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DOTTORATO DI RICERCA IN PATOLOGIA VEGETALE

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Studio dell'interazione *Colletotrichum acutatum* – frutto di fragola in relazione allo stadio fenologico dell'ospite

(Molecular bases of the interaction between the fungal pathogen *Colletotrichum acutatum* and strawberry fruits at different ripening stages)

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Chapter 1

Introduction

1.1 The strawberry anthracnose

Anthracnose disease of strawberry (*Fragraria x ananassa)* is caused by the fungal pathogens *Colletotrichum acutatum, C. fragrariae,* and *C. gloeosporioides* (Howard et al. 1992; Smith and Black; 1990). *C. acutatum* is the primary causal agent of strawberry anthracnose and causes severe economic losses on strawberry production all over the world. This fungal pathogen can affect most parts of the plant, such as roots, leaves, blossoms, twigs, and fruits, and causes symptoms as crown root rot, defoliation, blossom blight, and fruit rot (Fig. 1.1; Fig. 1.2). However, as for most *Colletotrichum* species, the most important losses due to *C. acutatum* infection occur when fruit is attacked (Bailey and Jeger; 1992).

Symptoms of anthracnose fruit rot appear as dark and sunken lesions on infected fruit; these can quickly enlarge and affect most of the fruit (Fig. 1.2). In humid conditions, the fungus can evade as salmon-colored spore masses covering the fruit lesions. Finally, infected fruit become completely rotten and dry up into a mummy.

Fig. 1.1: ***Colletotrichum acutatum*** **anthracnose on a strawberry blossom**

Fig. 1.2: ***Colletotrichum acutatum*** **anthracnose on a strawberry fruit**

Colletotrichum anthracnose can occur both on immature fruits at pre-harvest stage, or on mature fruits, at harvest or during post-harvest storage. In both cases, however, anthracnose symptoms become apparent only during storage or shelf-life, when products reached its highest commercial value. This is due to the ability of *Colletotrichum* species to develop latent infections on immature fruits, where the fungus becomes quiescent, and to resume its growth only when fruit has ripened (Prusky and Plumbey, 1992; Prusky, 1996).

It is commonly believed that the molecular factors inducing the fungi quiescence in unripe fruits evolved as plants defence mechanisms to protect fruits during growth (Prusky, 1996). Considering that the control of strawberry anthracnose is so far achieved mainly through agronomical practices, due to the lack of both active, non toxic agrochemicals and of resistant cultivars, the characterization of the fungal infection strategy on unripe and ripe fruits and of the molecular signals underneath the induction of fungi quiescence on immature fruits, can be crucial for developing of new control strategies.

1.2 Colonization strategy of *C. acutatum* on strawberry fruits and quiescence

With respect to the colonization strategy, fungal plant pathogens have been classified as biotrophs, necrotrophs or hemibiotrophs. Typically, biotrophic fungi maintain its host cell alive, while removing nutrients from them (Luttrell, 1974; Parbery, 1996); necrotrophs approach living host cells and kills them before entering their lumina (Luttrell, 1974; O'Connell et al., 1985, Parbery, 1996). Hemibiotrophy is a condition in which the fungus initially enters a biotrophic phase and then switches to a necrotrophic phase (Luttrel, 1974). Several studies reported on the colonization strategy of *Colletotrichum spp.* on fruits (Bailey et al. 1992; O'Connel et al., 2000), indicating that these species behave hemibiotrophic fungi, namely, after an initial biotrophic stage they switch to a necrotrophic behavior.

Although pre-penetration events are basically the same for all *Colletotrichum* species (deposition and attachment of conidia on host surface, germination and appressoria formation), major differences are found after penetration, when two different types of

infection strategy can be adopted, depending on both the *Colletotrichum* and host species. Theses consist of i) the 'intracellular hemiobiotrophic' strategy; here an early biotrophic phase, characterized by the production of intracellular primary hyphae, is followed by a destructive nectrotrophic phase associated with narrower secondary hyphae, which ramify throughout the host tissue, and ii) the 'subcuticular intramural' strategy, in which the fungus colonizes the most superficial epidermis layers intercellularly before spreading rapidly both intra- and intercellulary killing host cells (Fig. 1.3), (Bailey et al. 1992; O'Connel et al., 2000). This second necrotrophic phase is similar to the one described in the intracellular hemibiotrophic strategy, however, in contrast to this, in the 'subcuticular intramural' strategy, this phase is not so well morphologically distinguishable.

Consistently, *C. acutatum* invading the vegetative parts of the strawberry has been considered a generalist invader (Curry et al. 2002), since it displays the subcuticular intramural invasion without any intimate cytoplasmic interaction with the host cell, typical of hemibiotrophs. Indeed, the presence of a short biotrophic stage during the *C.acutatum* strawberry infection is still debated, due to the difficulty to morphologically probe it. On the other hand is not yet known which infection strategy is adopted by the *C. acutatutm* particularly during fruit infection.

