for selected times at 20ºC before treatment. After treatment, fruit were incubated for 6 days at the same temperature. Incidence of decay was calculated based on the number of infected fruit of the total number of fruit used. All treatment studies with inoculated fruit as described below were conducted two or three times.

Fungicides and sanitation agents. Fungicides used were formulated products of the DMI-triazoles cyproconazole (Alto 100SL; Syngenta Crop Protection, Greensboro, NC), metconazole, (Quash 50WDG, Valent USA, Walnut Creek, CA), propiconazole (Mentor 45WP, Syngenta Crop Protection), and tebuconazole (Elite 45WP, Bayer Crop Science, Research Triangle Park, NC), as well as a pre-mixture of the phenylpyrrole fludioxonil and the QoI azoxystrobin (Graduate A+ SL; Syngenta Crop Protection). Industrial grade sodium bicarbonate (FMC Wyoming Corp., Green River, WY) and the sanitizers sodium hypochlorite (household bleach, containing approximately 5.25% NaOCl) and HPPA (Perasan C5, 22.5% hydrogen peroxide; Enviro Tech Chemical Services, Inc., Modesto, CA) were used alone or in combination with fungicides. For treatment, fungicides and sanitizers were diluted to the desired concentrations with distilled water in laboratory studies or with tap water in experimental packingline studies.

Efficacy of selected DMI-triazole fungicides on reducing sour rot of lemons. Fruit were wound-inoculated with *G. citri-aurantii* (1×10^7 conidia/ml, prepared in sterile water with the addition of 2 µg cycloheximide/ml). Fungicide treatments at 256 µg/ml were applied to run-off using a hand-operated atomizer (Model 15-RD; DeVilbiss Health Care, Somerset, PA). Control fruit were sprayed with water.

Effect of treatment time after inoculation and treatment rate on the efficacy of propiconazole in managing sour rot of lemons and grapefruit. For evaluation of treatment timings after inoculation, fruit were wound-inoculated (5×10^5 conidia/ml, prepared in autoclaved

lemon juice and 2 µg/ml of cycloheximide added for lemons; 1 x 10^7 conidia/ml, prepared in autoclaved lemon juice for grapefruit) with *G. citri-aurantii.* Lemon fruit were incubated for 12, 16, 20, or 24 h and grapefruit were incubated for 18, 30, or 42 h before treatment. Treatments were applied by dipping fruit for 15 sec in aqueous propiconazole solutions at 256 µg/ml (lemons and grapefruit) or 384 µg/ml (lemons only). Control fruit were dipped in water.

For evaluation of treatment rates, fruit were inoculated with *G. citri-aurantii* using 1 x 10^7 conidia/ml (prepared in autoclaved lemon juice; lemons and grapefruit) or 5 x 10^5 conidia/ml (prepared in autoclaved lemon juice with the addition of 2 µg/ml of cycloheximide; lemons only). Treatments were applied by dipping for 15 sec in aqueous propiconazole solutions after 12 h (lemons) or 18 h (grapefruit). Concentrations were 64, 128, 256, 384, and 516 µg/ml for lemons and 32, 64, 128, and 256 µg/ml for grapefruit. Control fruit were dipped in water.

Effect of sanitizing treatments and SBC on the efficacy of propiconazole in reducing sour rot of lemons. In experiments with selected additives to the propiconazole treatments, lemon fruit were inoculated with 1 x 10^7 conidia/ml (prepared in autoclaved lemon juice). Aqueous treatment solutions of propiconazole (256 or 384 µg/ml) were prepared without or with the addition of sodium hypochlorite (100 µg/ml free chlorine), HPPA (80 µg/ml hydrogen peroxide), and/or SBC (1% w/v or 3% w/v). Dip treatments for 15 sec were done 14 h or 16 h after inoculation. Control fruit were dipped in water.

Laboratory and experimental packingline studies on the efficacy of propiconazole using different application strategies. Comparisons of application methods, application of with heated and non-heated solutions, as well as applications in combination with fruit coatings or other citrus postharvest fungicides were evaluated. In a laboratory study, lemon fruit were inoculated with *G. citri-aurantii* (1 x 10^7 conidia/ml, prepared in autoclaved lemon juice) and incubated for 14 h before treatment. Fruit were dip-treated for 15 sec or spray-treated with

propiconazole (256 µg/ml) to run-off as described above. Aqueous dip treatments were done either at ambient temperature (22ºC) or were heated to 48ºC (mean temperature) and the solution was re-heated for each of the replications. Control fruit were dipped in water at ambient temperature or in water heated to 52ºC.

In an experimental packingline study on the compatibility of propiconazole with storage and packing fruit coatings, lemon fruit were inoculated with *G. citri-aurantii* (1 x 10^7 conidia/ml, prepared in autoclaved lemon juice) After 14 to 16 h, fruit were treated on an experimental packingline at the University of California, Kearney Agricultural Center in Parlier, CA. This packingline is equipped to treat fruit using high- and low-volume application methods over moving roller beds with a 90 cm wide x 120 cm long treatment area. Propiconazole was applied as high-volume, aqueous in-line drenches at 250 µg/ml or as concentrate, low-volume spray applications at 2000 µg/ml in diluted storage fruit coating (Decco Lustr 202, US Post-Harvest, Inc., Monrovia, CA; diluted 1 part fruit coating diluted with 14 parts water) or undiluted packing fruit coating (Decco Pearl Lustr; Decco US Post-Harvest, Inc., Monrovia, CA). In-line drenches were applied to fruit by pumping a fungicide solution from a 70-L reservoir to a perforated steel distribution pan (91 cm x 91 cm area with 127 5-mm holes evenly distributed) over a moving roller bed. Treatment volumes for propiconazole (256 µg/ml) were equivalent to 33 liter/10,000 kg fruit. Drench treatments were followed by a low-volume spray with diluted storage fruit coating. Low-volume sprays were done with a controlled droplet applicator (CDA; Decco US Post-Harvest, Inc., Monrovia, CA) that was positioned in the center of the treatment area and ca. 20 cm above the roller bed. Treatment volumes for these spray applications were adjusted to 8.3 liter/10,000 kg fruit by regulating fruit coating output volumes and speed of fruit movement through the treatment area. Treatment times were generally between 12 and 15 s. Propiconazole was also applied in two-step applications, where the aqueous drench application (250 µg/ml) was followed by low-volume

sprays of propiconazole at 1000 μg/ml directly in a solution with diluted storage or packing fruit coating. Control fruit were treated only with water. Between treatments, fungicide reservoirs and tubing, as well as treatment beds were treated with a commercial alkaline detergent (PacFoam Plus; Pace International, Seattle, WA) and then thoroughly rinsed with water.

