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FINAL REPORT

PROJECT NO. IS-3494-03

**Characterization of the Biochemical Basis of Host
Specificity of *Penicillium digitatum* and *Penicillium
italicum* on Citrus Fruit**

S. Droby, T.R. Gottwald, T.G. McCollum, R. Stange, E. Lewinsohn

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Investigators Institutions

Principal Investigator (PI):
S. Droby

ARO, the Volcani Center

Co-Principal Investigator (Co-PI):
T.R. Gottwald

USHRL, USDA-ARS, USA

Collaborating Investigators:

T.G. McCollum

R. Stange

E. Lewinsohn

USHRL, USDA-ARS, USA

USHRL, USDA-ARS, USA

ARO, The Volcani Center

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Volatiles, growth promotion, recognition, pathogenicity, limonene, directional growth

Abbreviations commonly used in the report, in alphabetical order:

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Signature
Principal Investigator

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Authorizing Official, Principal Investigator
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Short Visits & Meetings	-----	2		2
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Description of Cooperation:

The research conducted during four years project (Extension of the project was approved without the addition of extra funds) was carried out in full cooperation between the two Israeli and the American laboratories. S. Droby (ARO) and T. Gottwald, G. McCollum and R. Stange (USDA-ARS, USHRL, FL) met three times: First meeting took place Prior to the official initiation of the research (August, 2004) followed by two meetings during the second and third year. Numerous discussions via the e-mail and phone were done to exchange protocols, explain results and plan experiments. Results e-mail. Plant material was exchanged by the two teams and used for experiments and analysis according to the expertise of each laboratory. Two manuscripts were already published (one in press) and additional two are in final stages of preparation. The results are planned to be presented jointly in summer 2008 in three international meeting.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	--	--	--	--
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Abstract

1. This research demonstrates that citrus fruit volatiles play an important role in host recognition by *P. digitatum* and *P. italicum*.
2. Volatiles derived from non-host fruits and vegetables (apple, pear, tomato, pepper, strawberry and avocado) had no effect on promotion of spore germination and growth of citrus pathogens.
3. Citrus volatiles have a specific stimulatory effect solely on *P. digitatum* and *P. italicum*. Non-citrus pathogens such as *P. expansum* and *B. cinerea* not affected or inhibited by the volatile materials. The specific stimulatory effect of fruit peel volatiles on citrus pathogens and inhibitory effect on non-pathogens indicate important role of volatile compounds in the host selectivity of citrus postharvest pathogens
4. Comparative CG-MS quantification was performed and identification of volatile constituents of citrus commercial oils, peel extracts and the headspace of the wounded fruits was completed. Monoterpene hydrocarbons (limonene, α -pinene, sabinene, and myrcene) were the most abundant in all volatiles regardless of the source.
5. Our results demonstrated stimulation of germination and germ tube growth in both *P. digitatum* and *P. italicum* by limonene, myrcene, α -pinene, and β -pinene). Limonene was shown to be the most efficient in induction of germination and growth in both pathogens.
6. *P. digitatum* spores placed on the surface of lemon fruit, adjacent to a wounded oil gland, were induced to germinate and grow, thus supporting all the in vitro results and demonstrating that the phenomenon of stimulation of germination and growth occurs on the fruit.
7. We established that *P. digitatum* is capable of biotransformation of limonene to α -terpineol. α -terpineol was proved to be involved in induction of fungal sporulation process.
8. Chemotropism (directional growth) of *P. digitatum* towards the volatiles released from the oil glands on fruit surface was demonstrated.
9. Citrus germplasm screening work for fruit susceptibility/resistance for *P. digitatum* infection showed no definitive results regarding host range and susceptibility. Although the sour orange selections appear to show higher resistance to infection and decay development.
10. We demonstrated that *P. expansum*, non citrus pathogen, is capable of germinating in citrus fruit surface wounds, but it strongly induced host resistance mechanisms which

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restrict its growth and prevented decay development. The host (citrus fruit) reacted strongly by production of ROS. On the other hand, *P. digitatum* seems to actively suppress host natural resistance mechanisms possibly through inhibiting the production of ROS production.