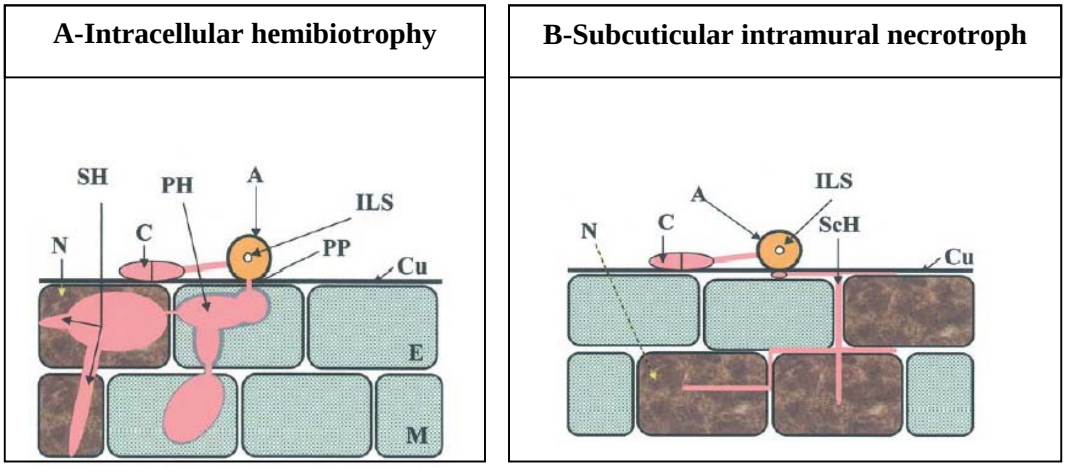


Fig. 1.3: **Infection strategies adopted by *Colletotrichum* species as reported by Bailey et al., 1992 and O'Connel et al. 2000** (described in Wharton and Uribeondo, 2004). The initial stages of early differentiation are similar for both strategies. A conidium (C) germinates and forms an appressorium (A). The appressorium produces a penetration peg (PP) which penetrates the cuticle (Cu) and as a result an internal light spot (ILS) can be seen in the appressorium. **A.** In intracellular hemibiotriphs the penetration peg penetrates the epidermal cell and swells to produces an infection vesicle and broad hyphae, named primary hyphae (PH), which may colonize adjacent epidermal (E) and mesophyll cells (M). During the early stages of this type of colonization, the interaction between the host and the pathogen is biotrophic (living cell represented in blue). The subsequent necrotrophic (N) (represented in brown) interaction is characterized by the formation of thin secondary hyphae (SH). These secondary hyphae grow intracellularly and itercellularly while secreting cell wall degrading enzymes and killing the host cells. **B.** In subcuticular intramural necrotrophs, host colonization is initially by subcuticular (ScH), and intramural hyphae the biotrophic phase is very short or does not occur. The fungus quickly spreads throughout the tissue and grows both inter- and intracellularly.

In most *Colletotrichum* species, the occurrence of the quiescent period can associate with different morphological stages, such as the appressoria or penetration peg (Muirhead and Deverall, 1984; Rappel et al., 1989; Prusky et al., 1991a; Prusky and Plumbey, 1992). In facts, in banana fruits, melanized appressoria of *C. musae* persisted until fruit ripened, indicating that these were responsible of the infections on ripe fruits (Muirhead and Deverall, 1984). On the other hand, Prusky et al. (1991a) and Rappel et al. (1989) found that the appressoria of *C. gloeosporioides* produced a short infection peg in the peel of unripe avocado fruit. Furthermore, germinated appressoria and subcuticular hyphae were recognized as quiescent elements of *C. acutatum* on unripe blueberries (Daykin and Milholland, 1984).
With respect to strawberry anthracnose, the hystopathology of *C.acutatum* infections in petioles and stolons has been well characterized (Curry et al. 2002), however the infection strategy adopted by this fungus on ripe or unripe fruits and in particular the quiescent stage, are not yet well defined.

The term quiescent infection describes a "quiescent or dormant parasitic relationship, which after a time, changes to an active one" (Verhoeff, 1974; Swinburne, 1983). The quiescent stage is designed as the period from host infection to the activation of fungal development and symptom expression (Prusky et al., 1996).
Extensive studies have been conducted in the past to disclose the biochemical basis of activation of quiescent pathogens and three hypothesis have been suggested (Prusky, 1996): i) deficiency of host nutritional resources necessary for pathogen development; ii) presence of preformed or inducible fungistatic antifungal compounds in unripe fruits; and iii) unsuitable environment for activation of fungal pathogenicity factors. Indeed, a variety of preformed or inducible fungistatic molecules have been shown to act as a major barrier against *Colletotrichum* supporting a major role of these factors in inducing quiescence for this pathogen species. Quiescent pathogens have developed a series of strategies to cope with these molecules, such as detoxyfication, efflux from its cells or suppression.

1.3 The phenylpropanoid and flavonoid metabolisms during fruit ripening and susceptibility

Among antifungal compounds, many phenylpropanoid and flavonoid metabolites have been shown to have major role in determining quiescence induction in unripe fruits (Ardi et al. 1998).The phenyl-propanoid and flavonoid metabolisms are key metabolisms during fruit ripening, providing important secondary compounds. Among these, there are anthocyanins for pigmentation, flavonoids, such as flavons, for protection against UV photo-damage, polymeric lignin, for structural support, and a series of antimicrobial compounds classified as inducible phytoalexins and preformed phytoanticipins. Moreover, phenylpropanoid and flavonoid products may play an important role as signal molecules, both in plant development and plant defence (Dixon et al., 2002). They can therefore directly or indirectly determine a different susceptibility of fruits during ripening.

