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An Avirulent *Galactomyces* Species that Controls Green Mold of Citrus Caused by *Penicillium digitatum*

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ABSTRACT

Green mold, caused by *Penicillium digitatum*, is the most prevalent postharvest disease of citrus in Texas. An avirulent *Galactomyces citri-aurantii* E.E.Butler (anamorph *Geotrichum citri-aurantii* (Ferraris) Butler) and formerly known as *Geotrichum candidum*, significantly reduced the incidence of green mold on wounded grapefruit and oranges in three trials. Tests were run three times and each test included five replications of 10 fruit. Although disease incidence varied, the reduction in green mold in fruit treated with the avirulent *G. citri-aurantii* was always significant. The avirulent *G. citri-aurantii* was obtained by chance as a result of frequent subculturing on potato dextrose agar of a virulent isolate, originally isolated at the Citrus Center in Weslaco, TX. Antibiosis by the avir against *P. digitatum* was detected on one medium, but not on several others. A U. S. patent has been issued for this avirulent organism.

RESUMEN

El moho verde, causado por *Penicillium digitatum*, es la enfermedad post cosecha mas común de cítricos en Texas. Una cepa avirulenta de *Galactomyces citri-aurantii* E.E. Butler (anamorfo *Geotrichum citri-aurantii* (Ferraris) Butler) conocida anteriormente como *Geotrichum candidum*, redujo significativamente la incidencia del moho verde en naranjas y toronjas heridas en experimentos en árboles. Se realizaron experimentos en 3 ocasiones y cada uno incluyó 5 repeticiones de 10 frutos. Aunque varió la incidencia de la enfermedad, la reducción del moho verde en la fruta tratada con la cepa avirulenta de *G. citri-aurantii,* fue siempre significativa. La cepa avirulenta de *G citri-aurantii* se obtuvo casualmente como resultado del frecuente subcultivo en agar papa dextrosa de un aislamiento virulento, aislado originalmente en el Citrus Center en Weslaco, TX. Se detectó antibiosis del aislamiento avirulento en contra de *P. digitatum* en un medio de cultivo, pero no en varios otros. El organismo avirulento ha sido patentado en Estados Unidos.

Additional keywords: biological control, grapefruit, green mold, oranges, sour rot, Galactomyces, Geotrichum

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Green mold of citrus fruit, caused by *Penicillium digitatum* (Pres.: Fr.) Sacc., is the major postharvest disease of grapefruit and oranges in south Texas, and is a common problem in California, Arizona, and Florida (Dawson and Eckert, 1977; Eckert and Eaks, 1989; Eckert et al., 1994; Wardowski and Brown, 1993). Control of green mold traditionally has relied on sanitation, avoidance of fruit wounding, and use of preharvest and postharvest fungicides. *P. digitatum*, however, frequently develops resistance to the commonly applied fungicides such as, benomyl, thiabendazole (TBZ) and imazalil (Eckert et al., 1994; Wild, 1994). Some isolates of *P. digitatum* are resistant to both benzidmidazoles and imazalil (Bus, 1992; Bus et al., 1991; Homes and Eckert, 1995). Furthermore, benomyl is no longer labeled for postharvest use in packing houses and will not be available for use in orchards after 2003. Additional control measures are needed for use in combination with, or instead of, fungicides.

Several organisms are reported to control green mold of citrus. A recent review article by El Ghaouth et al. (2002)

covers biological control of postharvest disease of citrus. They report that postharvest diseases of citrus are still controlled mainly with imazalil and thiabendazole, but that substantial progress has been made in the development of antagonistic microorganisms. Several yeasts and bacteria have been shown to reduce postharvest decay in citrus, including *Debaryomyces hansenni*, *Pichia guilliermondii*, *Saccharomyces cerevisiae*, *Candida* spp., and *Pseudomonas* spp. Several biological control agents are commercially available (El Ghaouth et al., 2002, Filonow et al., 1996). An isolate of *Pseudomonas cepacia* was reported to control green mold on oranges (Huang et al., 1993). This organism is taxonomically related to pathogenic strains of *P. cepacia*. In a review of biological control of postharvest diseases, Wisniewski and Wilson (1992) note that desirable characteristics in a biological control agent for postharvest use include nonpathogenic to host commodity, and compatibility with commercial procedures.