In another study to evaluate the efficacy of propiconazole applied by itself or in mixtures with azoxystrobin/ fludioxonil, lemon fruit were inoculated with isolates of *P. digitatum* (10^6 conidia/ml) either sensitive or less-sensitive to imazalil or with *G. citri-aurantii* ($5x10^5$ conidia/ml, prepared in autoclaved lemon juice with the addition of 2 μg/ml cycloheximide). Fungicide treatments were done after 12 or 16 h (except for the less sensitive isolate of *P. digitatum* that was only evaluated at 12 h) using in-line drenches followed by a low-volume spray application with diluted storage fruit coating as described above. Propiconazole was used at 256 μg/ml when applied by itself or at 516 μg/ml when applied together with azoxystrobin/fludioxonil. Azoxystrobin/fludioxonil was applied using the commercial pre-mixture Graduate A+ with each fungicide at 300 μg/ml.

Statistical analysis of data. Data for decay incidence were arcsine transformed for studies on the efficacy of DMI fungicides, the effect of sanitizers, SBC, or fruit coatings on propiconazole treatments, and the effect of temperature on the efficacy of propiconazole in reducing postharvest fruit decay. Bartlett's test for homogeneity of variances was performed for repeated experiments. In each study, variances were homogeneous ($P<0.05$) and thus, combined data sets were analyzed using a balanced one-way treatment structure in a randomized complete block design. Transformed values were analyzed using general linear model or analysis of variance and least significant difference mean separation procedures of SAS version 9.2 (SAS Institute, Cary, NC).

Regression analyses were used to analyze timing of application after inoculation and rate effects of propiconazole applications on transformed mean incidence of decay using SAS. Decay incidence was first normalized for each experiment by dividing the incidence value of treatment rates and timings by the decay incidence of the non-treated control. Standard errors were calculated as the square root of the variance of the experimental means. An analysis of covariance was done for comparing slopes and adjusted means of multiple regression lines. For this, the following strategy was used: 1) test the hypothesis that all slopes are equal to zero (H_0: $\beta_1 = \beta_2 = \beta_3 = \beta_4 = 0$ vs H_a: (not H_0)); 2) determine if a common slope model will adequately describe the data and test the hypothesis that the slope is equal to zero (H_0: $\beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta$ vs H_a: (not H_0)); and 3) Fit a common slope model and determine if linear, quadratic, or cubic estimates best describe the data. Additionally, regression lines with common slopes were compared at the midpoint using adjusted means (least squares means or LSMeans) of the regression line using mixed model procedures (SAS Version 9.2).

RESULTS

Efficacy of selected DMI-triazole fungicides in reducing postharvest sour rot of lemons. In a comparative laboratory study using inoculated lemon fruit, the incidence of sour rot was significantly ($P < 0.05$) reduced from the control using all four compounds applied at 256 µg/ml (Fig. 1). Treatments of cyproconazole and propiconazole applied 14 hour after inoculation were significantly more effective than those with metconazole or tebuconazole. The incidence of decay after six days of incubation at 20°C was 93.7% for the water-treated control, and 11.4%, 13.0%, 31.3% and 35.1% for propiconazole, cyproconazole, metconazole, and tebuconazole, respectively.

Effect of treatment time after inoculation and treatment rate on the efficacy of propiconazole in managing sour rot of lemons and grapefruit. Decay incidence increased when applications were delayed after inoculation. In the regression analysis, the models for both rates of propiconazole tested on lemons (256 and 384 µg/ml), as well as the one rate tested on grapefruit were significant ($P < 0.01$ for all regression models) and coefficients of determination of the regression lines ranged from 0.86 to 0.98. The slopes of the regressions of transformed decay incidence on application time after inoculation were positive (Fig. 2A,B). In the analyses of covariance, slopes for the regression lines were not significantly different ($P = 0.74$) for the two application rates used on lemons and the model estimates indicated a strong linear relationship between LSMeans of decay incidence and time of application of propiconazole after inoculation for the range of times evaluated. Thus, the efficacy of the two rates decreased similarly over time. Decay incidence ranged from 12.4% to 8.7% at the 12-h timing and increased from 33.0 % to 39.4% at the 24-h timing using the 256-µg/ml and 384-µg/ml rates, respectively (Fig. 2A). For comparison, an average of 95.0% of the fruit in the water-treated control developed sour rot. For grapefruit, treatments with 256 µg/ml propiconazole 18 h after inoculation still resulted in a high efficacy with 2.2% decay incidence as compared to the untreated control where 80.9% of the fruit had sour rot (Fig. 2B). When treatments were delayed 30 or 42 h after inoculation, the incidence on treated fruit increased to 17.6% or 78.8%, respectively.

In evaluations of the effect of treatment rate on the efficacy of propiconazole against sour rot, the regression models for both inoculum levels tested on lemons, as well as for the one inoculum concentration tested on grapefruit were significant ($P < 0.05$) and coefficients of determination of the regression lines ranged from 0.91 to 0.99. The slopes of the regressions of transformed decay incidence on propiconazole concentration were negative (Fig. 3A,B). In the analyses of covariance, slopes for the regression lines were not significantly different ($P = 0.76$)

for the two inoculum concentrations used on lemons and the model estimates indicated a strong linear relationship between LSMeans of decay incidence and concentrations of propiconazole evaluated. The predicted LSMeans from the parallel line regression models evaluated at the mean of the covariate were significantly different ($P < 0.001$).Thus, efficacy of propiconazole using the lower inoculum concentration was significantly higher than when fruit were inoculated with a higher inoculum concentration. Using the lower inoculum concentration, decay was reduced to 6.4% using 64 µg/ml and to 1.5% using 256 µg/ml of propiconazole as compared to the control with 85.0% incidence. Using the higher inoculum concentration, decay incidence ranged from 3.9% using 516 µg/ml to 37.2% using 64 µg/ml of propiconazole, as compared to the control with 90.4%. For grapefruit, decay incidence also decreased linearly from 56.2% using 64 µg/ml to 9.7% using 256 µg/ml of propiconazole as compared to the control with 67.4% (Fig. 3B).

