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The infection capacity of *P. expansum* and *P. digitatum* on apples and histochemical analysis of host response

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ABSTRACT

Fruit ripening is a complex process that involves a variety of biochemical changes and is also associated with increased susceptibility to pathogens. The present study determined the effects of fruit maturity and storage conditions on the infection capacity of a host (*P. expansum*) and non-host (*P. digitatum*) pathogen on apple. A range of inoculum concentrations and two different storage temperatures were utilized. Exposure to *P. expansum* at 20 °C resulted in significant differences in rot dynamics in apples collected at the earliest harvest date compared to all later harvest dates and inoculum concentrations assayed. Greater differences in infection capacity between harvests were obtained when fruit was stored at low temperature (0 °C). In contrast, *P. digitatum* was able to infect apples only under specific conditions and disease symptoms were limited to the initial wound inoculation site. When apples were resistant to *P. digitatum*, a visible browning reaction around the infection site was observed. Histochemical analyses of tissues surrounding the wound site were conducted. A positive reaction for lignin was observed in immature apples as early as 1 day after inoculation with either pathogen. Experiments conducted with the non-host pathogen indicated that lignification was an essential component of resistance in apples harvested prior to maturity or at commercial maturity. Apples harvested at an over-mature stage and inoculated with *P. digitatum* did not show evidence of staining for lignin until 7 days post-inoculation. Control samples only showed positive reaction in immature harvest. Results demonstrated that the maturity stage of fruit is an important factor in apple resistance to both *P. expansum* and *P. digitatum* and that lignin accumulation seems to play an important role when resistance is observed. Moreover, this is the first report demonstrating that *P. digitatum*, a non-host pathogen, has a limited capacity to infect apples.

1. Introduction

Blue mould, caused by *Penicillium expansum*, and green mould, caused by *Penicillium digitatum*, are the most important postharvest diseases of apples and citrus fruits, respectively. Both pathogens are necrotrophs that require wounds to enter the fruit (Kavanagh and Wood, 1967; Spotts et al., 1998). Mechanical injury caused during harvesting and postharvest handling provides an optimal locus for infection. The use of chemical fungicides is one of the primary means of controlling these postharvest diseases; however, fungicides may have a negative impact on the environment and both human and animal health. Their long-term use also leads to the development of fungicide-resistant strains. These problems have motivated the search for alternative approaches and the study of host–pathogen interactions to provide a better understanding of the virulence mechanisms of the pathogens as well as the defence responses of the hosts in order to design new and safer control strategies.

A host–pathogen interaction may be categorized as compatible if a pathogen overcomes plant defence barriers and establishes disease symptoms, whereas in a non-host or incompatible pathogen interaction, plants deploy an array of defences that prevent or significantly limit pathogen growth (Glazebrook, 2005). Resistance responses involve a complex and dynamic communication system that is established during the first steps of infection.

One of the most rapid defence reactions is the oxidative burst that is characterized by a rapid and transient accumulation of reactive oxygen species (ROS) (Torres et al., 2006) composed primarily of superoxide anion and hydrogen peroxide at the site of the invasion (Apel and Hirt, 2004). Research has shown that avirulent pathogens induce a biphasic ROS production in plants, consisting of a low amplitude first phase, followed by a much higher and sustained accumulation during the second phase (Lamb and Dixon, 1997; Torres et al., 2006). However, only the first phase has been detected during interactions with virulent pathogens (Bolwell et al., 2001). In oranges inoculated with *P. digitatum* the production of ROS seems to be

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suppressed whereas inoculation with *P. expansum*, a closely related non-host species, triggers the production of ROS at attempted penetration sites (Macarisin et al., 2007).

ROS production also has been associated with the formation of physical defensive barriers against the pathogens (Huckelhoven and Kogel, 2003) established at the site of the infection. Changes in gene expression involving increased expression of phenylpropanoid metabolism genes have also been detected in tissues undergoing a resistance response (Hutcheson, 1998). Phenylalanine ammonia lyase (PAL) is a key enzyme in this pathway, and is directly involved in the synthesis of phenols and lignin (Yao and Tian, 2005). PAL contributes to the disease resistance response in many fleshy fruits (Singh et al., 2010). Vilanova et al. (2012) demonstrated a positive reaction for lignin in immature oranges in both host (*P. digitatum*) and non-host (*P. expansum*) pathogen interactions.

Plant defence strategies against pathogen invasion may be modulated by fruit ripening (Su et al., 2011) which is itself a complex, developmentally regulated process encompassing alterations in gene expression and chemical and physiological changes (Cantu et al., 2008). However, some questions remain unanswered as to how fruit maturity may affect the infection capacity of both host and non-host pathogens. Torres et al. (2003) reported that apples harvested 7 days after commercial harvest were more susceptible to *P. expansum* than apples harvested 7 days before commercial harvest. Beno-Moualem and Prusky (2000) correlated higher levels of ROS found in unripe avocado with a lower susceptibility to *Colletotrichum gloeosporioides* compared to ripe fruit. In contrast, Davey et al. (2007) reported that the susceptibility of different apple genotypes to *Botrytis cinerea* decreased when the harvest date was extended.