As mentioned in the recent review, it is important to understand the mode of action of biological control agents, and to understand if antibiotic production is involved (El Ghaouth et al., 2002). Screening for antibiosis is often done with one culture medium, and not under a variety of nutrient and environmental conditions.

In a separate study, fruit that had been inoculated with an avirulent strain of *G. citri-auranti* had lower incidence of green mold from naturally occurring *P. digitatum* inoculum (Eayre and Skaria, *unpublished*). *G. citri-aurantii* is known to include strains that cause sour rot of citrus, rot of ripe tomato fruit, and noncitrus isolates that are weakly virulent on lemons (Butler, 1960; Butler et al., 1965). The purpose of the research reported here was to determine; 1) if *G. citri-auranti* strain avir can reduce development of green mold on grapefruit (*Citrus paradisi* Macf.) and oranges (*C. sinensis* (L.) Osb.), 2) if strain avir is compatible with postharvest citrus fungicides, 3) that strain avir does not incite sour rot disease on citrus fruit, and 4) if strain avir produces antibiotic compounds that inhibit the growth of *P. digitatum*.

MATERIALS AND METHODS

Avirulent isolate. *G. citri-auranti* was isolated from decayed grapefruit, and subcultured on potato dextrose agar (PDA) for 8 mo. In an experiment designed to test the effect of gibberellic acid treatments on control of sour rot, it was inadvertently found that the laboratory strain of *G. citri-aurantii*, (avir) did not cause sour rot, but did reduce the incidence of green mold.

Efficacy trials. To determine if avir controls green mold in harvested citrus, efficacy trials were performed with 'Rio Red' grapefruit and 'Marrs' orange. Fruit were harvested from orchards at the Texas A&M University-Kingsville Citrus Center in Weslaco, TX. Fruit were used for studies within 24 hr of harvest, without washing, allowing naturally occurring *P. digitatum* inocula to provide disease pressure for the trials. Experimental design was a randomized complete block that included five replications of the avir treatment and water treated control. The experiment was conducted three times. Fruit were randomly assigned to boxes containing 10 fruit each. Each fruit was punctured twice with a nail 6 mm deep, and each wound was treated with 10 µl of a *G. citri-auranti* arthrospore suspension (10^4 spores / l) of the avirulent culture. Control fruit were wounded and treated with sterile distilled water. Fruit were stored in plastic bags at 22º C. Disease incidence was recorded after 7 d. Three additional tests with nonwounded fruit were performed by dipping fruit in a suspension of $4x10^4$ cells avir ml^{-1} before storing. Results from six tests were combined for analysis using the SAS GLM macro, which fits a generalized, linear, mixed model to the binary responses using a logit link (Littell et al., 1996). Treatment is the fixed effect, while trial, trial x treatment and replications within trials are the random effects.

Isolate avirulence. To confirm that the isolate was avirulent, 'Rio Red', 'Marsh', and 'Ruby Red' grapefruits, 'N-33 Navel' and 'Marrs' oranges were wounded 5 mm deep with a sterile nail. Using a sterile spatula, the wounded fruit were inoculated with 3.8 mg avir arthrospores, stored at 22º C in plastic bags with moist paper towels for 9 d, and disease incidence recorded. As a positive control, fruit were inoculated with a known virulent isolate of *G. citri-auranti*. For negative controls, fruit were wounded, but not inoculated. In an additional experiment, navel orange fruit were soaked in water overnight to increase susceptibility before inoculation, and then treated as described above.

Fungicide sensitivity. To determine if *G. citri-auranti* strain avir can be used in combination with commonly used fungicides in the packinghouse, a fungicide sensitivity trial was performed using fungicide amended agar. Fungicide concentrations chosen did not exceed concentrations expected in packinghouse dump tanks, and were low enough to detect modest tolerance to the compounds. A 10 µl drop of spore suspension, containing 10^6 spores per ml was placed on the center of plates of potato dextrose agar (PDA) amended with imazalil, thiabendazole (TBZ), or ortho-phenylphenate (OPP) at several concentrations, and on unamended PDA. In each

Table 1. Effect of *G. citri-auranti* strain avir on incidence of green mold caused by *P. digitatum* summary of six trials.

