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Host Defenses Associated with Fruit Infection by *Colletotrichum* Species with an Emphasis on Anthracnose of Blueberries

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Abstract

Defense mechanisms in fruit against invasion by fungal pathogens fall into several categories, including fruit physiological factors, passive defense responses, and active defense responses. *Colletotrichum* spp. are common fruit rot pathogens on a variety of crops. The infection strategy of *Colletotrichum* pp. varies depending upon the host and the tissue type being colonized and range from hemibiotrophy to necrotrophy. Anthracnose fruit rot caused by *C. acutatum* is the most important postharvest disease of blueberries (*Vaccinium corymbosum*). In order to better understand host resistance mechanisms in blueberry fruits, the resistant cultivar Elliott was compared with the susceptible cultivar Jersey. Higher levels of hydrogen peroxide and differentially expressed defense-related genes were found in 'Elliott' than in 'Jersey' fruit after inoculation. Furthermore, higher levels of anthocyanins and flavonols, including two distinctive compounds, were observed in Elliott fruits and two unique flavonols were present in Elliott. Additionally, pH and sugar content were implicated as being involved in host resistance in blueberry fruits. The results from these studies provide a significant contribution to the understanding of the multiple factors that contribute to anthracnose fruit rot resistance in blueberry.

Introduction

Colletotrichum (teleomorph: *Glomerella*) species are ubiquitous fungi in the phylum Ascomycota and have been recovered from almost every plant species (47). Several members of the genus commonly cause anthracnose fruit rot of fleshy fruits, including *C. acutatum* J.H. Simmonds, *C. coccodes* (Wallr.) S. Hughes, and *C. gloeosporioides* (Penz.) Penz. & Sacc. Since symptoms often appear when fruit starts to ripen, the disease may also be termed ripe rot. The disease cycles for *Colletotrichum* spp. that infect fruit vary by host and climate. In temperate regions, the fungus overwinters on infected host tissue and releases conidia that infect green fruit during rainy periods the following spring. The infections remain latent until the fruit ripens (51). On strawberry leaves, the fungus can also survive and reproduce without causing symptoms during this stage (24). The latent phase of the infection often causes problems in estimating whether the disease is present and when the infection actually takes place.

When fruits ripen, particularly in strawberry and blueberry, the initial symptoms of infection are softening of the fruit, followed by the appearance of acervuli erupting through the fruit surface (Fig. 1). Lesions become sunken and the fruit eventually shrivels up. Secondary infections on adjacent fruit occur via splash dispersal of conidia produced on the surface of infected fruit (21,50).

The intent of this review is to discuss the literature on host defense mechanisms associated with fruit infections by *Colletotrichum* species of several plant species with a focus on recent advances in our understanding of resistance to anthracnose fruit rot in blueberries.

The Infection Process

The infection process typically starts when a conidium lands on the host, attaches itself, and begins to germinate by forming a germ tube, which gives rise to an appressorium. The appressorium, eventually becomes melanized and develops an internal light spot that corresponds to the penetration pore (13). The melanin inside the appressorium alters the permeability of the cell wall, creating a hypertonic environment that allows the fungus to directly penetrate the host epidermis using turgor pressure (51).

Many studies have investigated the infection strategies of *Colletotrichum* species on a variety of fruit crops, including almond (14), avocado (10), blueberry (52), olive (15), and strawberry (11). Following direct penetration of host tissues, *Colletotrichum* species generally two different host infection strategies depending on the tissue or host being colonized: intracellular hemibiotrophy and or subcuticular intramural necrotrophy (32). Intracellular hemibiotrophy is used to describe the direct invasion of the initial host cell by a primary infection vesicle, followed by the proliferation of thick primary hyphae and thin secondary hyphae. Only in later stages of the infection process does the fungus become necrotrophic. In subcuticular intramural necrotrophy, Colletotrichum spp. grow superficially under the plant cuticle, generally producing thinner necrotrophic hyphae sooner that do not invade the host tissue intracellularly.