Effect of sanitizing treatments and SBC on the efficacy of propiconazole in reducing sour rot of lemons. In two laboratory studies using dip applications, sodium hypochlorite did not reduce decay, whereas HPPA slightly reduced the incidence of sour rot in wound-inoculated fruit (Figs. 4,5). SBC was evaluated at rates that are commonly used commercially and was significantly more effective when used at 3% than at 1% (Fig. 4). When applied at 3% 16 h after inoculation, sour rot was reduced from over 97.5% incidence in the control to 46.2%. The efficacy of propiconazole was not affected when mixed with 1% or 3% SBC or with 100 µg/ml sodium hypochlorite although a trend for reduced decay was observed with the mixtures (Fig. 4). When propiconazole was mixed with 3% SBC plus sodium hypochlorite, the efficacy in reducing sour rot was significantly increased as compared to propiconazole alone (Fig. 4). When propiconazole was mixed with HPPA, decay incidence was slightly increased as compared to propiconazole alone (Fig. 5).

Laboratory and experimental packingline studies on the efficacy of propiconazole using different application strategies. In laboratory comparisons of dip and spray applications with propiconazole, the incidence of sour rot was slightly, but not significantly reduced in the dip treatment (Fig. 6). When heated (48°C) and non-heated propiconazole treatments were compared, the efficacy of the fungicide was slightly, but not significantly improved by the heated treatment (Fig. 6).

In a comparative experimental packingline study using propiconazole as aqueous in-line drench applications that were followed by an application of fruit coating, as spray applications in mixtures with fruit coatings, or as staged double fungicide treatments where the aqueous application was followed by a second application in a fruit coating, significant differences were obtained in the effectiveness of the fungicide. The aqueous drench application by itself or in combination with an application in storage or packing fruit coating was highly effective with an incidence of decay of 0% to 1.2% as compared to the control were 83.8% of the fruit showed symptoms of sour rot (Table 1). In contrast, single propiconazole applications that were made directly in storage or packing fruit coating were significantly less effective and resulted in 49.1.0 or 57.1% decay, respectively.

In-line drench applications with propiconazole at 256 μg/ml applied 12 or 16 h after inoculation in another experimental packingline study reduced sour rot from an incidence of 52.5% in the control to 0%, 0%, or 3.7% for the three timings, respectively (Table 2). The azoxystrobin-fludioxonil pre-mixture was not effective against sour rot and had a decay incidence statistically similar to the control. In this study we also evaluated the efficacy of these treatments against citrus green mold. When fruit were inoculated with an isolate of *P. digitatum* determined to be sensitive against the DMI-imidazole imazalil, propiconazole applications timed 12 and 16 h after inoculation reduced decay to 6.3% and 9.2%, respectively, as compared to the control with

65.1% green mold (Table 2). Propiconazole, however, was not effective against green mold caused by an isolate of *P. digitatum* moderately resistant to imazalil. The triple fungicide mixture of propiconazole (256 µg/ml or 512 µg/ml), azoxystrobin (300 µg/ml), and fludioxonil (300 µg/ml) effectively reduced sour rot and green mold caused by isolates of *P. digitatum* sensitive and moderately resistant to imazalil (Table 2).

DISCUSSION

In this study we provide new evidence that the fungicide propiconazole can be effectively used for the postharvest management of sour rot of citrus caused by *G. citri-aurantii* and we present information that will help to design use strategies to obtain maximum decay control. The efficacy of DMI fungicides against sour rot was demonstrated previously by others. For example, the triazole etconazole reduced the incidence of sour rot of oranges by 53.4% (Brown, 1986). The morpholine fenpropimorph and the triazole flutriafol showed comparable reductions in disease incidence of inoculated oranges in another study (Cohen, 1989). These fungicides, however, were never registered in the United States. In our own comparative evaluations, propiconazole and cyproconazole were similarly effective, and more effective than metconazole and tebuconazole at equivalent rates. These DMI compounds were chosen because they were the most effective in laboratory sensitivity studies with *G. citri-aurantii* and *G. geotrichum* among the members of this fungicide group evaluated (McKay et al., 2007; and manuscript in preparation). Propiconazole has been available for preharvest use on a variety of crops for numerous years and is planned for registration on stone fruit and other crops for the postharvest management of sour rot of caused by *G. geotrichum* and of other decays. The fungicide was found previously to be effective against citrus sour rot (Eckert et al., 1981) but registration was not pursued at this time. With the new

interest in this compound as a postharvest treatment, favorable toxicology characteristics for agricultural food crop registration, as well as support by the registrant, propiconazole will be the first highly effective treatment for the management of sour rot of citrus and other crops in the United States.

Our evaluations were done using inoculated lemons and grapefruit. The incidence of decay in the non-treated control varied widely among experiments and sometimes was too low to obtain meaningful data. The difficulty of obtaining consistent sour rot decay of inoculated fruit was recognized previously by others and this was associated initially with fruit maturity and water content of the rind (Baudoin and Eckert, 1982). The exact mechanism involved in fruit susceptibility, however, has not been elucidated (Davis and Baudoin, 1986). To increase consistency in inoculations for sour rot, some researchers have tried to predispose citrus fruit by submerging in water (Cohen et al., 1991). Others have added various chemicals to the inoculum, and cycloheximide, blasticidin S, glyphosate, and pectinase were found to be most effective in increasing the incidence of decay (de Matos and Eckert, 1983). In still other studies, higher conidial concentrations were used that led to an increased activity of polygalacturonase (Nakamura et al., 2001); a macerate of decayed lemon peel was added to the inoculum suspension (Cohen, 1989); or inoculum was prepared in fresh lemon juice (Smilanick et al., 2008). In our inoculations, decay was most consistently obtained when inoculum was prepared in autoclaved lemon juice and when either a low conidial concentration (i.e., 5×10^5 conidial/ml) was used together with 2 µg/ml of cycloheximide or a high conidial concentration (i.e., 1×10^7 conidial/ml) was used. For most studies presented here, inoculum was prepared using both of these methods and data are presented for those experiments where decay incidence in the untreated control was at least 50%.