These natural products are mainly derived from the amino acid L-phenylalanine, through a three steps commitment of the carbon flow, from primary metabolism to the general phenylpropanoid pathway. The first step is catalyzed by the phenylalanine ammonia-lyase (PAL) enzyme, and consists of the deammination of the phenylalanine to produce cinnamic acid, the phenylpropanoid skeleton (Fig. 1.4). Cinnamic acid 4-hydroxylase (C4H) enzyme catalyzes the introduction of a hydroxyl group in the cinnamic acid, producing the 4-coumarate compound. Subsequently, p-coumaroyl:CoA ligase (4CL) converts the 4-coumarate to its corresponding CoA thiol ester. From this step on, the phenylpropanoid metabolism branches, to produce thousands of compounds, many of which are plant species specific. Among these, there is the flavonoid pathway branch, leading to the production of flavonoids. The commitment of the phenyl-propanoid pathway into the flavonoid branch starts at the level of p-coumaroyl-CoA, which can condense with the malonyl-CoA from carbohydrate metabolism (Fig. 1.4) (Forkmann and Heller, 1999). Such condensation is catalysed by the enzyme chalcone synthase (CHS) and results in generates the in the production of the yellow coloured chalcone. For most plants chalcones are not the end-products; pathway proceeds with several enzymatic steps to other classes of flavonoid, such as flavanones, dihydroflavonols and finally to the anthocyanins, the

major water-soluble pigments in flowers and fruits. Other flavonoid classes (i.e. isoflavones, aurones, flavones, pro-anthcyanidins (PA) and flavonols) represent side branches of the flavonoid pathway and are derived from intermediates in anthocyanin formation. Many of these compounds can also directly display antifungal action or be precursors for a variety of antimicrobial molecules. The PAL and the CHS are the key enzymes in phenylpropanoid and flavonoid assembly, respectively; for this reason they areoften investigated in studying variation of plant susceptibility to fungal pathogens (Davies and Schwinn, 2003).

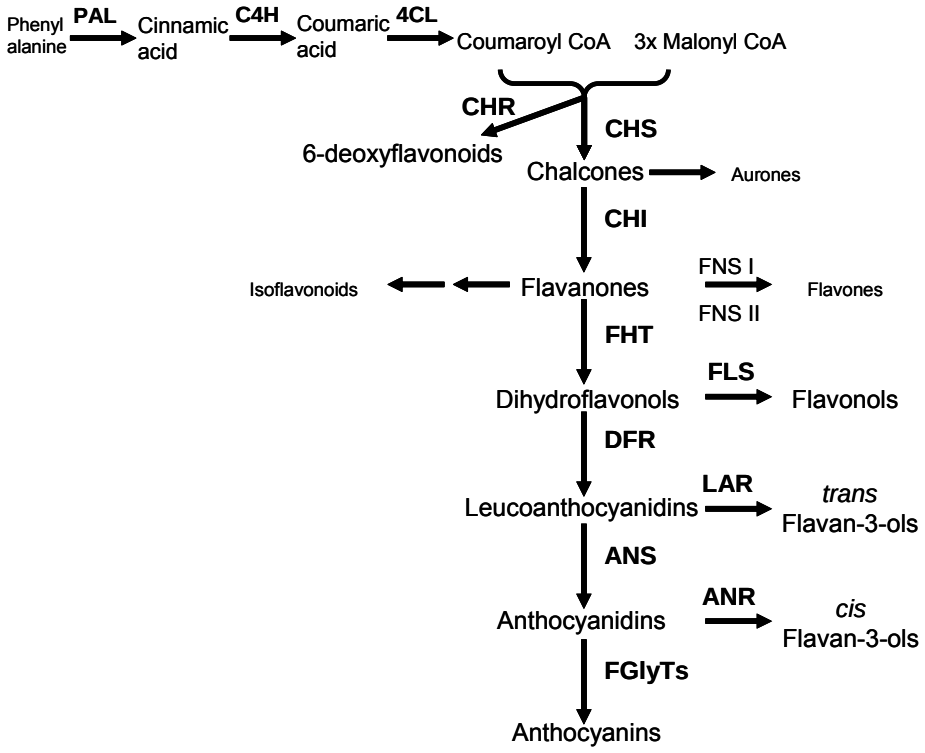


Fig. 1.4**: Phenylpropanoid and Flavonoid pathways**

In strawberry fruit the activity of PAL enzyme shows two peaks: the first at the immature green stage and a second one immediately before the full ripe stage (Given et al. 1998). The first activity peak could, in principle, be connected to the biosynthesis of some flavonoids, like condensed tannins, and phenols which are abundant in the first stage of fruit development, while the second peak, is certainly correlated to the anthcyanins accumulation (Cheng and Breen, 1991). Consistetly, the

same pattern of PAL activity characterizes the expression level of *dihydroflavonol 4-reductase* (*dfr*), and *flavonoid glycosyltransferase* (*fgt*) genes encoding enzymes of the antocyanin pathway l (Perkin-Veazie, 1995; Moyano et al., 1998).

In strawberry fruit there is a great variability in the composition and concentration of flavonoids. This is mainly the effect of different genetic backgrounds which determine the biosynthesis of specific enzymes and regulation factors. Besides this, developmental stages, environmental factors and biotic and abiotic stress may play a very important role for the differential accumulation of these metabolites. The way through which plants respond to these environmental stimuli is very complex and still largely unknown. Jin and Martin, (1999) revealed that a 187 amino acids polypeptide belonging to the big family of MYB transcription factors, named Fa-MYB1 negatively regulates the transcription of genes involved in flavonoids biosynthesis. Activation of FaMYB is essential for balancing the pigment accumulation in late ripening stages and/or to modulating the accumulation of different flavonoid end-products (Aharoni et al., 2001).