Intracellular hemibiotrophy requires a close cytoplasmic interaction between the host and the pathogen. Pathogens with this strategy are considered specialists, while subcuticular intramural necrotrophy does not require this closeness; therefore, these pathogens could be considered more generalists (36). Different infection strategies in strawberry appear to be associated with tissue type: intercellular hemibiotrophy occurs on leaves (2) and subcuticular intramural necrotrophy occurs on petioles, stolons, and leaves (2,11). In olives, while both these strategies were observed, it was not related to host plant resistance or tissue type (15). However, in blueberries, the type of infection strategy was associated with a susceptible or a resistant interaction (52). Additionally, a hypersensitive response was observed in the resistant cultivar but not in the susceptible cultivar around 96 hours post inoculation (29).

In blueberries, a lower rate of conidium germination and appressorium formation was observed on a resistant cultivar (Elliott) compared to a susceptible cultivar (Jersey) (52), but only on ripe fruit, as no differences were observed on immature fruit (26). The difference appears to be the result of a relative increase in the rate of appressorium formation on the susceptible cultivar Jersey as the fruit ripens, whereas the rate on the resistant cultivar Elliott remains steady across fruit development stages (26). A possible reason for this phenomenon could be changes in the structure or composition of the waxy cuticle in susceptible fruit that stimulate conidium germination and appressorium formation. In avocados, cuticular wax has been shown to trigger conidium germination and appressorium formation of *C. gloeosporioides*. However, waxes from non-host plants strongly inhibited appressorium formation (37).

Passive Host Defenses

Fruit physiological factors. The infection process of *Colletotrichum* spp. on fruits has been studied in a number of plant pathosystems. In general, as fruits start to ripen they become increasingly susceptible to infection (6,30,43,54). During fruit ripening, many physiological changes occur, such as a reduction in fruit firmness, changes in pH and cell wall composition, and an increase in soluble sugars and secondary metabolites, such as anthocyanins (3,44). In avocado, several factors have been associated with increased fruit susceptibility to infection by *Colletotrichum gloeosporioides* as fruit ripens, including an increase in fruit pH (43), a decrease in preformed antimicrobial compounds (41), and pathogenicity factor inhibitors such as epicatechin (18).

Soluble sugars may also play a role in defense responses during ripening. Guava cultivars that contained high levels of soluble sugars and ascorbic acid were also the most resistant to *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (anamorph: *C. gloeosporioides*) (46). In grapes, the accumulation of antifungal proteins and sugars during fruit ripening is an important defense mechanism against the fungal pathogens *Botrytis cinerea* Pers.:Fr. and *Guignardia bidwellii* (Ellis) Viala & Ravaz (45,49). In blueberries, our research has shown that there is a positive linear correlation between fruit sugar content and anthracnose fruit rot resistance which suggests that soluble sugars may play a direct or indirect role in the resistance response (27). However, it most likely is an additive effect as indicated by the relatively low *r* values and the fact that even some moderately susceptible cultivars had fairly high soluble sugar concentrations. We found that high sugar concentrations in artificial media had a negative impact on hyphal growth of *C. acutatum*, presumably by increasing osmotic stress. This reduction was more pronounced with D-glucose than with D-fructose (27). This suggests that internal sugar content in fruit may play a role in slowing the growth of *C. acutatum*, especially in combination with low pH, during the colonization of the fruit and should be investigated further.

Antimicrobial fruit volatiles have been investigated in relationship to anthracnose resistance. In strawberries, the effects of aldehydes, alcohols, and esters on mycelial growth of *C. acutatum* were investigated. (*E*)-Hex-2-enal was identified as the most biologically active. This compound altered the structure of the conidial cell wall and plasma membrane, causing disorganization and lysis of organelles, and eventually, cell death (1). In blueberries, many of these compounds were also identified but the quantity of the volatile compounds was not correlated with anthracnose fruit rot resistance in the various blueberry cultivars (38,39).

