BARD

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**Modulation of Pathogenicity of Postharvest Pathogens
by Environmental pH**

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Final Scientific Report

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Cooperation Summary (numbers)

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Description Cooperation:

Cooperation between the American and Israeli team was very high. Several aspect of the work was done in joint collaborations. But we had independent research in each specific pathogen. Beside that very fruitful work was done and published together. We intend to follow this work and present a future joint proposal.

Abstract

Until recently, environmental pH was not considered a factor in determining pathogen compatibility. Our hypothesis was that the environmental pH at the infection site, which is dynamically controlled by activities of both the host and the pathogen, regulates the expression of genes necessary for disease development in *Colletotrichum gloeosporioides* and *Sclerotinia sclerotiorum*. This form of regulation ensures that genes are expressed at optimal conditions for their encoded activities. Pectate lyase encoded by *pelB*, has been demonstrated to play a key role in virulence of *C. gloeosporioides* in avocado fruit. Polyglacturonase synergism with oxalic acid production is considered to be an essential pathogenicity determinant in the interactions of *S. sclerotiorum* with its numerous hosts. A common regulatory feature of these virulence and pathogenicity factors is their dependence upon environmental pH conditions within the host niche to create optimal conditions for expression and secretion. In this proposal we have examined, 1) the mechanisms employed by these fungi to establish a suitable pH environment, 2) the molecular levels at which genes and gene products are regulated in response to environmental pH, and 3) the molecular basis and functional importance of pH-responsive gene regulation during pathogenicity.

The specific objectives of the proposal were:

1. Characterize the mechanism of local pH modulation and the effect of ambient pH on the expression and secretion of virulence factors.
2. Provide evidence that a conserved molecular pathway for pH-responsive gene expression exists in *C. gloeosporioides* by cloning a *pacC* gene homologue.
3. Determine the role of *pacC* in pathogenicity by gene disruption and activating mutations.

Major conclusions

1. We determined the importance of nitrogen source and external pH in the secretion of the virulence factor pectate lyase with respect to the ambient pH transcriptional regulator *pacC*. It was concluded that nitrogen source availability and ambient pH are two independent signals for the transcriptional regulation of genes required for the disease process of *C. gloeosporioides* and possibly of other pathogens.
2. We also determined that availability of ammonia regulate independently the alkalization process and *pelB* expression, pectate lyase secretion and virulence of *C. gloeosporioides*.
3. Gene disruption of *pacC* reduced virulence of *C. gloeosporioides* however did not reduced fully *pelB* expression. It was concluded that *pelB* expression is regulated by several factors including pH, nitrogen and carbon sources.
4. Gene disruption of *pacC* reduced virulence of *S. sclerotiorum*. Creation of a dominant activating *pacI^c* mutation and introduced this allele into one of the *pacI* loss-of-function ($\otimes pacI$) indicated that *pacI* transcripts remained alkaline-inducible, although much reduced.

Agricultural implications

In work that has been done in a parallel systems it was described a clear agricultural implication where modulation of host pH by acid treatments resulted in decay control of postharvest diseases. This represents the first report of postharvest treatment of acid solutions for the modulation of host environment for prevention of decay development.

Achievements

Recent publications have suggested that environmental pH is considered a key factor in determining pathogen compatibility. Our early hypothesis was that the environmental pH at the infection site, which is dynamically controlled by activities of both the host and the pathogen, regulates the expression of genes necessary for disease development in *Colletotrichum gloeosporioides* and *Sclerotinia sclerotiorum*. This form of regulation ensures that genes are expressed at optimal conditions for their encoded activities. Pectate lyase encoded by *pelB*, has been demonstrated to play a key role in virulence of *C. gloeosporioides* in avocado fruit. In this proposal we have examined, 1) the mechanisms employed by these fungi to establish a suitable pH environment, 2) the molecular levels at which genes and gene products are regulated in response to environmental pH, and 3) the molecular basis and functional importance of pH-responsive gene regulation during pathogenicity.

Our first objective was to determine the relation between nitrogen source and pH alkalization. It was found that accumulation of ammonia and associated tissue alkalization predisposes fruit to attack by *Colletotrichum gloeosporioides*. As the external pH increases from 4.0 to 6.0, pectate lyase (PL) and other extracellular proteins are secreted and accumulate. At pH 4.0 neither *pelB* (encoding PL) transcription nor PL secretion were detected; however, they were detected as the pH increased. Nitrogen assimilation also was required for PL secretion at pH 6.0. Both inorganic and organic nitrogen sources enhanced PL secretion at pH 6.0, but neither was sufficient for PL secretion at pH 4.0. Sequence analysis of the 5'-upstream region of the *pelB* promoter revealed nine putative consensus binding sites for the *Aspergillus* transcription factor PacC. Consistent with this result, the transcript levels of *pac1* (the *C. gloeosporioides* *pacC* homologue) and *pelB* increased in parallel as a function of pH. Our results suggest that the ambient pH and the nitrogen source are independent regulatory factors for processes linked to PL secretion and virulence of *C. gloeosporioides*.