Thus, the establishing of these regulation mechanisms may result from the coordinated action of many regulation factors, mostly unknown, that finely modulate the abundance of specific metabolites on the basis of the plant physiological requirements.

1.4 Fruit Alkalinization and pathogen susceptibility

Production of reactive oxygen species (ROS) by the host upon pathogen infection have also been shown to play an important role in inducing *C. gleosporoides* quiescence on unripe avocado fruit (Beno-Moualem and Prusky, 2000). On ripe fruit, this fungus can indirectly participates to ROS suppression through the increase in the environmental pH (Beno-Moualem and Prusky, 2000; Wang et al. 2004). On the other hand, the environmental pH plays crucial role in determining quiescence of transition from the biothrophic to the necrotrophic stage of fungal pathogens and in particular, *C. gleosporoides*. Fruit pH normally increases during ripening, but pathogens are able to interfere with the physiologoical pH changes to favour their pathogenicity (Denison, 2000; Yakoby et al., 2000; Prusky et al., 2001; Eshel et al.,

2002; Prusky and Yakoby, 2003). Depending on their favoured environmental condition, fungi can either promote ambient acidification through secretion of organic acids, or alkalinization, through active secretion of ammonia, resulting from protein degradation and amino acids deamination (Prusky et al., 2003; Manteau et al., 2003 Ruijter et al., 1999).

For alkalinizing fungi, such as *Colletotrichum spp*., ambient ammonification has important effects on the host cells physiology, since it affect membrane function such as ion channels and plasma membrane ATPase (Gerendas and Ratcliffe, 2000), and the vacuolar and citoplasmic pH (Kosegarten et al., 1997), leading to host stress responses, such as ethylene synthesis. Specifically for *C. gloeosporioides* infection on avocado fruits, it has been demonstrated that ammonification is crucial for determining the activation of the necrotrophic phase, since it leads not only to destabilization of the host cell responses, but also induce activation of important fungal pathogenicity factors (Kramer-Haimovich et al., 2006). Whether similar mechanisms regulate also the activation of quiescent *C. acutatum* infection on ripe strawberry fruit is at present still unknown.

1.5 Variation of gene expression during strawberry-*C.acutatum* interaction

Regulation of the gene expression is one of the mechanism used by cells to respond to external stimuli. Although the enzyme activity determines the real level of a specific function, measurement of the transcripts level has proven to be valuable tool for highlighting the genes encoding for the important activities in each specific cellular process (Aharoni and Vorst, 2001). In a recent study, a suppression subtractive hybridization (SSH) technique was used to analyse differences in genes expression induced in the vegetative part (crowns) of *Fragraria x ananassa* (cv. Camarosa) plants upon infection with *C. acutatum* (Casado-Diaz et al. 2006). Among genes found to be differentially regulated, genes encoding proteins involved in plant stress defence, such as PR (pathogenesis related) -5 and PR-10 proteins, peroxidase, β 1-3 glucanase, and WRKY transcription factor, were found up-regulated in strawberry crown upon infection. In the same study, the transcription

levels of these genes was then analysed by Real Time PCR also in fruits infected with *C. acutatum* and found similarly regulated. This study provided important clues on the genes potentially involved in the interaction between strawberry and *C. acutatum*. However, a transcriptome analysis of strawberry fruits interacting with *C. acutatum* is still missing. Furthermore, no information is available about the genes putatively involved in the different susceptibility of unripe and ripe strawberry fruits to *C.acutatum*. These informations are fundamental to understand the molecular mechanisms underneath both the quiescence induction of *C. acutatum* on unripe white fruits, and the fungal invasion on ripe red fruits.

1.6 Microarray technology

The microarray transcriptome analysis performed in this study, was carried out using the Combimatrix microarray platform. This analysis represents the first transcriptome analysis made on *Fragraria x ananassa* using this particularly sensitive technique.

Combimatrix is the last generation of microarray synthetizers and, with respect to the traditional microarray synthetizers, it allows transcriptome analysis highly reliable for both quality and quantity signals.

This is due to the electrochemical synthesis approach (CombiMatrix, Bothel, WA) which uses small electrodes embedded into the chip support to manage individual reaction sites. Solutions containing specific bases are washed over the surface and the electrodes are activated in the necessary positions in a predetermined sequence that allows the sequences to be constructed base by base. With respect to photolithographic method (Affimetrix), Combimatrix synthesis is less expensive and more malleable because allows to modify the chip anytime.

These types of microarrays are generally hybridized using the 'one color approach' method. In this, each labeled sample is hybridized on one microarray individually (one-color approach), and the data analysis are perfomed on each single condition at a time. This avoids the artefacts brought by a likely different hybridization efficiency associated to the two different fluorescent dyes (Goryachev et al., 2001; Wang et al.,

2001). Furthermore, the “one color” approach allows to compare the measured gene expression output from a single a microarray directly across a number of other microarrays to generate new and multiple ratiometric measurements.