Pre-formed antimicrobial compounds. Several pre-formed compounds have been identified in fruit that may play a role in resistance to *Colletotrichum* infection. Resistance to *C. gloeosporioides* in unripe avocado fruit is correlated with the presence of fungitoxic concentrations of the preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxoheneicosa-12,15-diene (diene) in the pericarp of unripe fruits (41). A second antifungal compound was subsequently purified from unripe avocado fruit and identified as 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-ene (42). When fruits ripen, the activity of the enzyme lipoxygenase increases causing the degradation of these preformed antifungal compounds and fruit gradually becomes more susceptible (43). Interestingly, the lipoxygenase activity in avocado fruits is affected by the flavonoid epicatechin, which acts as a natural inhibitor (40). In green fruit, the concentration of epicatechin gradually decreases upon ripening until the fruit becomes completely susceptible.

Chemical host plant resistance in other *Colletotrichum* fruit pathosystems is less well studied. In bananas, resistance has been attributed to dopamine and its oxidation products, which were isolated from the peel of unripe banana in concentrations that inhibited *C. musae* (Berk. & M.A. Curtis) Arx *in vitro*; it was therefore presumed to be a possible preformed antifungal compound. However, the concentration of the compound was not synchronized with changes in decay development (31). In blueberries, several studies have been carried out into the antifungal properties of ripe blueberry fruit extracts from wild highbush blueberry plants (*Vaccinium corymbosum*) as they relate to fruit decay and herbivore preference (7,8,9). These studies indicated that the main antifungal compounds present in ripe blueberry fruit were water-soluble phenolics and acids. They also proposed that resistance in ripe blueberries may be due to an interaction between simple phenolic compounds and organic acids and not necessarily individual fungitoxic compounds (7).

In blueberry fruits, research has shown that anthocyanin levels increase at the site of infection in the anthracnose-resistant cultivar Elliott and peak around 96 h after inoculation (29). Additionally, Elliott contains more anthocyanins and other flavonoids in noninoculated fruit than the susceptible cultivar Jersey (Fig. 2) (28). Anthocyanins, which are derived from anthocyanadins (Fig. 2A), do not seem to play a direct role in the resistance response but may play an indirect role

by protecting host tissues from oxidative damage. The non-anthocyanin flavonoid fraction from the resistant cultivar Elliott seems to play a key role in suppressing growth and development of *C. acutatum*. This fraction contains two distinctive flavonol (Fig. 2B) compounds: quercetin-3-O-rhamnoside and a flavonol glycoside, putatively identified as syringetin-rhamnoside, which may be important in the resistance response because of increased antifungal activity (Fig. 2B).