Achievements:

Research Objectives as presented in the research proposal:

1. Identify the wound-induced volatile and constitutive non-volatile compounds of citrus fruit peel stimulatory to germination and growth of *P. digitatum* and *P. italicum*.
2. Determine how levels of the stimulatory compounds change during fruit development and correlate these changes with changes in susceptibility to disease.
3. Determine how stimulatory compounds affect gene expression in *P. digitatum* and *P. italicum* during early stages of the germination process and later growth.
4. Screen germplasm of citrus relatives to establish the host range of the pathogens and determine host susceptibility

As suggested by panels and reviewers, additional objective was included to evaluate the response of citrus fruit to non pathogen (e.g. *P. expansum*) and its effect on germination and growth.

1. Role of citrus volatiles in host recognition, germination and growth of *Penicillium digitatum* and *Penicillium italicum*

Volatiles emitted from wounded peel tissue of various citrus cultivars had a pronounced stimulatory effect on germination and germ tube elongation of both *P. digitatum* and *P. italicum*; however, *P. digitatum* appeared to be more sensitive to the stimulatory action of citrus peel volatiles. When exposed to volatiles from grapefruit peel discs, the percentage of germinated spores of *P. digitatum* and *P. italicum* was 75.1 % and 37.5 %, respectively, whereas germination of controls was 6.8 % and 14.7 %, respectively. In contrast, *B. cinerea* and *P. expansum* were either not affected or inhibited by the peel

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volatiles. GS-MS analysis of volatiles present in the peel of various citrus fruit cultivars revealed that limonene is the major fruit peel volatile. Its percentage ranged from 89 – 95% at the early stages of fruit development throughout the harvest season. Myrcene and α -pinene made up the second and third greatest amounts among the volatiles found in these oils, ranging from 2.12% to 2.33% and from 0.71% to 1.25 %, respectively. All four monoterpenes, limonene, α -pinene, β -pinene and myrcene were stimulatory to *P. digitatum* and *P. italicum* but inhibitory to or had no effect on *P. expansum* and *B. cinerea*. Germ tube elongation in *P. digitatum* responded most strongly to limonene and less strongly to α -pinene and β -pinene while myrcene had little effect. In *P. italicum*, myrcene stimulated germ tube elongation the most followed by limonene, with α -pinene, and β -pinene being about equal. Germination of *P. italicum* conidia was highest in response to myrcene with the effect of the other compounds being about equal at concentrations of 5 μ l or more per plate

This work was accepted for publication in "Postharvest Biology and Technology" (In press, see appendix for details).

2. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit

During the infection of citrus fruit by *Penicillium digitatum* there is little evidence of a host defense response. This suggests that *P. digitatum* has the ability to suppress host defenses. The current study demonstrates that *P. digitatum* suppresses a defense-related hydrogen peroxide (H₂O₂) burst in host tissue. In contrast, the non host pathogen, *Penicillium expansum*, triggers production of a significant amount of H₂O₂ in citrus fruit exocarp. Using laser scanning confocal microscopy, we demonstrated that *P. digitatum* suppressed an elevation in H₂O₂ up to 42 h after inoculation. Nevertheless, H₂O₂ levels around wounds inoculated with *P. expansum* increased by 63-fold above the control. *P. digitatum* continued to suppress H₂O₂ production in citrus fruit exocarp up to 66 h post inoculation and H₂O₂ levels were actually threefold below that of non inoculated

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controls. In contrast, the H₂O₂ level was still about 11-fold above the control value in wound sites inoculated with *P. expansum*.

Studies on the effect of organic acids (as pH modulators) on the response of citrus fruit to compatible and noncompatible pathogens indicated that pathogenicity was enhanced only when host-tissue acidification was accompanied by the suppression of H₂O₂. Additionally, pathogenicity of both *P. digitatum* and *P. expansum* on citrus fruit was significantly enhanced by the H₂O₂-scavenging enzyme catalase. Based on our study and previous reports regarding the potential involvement of citric acid and catalase in green mold pathogenesis, we suggest that these compounds are strongly associated with the virulence of *P. digitatum*.

This work was published in "Phytopathology" (see appendix for details).

