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Mechanisms modulating fungal attack in post-harvest pathogen interactions and their control

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Abstract As biotrophs, insidious fungal infections by post-harvest pathogens remain quiescent during fruit growth, but at a particular phase, during ripening and senescence, the pathogens transform to necrotrophs and elicit the typical decay symptoms. Exposure of unripe hosts to pathogens initiates defensive signal-transduction cascades that limit fungal growth and development. Exposure to the same pathogens during ripening and storage activates a substantially different signalling cascade that facilitates fungal colonization. This review will focus on modulation of post-harvest host-pathogen interactions by pH and reactive oxygen species (ROS). Modulation of host pH in response to a host signal is bidirectional and includes either alkalinisation by ammonification of the host tissue, or acidification by secretion of organic acids. These changes sensitise the host and activate the transcription and secretion of fungal hydrolases that promote maceration of the host tissue. This sensitisation is further enhanced at various stages by the accumulation of host or fungal ROS that can further weaken host tissue and amplify fungal development. Several specific examples of coordinated responses that conform with this scheme are described, followed by discussion of the means to exploit these mechanisms to establish new approaches to post-harvest disease control.

Keywords Fruit ripening · Fruit senescence · Pectolytic enzymes · Quiescent infections · pH regulation · PELB · PACC

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Introduction

Post-harvest fungal pathogens exploit three main routes to penetrate the host tissue: (a) through wounds caused by biotic and/or abiotic agents during growth and storage; (b) through natural openings such as lenticels, stem-ends and the pedicel–fruit interface; and (c) by directly breaching the host cuticle, which can occur throughout fruit growth. An active pathogen can start its attack process immediately after spores land on the wounded tissue, whereas other pathogens can breach the unripe fruit cuticle and then remain inactive for months until the harvested fruit ripens. The penetration process may go unnoticed by the host, or it may result in rapid defence signalling that results in the induction of defence molecules that will limit fungal development. The period from host infection to the activation of fungal development and symptom expression is designated the quiescent stage (Prusky 1996). After harvest, during ripening and storage, the mechanism that protects the fruit from fungal attack becomes non-functional. This transition

from a resistant to a susceptible state parallels physiological changes that occur during ripening, and which the pathogen senses and responds to. In the present review, we focus on some of the interactions between the fungus and the host environment, that serve the pathogen as a basis for fungal colonization. We also touch upon the signals that may activate the transition from quiescent to necrotrophic mode during fruit ripening.

The quiescent stage

During the colonization of plant hosts, post-harvest fungal pathogens exploit two main modes of nutrition: biotrophy, in which the nutrients are obtained from the living host cells, and necrotrophy, in which nutrients are obtained from dead host cells killed by the fungus (Perfect et al. 1999). Both of these nutritional modes are exhibited by post-harvest pathogens. Opportunistic post-harvest pathogens, in an inactive mode, may also be located within the fruit, awaiting fruit wounding, ripening or senescence. The length of each period varies among pathogens, hosts, and host developmental stages. Pathogens such as *Colletotrichum*, *Monilinia*, *Botrytis* and *Alternaria* may remain quiescent for long periods in developing fruit tissues, but initiate immediate necrotrophic development on ripening and senescing fruits.

Colletotrichum is one of the major post-harvest pathogens in which quiescence has been studied. *Colletotrichum* spores adhere to and germinate on the plant surface, where they produce germ tubes; the tip of the germ tube developing from the appressorium penetrates through the cuticle with an infection peg. Following penetration, *Colletotrichum* initiates subcuticular intramural colonization (Perfect et al. 1999) and spreads rapidly throughout the tissue with both inter- and intracellular hyphae that kill cells as they advance. After colonizing one or more host cells, the infecting hyphae, which can be described as biotrophic (Kramer-Haimovitch et al. 2006), subsequently give rise to secondary necrotrophic hyphae (Bailey and Jeger 1992; Coates et al. 1993; Latunde-Dada et al. 1996; Mendgen and Hahn 2001; O'Connell et al. 1985). *Botrytis* and *Monilinia* can penetrate through wounds and also breach the fruit cuticle by extending an infection peg from an appressorium, which then remains quiescent for long periods (Fourie and Holz 1995; Pezet et al. 2003). Depending on the physiological status of the organ, these hyphae may continue the infective process or remain quiescent.