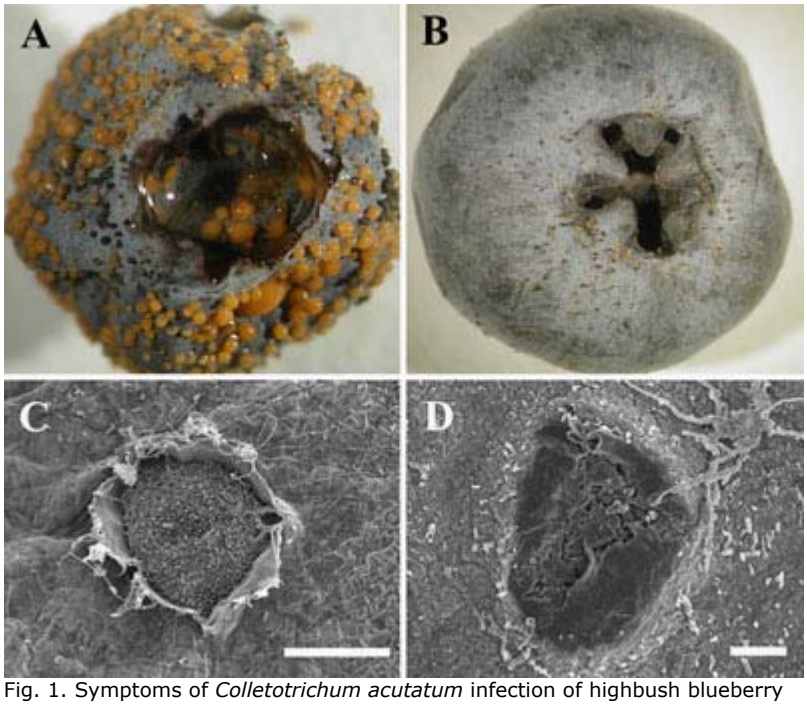

Fig. 1. Symptoms of *Colletotrichum acutatum* infection of highbush blueberry fruit. (**A**) Visual symptoms of infection 8 days after inoculation in the susceptible cultivar 'Jersey' and (**B**) the resistant cultivar 'Elliott.' Scanning electron micrograph of a typical acervulus on the fruit surface of (**C**) Jersey (bar = 100 µm) and (**D**) Elliott (bar = 20 µm).

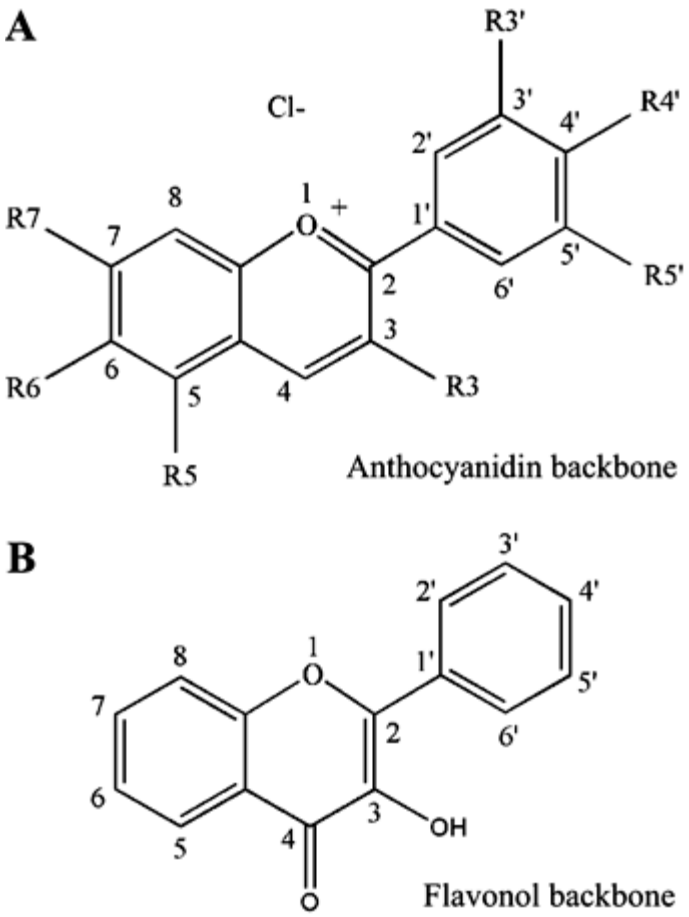

Fig. 2. Basic chemical structures of two common flavonoid groups (anthocyanidins and flavonols) present in fruit of highbush blueberries: (**A**) anthocyanidin backbone which for anthocyanins is typically glycosylated at the R3 position; and (**B**) flavonol backbone.

Active Host Defenses

Defense-related proteins. A variety of active defense mechanisms in *Colletotrichum*-plant interactions have been observed, including the production of host-derived, cell-wall-degrading enzymes such as chitinases and β-1-3-glucanases (5,16,23,53). When the genes coding for these products are over-expressed in plant tissue, the result is often increased disease resistance. For example, in transgenic tobacco plants, the overexpression of a chitinase gene led to broad resistance against the fungal pathogen *Rhizoctonia solani* J.G. Kühn, and the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (12). Upon infection of pepper fruits (*Capsicum annuum* L.) by *C. gloeosporioides*, multiple defense-related proteins like cytochrome P450 (33), defensin, thionin-like protein (34), thaumatin-like protein (20), and esterase (22) are induced in incompatible interactions.