The aim of the present study was to investigate the infection capacity of the host, *P. expansum* (compatible), and the non-host, *P. digitatum* (incompatible) pathogens in 'Golden Smoothee' apples at different (i) maturity stages; (ii) inoculum concentrations, and (iii) storage temperatures.

The infection capacity studies were combined with a histochemical analysis of apple fruit tissues at the site of inoculation to characterize the accumulation of suberin and lignin in order to define their role in host resistance against both pathogens.

2. Materials and methods

2.1. Fruits

'Golden Smoothee' apples were harvested at different maturity stages from August to October, 2009 (six harvests ranging from immature to over-mature) from a commercial orchard in Mollerussa (Catalonia, Spain). Harvests 1 and 2 were considered as prior to commercial maturity (immature fruit), harvests 3–5 were considered as commercial maturity (mature fruit), and harvest 6 was considered as past maturity (over-mature fruit). Apples were used immediately after harvest. Data obtained for quality as described below confirmed that the harvest dates represented different levels of maturity.

2.2. Fungal cultures

P. expansum CMP-1 and *P. digitatum* PDM-1 are the most aggressive isolates from our collection capable of infecting pome fruits and citrus, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extracts, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded pome fruits (*P. expansum*) or citrus (*P. digitatum*) and then reisolated to maintain virulence. Conidia from 7- to 10-day-old cultures grown on PDA were collected by rubbing the surface of the agar with sterile glass rod. The concentration was determined with a haemocytometer and diluted to different concentrations (10^7, 10^6, 10^5 or 10^4 conidia/mL) and then used for the determination of infection capacity.

2.3. Infection capacity

The effects of fruit maturity, inoculum concentration, and storage temperatures were assessed for both the compatible interaction (*P. expansum*-apples) and the incompatible interaction (*P. digitatum*-apples). Apples were washed thoroughly with tap water and allowed to dry before artificial inoculation. Apples were wounded with a nail (1 mm wide and 2 mm deep) and inoculated with 15 μL of an aqueous conidial suspension of either pathogen at four different concentrations; 10^7 and 10^6 conidia/mL are considered in this work as high inoculum concentrations, and 10^5 and 10^4 conidia/mL are considered as low inoculum concentrations. The infection capacity of each pathogen was assessed at two different storage temperatures (0 and 20 °C) and 85% relative humidity. The diameter of rot was measured over the duration of each experiment in order to obtain information on the rot dynamics of each pathogen as affected by inoculum concentration, temperature, and fruit maturity. Five apples constituted a single replicate and each treatment was repeated four times.

2.4. Determination of quality parameters

Colour development, flesh firmness, starch index, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality.

Colour was measured using hue values, which were calculated from a^* (red-greenness) and b^* (yellow-blueness) parameters measured with a CR-200 chromameter (Minolta, Japan) on both the exposed and the shaded sides of each fruit, using standard CIE illuminant and 8 mm viewing aperture diameter. Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11 mm diameter plunger tip. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping of cross-sectional fruit halves in 0.6% (w/v) I_2–1.5% (w/v) KI solution for 30 s. Data on maturity indexes represent the mean of 20 individual fruits.

2.5. Histochemical tests

The development of resistance was studied by wounding 'Golden Smoothee' apples at three maturity stages: immature (harvest 1), commercial (harvest 4) and over-mature (harvest 6). Apples were inoculated with *P. expansum* or *P. digitatum* at either 10^7 or 10^4 conidia/mL. Control fruits were wounded but not inoculated. Fruits were stored at 20 °C and 85% RH and samples collected for histochemical analyses at 1, 3, 5, 7, and 9 days.

At each collection time, excised peel and pulp tissue cylinders (8 mm inside diameter and 4 mm deep) encompassing the wound site were infiltrated with FAA (formalin, glacial acetic acid, 96% ethanol, and water 10:5:50:35 v/v) and fixed for up to 48 h. Cylinders were dehydrated in an ethanol-xylene series, embedded in paraffin, sectioned transversely along the long axis at a thickness of 20 μm with a rotary microtome and fixed to glass-slides with Haupt adhesive and heat. Sections were deparaffinised with xylene and brought to miscibility with water to apply the following histochemical tests:

I. A Maüle reaction for lignin was performed according to the method described by Thomson et al. (1995) with slight modifications. Sections on slides were stained with 1% (v/v) aqueous potassium permanganate for 15 min, rinsed three times with distilled water (30 s each rinse), placed in 1% (v/v) HCl for 4 min, rinsed in water and then placed in 0.025% (v/v) ammonia for 5 s. The sections were rinsed in distilled water for