Our second objective was to provide evidence that a conserved molecular pathway for pH-responsive gene expression exists in *C. gloeosporioides* and determine its relation to *pelB* expression. It was found that secretion of ammonia by *C. gloeosporioides* in the presence of KNO_3 was induced by decreasing the pH from 7.0 to 4.0. When the fungus was grown at pH 4.0 or 6.0 in the absence of a nitrogen source, ammonia did not accumulate, and neither *pelB* (encoding pectate lyase) transcription nor pectate lyase secretion was detected. Under these nitrogen starvation conditions, only transcriptional activation of *areA*, which encodes the global nitrogen regulator, was detected. *pelB* transcription and pectate lyase secretion were both detected when *C. gloeosporioides* was grown at pH 6.0 in the presence of ammonia.

accumulated from different nitrogen sources. The early accumulation of ammonia induced early *pelB* expression and pectate lyase secretion. As the external pH increased from 4.0 to 6.0, transcripts of *pac1*, the *C. gloeosporioides* *pacC* homolog, also could be detected. Nit mutants of *C. gloeosporioides*, which cannot utilize KNO_3 as a nitrogen source, did not secrete ammonia, alkalinize the medium, or secrete pectate lyase. If Nit mutants were grown at pH 6.0 in the presence of glutamate, then pectate lyase secretion was induced. Infiltration of 0.1 M ammonium hydroxide at pH 10 into ripening avocado fruits enhanced the activation of quiescent infection and symptom development by *C. gloeosporioides*. These results suggest that ambient pH alkalization resulting from ammonia accumulation and the availability of ammonia as a nitrogen source independently regulate *pelB* expression, pectate lyase secretion, and virulence of *C. gloeosporioides*.

Our third objective was to provide direct evidences of the role of *pacC* in pathogenicity by gene disruption and activating mutations. Gene disruption was done both on *C. gloeosporioides* and *S. sclerotiorum*. In *C. gloeosporioides* a new method vector construction for gene disruption was developed by Dr. Amir Sherman. Using this method *pacC* disruption achieved. The disrupted mutant showed a total reduction of *pacC* relative expression and 85% inhibition of decay development on avocado fruits. The mutant and the wild type isolates did not differed in spore germination, appressoria formation and fungal growth. When the mutant and the wild type were grown in culture media at a pH range of 4.5-7.5 no significant differences were recorded between them. Relative expression of *pelB* and PL-B secretion in Δ *pacC* mutant was reduced fold but not fully inhibited. Our present result suggests the importance of *pacC* in *C. gloeosporioides* pathogenicity by regulation the regulation of PL-B production. However the fact that *pelB* was not fully inhibited in the Δ *pacC* mutant might suggest that *pelB* is regulated as well by other factors than pH as carbon source.

In *S. sclerotiorum* the synergistic activities of oxalic acid and endopolygalacturonases are thought to be essential for full virulence of *Sclerotinia sclerotiorum* and other oxalate-producing plant pathogens. Both oxalic acid production and endopolygalacturonase activity are regulated by ambient pH. Since many gene products with pH-sensitive activities are regulated by the PacC transcription factor in *Aspergillus nidulans*, we functionally characterized a *pacC* gene homolog, *pac1*, from *S. sclerotiorum*. Mutants with loss-of-function alleles of the *pac1* locus were created by targeted gene replacement. In vitro mycelial growth of these *pac1* mutants was normal at acidic pH, but growth was inhibited as culture medium pH was increased. Development and maturation of sclerotia in culture was also aberrant in these *pac1* replacement mutants. Although oxalic acid production remained alkaline pH-responsive, the

kinetics and magnitude of oxalate accumulation were dramatically altered. Additionally, maximal accumulation of endopolygalacturonase gene transcripts (*pgl*) was shifted to higher ambient pH. Virulence in loss-of-function *pac1* mutants was dramatically reduced in infection assays with tomato and *Arabidopsis*.

Further work in *S. sclerotiorum* was applied to change the *pac1* coding sequence by site-directed mutagenesis to create a dominant activating *pac1^c* mutation and introduced this allele into one of the *pac1* loss-of-function ($\otimes$ *pac1*) strains. Consistent with a constitutive activation of Pac1 function, oxalic acid accumulation in recovered $\otimes$ *pac1-pac1^c* strains was largely independent of ambient pH. Accumulation of *pac1* transcripts remained alkaline-inducible, although much reduced. Surprisingly, the accumulation of *pgl* and *acpl* transcripts, normally favored by low pH conditions, were up-regulated in $\otimes$ *pac1-pac1^c* strains across all pH conditions. The transcript accumulation pattern for *pg6*, an alkaline-expressed endopolygalacturonase gene, however, did not differ from wild type. In pathogenicity assays using *Arabidopsis* and detached tomato leaflets, activating *pac1^c* strains showed reduced virulence compared to the wild type and to control strains despite the ability to accumulate oxalic acid independent of prevailing ambient pH conditions. These results support the hypothesis that appropriate gene regulation in response to ambient pH is important for full virulence of *S. sclerotiorum* independent of oxalic acid accumulation.

Overall this project has contributed to our understanding of the role of ambient pH and ambient pH-dependent gene expression on pathogenic development in Sclerotinia and Colletotrichum. A mutation in the ambient pH regulatory pathway has most profound effects on pathogenesis.

Other closely related research was carried out as well in relation to another acidifying pathogen, *Penicillium expansum* and is reported in the list of publications. This project has resulted in clear economic impacts for agriculture, based on the use of acid solutions for the control of postharvest disease caused by *A. alternata* as pathogen that alkalize the host environment. This method is been used as a commercial postharvest treatment of mango fruits for the control of *A. alternata*.

Concerning the cooperation this proposal has been a clear basis for joint cooperation. Several parts of the joint manuscripts were carried out in parallel in both labs and had resulted in joint publications. Scientific meetings took place during several of the annual Phytopathological meetings beside of a specific visit of D. Prusky to Florida.

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Published papers.

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12. Kim, Y., Prusky, D. & Rollins, J. 2006. An activating mutation of the *Sclerotinia sclerotiorum* pac1 gene increases oxalic acid production at low pH but decreases virulence. Mol. Plant Pathol. In preparation.