Research has shown that infection by *C. acutatum* is reduced significantly by a polygalacturonase inhibitor protein extracted from apples (17). Host plant resistance to fungi in apple (55), raspberry (19), and tomato (48) fruits has been linked to these inhibitor proteins. Additional research has shown that these proteins are predominately expressed in the epidermal layers of the fruit and regulated in response to infection and wounding (55). A novel protein that has been identified in pumpkin rinds is capable of inhibiting the growth of *C. coccodes in vitro* at 10-20 μM. This protein was found to be nonphytotoxic and heat-stable, and is proposed to be a natural antifungal agent (35).

In blueberries, 37 differentially expressed sequence tags (ESTs) used to identify gene transcripts, were detected in Elliott versus Jersey upon infection by *C. acutatum*. Several of the ESTs had homology to known plant defense genes,

such as a class II chitinase, pathogenesis-related protein 10 (PR10), and β-1-3 glucanase. Two putative genes involved in oxidative stress were also identified: a metallothionein-like protein and monodehydroascorbate reductase (26). A more detailed investigation of gene expression during the early stages of infection, including pre-penetration events, will help to pinpoint when the host first recognizes that it is being attacked by *C. acutatum* and initiates the resistance response.

Reactive oxygen species. Reactive oxygen species, which cause cellular damage such as hydrogen peroxide (H_2O_2) are important in the resistance response in the *C. coccodes*-tomato fruit interaction. Hydrogen peroxide generation occurs around 24 to 48 h after inoculation, corresponding with melanized appressorium formation and attempted fungal penetration (25). In strawberries, H_2O_2 generation plays a role in restricting fungal penetration and inhibiting fungal invasion, leading to the hypersensitive response and triggering rapid necrosis at infection sites or activating defense-related genes (4).

In the *C. acutatum*-blueberry fruit pathosystem, research has shown that an oxidative burst similar to that in the *C. coccodes*-tomato fruit interaction occurs (25) and seems to correlate well with the formation of melanized appressoria (25,28,52), indicating attempted penetration. However, reactive oxygen species may be plant or pathogen derived. If it is plant derived, H_2O_2 may be important in the resistance response in Elliott by preventing fungal penetration. On the other hand, if the H_2O_2 is pathogen derived this could indicate preferential necrotrophy of *C. acutatum* on Elliott fruit. Since H_2O_2 can be a pathogenicity factor for necrotrophic pathogens, it may be important in the initial colonization of Elliott fruit. However, because of the relatively short duration of the H_2O_2 boost and coincident timing with peak appressorium formation (28,52), it seems likely that it represents a host response and serves to prevent pathogen ingress. In either case, the oxidative stress genes that were identified are likely up-regulated in Elliott fruit are likely up-regulated to prevent oxidative damage to plant tissues (26).

Conclusions

The infection processes of *Colletotrichum* spp. on fruit are complex and potentially involve different infection strategies based on plant tissue type and host cultivar. Host plant resistance against *Colletotrichum* spp. involves multiple mechanisms. Fruit physiological factors like sugar content, pH and surface waxes can affect fungal growth. Preformed antimicrobial compounds like antifungal dienes, resorcinols, or flavonoids may be extremely important in unripe- as well as ripe-fruit resistance. Active defenses including induced antimicrobial compounds, reactive oxygen species and defense-related proteins may also be important in the resistant response in ripe fruit.

The recent research on blueberries has highlighted several of these aspects in terms of host resistance. Based on this research we propose a simplistic model of the primary differences between the infection process on fruit of a susceptible and resistant cultivar (Fig. 3). An improved understanding of the underlying molecular mechanisms will lead to novel strategies for management of anthracnose fruit rot in blueberry and possibly other diseases caused by fruit-infecting *Colletotrichum* species.