The close association between the infection peg and the host fruit is likely to involve exchanges of chemical and physical signals that control transport of nutrients and modulate defence responses. This type of interaction has been demonstrated in rust haustorial penetration (Heath 1997) but not in systems involving post-harvest pathogens.

In the light of published reports, three major modes for the activation of quiescent biotrophic pathogens have been hypothesized (Prusky 1996): (a) a lack in the host of the nutritional resources required for pathogen development; (b) the presence of preformed or inducible fungistatic antifungal compounds in resistant unripe fruits; and (c) an unsuitable environment for the activation of fungal pathogenicity factors.

Factors facilitating pathogenicity

Quiescence may result from a localized host response that is often associated with an oxidative burst, i.e., the production of reactive oxygen species (ROS). Localized generation of ROS during quiescence was found to be one of the earliest (within 2–3 h) detectable cytological defence responses to attempted penetration by *Colletotrichum gloeosporioides* into unripe, resistant avocado fruits (Prusky et al. 1988). During quiescent infections of tomato leaves by the hemibiotroph *Colletotrichum coccodes*, a localized accumulation of H_2O_2 could be clearly seen during the initial stages of fungal penetration. In addition, treatment of tomato fruits with the H_2O_2-scavenging protein catalase, prior to inoculation with *C. coccodes*, resulted in a significant increase in fungal penetration efficiency. At the same time, less H_2O_2 was detected in the infection zone, which suggests that ROS production by the host may be important in inducing resistance and maintaining fungal quiescence (Beno-Moualem and Prusky 2000). Similar behaviour was observed when another hemibiotroph, *Septoria tritici* infecting wheat was inhibited by H_2O_2 during the biotrophic phase (Shetty et al. 2007). Decomposition of H_2O_2 by infiltrating catalase increased susceptibility whereas addition of H_2O_2 to the wheat increased tolerance to fungal attack.

What possible factors could affect ROS production in ripening fruits? ROS production is dependent on the physiological stage of fruit ripening. *Colletotrichum gloeosporioides* infection of unripe, resistant avocado fruits (quiescent interaction) activated a threefold increase in the level of oxygen radicals, with a corresponding increase in NADPH oxidase activity (Beno-Moualem and Prusky 2000), whereas no significant enhancement of ROS production was observed in ripened fruits.

ROS production by pathogens that induce quiescent infections may depend on the environmental pH (Mellersh et al. 2002), a factor that changes naturally during fruit ripening and storage. The pH is also changed, or induced to change, by the pathogens around the infection site (Beno-Moualem and Prusky 2000; Wang et al. 2004). Thus, an increase in the natural pH during fruit ripening or as a result of fungal activity can suppress oxidative responses of either the pathogen or the host, and so facilitate activation of the quiescent infection.

ROS can be produced, perceived and detoxified by fungi, whose growth, virulence and differentiation may be profoundly affected by these metabolites (Aguirre et al. 2006). However, little is known about the exact spatial and temporal mechanisms that protect fungal cells from self-produced or host-produced ROS during the interaction *in planta*. Fungal pathogens have also evolved strategies to compromise or delay the activation of efficient host defence responses. Cessna et al. (2000) suggested that secretion of oxalic acid by *Sclerotinia sclerotiorum* suppresses the initiation of the oxidative burst that marks the activation of defence responses. *Botrytis cinerea* possesses an arsenal of genes which are capable of detoxifying ROS in axenic media (Gil-ad et al. 2000), and it is likely that these enzymes can prevent ROS damage to fungal cells *in planta*. Also in *Botrytis*-bean cell interactions, suppression of a second specific ROS burst following the first non-specific ROS production was observed in aggressive isolates of the pathogen (Unger et al. 2005). In this case, the suppressor of ROS production was identified as 2-methyl succinate, and was able to suppress the hypersensitive response-like necrosis that reduces the virulence of *B. cinerea*. Proline was also shown to be an effective scavenger of intracellular ROS in *Colletotrichum trifolii* (Chen and Dickman 2005). This ability of proline to function as a potent antioxidant and inhibitor of the hypothesized programmed cell death process may represent an important and broad-based function in the cellular response to stress.

