**BARD Final Scientific Report
Cover Page**

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Project Title: Redox Climate in Quiescence and Pathogenicity of Postharvest Fungal Pathogens

Investigators

Institutions

Principal Investigator (PI):

Richard M. Bostock

University of California, Davis

Co-Principal Investigator (Co-PI):

Dov Prusky

Martin Dickman

Volcani Institute

University of Nebraska

Collaborating Investigators:

Stephen Marek (postdoctoral associate)

Miin-Huey Lee (Ph.D. candidate)

University of California, Davis*

University of California, Davis

*current title and address: Assistant Professor, Department of Entomology and Plant Pathology
127 Noble Research Center, Oklahoma State University, Stillwater, OK 74078

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

Brown rot of stone fruits, *Monilinia fructicola*, anthracnose of avocado, *Colletotrichum gloeosporioides*, cutinase, antioxidants, Agro-transformation

Abbreviations commonly used in the report, in alphabetical order: CA, caffeic acid; CGA, chlorogenic acid; GFP, green fluorescent protein; GSH, glutathione.

Budget: IS: \$111,000

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Total: \$ 270,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		3		
Submitted, in review, in preparation		2		
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Book chapters				
Books				
Master theses				
Ph.D. theses		2		
Abstracts		3		
Not refereed (proceedings, reports, etc.)		1		

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Stephen Marek (ss# is confidential and we choose not to disclose that information)

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1	2	3
Longer Visits (Sabbaticals)	0	0	0	0

Description of Cooperation:

The research on this project was conducted in three laboratories. Work in the Prusky lab (Volcani, Israel) focused on the effects of antioxidant phenolics and pH on pectolytic, proteolytic and laccase enzymes produced by the avocado pathogen, *Colletotrichum gloeosporioides* (reported in previous annual report). Work in the Bostock lab (UC Davis, USA) focused on related antioxidant effects on cutinase and cell wall degrading enzymes produced by *Monilinia fructicola* and other post-harvest pathogens, and the research summarized in this final report. Work in the Dickman lab (Nebraska, USA) provided support for molecular analyses of the fungal pathogens used in this project.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0

**Final Report
2003
BARD Project No. US-3039-98**

Redox Climate in Quiescence and Pathogenicity of Postharvest Fungal Pathogens

Richard M. Bostock (University of California, Davis)

Martin B. Dickman (University of Nebraska)

Dov Prusky (Volcani Institute)

Stephen M. Marek (University of California, Davis)

Miin-Huey Lee (University of California, Davis)

Abstract

Monilinia fructicola causes brown rot blossom blight and fruit rot in stone fruits. Immature fruit are highly resistant to brown rot but can become infected. These infections typically remain superficial and quiescent until they become active upon maturation of the fruit. High levels of chlorogenic acid (CGA) and related compounds occur in the peel of immature fruit but these levels decline during ripening. CGA inhibits cutinase expression, a putative virulence factor, with little or no effect on spore germination or hyphal growth. To better understand the regulation of cutinase expression by fruit phenolics, we examined the effect of CGA, caffeic acid (CA) and related compounds on the redox potential of the growth medium and intracellular glutathione (GSH) levels. The presence of CA in the medium initially lowered the electrochemical redox potential of the medium, increased GSH levels and inhibited cutinase expression. Conidia germinated in the presence of CA, CGA, or GSH produced fewer appressoria and had elongated germ tubes compared to the controls. These results suggest that host redox compounds can regulate fungal infectivity.

In order to genetically manipulate this fungus, a transformation system using *Agrobacterium* was developed. The binary transformation vector, pPTGFPH, was constructed from the plasmid pCT74, carrying green fluorescent protein (GFP) driven by the *ToxA* promoter of *Pyrenophora tritici-repentis* and hygromycin B phosphotransferase (*hph*) under control of the *trpC* promoter of from *Aspergillus nidulans*, and the binary vector pCB403.2, carrying neomycin phosphotransferase (*nptII*) between the T-DNA borders. Macroconidia of *M. fructicola* were co-incubated with *A. tumefaciens* strain LBA 4404(pPTGFPH) on media containing acetosyringone for two days. Hygromycin- and G418-resistant *M. fructicola* transformants were selected while inhibiting *A. tumefaciens* with cefotaxime. Transformants expressing GFP fluoresced brightly, and were formed with high efficiency and frequency of T-DNA integration frequency. The use of these transformants for *in situ* studies on stone fruit tissues is discussed.