Description of non published data

1. Composition of Volatiles of infected vs. healthy fruit tissue

■

Quantitative determination of volatiles was carried out in the head space of wounded and infected fruit and only wounded fruit after 7 days of incubation at 20 C. The volatiles released to the head space were trapped by special column containing Porapak-Q attached to air flow system and analyzed by GC-MS following extraction from the column. In addition, volatile materials were determined in decayed and healthy fruit peel tissue following extraction with hexane.

As presented in Fig. 1 the volatile profiles of infected and healthy (only wounded) fruit that the most pronounced difference was in the amount of Limonene that was most significant in the infected fruits compared to the healthy tissue. Myrcene and linalool was also found in higher amounts in the head space of infected tissue.

Analysis of volatiles in the decayed (soft) and healthy fruit tissue showed (Fig. 2) that amount of limonene was lower in the soft tissue compared with the healthy fruit peel tissue. As for the other volatile compounds, α -terpineol was present only in the soft infected peel tissue. This material is a product of limonene breakdown.

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Fig. 1 Volatile profile in heads space of infected (■) and healthy (□) fruit tissue after 7 days of incubation at 20°C.

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Tissue type

C

Compound

Fig. 2: volatile profile of soft infected and healthy orange fruit peel tissue

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2. Biotransformation of limonene by *P. digitatum*

Based on the results obtained from the analysis volatiles released to the head space and infected and health fruit and those present in soft decayed and healthy fruit tissue it was assumed that the fungus (*P. digitatum*) may be involved in transformation of limonene to α -terpineol and other products. Tests conducted to evaluate the ability of *P. digitatum* to transform limonene to α -terpineol in vitro. In the first set of tests, the transformation of limonene added to liquid medium was evaluated. Results are presented in Fig. 3 The break down product α -terpineol was detected in the medium indicating that *P. digitatum* is capable to transforming limonene.

Fig. 3: Biotrasformation of Limonene added to liquid medium by *P. digitatum*.

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Additional experiments were done to determine the capability of *P. digitatum* to transform limonene added to the head space of the culture using the SPME system. In these experiments the fungus was grown in SPME tubes containing PDA for 4 days and then exposed for 24 or 48 h to limonene spotted on paper disc glued to the tubes' cover. Results presented in Fig.12 clearly show the concentration of limonene decreased to 58.8% of the initial amount and α -terpineol increased from 0% to 41.1% after 24 h (Fig. 4).

Fig. 4: Biotransformation of limonene by *P. digitatum* in the SPME system.

3. Biological activity of α -terpineol against spore germination and germ tube growth

The effect of the biotransformation product α -terpineol of spore germination and germ tube elongation of the *P. digitatum*, *P. expansum*, *Italicum* and *B. cinerea* was tested using the Petri dish bioassay. Results presented in Fig. 5 indicate that α -terpineol at a concentration of 0.5 μ l/plate promoted spore germination of *P. digitatum* to 46% compared to 14% germination in the control. *P. Italicum* was not affected at this concentration. On the other hand, *P. expansum* and *B. cinerea* were inhibited at the same concentration. Higher concentrations of α -terpineol inhibited all fungi tested.

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Fig. 5: Effect of α -terpineol on spore germination and germ tube length.

— *P. italicum* - - - *B. cinerea* — *P. digitatum* — *P. expansum*

The effect of α -terpineol against *P. digitatum* was compared to limonene. Results demonstrated that limonene promoted spore germination and growth at all concentration tested while α -terpineol inhibited germination at concentration of 5 μ l/plate and above. Promotion of germination by α -terpineol was evident at 0.5 μ l/plate (Fig. 6).

Fig. 6: Effect of limonene and α -terpineol on spore germination and germ tube length of *P. digitatum*.

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4. Effect of α -terpineol on sporulation of *P. digitatum*

Following the earlier findings regarding the breakdown of limonene to α -terpineol it was hypothesized that the presence of α -terpineol in the decayed fruit tissue may be involved in inducing sporulation of the fungus. To test this hypothesis, we used in vitro system in which PDA plates were seeded with spore suspension of the fungus and placing different concentrations of α -terpineol on filter paper discs in the center of the plate. As shown in fig. 7 only the discs containing 10 μ l markedly induced sporulation while higher concentrations caused inhibition indicating that this effect is concentration dependent. Limonene has no effect on sporulation.