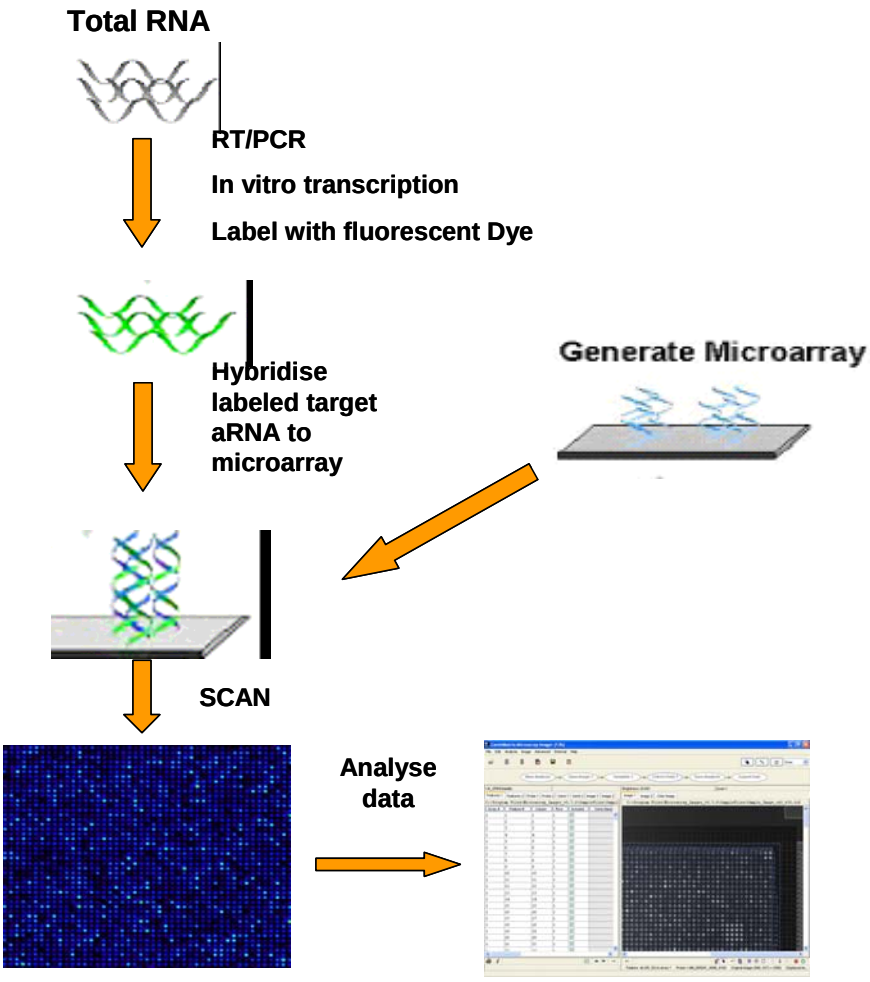


Fig. 1.5**: Schematic diagram of the microarray technique**

Microarrays take advantage of existing EST collections and genome sequence data and are thus limited by the availability of the same; for Arabidopsis and the other plant sequenced genome, chips with sequences represent almost the complete 'transcriptome'. In Arabidopsis, the genes up- and down-regulated during stress defence and upon wounding were revealed using DNA microarray (Malek et al., 2000; Reymond et al., 2000; Schenk et al., 2000).

The Combimatrix microarray used in this study contains all the 4197 expressed sequences of *Fragraria x ananassa* existing on EST databases and the 13366 from *Fragraria vesca.* These sequences were chosen from the TIGR Plant Transcript Assemblies database (http://plantta.jcvi.org/). Because the genome sequence of *Fragraria x ananassa* is not yet available, the data obtained with this method can be partial.

Chapter 2

Materials and methods

2.1 Fruits and fungal materials

In April 2007, strawberry (*Fragraria x ananassa*) red and white fruits, *cv.* Alba were harvested from a local fungicide-free orchard (Cesena, Italy).

C. acutatum was grown on potato dextrose agar (PDA) (Sigma St. Louis, MO, USA) at 20°C for 10 days. Conidial suspensions of pathogen were prepared by washing the colonies with sterile distilled water containing 0.05% (v/v) Tween 80, quantified through haemocytometer, and properly diluted to a concentration of about 10^6 conidia/ml for the fruit infection trials.

Three replicates of 20 fruits each, were dipped for 1 min in the *C.acutatum* conidial suspension or in distilled water as control. The fruits were conserved at 20 °C and 70% of humidity for 24 hours and then peeled with a knife; the skin was immediately frozen in liquid nitrogen and transferred to -80 °C until use.

Similarly, for the analysis of susceptibility, three replicates of 20 fruits each, were inoculated with 10^6 conidia/ml suspension or with distilled water as negative control, and stored at 20 °C and 70% of humidity for three days.

After that time, the degree of susceptibility of strawberry fruit to the pathogen was determined as percentage of infected fruits.

2.2 Analysis of pH and ammonia concentration

The white and red fruits were inoculated with about 10^6 conidia/ml suspension or with distilled water as negative control, and stored at 20 °C and 70% of humidity for 24 hours.

The pH of white and red strawberry fruits, was measured at different times (0 to 24 h) after fungal inoculation or water as control. pH measurements were determined by placing a suited electrode (Mettler Toledo-InLab427-412) directly on exposed strawberry fruit surface to a depth of about 2mm. All measurements were repeated on three fruits in correspondence of two different fruit surface areas. To test the hypothesis that direct pH measurement is a reliable indicator of the environment within the fruit, the direct measurement was compared with the pH determined by the common homogenization method (Lurie and Pesis 1992) in which 2 g of pericarp tissue is crushed with a plunger and filtered through a 4-ml, 0.45-µm centrifuge filter at 4,000 × *g*. Direct and homogenate pH measurements were compared in all fruits used in the present work. Only measurements with a regression coefficient between the measurements $r = 0.999$ were taken into account.