Summary of principal achievements of the project (3 years)

- Adapted/developed a novel affinity purification scheme for fungal cutinases (showed proof of concept; U.S.).

- Cloned and characterized a full-length cutinase cDNA from *Monilinia fructicola*, information that now has enabled isolating a genomic clone with upstream regulatory sequences (U.S.).
- Demonstrated redox regulation of *Monilinia* cutinase expression and infection structures (U.S.), and of *Colletotrichum gloeosporioides* cell wall degrading enzymes (Israel).
- Developed a facile transformation system for *M. fructicola*, and enabling expression of reporter genes for in situ studies of the host-parasite interaction and other transgenes of interest (U.S.).

Introduction and Objectives

Monilinia fructicola and *Colletotrichum gloeosporioides* are important fungal pathogens in the United States and Israel that limit the post-harvest life of stone fruits and subtropical fruits, respectively. Control of the diseases caused by these pathogens is complicated by quiescence of fruit infections that can lead to severe disease losses upon activation of the pathogen during storage and shipment. Fruit become increasingly susceptible during ripening with a corresponding decline in the levels of reducing phenols in the fruit epidermis. Our previous studies indicate that catecholic phenolics, particularly chlorogenic acid and epicatechin which decline during ripening, can inhibit production or action of degradative enzymes secreted by these pathogens, with at least part of the effect evident at the level of gene expression.

The objective of this project was to assess the influence of redox climate on the ability of these fungi to express a representative set of degradative enzymes associated with decay of fruit. We propose that this redox climate is determined in part by levels of fruit phenolics. We also propose that changes in the external redox environment are sensed by pathogenic fungi, which result in fungistasis or active growth and colonization of fruit depending on the direction of the change. The overall objectives of this project were to 1) determine if extracellular redox status influences the expression of cutinolytic and cell wall degrading enzymes, and, if so, determine the level at which expression is affected; 2) identify redox-responsive genes in these fungi by differential cDNA display and suppression subtractive hybridization; and 3) functionally test selected redox-responsive genes in transformed fungal strains for effects on pathogenicity and response to redox agents. With respect to these objectives, progress was made primarily on objectives 1 and 3 during the period of the grant. The following is a summary of work completed since our previous report and constitutes the final report for this project.

Review of work completed in U.S. (Bostock's lab during final project year)

In the last year of the project we focused on four topics related to objectives 1 and 3. These topics were 1) to determine the effect of caffeic acid on the extra- and intracellular redox status of *Monilinia fructicola*, 2) to determine the effect of redox compounds, including caffeic acid, chlorogenic acid and glutathione, on *M. fructicola* germ tube growth and brown rot disease development, 3) to isolate the 5' and 3' flanking regions of the *M. fructicola* cutinase gene (*Mfcut1*) to make *Mfcut1* knockout mutants, and 4) to develop Agro-transformation in *Monilinia* as a facile method to transform strains with genes of interest (e.g., GUS driven with the *Mfcut1* promoter for studying *in situ* regulation of cutinase) and to generate mutants by T-DNA insertion. We have measured the changes in pH, redox potential, cutinase activity, and

intracellular glutathione levels over time in the external medium and mycelia exposed to caffeic acid. Also, we have measured appressorium formation and brown rot symptom development in the presence of antioxidants. We currently are screening *Mfcut1* knockout mutants and GUS expression mutants.