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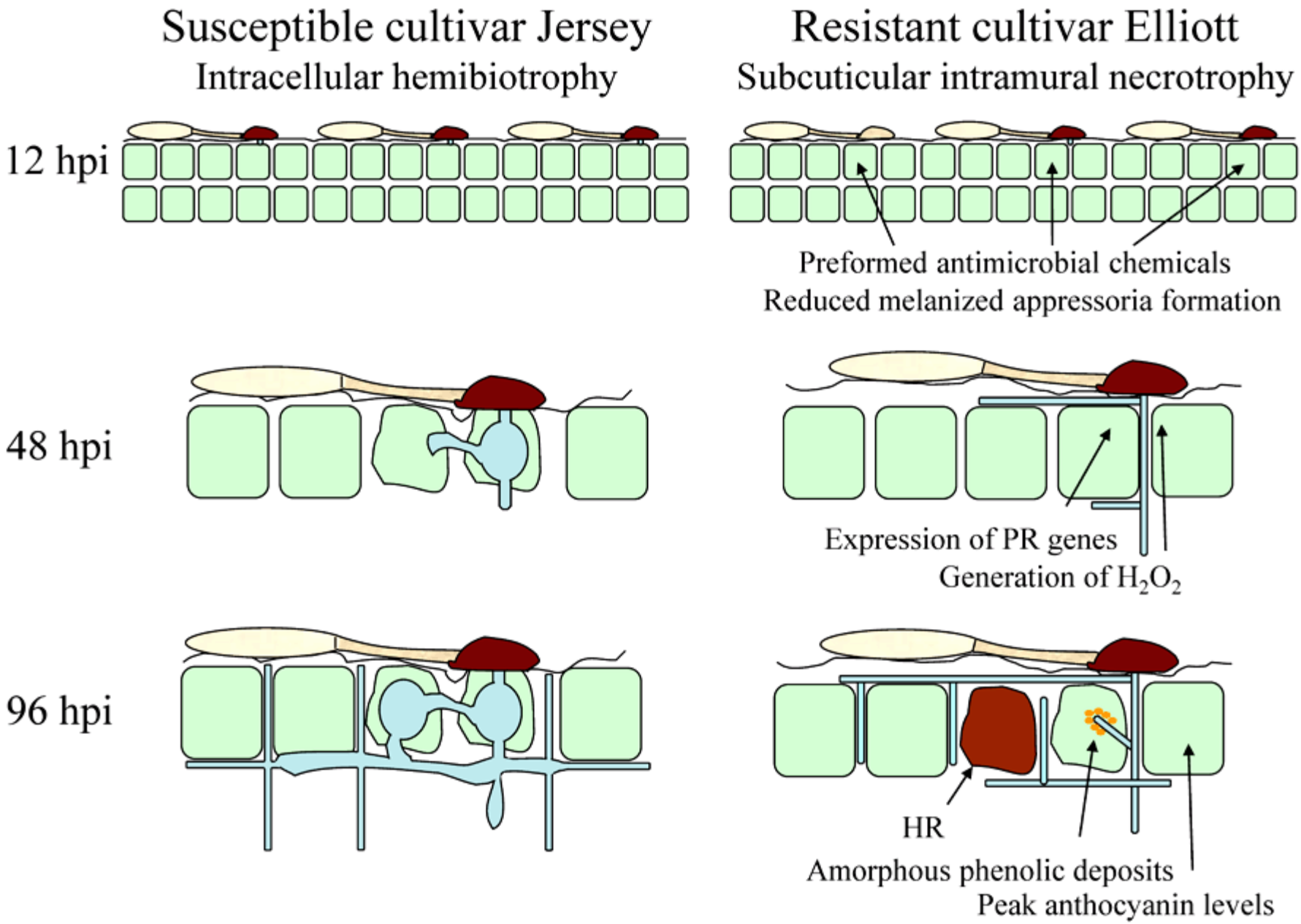

Fig. 3. Proposed model of the defenses responses present during the infection process of *C. acutatum* on fruit of susceptible ('Jersey') and resistant (Elliott) blueberries 12, 48, and 96 h post inoculation (hpi). HR = hypersensitive response and PR = pathogenesis-related.

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