Similarly, ammonia ions concentration was determined from time 0 to time 24 HPI with *C. acutatum* or water, as control. Ammonia was measured with an ammonia electrode (Mettler Toledo- DC218-NH_4) by placing it directly on exposed fruit surface to a depth of about 2mm.

Different concentration (0 to 600 uM) of NH_4Cl were used as standards. Experiments were repeated at least three times on three biological replicates. The results of one representative experiment are presented. Standard errors of means were calculated.

2.3 Tissue processing for histological analysis

The white and red fruits were inoculated with about 10^6 conidia/ml suspension or with distilled water as negative control, and stored at 20 °C and 70% of humidity. Histological analysis were assessed after 8, 16, 24 and 32 hours of inoculation on three replicates of five fruits each. To analyse germination of the spore on the fruit surface, after these times of incubation,the epidermal layer of white and red strawberry fruits was cut with a knife to a depth of about 1 mm, in three different positions of each fruit surface, and the tissue was placed on a glass microscope slide surface. The conidia were stained with 1-2 drops of lactophenol blue solution, covered, incubated for 5 min and finally fixed under the flame. The conidia were visualized on a Nikon Eclipse, TE2000-E microscope (Nikon, Melville, NY, USA).

In order to investigate if the fungus was penetrated on epidermal strawberry layer, the middle portion of fruits was sliced transversely along the axis immediately after the time of incubation and fixed in FAA solution (formalin: acetic acid: 50% ethanol, 5: 5: 90, v/v/v). After a minimum of 24 hours of fixation period, samples were dehydrated through a graded ethanol series for 30 min (ethanol 70%, 80%, 90% and 100%) and then infiltrated with paraffin (Oxford Labware, St Louis) in a 60°C overnight and then maintained at 4°C. Embedded samples were cut with a rotary microtome (Leitz 1512 microtome) in to 6 μn thick sections longitudinally along the axis and mounted on a glass microscope slide surface. The paraffin was removed with limonene 100% and the sections were rehydrated in a graded ethanol series (ethanol 100%, 90%, 69% and 70%) and finally with distilled water. Samples were stained with Gill hematoxylin and eosin Y, to stain nuclei and cytoplasmic proteins respectively, and the dye in excess was removed using Ethanol 95%. Finally samples were covered with a coverslip slide, fixed and examined under a optical Nikon Eclipse, TE2000-E microscope (Nikon, Melville, NY, USA).

2.4 RNA extraction for qRT-PCR and Microarray analysis

Total RNA was extracted following the protocol published by Lopez Gomez and Gomez-Lim (1992) with several modifications: 2g of frozen strawberry skin were ground to a fine powder in liquid nitrogen with mortar and pestle and the ground tissue was suspended in 12 ml of extraction buffer (150 mM Tris-borate pH 7.5, 50 mM EDTA pH 8, 2% SDS, 1% β-mercaptoethanol)

The homogenate was immediately vortexed for 1 min with 0.11 volume of 5M potassium acetate and 0.25 volume of absolute ethanol. Then, 0.33 volume of phenol-chlorophorm (1:1) was added. After centrifugation at 15000 x g for 10 min, the aqueous phase was transferred in a new tube and the RNA was precipitated in a equal volume of 6M LiCl. After incubation overnight at 4 °C, the sample was centrifuged at 20000 g for 30 min at 4 °C, and washed with 200 µl of 80% ethanol. The pellet was resuspended in 1 ml of 0.1% DEPC water, transferred in a 2 ml eppendorf tube and precipitated with one volume isopropyl-alcohol and 0.1 volume of 3M Sodium Acetate pH 5.2 at -80 °C for 60 min. After a centrifugation at 14000 x g for 5 min and one washing step in 80% ethanol, the pellet was air dried and resuspended in 50 µl of 0.1% DEPC water. DNA was removed from the samples by Turbo DNase treatment (Ambion, USA) following the manufacturer's instructions. Two additional purification steps with one volume of phenol : chloroform 1:1 and with one volume of chloroform were always performed. The aqueous phase was transferred in a new tube and the RNA was precipitated in NaCl 5M (0.25 M final concentration) and 2 volumes of 100% ethanol, in ice for 30 min. After centrifugation at 20000 x g for 20 min at 4 °C, the pellet was washed with ethanol 80%, air dried and resuspended in 50 µl of 0.1% DEPC water.

RNA purity was analyzed by measuring the A_{260}: A_{230} and A_{260}: A_{280} ratios using ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, USA); RNA quantity was obtained from the absorbance at 260 nm; to analyze the integrity of the samples and to confirm the quantification, 0.5 µg of RNA was run on agarose

gel (fig. 2.1). Only high quality RNA with A_{260}: A_{230} and A_{260}: A_{280} ratios ≥ 2 was used for qRT-PCR and microarray analysis.

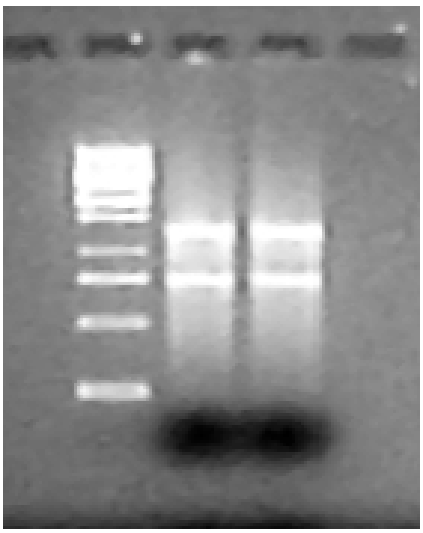


Fig. 2.1: **Agarose gel of total RNA.** The integrity of RNA was indicated by the presence of the two ribosomal RNA bands. In the first lane the 1Kb Gene Ruler (Fermentas) was loaded. RNA yield ranged from 40 to 80 μg, from 2 g of tissue.