	Incidence of green mold	
	G. citri-auranti strain avir treated	control
	%	
trial 1	90	98
trial 2	42	90
trial 3	42	58
trial 4	5	18
trial 5 N-33 navel	17	34
trial 6 Rio Red	2	9
95% C.I.	(17,36)	(42,66)

Pr>f=.0052

Trials 1-3 were performed with wounded 'Rio Red' grapefruit and 'Marrs' orange, trial 4 was performed with non-wounded 'Rio Red' grapefruit, and trials 5 and 6 were performed with wounded 'N-33' navel orange and 'Rio Red' grapefruit.

[a]Each trial included five replications. Differences between numbers of rotted fruit in treated and control groups were significant at the Pr>f= 0.0052 as determined by Type 3 test of fixed effects and 95% confidence intervals did not overlap

case, technical grade material was dissolved in methanol and added to autoclaved, cooled, PDA. The test was performed three times; each trial included 3 plates of each concentration. The concentrations of each compound were: OPP and imazalil: 5 ppm, 25 ppm, 50 ppm, 75 ppm and 100 ppm; and TBZ: 5 ppm, 50 ppm, 100 ppm, and 200 ppm. Plates were incubated at 25º C and colony diameter was recorded 2 and 5 d after spore suspensions were transferred to the plates.

Temperature range. To determine the optimum temperature for radial growth, *G. citri-aurantii* strain avir, wild type *G. citri-aurantii*, and *P. digitatum* were grown on PDA at 5, 10, 15, 20, 25, 27, 30, 32, 35 and 37º C. Cultures were transferred to PDA by placing a 20 µl drop of spore suspension on the center of the PDA plate, and the plates were wrapped in aluminum foil. The temperature range trial was performed three times, and each trial included three replications. To

Table 2. Production of antifungal compounds by *G. citri-auranti*.

	Indicator organismsz and Producer strainsy					
	G. citri-auranti			*P. digitatum*		
mediumw	avir	485-10x	DH5a	avir	485-10	DH5a
PDA	-v	+	NG	-		
PDA+	-	+	NG	-		
GYP	-	+	-	+		
LAA	-	+	NG	-		
SRM-af	-	+	-	poor growth of *P. digitatum*		
TSA	-	+	-	poor growth of *P. digitatum*		
MA	-	+	NG	-	-	NG
YMA	-	+	-	-	+	-
SYMS	-	+	NG	-	-	NG

zIndicator organisms were used to indicate production of antifungal compounds by producer strains. Zones of inhibition in the growth of the indicator organisms surrounding a producerstrain suggested antibiotic production by a producer strain.
yProducer strains were inoculated onto the test media 5 days prior to overspraying with the indicator organisms.
x*P. syringae* strain 485-10 was used as a positive control since it produces syringomycin which is inhibitory to *P. digitatum* and *G. citri-aurantii*.
wMedia tested included, malt agar (MA), special yeast and mold agar (SYM), tryptic soy agar (TSA), PDA, PDA suplemented with 1.5% glucose and 0.4% casamino acids (PDA+), glucose yeast extract-peptone agar containing 5 g glucose, 3 grams yeast extract, 5 g peptone, and 18 g agar per litre (GYP), Yeast and mold agar (YM; Difco), syringomycin media (SRM-af; Bull et al., 1997), and lemon albedo agar containing 28 g albedo peelings blended in 400 ml dH20 and filtered, 2% agar (LAA).
vInhibition in growth of indicator organisms surrounding a producer strain indicated that an antifungal compound was produced by the producer strain. Strains surrounded by a zone of inhibition or not surrounded by a zone of inhibition were indicated by + and -, repectively. NG signifies that the producer strain did not grow on that medium. In all cases, results were the same on three replicate plates. The experiment was performed three times.

Table 3. Radial growth of avir, *G. citri-auranti* and P. digitatum on fungicide amended media.