We have hypothesized that the changes in the biochemical composition of peach fruit during ripening are likely important in the regulation of quiescent infections. We further hypothesize that the decline in antioxidant phenolic compounds as fruit mature may trigger the fungus to assume an active invasive, colonization habit. We have shown that the predominant phenolics in the fruit surface inhibit cutinase expression but not fungal growth. To understand how the fruit phenolic compounds negatively regulate cutinase expression, we examined the effect of selected phenols on the redox potential of the medium and intracellular glutathione (GSH) levels. Stationary liquid cultures of *M. fructicola* were started from conidial suspensions and grown in Czapek-Dox medium supplemented with 0.1% yeast extract with 0.1% apple cutin as the sole carbon source (Fig. 1). Stock solutions of 100 mM caffeic acid were prepared in methanol and used to bring cultures to 1 mM caffeic acid for treatments, with an equal concentration of methanol used in controls. Mycelium and culture filtrates sampled over time and were separated by filtration and analyzed. The pH and redox potential of each culture filtrate was determined using an Ag/AgCl pH electrode and platinum-Ag/AgCl combination electrode, respectively. Total glutathione levels were determined according to (2) and the p-nitrophenol butyrate esterase activity was determined according to (4).

Phenolic compounds initially reduced the redox potential of the medium, increased intracellular GSH levels and inhibited cutinase expression in the inductive medium (Fig. 1). In other words, caffeic acid cultures were more reducing than control cultures at the beginning of growth but became more oxidizing than control cultures after fifteen days growth (Fig. 1B). Intracellular glutathione levels of *M. fructicola* increase with growth in both caffeic acid containing and control cultures, but were maintained at somewhat higher levels in the caffeic acid cultures (data shown in previous report). After fifteen days growth in the presence of cutin, fungal cultures in the presence of caffeic acid secreted significantly less esterase activity into culture filtrates and contained higher glutathione levels in mycelia than control cultures (Fig. 1A).

Green fruit infections by *M. fructicola* often become quiescent that then can become active in mature fruit to cause pre- and post-harvest brown rot and severe yield losses. The quiescent infections sometimes show tiny restricted necrotic spots, but mostly are asymptomatic. The resting structures of *M. fructicola* during quiescent infection remain unknown. In *Colletotrichum*, appressoria and infection pegs have been suggested to serve as resting structures in quiescent infections. We have found *M. fructicola* can form an atypical appressorium on fruit and polystyrene surfaces (Fig. 2). *M. fructicola* spores were collected from V-8 agar plates or from infected fruit. The spores were collected by spreader or by brush with sterile water and passed through 3-layers of cheesecloth. The spores were then washed by centrifugation and resuspended in sterile water for three cycles. Over 90% of the washed spores collected from V-8 medium plates formed appressoria on a polystyrene surface, whereas less than 40 % of the spores formed appressoria on glass slides. Non-washed spores collected from V-8 medium plates showed almost no appressoria formation on a polystyrene surface. Approximately 70% of unwashed spores harvested from infected fruit formed appressoria on a polystyrene surface and the spores

germinated poorly after washing (data not shown). These data suggest that washed spores harvested from medium behave as spores in nature.

M. fructicola appressoria formed on the skin of unripe fruit of three nectarine varieties (Fantasia, Independence, and Dwarf), but did not form on skin of ripe fruit (var. Fantasia, Independence, and Star Bright) (Figs. 2A&B). The penetration peg formed from a *M. fructicola* appressorium from a washed spore can penetrate an onion epidermal cell. An adhesion zone also can be observed to surround an appressorium (data not showed). These observations suggest that *M. fructicola* appressoria are capable of penetration and attachment on green fruit.