Fig. 7 Effect of limonene and α -terpineol on sporulation of *P. digitatum*.

A -control ,E-B -5-40 μ l/plate α -terpineol ,F-I -5-40 μ l/plate limonene.

5. Germination and (directional growth) of *P. digitatum* towards citrus peel wounds

Collectively, the findings of this work supported our hypothesis regarding the important role of citrus fruit volatiles in specifically promoting germination and germ tube growth of the two citrus pathogens *P. digitatum* and *P. italicum*. To support the in vitro work we have done extensive microscopic observations about the effect of fruit wounds to promote germination of fungal spores in vivo on the fruit peel. The micrographs presented in Fig.8 clearly illustrate that fungal spores placed on the surface of lemon fruit, adjacent to a wounded oil gland, were induced to germinate and

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grow. As shown in Fig 8 B, the vast majority of the spores germinated and formed long germ tubes within 40 hours after incubation even though they were not in contact with the oils that may or may not have leaked from the punctured glands. Massive growth of the fungus and further colonization of the wounded oil glands were observed after 140 h. (Fig 8D) while spores placed on an intact fruit surface did not germinate or grow even after 140 h of incubation.

Fig 8: Micrographs illustrating developmental changes of conidia of *P. digitatum* on the surface of wounded (B and D) and intact fruits (A and C) at 40 h (A and B) and 140 h (C and D) post inoculation (P.I.). Magnification X 60.

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Fig. 9: SEM Micrograph illustrating spore germination and directional growth of *P. digitatum* spores towards the wounds in fruit peel.

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Fig. 9: SEM Micrograph illustrating spore germination and directional growth of *P. digitatum* spores towards the wounds in fruit peel.

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6. Screening Citrus germplasm (fruit) for resistance to *Penicillium digitatum*

Citrus germplasm screening work for fruit susceptibility/resistance for *P. digitatum*

infection showed no definitive results regarding host range and susceptibility. Although the sour orange selections appear to show higher resistance to infection and decay development.

First season:

Exp. 1

Selection	Decay 6 DAI Mean (%)	
Seville	5.6	a
Bittersweet	6.1	a
Sour #2	11.1	ab
Brazil	15.6	abc
Bergamot	22.2	abc
Flame/FD	29.3	bcd
Hamlin	32.5	cd
Natsudaiddai	33.3	cd
Flame/827	41.4	d
Fairchild	77.8	e

Exp. 2

Selection	Decay 6 DAI Mean (%)	
Seville	30.3	a
Bittersweet	31.3	a
Palestine	33.3	a
Sour		
Brazil	41.1	ab
Sour #2	56.6	abc
Natsudaiddai	66.7	bc
Flame/SO	68.7	bcd
Flame/FD	81.8	cd
Bouquet	83.3	cd
Butwal	89.9	d

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Second Season:

Exp. 1

Selection	Decay 6 DAI Mean (%)	
Brazil (12-1)	5.6	a
Bittersweet (11-7)	7.1	a
Seville (11-6)	7.4	a
Bergamot (12-15)	8.3	a
Hamlin (3-14)	10.3	a
Scillian 2n (12-16)	11.1	a
Ambersweet (10-4-20)	11.1	a
Palestine (12-9)	13.0	a
Sauvage (12.4)	13.0	a
Marsh (5-22)	13.0	a
Ruby (6-26)	18.2	a
Butwal (6-6)	25.3	a
Page (10-20)	54.8	b
Orlando (9-22)	68.5	b

Exp. 2

Selection	Decay 6 DAI Mean (%)	
Seville	17.8	a
Sauvage	20.0	ab
Bittersweet	21.1	ab
Flame	21.1	ab
Cadenera Fina	30.0	ab
Bergamot	32.2	ab
Palestine Sour	32.2	ab
Natsudaikai	36.7	ab
Scillian (2N)	42.2	ab
Ambersweet	43.3	b
Orlando	77.8	c
Swingle Tangor	78.9	c
Butwal	81.1	c

Sour orange varieties are shown in **boldface**.