		Average radial growth					
		Concentration (ppm)					
Fungus	Fungicide	0	5	25	50	75	100
		mm					
Avir	OPP	35.3	28.7	8.0	0	0	0
G. candidum		36.3	29.0	8.3	0.3	0	0
P. digitatum		28.7	10.7	0	0	0	0
Avir	MZL	-	34.3	19.0	5.0	1.0	0
G. candidum		-	32.0	21.0	11.0	2.7	0.7
P. digitatum		-	0	0	0	0	0
Avir	TBZ	-	34.0		35.7		22.3
G. candidum		-	33.0		35.3		19.7
P. digitatum		-	0		0		0

A 10 µl drop of spore suspension, containing 10^6 spores per ml was placed on the center of plates of potato dextrose agar (PDA) amended with imazalil, thiabendazole (TBZ), or ortho-phenyl phenate (OPP) at several concentrations, and on unamended PDA. In each case, technical grade material was dissolved in methanol and added to autoclaved, cooled, PDA. Plates were incubated at 25º C and colony diameter was recorded 2 and 5 d after spore suspensions were transferred to the plates. The test was performed three times; each trial included three plates of each concentration. The results of the tests were similar, results of the first trial are shown here.

determine if *G. citri-aurantii* strain avir was capable of growth or survival at human body temperature, plates were incubated at 37º C for 7 d and were removed and incubated at ambient temperature for 5 d days and colony growth observed.

Test for fungal inhibition. *Pseudomonas syringae* pv. *syringae* strain 485-10, a citrus black pit isolate, and *Escherichia coli* strain DH5∝ were used as positive and negative controls, respectively, for antibiotic production. Bacterial cultures were stored at -80º C in 1:1 glycerol:nutrient broth (NB; Difco Laboratories, Detroit, MI), and were routinely cultured on nutrient agar (NA; Difco). *P. digitatum* isolate M6R, which is sensitive to imazalil and TBZ, and *G. citri-aurantii* strain 93-4a were used (Bull et al., 1998). Fungi were stored on silica gel at 4º C and routinely cultured on PDA. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

The media used in this experiment were: malt agar (MA; Difco), special yeast and mold agar (SYM, Difco) , tryptic soy agar (TSA, Difco), PDA, PDA supplemented with 1.5% glucose and 0.4% casamino acids (PDA+), glucose yeast extract-peptone agar containing 5 g glucose, 3 g yeast extract, 5 g peptone, and 18 g agar liter^{-1} (GYP), yeast and mold agar (YM; Difco), syringomycin minimal media amended with arbutin and fructose (SRM-af) (Mo and Gross 1991), and lemon albedo agar containing 28g albedo peelings blended in 400ml dH_2O and filtered 2% agar (LAA).

Bacterial cultures grown 48 h at 27º C on NB, and fungal cultures grown one week on PDA were used as inocula. Test organisms were suspended in 10 ml of water and vortexed to disperse clumps. A 5 µl drop of the spore or bacterial suspension was placed on the test medium. Each plate had one spot each of strain 485-10, *E. coli* and *G. citri-aurantii* strain avir. Plates were incubated for 5 d days at 27º C. The test organisms were then killed by incubating the plates under UV light for 20 min. Inoculum of strains M6R and 93-49 were made by adding 3 ml of water containing one drop of Triton X to the PDA on which they were growing and gently rubbing the colonies with a glass rod. The suspension was then vortexed to break up spore chains and arthrospores and adjusted to 0.1 OD at 420nm. Plates with test organisms were then over sprayed with either *G. citri-auranti* or *P. digitatum*. After 2 d at 27º C, colonies with a zone of inhibition 2 mm or larger, devoid of fungal growth were considered to be positive for production of antifungal compounds. Three replications were made of each plate, and the test was conducted three times.

RESULTS

Efficacy trials. Disease incidence in wounded, *G. citri-auranti* strain avir treated fruit was consistently and significantly lower than in wounded control fruit. Disease incidence in nonwounded, *G. citri-auranti* strain avir treated fruit was low. Differences between numbers of rotted fruit in treated and control groups were significant at the Pr>f= 0.0052 as determined by Type 3 test of fixed effects and 95% confidence intervals did not overlap when variance among trials was excluded (Table 1).