The production of ROS by the host has two mutually conflicting effects during the transition of the pathogen from quiescent to active infection. On the one hand, the accumulation of ROS is a potential trigger of defence mechanisms that confine the pathogen during the biotrophic phase. On the other hand, cell death induced by ROS can lead to the formation of necrotic tissue from which quiescent pathogens obtain the nutrients that will fuel their development during the necrotrophic phase of either the hembiotrophic or the necrotrophic pathogen (van Baarlen et al. 2004). Which of the two effects is more important in modulating the activation of a quiescent infection is still unknown. For example, in *Botrytis*, modulation of ROS was correlated with necrosis and pathogenicity: deletion of a Cu–Zn superoxide dismutase gene retarded the development of necrosis (Rolke et al. 2004). Govrin and Levin (2000) used another approach to show that inhibitors of NADPH oxidase reduced the level of ROS in *Arabidopsis*, and restricted necrosis caused by *B. cinerea*: infiltration of solutions that increase ROS production consistently stimulated leaf necrosis caused by *Botrytis* and *S. sclerotiorum*.

Effect of pH on the expression of fungal virulence factors

During fruit ripening and senescence, pH levels change as part of the ripening process: for instance, the pH of avocado fruit increased from 5.2 to 6.0 during ripening (Yakoby et al. 2000). Pathogens can also alter the pH in the vicinity of the infection site, and the change in pH can modulate the expression of pathogenicity factors (Denison 2000; Yakoby et al. 2000; Prusky et al. 2001b; Eshel et al. 2002; Prusky and Yakoby 2003). Expression of the endoglucanase gene *AAK*1 in *Alternaria alternata* was found to be maximal at pH levels above 6.0, which are characteristic of decayed tissue; it was not expressed at the lower pH values at which the pathogen was quiescent (Eshel et al. 2002). In the pathogen *C. gloeosporioides*, the gene *PEL*B was expressed when the pH was above 5.7, a value similar to that of decaying tissue (Yakoby

et al. 2000, 2001). The transcription factor PacC, which is involved in pH regulation, exhibits a similar expression pattern to that of *PEL*B, which suggests that they are co-regulated or that *PAC*1 regulates the expression of *PEL*B (Fig. 1) (Drori et al. 2003). Disruption of *PAC1*, the orthologue of *PACC*, in *C. gloeosporioides* resulted in loss-of-function mutants with severely attenuated virulence, suggesting that pH responsiveness is critical for the activation of the pathogenicity of this fungus (Miyara et al., unpublished). In another example, expression of the

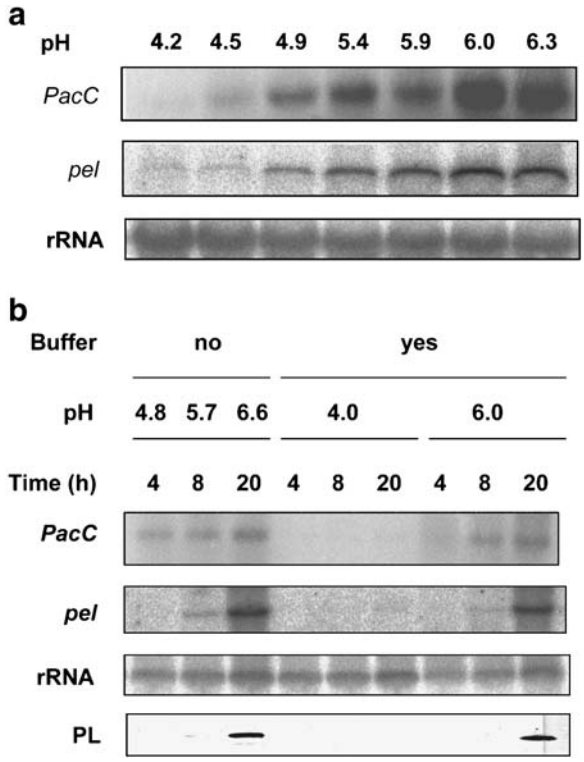

Fig. 1 Transcriptional activation of *pel*B and *PacC* by *C. gloeosporioides* as a function of pH. **a** Expression as a function of pH. Northern analysis of total RNA isolated from *C. gloeosporioides* mycelia 16 after transfer to fresh secondary cultures buffered with phthalate to the indicated pH values. Blots were probed with *pelB* (*middle panel*), and then sequentially stripped and reprobed with *pac1* (*top panel*) and ribosomal DNA (rDNA – *bottom panel*) probes as indicated. **b** Expression as a function of time and pH. Growth medium was buffered with phthalate to pH 4.0 or 6.0, or was unbuffered. At different times the culture media were harvested, hyphae were subjected to RNA extraction and blots were probed with *pelB*, and then sequentially stripped and reprobed with *pac1* and ribosomal DNA (rDNA) probes as indicated. Western analysis of secreted PL was performed on concentrated and dialysed culture medium