All mean separations used Tukey (0.05%) and were calculated based on arcsin transformed decay data.

Exp. 3

Selection	Decay 6 DAI Mean (%)	
Seville	26.3	a
Flame	30.3	a
Bittersweet	33.3	a
Brazil	33.3	a
Scillian (2N)	37.4	ab
Palestine	40.4	ab
Sauvage	46.5	ab
Cadenera Fina	47.2	ab
Ridge Pineapple	47.5	ab
Natsudaikai	65.1	bc
Bergmott	66.7	bc
Butwal	84.4	cd
Sexton	88.3	de
Sunburst	97	e

Exp. 4

Selection	Decay 6 DAI Mean (%)	
Redblush	27.8	a
Scillian 2n	29.6	a
Bittersweet	31.3	a
Seville	31.3	a
Sauvage	40.7	ab
Sour #5	41.7	ab
Brazil Sour	43.4	ab
Cadenera Fina	48.1	ab
Palestine Sour	50.9	ab
Natsudaikai	53.7	ab
Valencia	54.6	abc
Bergamont	69.4	bcd
Ortanique	87.8	cde
Wilking	90	de
Butwal	97.2	e

Sour orange varieties are shown in **boldface**.

All mean separations used Tukey (0.05%) and were calculated based on arcsin transformed decay data.

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Orlando = 'Duncan' X 'Dancy' [Tangelo]

Ortanique = Chance seedling; presumed SwO X M cross. [tangor]

Page = 'Minneola' ('Duncan' x 'Dancy') X 'Clementine'

Sexton = Chance seedling; presumed Gf X M cross. [tangelo]

Sunburst= {'Robinson' ['Clementine' X 'Orlando' ('Duncan' X 'Dancy')]} X 'Osceola'
Clementine' X 'Orlando' ('Duncan' X 'Dancy')

Wiling = C. deliciosa 'Willow Leaf' X C. nobilis 'King'

7. The role of citrus peel compounds in host recognition by *Penicillium digitatum* and *P. italicum*

Components of citrus peel stimulated the growth of *Penicillium digitatum* and *P. italicum*, suggesting that components of the host play a role in recognition by the pathogen. To determine which components of the peel are responsible in the peel are responsible for this stimulation, flavedo of *Citrus medica* and *C. paradisi* was extracted with 80% acetone. These extracts were further separated into hexane, or ethyle acetate-partitioning, and aqueous remaining fractions. The effect of these fractions, in factorial combination, was assessed using a vital stain (MTT) to quantify the fungal growth. Optimal growth was obtained only when hexane and aqueous were combined. Bioassay-guided purification of the active components in the hexane fraction was performed using aqueous fraction as background. Following chromatography on silica gel and c-18, the components in active fractions were identified by NMR. **Epoxybergamittin** was identified from *C. paradise*. The active fraction from *C. medica* contained related compound, **prangolarin**, and **citropene**. This suggests that host recognition by *P. digitatum* and *P. italicum* may involve a small number of related compounds.

Manuscript describing these findings in preparation.

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Cooperation

List of Publications:

Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M., and Droby, S. 2007. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. *Phytopathology* 97:1491-1500.

Droby, S., Eick, A., Macarisin, D., Cohen, L., Rafael, G., Stange, R., McCollum, G., Dudai, N., Nasser, A., Wisniewski, M. and Shapira, R. 2008. Role of citrus volatiles in host recognition, germination and growth of *Penicillium digitatum* and *Penicillium italicum*. *Postharvest Biology and Technology* 48: in press.

Stange, R.R., McCollum, T.G., Midland, A.L., Sims, J.J. and Droby, S. 2008. The role of citrus peel compounds in host recognition by *Penicillium digitatum* and *P. italicum*. In preparation.

Droby, S., Eick, A., Macarisin, D., Cohen, L., Rafael, G., Stange, R., McCollum, G. 2008. Biotransformation of citrus volatiles by *Penicillium digitatum* and role of metabolites in inducing sporulation. In preparation.