2.5 Expression of genes of the phenylpropanoid and flavonoid pathways: qRT-PCR analysis

All the qRT-PCR experiments were carried out at the BAS-BIOTEC-GEN laboratory of ENEA Trisaia Centre. Both type of fruits, inoculated with the pathogen (WI and RI) or mock inoculated (WH and RH) were analyzed. Three replicates of RNA extraction and c-DNA firs strand synthesis were made.

To generate first strand cDNA, 1 μg of total RNA was used with Superscript III Reverse Transcriptase (Invitrogen, Milan, Italy) and oligo(dT) as primers in a 30 μl of total volume according to manufacturer's instructions. cDNA concentration in the RT mix was quantified using a ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, USA)

The primers sequences were designed on the basis of those from Almeida et al. (2007) (tab. 2.1).

Name	Sequence (5’ to 3’)
PAL for	TTGAAGCTCATGTCTTCCAC
PAL rev	CAAGTTCTCCTCCAAATG
C4H for	TGCCCTTGGCTTCATGACT
C4H rev	GCTTGACACTACGGAGAAAGGT
CHS for	GGCTCACCGTCGAGACCG
CHS rev	GGAGAAGATCACTCGAATCA
CHI for	GCCGGAAATGGGAAAGTG
CHI rev	GCTCAGTTTCATGCCTTGAC
FHT for	ATCACCGTTCAACCTGTGGAAG
FHT rev	TCTGGAATGTGGCTATGGACAAC
DFR for	GGGCCGCTACATCTGTTC
DFR rev	GGAACATTATACTTGGGGTAT
ANS for	GACTTGTCCATTTGGCCTC
ANS rev	CCCCCTCAGTTCCTTAGCATACTC
FGT for	CAAGCAGTCCAACAGCTCAATC
FGT rev	GAAAACATACCCCTCCGGCAC
LAR for	CAACTTCCCCATTGAAGG
LAR rev	ATTGAAGCACTCATCCAAAG
ANR for	CCTGAATACAAAGTCCCGACTGAG
ANR rev	GTACTTGAAAGTGAACCCCTCCTTC
MYB1 for	GCAACTTGAGGATCAGCC
MYB1 rev	GGTGCCTGAGTTGAATCTC
EF for	TGCTGTTGGAGTCATCAAGAATG
EF rev	TTGGCTGCAGACTTGGTCAC

Tab. 2.1: **PCR primers used for qRT-PCR experiments.**

Real Time PCR was performed on a ABI Prism 7900HT thermocycler (Applied Biosystems, Monza, Italy) using Platinum Sybr- Green Master mix (Invitrogen, Milan, Italy). Each reaction mixture contained 1 μg of cDNA first strand as template, 375 nM of each specific primer, 1X Platinum Sybr- Green Master mix (Invitrogen, Milan, Italy) and water to a total volume of 4 μl. An annealing temperature of 58 °C was used for all genes analyzed. Three replicates of each reaction were always run in the same experiment.

All thermal cycles started with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of a denaturation step at 95 °C for 30 sec, an annealing step at a specific temperature and an extension step at 72 °C for 1 min.

Fluorescence was monitored at the end of each annealing step. To asses the amplification specificity, melting curve analysis was always performed at the end of each experiment by monitoring the fluorescence from 55 °C to 95 °C every 0.1 °C. Amplified products were then checked by agarose gel electophoresis (1,5%); quantification was carried out using the standard curve generated by serial dilutions of a PCR product of each gene. Data were analyzed using SDS Software, Version 2.0 (Applied Biosystems, Monza, Italy).

An *eleongation factor 1* α gene, having constant expression levels (data not shown), was used to normalize raw data and calculate relative transcript levels.

Data were analyzed by ANOVA and means were separated by the least significative difference test at the 0,05 probability level. STATISTICA software was used (Statsoft Inc., Tulsa, OK, USA). Unless noted otherwise, only results significant at $P \leq 0.05$ are discussed.

2.6 Microarray analysis

White and red strawberry fruits inoculated with the pathogen (WI and RI) or mock inoculated (WH and RH) were compared on microarray analysis.

All the microarray experiments were carried out at the BAS-BIOTEC-GEN laboratory of ENEA Trisaia Centre.

The Combimatrix microarray technique together with the suited protocols were used. Each microarray experiment was run in three replicates, using three independent preparations of labeled cRNA (complementary RNA) named also aRNA (amplified RNA), generated from as many RNA extractions, for each sample.

2.6.1 Microarray fabrication

A DNA microarray of more than 93,300 oligo-probes was produced including 4.197 expressed sequences of *Fragaria x ananassa* and 13.366 of *Fragaria vesca.* These sequences were chosen from the TIGR Plant Transcript Assemblies database (http://plantta.jcvi.org/). Of the selected sequences, 9,814 are singletons clones and retain their GenBank accession numbers as identifiers, while the others are TIGR plant transcript assemblies (TAs). The oligo-probes of about 35-mers have been designed and synthesized *via in situ* on chips based on CustomArray™ semiconductor technology (CombiMatrix Corporation, www.combimatrix.com) by using a CustomArray™ Synthesizer (CombiMatrix Corporation). Moreover, microarray layout included 462 oligo-probes corresponding to 18 different phage Lambda sequences and 2781 oligo-probes corresponding to 63 different quality controls (QC) CombiMatrix.