*Fusarium oxysporum PG*1 and *PG*5 genes was enhanced in an acidic environment, and a sequence that controls the positive activation of genes by pH, the PACC recognition site, was found in *PG*1 (Caracuel et al. 2003). Although a PACC homologue in *B. cinerea* has not been described to date, the sequence corresponding to the PACC consensus recognition site has been identified in all endo*PG* genes (Wubben et al. 1999; Manteau et al. 2003). Furthermore, the differential expression of endo*PG* by *B. cinerea* (ten Have et al. 2001) correlated well with the pH characteristics of apple and zucchini, which have low and neutral pH, respectively. In the fungus *Penicillium expansum*, PG (*PEPG*1) showed high expression at pH 4.0 and minor expression above pH 5.0. Similar results were also obtained for the endo*PG* gene, *PG*1, of *S. sclerotiorum*: transcription of the PACC homologue, *PAC*1, declined during acidification, concomitant with an increase in *pg*1 expression, and this gene was found to contain the PACC recognition site in its promoter (Rollins and Dickman 2001).

Other putative virulence factors of *B. cinerea*, including oxalic acid, laccase and protease, are also released in a pH-regulated manner at pH values in the range of 3.1–6.0. Activation of these factors at pH levels within the natural range of the host tissue is indicative of the significance of the differential expression of virulence factors in different hosts (Lumsden 1976; Movahedi and Heale 1990; Manteau et al. 2003; Vernekar et al. 1999; Ye and Ng 2002; Caracuel et al. 2003). The ability of different races of the fungus to fine tune enzyme expression in response to the ambient pH in the host further highlights the importance of the specific regulatory system that is activated under a changing environmental pH which can lead to the activation of quiescent infections (Prusky and Yakoby 2003).

The ability to modify pH may be expressed in either direction, and fungi that raise or reduce it are described as ‘alkalinizing fungi’ or ‘acidifying fungi,’ respectively.

Alkalinizing fungi

Alkalinization of the infection site by fungi is achieved by active secretion of ammonia, which is largely the product of protease activity and deamination of amino acids. The pathogenicity of *C. gloeosporioides* and expression of the virulence factor PL-B both depend on

raising the ambient pH. In the case of polyphage pathogens such as *A. alternata*, a threefold to tenfold increase in ammonia concentration, and a pH elevation of 0.2 to 2.4 pH units were detected in several hosts: tomato, pepper, melon, cherry and persimmon (Eshel et al. 2002).

Acidifying fungi

Other post-harvest pathogens, such as *P. expansum*, *P. digitatum*, *P. italicum* (Prusky and Yakoby 2003), *B. cinerea* (Manteau et al. 2003) and *S. sclerotiorum* (Bateman and Beer 1965; Ruijter et al. 1999), utilize tissue acidification to support their attack via the secretion of organic acids. *Sclerotinia sclerotiorum* and *B. cinerea* decrease the host pH by secreting large amounts of oxalic acid (Rollins and Dickman 2001; Manteau et al. 2003), whereas *Penicillium* (Prusky and Yakoby 2003; Prusky et al. 2004) (Fig. 2) and *Aspergillus* (Ruijter et al. 1999) secrete mainly gluconic and citric acids. *Penicillium expansum* isolates expressing higher activities of glucose oxidase and ability to secrete gluconic acid, showed increasing decay development in apple fruits. In contrast, reduction of glucose oxidase activity, by lowering the concentration of oxygen in the atmosphere, inhibited gluconic acid accumulation and reduced the decay development of *P. expansum* (Hadas et al. 2007). Acidic pH-specific expression of other members of the PG family was found in *S. sclerotiorum* (Lumsden 1976) and *BCPG3* in *B. cinerea* (Wubben et al. 2000). Taken together, these results suggest that environmental acidification is important for fungal attack. It should also be noted that acidifying fungi possess the capacity to raise low pH levels to a favourable optimum (Zhang et al. 2005).