As mentioned above, green fruit skin has significantly higher levels of phenolics (e.g., caffeic acid and chlorogenic acid) than mature fruit. We have studied the effect of phenolics and other antioxidants on *M. fructicola* germ tube development. *M. fructicola* spores were collected and washed from 10-day-old V-8 agar cultures. The washed spore suspension was incubated with 0.5 mM sucrose and various concentrations of antioxidants as indicated. Appressorium formation and spore germination rates were counted after 17 hr incubation on a polystyrene surface at room temperature. Antioxidants, including caffeic acid, chlorogenic acid and reduced glutathione (GSH) inhibited appressorium formation, but only slightly inhibited spore germination (Figs. 3&4). It has been well documented that *M. fructicola* has lower infection rates on green fruit than on mature fruit (Biggs and Northover 1988; Ibbotson-Darhower *et al.* 1998). Regardless of whether appressoria can serve as resting, infection or adhesion structures in the establishment of quiescent infections, caffeic and chlorogenic acids could affect *M. fructicola* infection through an effect on appressorium formation and action. We also examined the direct effect of these redox compounds on brown rot development. Washed *M. fructicola* spores were co-inoculated with antioxidants by applying the mixture on mature fruit and then incubating the inoculated fruit in a moisture chamber at room temperature. GSH (1 mM) was able to restrict brown rot symptom development (Fig. 5), whereas caffeic acid and chlorogenic acid could not restrict the symptom development even at concentrations as high as 2 mM under our experimental conditions (data not shown).

Portions of this work were reported in abstract/poster form Lee, M.H., Marek, S.M., and Bostock, R.M. 2002. Redox regulation of *Monilinia fructicola* cutinase expression and germ tube development. *Phytopathology* 92: No. 6 (Suppl.) Publication no. P-2002-0672-AMA.

Fig. 2. Appressoria of *M. fructicola* isolate MUK1. Appressoria were formed by washed conidia in water 9 hr after inoculation of immature “Fantasia” nectarine fruit (A), but only hyphal germ tubes were formed on mature nectarine fruit (B). For comparison, appressoria (C) and hyphal germ tubes (D) formed in a 96-well plate after 9 hr in 0.5 mM sucrose.

A

B

Fig. 3. (A) Appressorium formation by *M. fructicola* was inhibited by CA (caffeic acid) and ChA (chlorogenic acid), both at 0.5-1 mM. (B) Germination was slightly inhibited by 0.5 mM ChA and 1 mM of CA and ChA. Appressorium formation and germination were counted after 17 hr incubation in 96-well plates containing washed spores amended with 0.5 mM sucrose.

Development of *Agrobacterium tumefaciens*-mediated transformation in *M. fructicola*.

In order to genetically manipulate this fungus, a transformation system using *Agrobacterium* was developed. The binary transformation vector, pPTGFPH, was constructed from the plasmid pCT74, carrying green fluorescent protein (GFP) driven by the ToxA promoter of *Pyrenophora tritici-repentis* and hygromycin B phosphotransferase (*hph*) under control of the *trpC* promoter of from *Aspergillus nidulans*, and the binary vector pCB403.2, carrying neomycin phosphotransferase (*nptII*) between the T-DNA borders (see figure below). Macroconidia of *M. fructicola* were co-incubated with *A. tumefaciens* strain LBA 4404(pPTGFPH) on media containing acetosyringone for two days. Hygromycin- and G418-resistant *M. fructicola*

transformants were selected while inhibiting *A. tumefaciens* with cefotaxime. Transformants expressing GFP fluoresced brightly.

Strain	Background	Helper pTi	Plasmid	Markers
<i>At</i> LBA4404	TiAch5	pAL4404	none	
<i>At</i> LBA4404	TiAch5	pAL4404	pCB403.2	<i>nptII</i> ^a
<i>At</i> LBA4404	TiAch5	pAL4404	pPTGFPH	<i>nptII</i> , <i>hph</i> ^b , <i>sgfp</i> ^c
<i>At</i> EHA105	C58	pTiBo542?T-DNA	none	
<i>At</i> EHA105	C58	pTiBo542?T-DNA	pCB403.2	<i>nptII</i>
<i>At</i> EHA105	C58	pTiBo542?T-DNA	pPTGFPH	<i>nptII</i> , <i>hph</i> , <i>sgfp</i>

^a *nptII* = neomycin phosphotransferase under control of the nopaline synthase (*nos*) promoter and terminator, confers resistance to neomycin, kanamycin, and G418. ^b *hph* = hygromycin phosphotransferase under control of the *trpC* promoter from *Aspergillus nidulans*. ^c *sgfp* = modified green fluorescent protein (S65T; Blue-SGFP-TYG) under control of the *ToxA* promoter from *Pyrenophora tritici-repentis* and the *nos* terminator

The following results were obtained from these experiments.