Our experiments identified critical application timings and rates for propiconazole treatments of inoculated fruit. For lemons, using two treatment rates (256 and 512 µg/ml), decay incidence increased rapidly when treatments were delayed for more than 12 h after inoculation. In studies by others with fenpropimorph, no decay developed when treatments were done within 6 h of inoculation, but incidence was 92% when applied after 24 h (Cohen, 1989). Based on our results, commercial treatments with propiconazole to lemons should not be delayed for more than 16 h after harvest to obtain a high efficacy. In contrast, for grapefruit, treatments done 18 h after inoculation still provided a very high efficacy. Reduced performance due to delayed application of postharvest fungicides is largely due to, i) the rate of development of the fungus in the host tissue and, ii) the apoplastic and symplastic mobility of the fungicide in the host tissues (Hewitt, 1998). It is unclear if the differences we observed between the two citrus species can be generalized. Grapefruit are considered very susceptible to sour rot. Possibly, the 'Oroblanco' cultivar used in our studies is more resistant to sour rot and the pathogen advances more slowly into the tissue. Alternatively, the fungicide may have penetrated deeper into the rind tissue of grapefruit than of lemon.

In the rate studies, fungicide concentrations were used that were suggested by the registrant. A rate of 256 µg/ml was sufficient to reduce decay to very low levels on lemons inoculated with a low conidial concentration and for grapefruit inoculated with a high concentration. For lemons using the high inoculum concentration, to achieve a similar level of sour rot control, a rate of 512 µg/ml was required. This suggests that commercial applications to fruit that are not being stored could be done at a lower rate because infections occurring in the field mainly originate from inoculum adhering to soil particles where conidial concentrations can be considered low. In long-term storage, a common practice for lemons in California, however, infections are mainly initiated from juices dripping from infected fruit where conidial

concentrations will be very high. Thus, for fruit being stored, a higher rate of propiconazole will be beneficial. In our postharvest studies using different application strategies, fruit were treated with a sub-optimal rate of 256 µg/ml and after up to 16 h after inoculation. This was done to see fungicide-treatment interactions more clearly.

Numerous studies have demonstrated the effective management of postharvest pathogens of citrus using drench or soak treatments with alkaline solutions of carbonate, bicarbonate, or sorbate salts (Eckert and Eaks, 1989; Palou et al., 2001; Kanetis et al., 2008; Smilanick et al., 1999, 2008) and these treatments have been in use for over 50 years (Adaskaveg et al., 2002). A standard practice in California packinghouses is to drench fruit over rotating brushes or soak fruit in a tank using a heated (46 to 52°C) re-circulating solution of 1 to 3 % sodium bicarbonate with sometimes 100 to 200 µg/ml sodium hypochlorite (chlorine) added, and then rinse fruit with water. Used alone, however, these treatments have limitations for postharvest decay control. Thus, other fungicides are sometimes added to the solution or are applied subsequently. We found that SBC applied at 3% by itself or in combination with chlorine at ambient temperature significantly reduced the incidence of sour rot of inoculated fruit. When combined with propiconazole, a slight additive effect of the SBC or SBC-chlorine and propiconazole treatments was observed. SBC used at 1%, a concentration that is often preferred in commercial use to avoid dehydration of fruit (Eckert and Eaks, 1989), only reduced sour rot relative to the propiconazole treatment alone in one of the studies, but not in the other one. Still, no negative interaction with propiconazole was observed.

The increased efficacy of some citrus postharvest fungicides against green mold when applied at higher temperature (D'Aquino et al., 2006; Schirra et al., 2005; Smilanick et al., 2006) led to the common practice of using heated fungicide solutions. Heated dip treatments with propiconazole in our study did not improve the efficacy against sour rot as compared to non-

heated solutions. Application times of 15 sec., may not have been long enough for treating fruit with a temperature of 20 to 25°C, and possibly longer timings could improve efficacy. In soak applications of fruit in tanks with SBC and/or fungicides in packinghouses, fruit generally remain submerged for 2 to 4 min.

In packinghouse operations, sanitizing treatments are used to inactivate inoculum on fruit surfaces and to clean equipment. They are also used to prevent the build-up of microorganisms in fungicide solutions for re-circulating, high-volume applications (Eckert and Eaks, 1989), however, some fungicides are not stable under these oxidizing conditions. For example, among the citrus postharvest fungicides, fludioxonil and thiabendazole are stable in solutions of chlorine or SBC-chlorine, whereas imazalil and pyrimethanil are not (Kanetis et al., 2008). The use of chlorine is currently subjected to tighter restrictions due health safety and environmental concerns because of the potential formation of toxic chlorinated by-products, including chloroform. New sanitizers are being developed for use by the food industry, and one of these products is HPPA. Both ingredients of HPPA (hydrogen peroxide and peroxyacetic acid) have antimicrobial activity and leave no toxic residuals. Unlike chlorine, however, peroxyacetic acid remains effective in the presence of organic material (Block, 2001). In direct exposures of conidia of *P. digitatum* to HPPA, higher concentrations and longer exposure times were needed to inactivate spores than when using chlorine (Kanetis et al., 2008). The requirement for long exposure times, however, was not considered a limiting factor for sanitation of recirculating systems if a large reservoir is used.

HPPA at 2700 µg/ml was previously shown to have no negative interactions with thiabendazole, imazalil, pyrimethanil, and fludioxonil (Kanetis et al., 2008). In our study with propiconazole using HPPA at 80 µg/ml, efficacy of propiconazole was slightly reduced. A lower concentration of HPPA was used in the current study because fruit treated with HPPA at 80 µg/ml

do not require a water rinse and thus, HPPA can be directly incorporated into the fungicide solution. HPPA at 80 µg/ml has been successfully used for surface sanitation of cherry and peach fruit (Adaskaveg, *unpublished*).