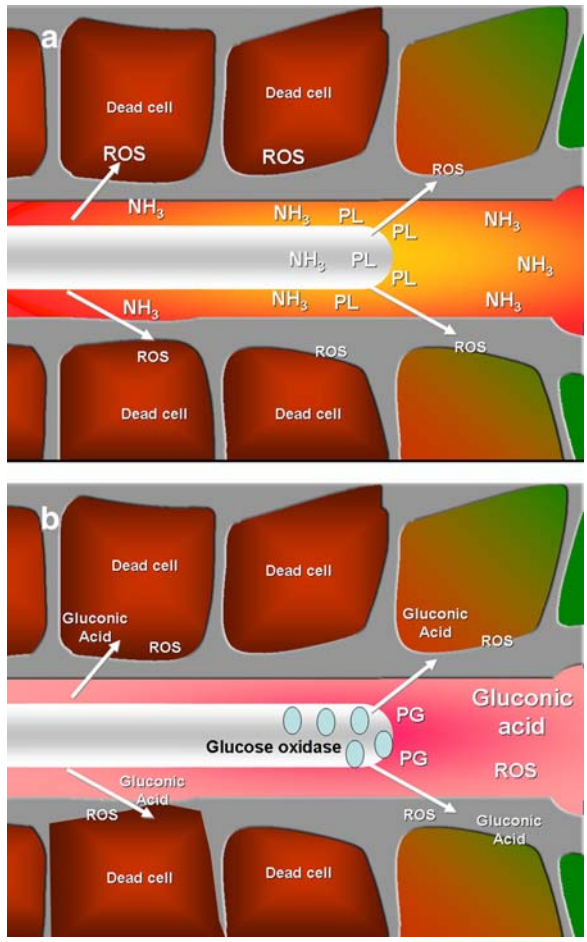

Fig. 2 Model for the activation of quiescent infections in post-harvest pathogens. In this model, two options are required for the transformation of quiescent to active infections pending on the type of pathogen (alkalinize or acidify the host tissue). The module A describes the increase in pathogenicity by the ammonification of the host tissue and ROS production by *Colletotrichum* and *Alternaria*; PL: pectate lyase. Module B describes the increase in pathogenicity by acidification of the host tissue, secretion of gluconic acid and ROS accumulation by *Pencillium expansum*; PG: polygalacturonase

Modulators of the activation of quiescent infections and signal activation

The transition from biotrophism to a necrotrophic–saprophytic stage appears to be related to factors that are modulated at the intracellular level, and that are affected by nutrients and ambient pH. Each of the secreted compounds (organic acid or ammonia) plays a critical physiological role in the initiation of necrotrophic development. Speculation regarding the mechanisms by which secretion of organic acids enhances virulence centers on three modes of action. First, oxalate may be directly toxic to host plants and may weaken them, thereby facilitating invasion. Second, it has been hypothesized that chelation of cell-wall Ca^{2+} by oxalic and gluconic acids loosens the plant cell wall (Bateman and Beer 1965; Hadas et al. 2007). Finally, oxalate secretion may suppress ROS generation together with the associated plant defence responses, thereby contributing to activation of the necrotrophic mode of development (Cessna et al. 2000).

Ammonification of the tissue also has significant physiological and biochemical effects on the host.

Recent results have shown that ammonification of the tissue enhances the accumulation of ROS in infected tissue (Alkan et al., unpublished data). Furthermore healthy plant cells have an electrochemical proton gradient across the plasma membrane, that is important for ion uptake, solute transport, and cell-wall growth. Several studies have demonstrated that transient extracellular alkalinization is essential for the induction of defence responses by fungal elicitors (Schaller and Oecking 1999). However, an essentially different feature of the activation of necrotrophic processes in *Colletotrichum* and *Alternaria* is the extreme elevation of pH that accompanies the accumulation of ammonia. The increased pH of the medium can affect all membrane functions, such as ion channels and plasma membrane ATPase (Gerendas and Ratcliffe 2000). Ammonium-induced alkalization leads to accumulation of weak bases and, subsequently, to elevation of the cytoplasmic and vacuolar pH (Kosegarten et al. 1997). Ammonium-toxicosis symptoms can lead to host stress responses that are expressed as elevated ethylene synthesis and various changes in membrane flux (Ingermarsson et al. 1987). Ammonium toxicosis of the host can be aggravated by the fungus, because ammonia can activate the expression of genes encoding pathogenicity factors, such as *PEL*B and PL secretion, in *C. gloeosporioides* (Kramer-Haimovich et al. 2006). This suggests that ammonification by *Colletotrichum* and *Alternaria* at the edge of the infection site may lead to deregulation of the host responses that, in turn, would facilitate the activation of fungal pathogenicity genes.