<i>Agrobacterium</i> strain	<i>Monilinia fructicola</i> MUK1 transformants/spore conc.			Transformation Efficiency (%) Mean ($\pm$ SD)
	10^2	10^3	10^4	
LBA4404	0	0	0	0 (0)
LBA4404(pCB403.2)	18	70	207	9 (8)
LBA4404(pPTGFPH)	23	100	205	12 (11)
EHA105	0	0	0	0 (0)
EHA105(pCB403.2)	12	60	170	7 (5)
EHA105(pPTGFPH)	11	ND ^a	180	4 (6)

^aND = not determined

GFP Expression in Transformants

***Monilinia fructicola* transformed with T-DNA from pPTGFPH.** (A). Hyphae ramified at edge of cherry petal, 200x. (B) Conidiophore developing on colonized cherry stamen, 400x. (C) Microconidiophores on cherry sepal, 300x. (D) Sporodochia emerging from colonized cherry stamen, 400x. (E) Macroconidia from culture, 200x. (B&D = confocal micrographs)

There are a number of significant findings from this work. This is the first report of the genetic transformation of *Monilinia fructicola* (reported in abstract/poster form Marek, S.M., Pan, Z., Ciuffetti, L.M., and Bostock, R.M. 2002. *Agrobacterium tumefaciens*-mediated transformation of *Monilinia fructicola* with green fluorescent protein. *Phytopathology* 92: No. 6 (Suppl.) Publication no. P-2002-0673-AMA.). In order to accomplish this, we have utilized *nptII* selection using G418 selection, the first known use of this strategy in filamentous fungi. The use of rich media (e.g. V-8, PDA) suppressed sensitivity to G418 observed on defined minimal media. Hygromycin-resistance of putative transformants was marginal (10 µg/ml) and may be due to the weak expression of *hph* under the *trpC* promoter in *M. fructicola*.

Facile *Agrobacterium*-mediated transformation obviates the need to optimize spheroplast/protoplast production for conventional PEG-assisted transformation. We achieved transformation efficiency (~10%) sufficient for T-DNA tagging insertional mutagenesis. We also found transformation to be stable even after subculturing several times on nonselective media. In general, GFP expression appeared brighter in conidiophores than in vegetative hypha. A potential limitation to the use of GFP as a reporter gene to examine the interaction of *M. fructicola* with *Prunus* spp. is that background autofluorescence of some host tissues may interfere with the GFP fluorescence.

In summary, we found that *Agrobacterium*-mediated transformation provided a facile, efficient method for the transformation of *M. fructicola*.

Review of work completed in Israel

The major portion of this work was conducted during 2001 and early 2002, and discussed in more detail in the previous annual report. To briefly summarize, the studies with *C. gloeosporioides* indicate that the antioxidants epicatechin and catechin, present in avocado fruit, can inhibit the fungal pectate lyase (PL) directly, with possible indirect effects at the level of synthesis or secretion. Pro-oxidants, such as ozone and H₂O₂, had variable effects on *C. gloeosporioides* growth and cell wall pectin-degrading activities. H₂O₂ strongly inhibited PL activity directly. Polygalacturonase (PG) activity was relatively unaffected directly by antioxidants or oxidants. PL and PG activities were inhibited by H₂O₂ in a parallel fashion in cultures initiated by spores. In cultures initiated with mycelium the effects of oxidants and antioxidants on PG and PL activities were variable and depended somewhat on pH of the medium.

Publications and abstracts resulting from this work

Wang, G. Y., T. J. Michailides, B. M. Hammock, and R. M. Bostock. 1999. Cloning and characterization of a redox-responsive cutinase from *Monilinia fructicola*. *Phytopathology* 89:S82. Publication no. P- 1999-0587-AMA. 72.

Bostock, R.M., S.M. Wilcox, G. Wang, J.E. Adaskaveg. 1999. Suppression of *Monilinia fructicola* cutinase production by peach fruit surface phenolic acids. *Physiological and Molecular Plant Pathology* 54: 37-50

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