Experimental packingline experiments were conducted to evaluate the performance of propiconazole under conditions similar to commercial packinghouse practices and to evaluate additional application strategies. An important component of postharvest applications is the use of fruit coatings. Fruit that are marketed soon after harvest (most oranges, grapefruit, and mandarins) are commonly treated with a fungicide and a packing fruit coating. Lemons in California, however, are often being stored for several months before marketing. Before storage, they are treated with a fungicide and a storage fruit coating. Before marketing, a second fungicide application in combination with a packing fruit coating is done (Eckert and Eaks, 1989). Although fungicides can be directly mixed with fruit coatings, the efficacy in reducing decay is often decreased as compared to an aqueous application, and this was also observed in our studies with propiconazole. This reduced efficacy has been attributed to a reduced movement of the fungicide and limited penetration into the fruit rind and into sites with developing infections (Eckert and Brown, 1986; Kanetis et al., 2008). Because of the higher fungicide efficacy in aqueous solutions and the need for using a fruit coating, staged applications have become a common practice. In these applications, an aqueous fungicide treatment is followed by an application with fruit coating that may also contain a fungicide. Using this strategy in our experimental packingline study, sour rot was reduced to very low levels, similar to an aqueous treatment alone. The two-stage application with incorporation of a fungicide in the fruit coating can have a specific advantage for managing Penicillium decays where control of sporulation on decaying fruit is another important goal of postharvest treatments. Although decay control may be compromised when directly applied in the fruit coating, the anti-sporulation activity of some

fungicides such as fludioxonil and azoxystrobin may be increased (Kanetis et al., 2007). Thus, the two-stage application maximizes decay as well as sporulation control of some decay organisms (e.g., *Penicillium* spp.).

Several fungicides are currently registered to manage Penicillium decays of citrus fruit. The recent registration of azoxystrobin, fludioxonil, and pyrimethanil introduced new classes of fungicides with new modes of action that allow the effective control of decay caused by widespread populations of the pathogens that are resistant against the older fungicides thiabendazole and imazalil. The current trend for strategies to minimize the development of resistance includes the use of fungicide mixtures and pre-mixtures where at least two components are effective against the pathogens. Isolates of *Penicillium* spp. resistant to imazalil, however, are cross-resistant to propiconazole (McKay et al., manuscript in preparation). Also, as we demonstrated in the current study, decay caused by imazalil-resistant isolates of *P. digitatum* cannot be managed using propiconazole and thus, fungicides with different modes of action have to be used. Propiconazole by itself could be used for early-season fruit when the incidence of imazalil resistance is generally low (Adaskaveg, *unpublished*). Thus, ideally a triple-fungicide mixture should be applied, with two components active against *Penicillium* spp. and with propiconazole as the sour rot fungicide. As we demonstrated, this mixture had a very high efficacy against sour rot as well as green mold caused by imazalil-sensitive and –resistant isolates of *P. digitatum*. With reduced usage of SOPP fungicides and the need for a sour rot control material other than the carbonate salts that can desiccate fruit and reduce storage time of fruit when used at high rates, propiconazole will be an excellent fungicide for the integrated postharvest management of citrus sour rot and of decays caused by DMI-sensitive isolates of *Penicillium* spp.

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Table 4.1. Efficacy of propiconazole for control sour rot of lemon fruit in an experimental packingline study

	First application				Second application				
No.	Treatment[a]	Rate (µg/ml)	Application method	Fruit coating	Treatment	Rate (µg/ml)	Application method	Fruit coating	Decay incidence (%)[b]
1	Water	---	Drench	None	---	---	---	Storage	83.8 a
2	PPZ	2000	LV-Spray	Storage	---	---	---	---	49.1 b
3	PPZ	2000	LV-Spray	Packing	---	---	---	---	57.1 b
4	PPZ	250	Drench	None	---	---	---	---	0 c
5	PPZ	250	Drench	None	PPZ	500	LV-Spray	Storage	0.6 c
6	PPZ	250	Drench	None	PPZ	500	LV-Spray	Packing	1.2 c

[a] Treatments were applied 14 to 16 h after inoculation with *Galactomyces citri-aurantii* (1 x 10^7 conidia/ml, prepared in autoclaved lemon juice). Propiconazole (PPZ) was applied as a high-volume in-line drench applications or as a low-volume spray on a roller bed as aqueous or fruit coating treatments (see methods). After treatment, fruit were incubated for 6 days at 20ºC.

[b] Values with the same letters indicate that treatment means were not significantly different ($P < 0.05$) following an analysis of variance and least significant difference means separation test procedures.

Table 4.2. Efficacy of aqueous in-line drenches with propiconazole applied by itself or in a mixture with azoxystrobin and fludioxonil against green mold caused by imazalil-sensitive or -resistant isolates of *Penicillium digitatum* and against sour rot of lemon fruit

Treatment[a]	Time after inoculation (h)	Decay incidence (%) Sour rot	Green mold-S[b]	Green mold-MR
Control	12	52.5 a[c]	65.1 a	83.3 a
PPZ 256 µg/ml	12	0 b	6.3 b	73.8 a
PPZ 256 µg/ml	16	3.7 b	9.2 b	NT
Fludioxonil 300 µg/ml + azoxystrobin 300 µg/ml	12	37.7 a	2.7 bc	NT
Fludioxonil 300 µg/ml + azoxystrobin 300 µg/ml + PPZ 256 µg/ml	12	2.8 b	NT	1.2 b
Fludioxonil 300 µg/ml + azoxystrobin 300 µg/ml + PPZ 512 µg/ml	12	0 b	0 c	1.2 b

[a] Aqueous fungicide treatments were followed by a low-volume spray application with diluted storage fruit coating. Fludioxonil and azoxystrobin were applied as the commercial pre-mixture Graduate A+. PPZ = propiconazole. NT = not tested.

[b] Fruit were inoculated with isolates of *P. digitatum* (10^6 conidia/ml) sensitive (S; EC_{50} = 0.05 µg/ml) or moderately resistant (MR; EC_{50} = 0.32 µg/ml) to imazalil or with *Galactomyces citri-aurantii* ($5x10^5$ conidia/ml, in autoclaved lemon juice plus 2 µg/ml cycloheximide).

[c] Mean values of decay incidence in each column followed by the same letter are not statistically different (P<0.001) according to analysis of variance and least significant difference mean separation procedures.