Isolate avirulence. Grapefruit inoculated with *G. citri-auranti* strain avir did not rot. Of 50 N33 navel orange fruit tested, the majority were infected with *P. digitatum*, and the identity of the pathogen in one infected fruit was not clear. A total of 310 fruit were treated with avir, and an equal number were included in the positive control consisting of inoculation with a virulent strain of *G. citri-auranti*, and in the water control. Fruit were observed for symptoms of sour rot for up to 10 d. Tests of virulence on soaked fruit were run three separate times. Wild type *G. citri-auranti* caused sour rot in 50%, 38%, 80%, and 60% of grapefruit, oranges, soaked grapefruit, and soaked oranges, respectively. Avir and water controls did not cause sour rot in any of the fruit tested.

Fungicide sensitivity and temperature optimum. Growth of *G. citri-auranti* strain avir did not occur on PDA amended with SOPP or imazalil. Growth of *G. citri-auranti* strain avir was reduced on PDA amended with TBZ (Table 3). Results from three trials were similar, and one trial is shown in the table.

Optimum temperature for growth of *G. citri-auranti* strain avir was 30º C, while optimum temperature for growth of *P. digitatum* was 27º C. None of the strains grew at 37º C.

Production of antifungal compounds. On PDA, radial growth of *P. digitatum* was not influenced by coinoculation with *G. citri-auranti* strain avir. In addition, inhibition of *P. digitatum* was not detected when *G. citri-auranti* strain avir was incubated on PDA five days prior to transfer of *P. digitatum* culture to the plate. However, large zones of inhibition (over 10 mm) were present on GYP agar when *G. citri-auranti* strain avir was incubated for 5 d prior to overspray with *P. digitatum* spores. Under the same conditions, no zones of inhibition were observed on the other media tested. Results using wild-type *G. citri-auranti* strain 93-49 were identical to those of *G. citri-auranti* strain avir. Strain 93-49 produced antifungal compounds on GYP agar inhibitory to *P. digitatum* (Table 2).

DISCUSSION

As part of an integrated approach, *G. citri-auranti* strain avir could play a significant role in managing green mold, a serious postharvest disease, with reduced dependence on chemical fungicides. A patent has been issued for the use of this strain in the biological control of postharvest diseases (Eayre and Skaria, 1996). Since *G. citri-auranti* strain avir was found to be resistant to benzidamazole fungicides, it may be possible to apply it in combination with reduced rates of the fungicides to obtain satisfactory disease control. Future studies should address compatibility with commercial practices, for example, the effect of various waxes on avir. It is also important to understand mode of action, ability to colonize fruit, and the effect on host physiology (El Ghaouth et al., 2002).

Wild type *G. citri-auranti* is a common pathogen of citrus fruit, and can be expected to survive well on the fruit surface. Avir was less sensitive to OPP, imazalil, and TBZ than *P. digidatum*. Future work should address the use of avir in combination with postharvest fungicides for enhanced control and reduced risk or resistance to fungicides developing in pathogen populations. *G. citri-auranti* strain avir was not found

to infect grapefruit or oranges, insuring that there is no risk that this organism will cause disease on the fruit it would be used to protect. Additionally, it did not grow at 37º C, and therefore, will not grow at human body temperature. Avir differs from the noncitrus isolates described by Butler et al. (1965) in that it is not even weakly virulent on citrus.

Yeasts controlling postharvest diseases are generally hypothesized to act by nutrient competition, mycoparasitism and induced resistance (Bull, 1999). However, yeasts have been shown to produce antibiotics or antifungal compounds (Da Silva and Pascholati, 1992; McCormack et al., 1994; Takesako et al., 1991). In many instances scientists have ruled out antibiosis after a cursory search. In this study we demonstrated that antibiosis experiments conducted with one medium and without preinoculation of the antagonist may not detect antibiosis. It is important to test for antibiosis under a variety of conditions and with several different media. However even a rigorous search may not reveal antibiotic production by some yeasts (Filonow et al., 1996).

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