Present results suggest that one of the signals affecting secretion of organic acid and/or ammonia is the host environment pH, and this same signal may activate ammonium transporters in fungi. It is hypothesized that proteins such as the putative yeast outward transporter YaaH (Guaragnella and Butow 2003) are responsible for the elimination of excess ammonia. Other proteins, such as ammonium permeases (MEPs) of *Saccharomyces cerevisiae*, are members of a unique family of cytoplasmic membrane transporters that are specific for the ammonium ion (Pao et al. 1998). It has been suggested that members of the MEP/Amt family actively transport the charged species NH_4^+ across the cytoplasmic membrane (Howitt and Udvardi 2000), but no published reports have addressed the role of these transporters in fungal pathogenicity. Further studies of these transporters could help to explain the mechanism(s) underlying ammonium secretion by various post-harvest pathogen species.

New approaches for disease control of post-harvest pathogens

Harvested fruit and vegetables are agricultural produce that require stringent control of post-harvest handling, to avoid disease and to preserve the quality of the produce (Kobiler et al. 2001; Prusky et al. 2001a). This can be achieved by integration of safer synthetic fungicides, biological antagonists (Janisiewicz and Korsten 2002) and physical treatments (Prusky et al. 2001a). The studies that revealed host environment alkalinization by ammonia secretion during *A. alternata* and *C. gloeosporioides* colonization of fruits have opened a new approach to the modulation of disease development and control (Prusky et al. 2001a, b, Prusky and Yakoby 2003). For example, post-harvest treatment of mango fruits with HCl reduced the incidence of *Alternaria* rot after storage and shelf life (Prusky et al. 2006). These results suggest that acidic solutions may reduce the incidence of *Alternaria* rot by modulation of the pH at the infection court. In vitro and in vivo studies demonstrated that acidic solutions were also very efficient in inhibiting spore germination and germ-tube elongation.

Prusky et al. (2006) suggested that acidification of the fruit surface would provide an attractive approach for control of alkalinizing post-harvest pathogens, such as *Alternaria alternata*. This principle can apply to the use of alkalinizing agents, e.g., $NaHCO_3$, against acidifying pathogens such as *P. expansum*, *P. italicum*, and *P. digitatum* (Prusky et al. 2001b; Porat et al. 2002; Smilanick et al. 1999, 2005). It should be acknowledged that decreasing external pH to control alkalinizing fungi might also result in more favourable conditions for acidifying fungi (Zhang et al. 2005). An alternative approach for prevention of major pathogens in harvest produce could be by breading the fruit to unfavourable pH to the pathogens; however, this might lead to changes in the quality and taste of the fruits.

Summary

It has become clear in recent years that the activation of quiescent infections is not a simple process whereby a

decline in host resistance leads to activation of fungal attack. Rather, activation of quiescent biotrophic infections seems to involve a coordinated series of events (Fig. 2). One component in this complex process lies in the physiological and biochemical changes that occur in the host during ripening and senescence and that lead to a decreased host response and increased susceptibility. In parallel, activation of quiescent fungal infections involves processes that compromise host defences, directly or indirectly, by detoxification of antifungal agents (Prusky 1996). The physiological changes that accompany fruit ripening and host senescence – changes in, e.g., host pH, sugar content and cell-wall components, and oxidation of wounded tissue – trigger responses by the infecting fungus. The acidification of the tissue by organic acids (oxalic and gluconic) or its alkalinization by ammonia, and the possible modulation of the host ROS response and fungal ROS production, may contribute to rapid necrotization of the tissue. Further amplification of the decay can result from activation of gene expression and release of cell wall-degrading enzymes.

Despite intensive research, we still do not have sufficient knowledge of the range of tools utilized by post-harvest pathogens to elicit the transition from a quiescent biotrophic stage to an active necrotrophic one. In *C. gloeosporioides*, alkalinization resulted in significant changes in the expression of transcription factors, sugar transporters, and a wide range of primary metabolic genes (Miyara et al. 2005). For example, the elevated expression of glutamine synthase may reflect a requirement to handle the tremendous amount of ammonia secreted into the host tissue (Eshel et al. 2002). Further clarification of the role of putative signals (pH, nitrogen and sugar) in post-harvest pathogenesis during fruit ripening is clearly needed. Nevertheless, the current state of knowledge of fungal modulation of host pH has already opened new avenues for the control of post-harvest pathogens (Prusky et al. 2006).

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