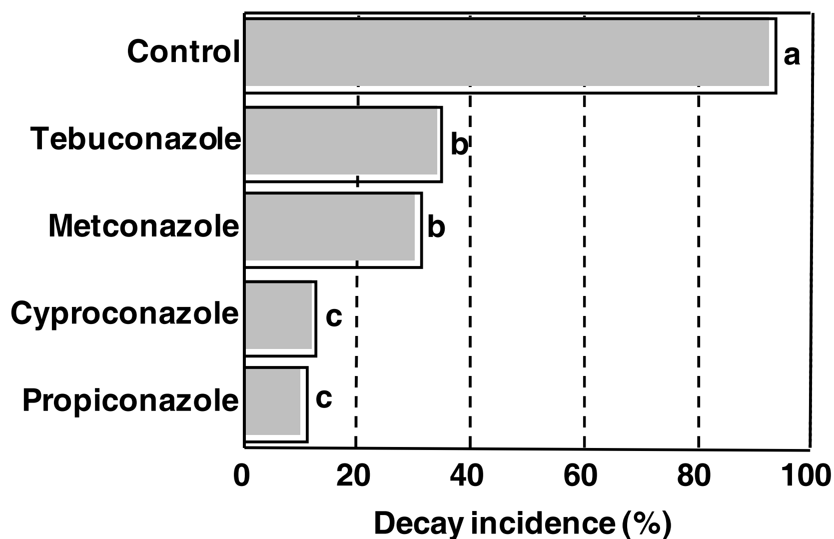

Figure 4.1. Effect of selected DMI-triazole fungicides on incidence of postharvest sour rot of lemon fruit. Spray applications to run-off with aqueous fungicide preparations (256 µg/ml) were done 14 h after wound-inoculation with 1 x 10^7 conidia/ml of *Galactomyces citri-aurantii* prepared in sterile water and addition of 2 µg/ml of cycloheximide. Decay incidence was evaluated after 6 days of incubation at 20ºC. Horizontal bars with the same letter indicate that treatment means were not significantly different ($P < 0.05$) following an analysis of variance and LSD means separation procedures.

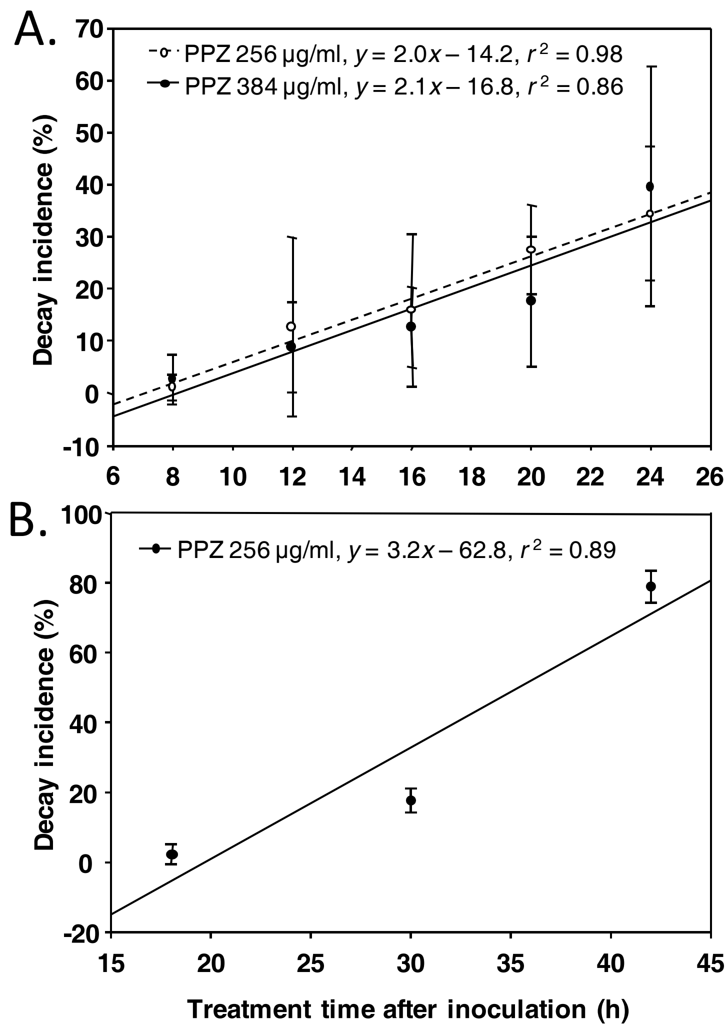

Figure 4.2. Effect of treatment time after inoculation on the efficacy of propiconazole in managing sour rot of **A,** lemons or **B,** grapefruit in laboratory studies. Fruit were dipped for 15 sec in aqueous propiconazole (PPZ) solutions at 256 µg/ml (open circles; lemons only) or 384 µg/ml (closed circles) at selected times after wound-inoculation with conidia of *Galactomyces citri-aurantii* (5 x 10^5 conidia/ml, prepared in autoclaved lemon juice and 2 µg/ml of cycloheximide added for lemons; 1 x 10^7 conidia/ml, prepared in lemon juice for grapefruit) and incubation at 20ºC. Decay incidence was regressed on treatment time after inoculation. Vertical bars represent standard errors for each treatment time from two experiments.

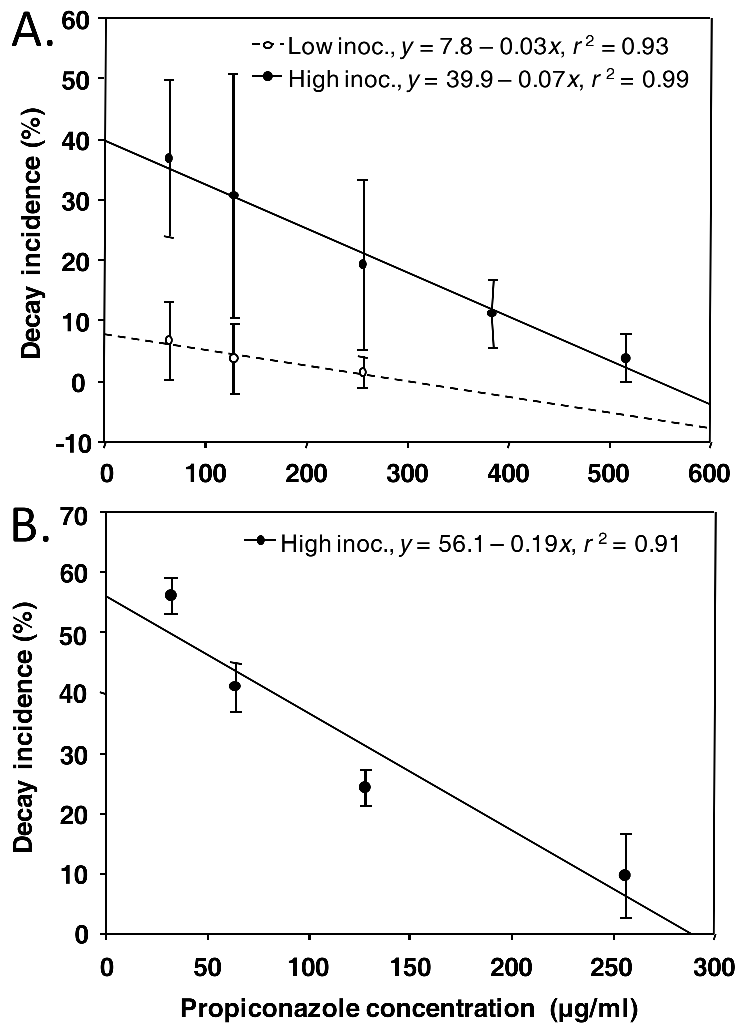

Figure 4.3. Effect of treatment rate on the efficacy of propiconazole in managing sour rot of **A,** lemons or **B,** grapefruit in laboratory studies. Fruit were dipped for 15 sec in aqueous propiconazole solutions 12 h (lemons) or 18 h (grapefruit) after wound-inoculation with *Galactomyces citri-aurantii* at 1 x 10^7 conidia/ml (high inoc.) prepared in autoclaved lemon juice (closed circles) or at 5 x 10^5 conidia/ml (low inoc.) in autoclaved lemon juice and addition of 2 µg/ml of cycloheximide (open circles; lemons only). Fruit were incubated for 6 days at 20ºC. Decay incidence was regressed on propiconazole concentration. Vertical bars represent standard errors for each treatment time from two experiments.

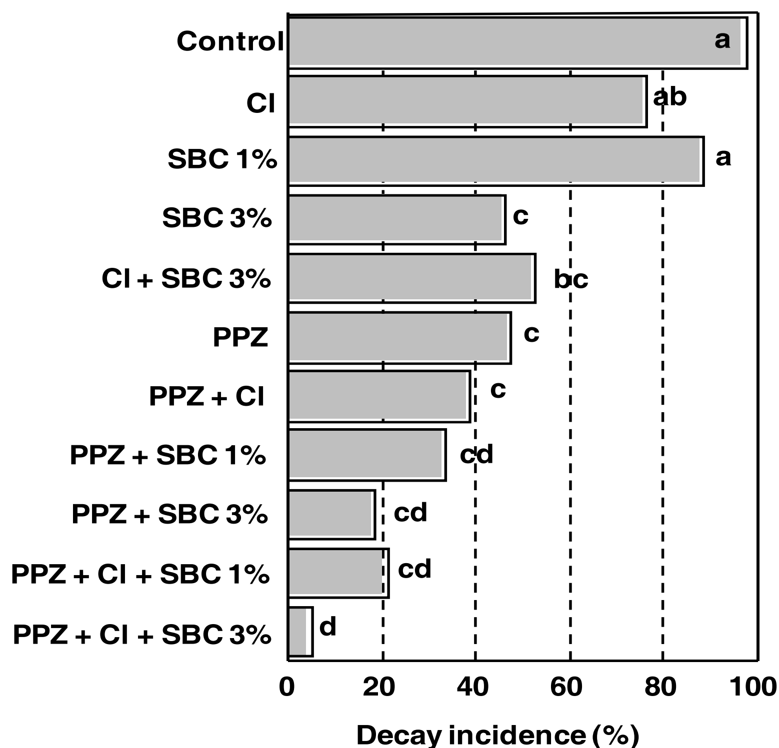

Figure 4.4. Effect of sodium hypochlorite and sodium bicarbonate on the efficacy of propiconazole to control sour rot of lemon fruit. Fruit were dip-treated for 15 sec 16 h after wound-inoculation with *Galactomyces citri-aurantii* (1 x 10^7 conidia/ml, prepared in autoclaved lemon juice). Aqueous treatment solutions of propiconazole (PPZ; 384 µg/ml) were prepared without or with the addition of sodium hypochlorite (Cl; 100 µg/ml free chlorine) and/or sodium bicarbonate (SBC; 1% w/v or 3% w/v). Fruit were incubated for 6 days at 20ºC. Horizontal bars with the same letter indicate that treatment means were not significantly different ($P < 0.05$) following an analysis of variance and least significant difference means separation test procedures.

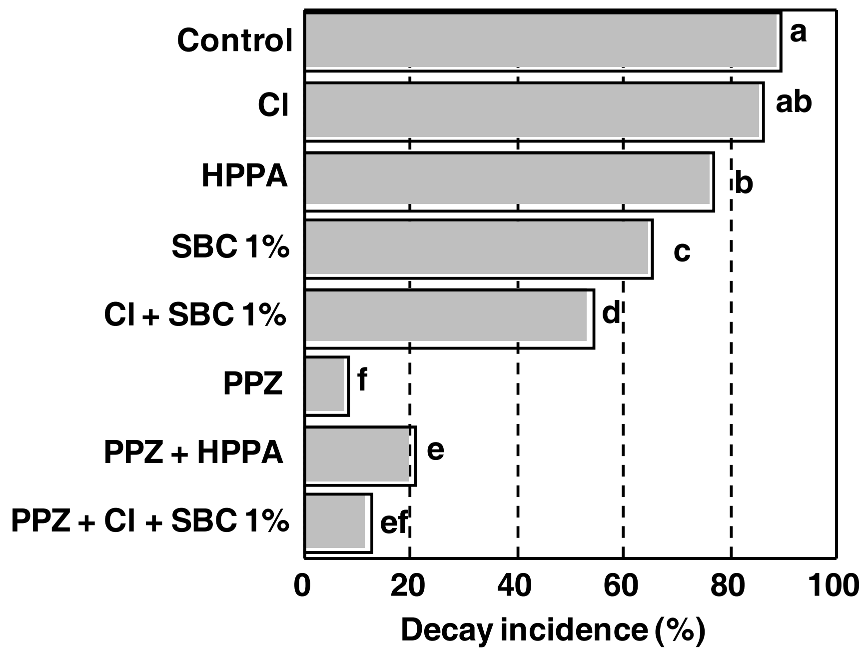

Figure 4.5. Effect of sanitizing agents and sodium bicarbonate on the efficacy of propiconazole to control sour rot of lemon fruit. Fruit were dip-treated for 15 sec 14 h after wound-inoculation with *Galactomyces citri-aurantii* (1 x 10^7 conidia/ml, prepared in autoclaved lemon juice). Aqueous treatment solutions of propiconazole (PPZ; 256 µg/ml) were prepared without or with the addition of sodium hypochlorite (Cl; 100 µg/ml free chlorine), hydrogen peroxide (HPPA; 80 µg/ml, using a hydrogen peroxide-peroxyacetic acid mixture), and/or sodium bicarbonate (SBC; 1% w/v). Fruit were incubated for 6 days at 20ºC. Horizontal bars with the same letter indicate that treatment means were not significantly different ($P < 0.05$) following an analysis of variance and least significant difference means separation test procedures.

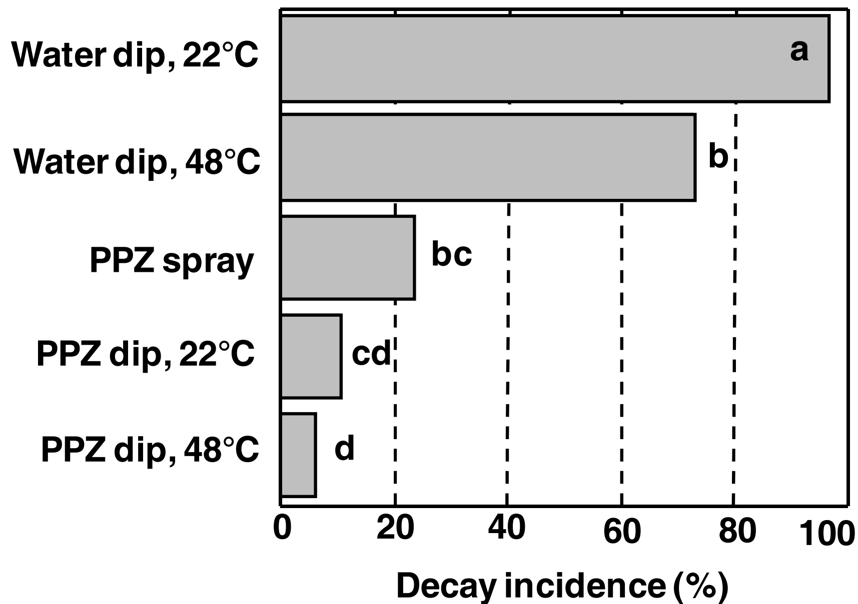

Figure 4.6. Efficacy of propiconazole for control sour rot of lemon fruit using applications at two temperatures. Fruit were dip-treated for 15 sec or spray-treated to run-off 14 h after wound-inoculation with *Galactomyces citri-aurantii* (1×10^7 conidia/ml, prepared in autoclaved lemon juice). Aqueous dip treatments were done either at ambient temperature (22ºC) or heated (48ºC), whereas sprays were done only at ambient temperature. Propiconazole was applied at 256 µg/ml. After treatment, fruit were incubated for 6 days at 20ºC. Horizontal bars with the same letter indicate that treatment means were not significantly different ($P < 0.05$) following an analysis of variance and least significant difference means separation test procedures.

CHAPTER V. GENERAL CONCLUSIONS

Outbreaks of sour rot have resulted in substantial economic losses to California's $1.7 billion (annual 2009) citrus industry because there have been no practical treatments available to effectively manage the disease. We evaluated DMI-triazole fungicides, in particular propiconazole, as potential new postharvest disease management tools for use by the citrus industry. The major objectives of the research reported in this dissertation were:

i. To identify and distinguish two species of *Galactomyces* that cause postharvest sour rot diseases of citrus and other fruits.
ii. To characterize the genetic diversity, population genetic structure, and reproductive strategy of diverse populations of *G. citri-aurantii* and *G. geotrichum* collected from California other parts of the United States and from twelve countries around the world.
iii. Determine in vitro sensitivities of the two *Galactomyces* spp. and *Penicillum digitatum* that causes green mold of citrus to selected DMI-triazole fungicides and estimate natural resistance frequencies to propiconazole.
iv. Determine the best strategies to apply the postharvest fungicide propiconazole to effectively manage sour rot and green mold diseases of citrus.

Our research provides new information that will be useful for developing propiconazole as an effective and robust postharvest fungicide. Results and conclusion were as follows:

i. We developed PCR primers using β-tubulin and endo-polygalacturonase gene sequences that effectively differentiate *G. citri-aurantii* and *G. geotrichum.*

ii. Using mating type segregation ratios for *G. citri-aurantii* as well as multilocus genetic analysis for both *Galactomyces* spp., we determined that the pathogens use a mixed reproduction strategy with sexual and asexual reproduction. I_A and PTLPT analyses were generally consistent with random mating for both species.

iii. Haplotypic diversity and the *Nm* estimate of gene migration were determined to be high for populations of the two *Galactomyces* spp., indicating a high genetic potential for adaptability including developing resistance to fungicides.

iv. We demonstrated that a diverse population of *G. citri-aurantii* is highly sensitive to propiconazole, reducing mycelial growth in vitro with a mean EC_{50} value of 0.34 μg/ml for 139 isolates. Propiconazole was also highly effective against *G. geotrichum* and against imazalil-sensitive isolates of *P. digitatum* with mean EC_{50} values of 0.14 μg/ml and 0.008 μg/ml, respectively. Propiconazole most effectively controlled the growth of *P. digitatum* and *G. citri-aurantii* at a pH of 5.0.

v. Using a procedure that effectively detects resistance frequencies as low as 9.7×10^{-8} in other fungi, we were unable to determine any natural resistance to propiconazole for two *Galactomyces* spp. For *P. digitatum,* resistance frequencies for propiconazole ranged from 8.0×10^{-8} to 1.6×10^{-7} using the same procedure.

vi. We developed effective application strategies for propiconazole that will maximize the effectiveness and reduce the potential for field resistance to develop:

a) Propiconazole is best used within 12 h of infection. b) The fungicide is most

effectively applied as an aqueous solution or as a combination treatment using an aqueous application followed by a fruit coating (mixed or not mixed with propiconazole), rather than applied directly in fruit coatings alone. c) The fungicide showed no loss of efficacy when mixed with chlorine or sodium bicarbonate. Hydrogen peroxide/peroxyacetic acid slightly decreased the efficacy of propiconazole. d) When propiconazole was mixed with azoxystrobin and fludioxonil, the mixture provided greater than 95% reductions of both green mold (caused by imazalil-sensitive or –resistant isolates of *P. digitatum*) as well as sour rot of inoculated lemons.