The widely accepted Minimum Information About a Microarray Experiment (MIAME) guidelines for microarray analysis and verification was followed, and microarray experiments have been deposited to the EBI public repository ArrayExpress (Accession number E-MEXP-2274).

2.6.2 cRNA synthesis, labeling and quantification

For each experiment, 2 μg of high quality RNA (OD ratios higher than 2.0) was reverse-transcribed amplified and labeled in the presence of Cy5 using the ULS RNA ampULSe kit (Kreatech) following the manufacturer's instructions. (Fig. 2.2).

Differently from previous labeling systems, in which the fluorophore labeled aminoallyl-modified nucleotides in the cDNA, creating molecules with a great steric encumbrance with the risk of interference with downstream hybridization, the advantage of Kreatech system refers to the fact that fluorophore Cy5 exclusively recognizes and labels the guanine base of cRNA. This allows a better representation of the transcriptome and a major reproducibility inter- and intra chip. Furthermore, since unmodified nucleotides are used, the Kreatech system allows the reuse of cRNA for other experiments.

A purification step was performed using KREApure columns (Kreatech) associated with the system, to remove unincorporated dye.

The efficiency of Cy5 label incorporation into the cRNA and the quality and amounts of labelled cRNA were verified using a ND-1000 UV spectrophotometer (Nanodrop Technologies).

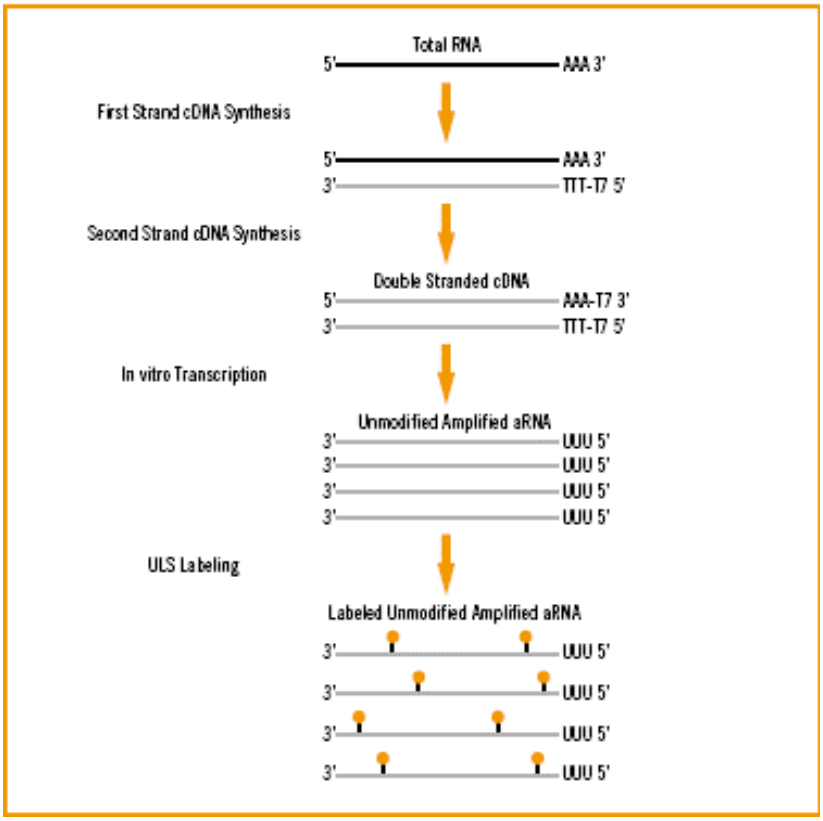


Fig. 2.2: **Schematic overview of RNA ampULSe: Amplification and Labeling procedure.** A cDNA first strand was synthetized from 2 µg of total RNA using reverse transcriptase and oligo (dT). The second Strand cDNA synthesis was performed using DNA polymerase associated to the kit. The generated doubled strand cDNA was *in vitro* transcripted with T7 Enzyme Mix containing RNA polymerase and RNase hinibitors. The cRNA (complementary RNA), also named aRNA (amplified RNA, since its quantity is higher than the starting RNA), was quantified, purified, and then labeled to Cy5 following the manufacturer's instructions.

2.6.3 Microarray hybridization

The microarray was pre-hydridized for one hour at 45 °C with the pre-hybridization solution (6X SSPE, 0,05% Tween-20, 20mM EDTA,5X Denhardt's solution, 100 ng/µl Salmon sperm, 0.05% SDS). Cy5-labelled cRNAs were fragmented prior to hybridization with Tris Acetate pH 8.1 (200mM), KOAc (500mM) and MgOAc (150mM) for 20 minutes at 95 °C, to maximize binding specificity and detection sensitivity. Labeled and fragmented cRNAs were re-suspended in hybridization buffer containing 25% formamide, 6X SSPE, 0.05% Tween-20, 20mM EDTA, 0.04% SDS and 100 ng/ul Salmon sperm DNA. The hybridization chamber was filled with the hybridization solution and the microarray was incubated at 45 °C overnight with gentle rotation in a rotisserie oven. Microarray were washed in decreasing SSPE concentrations and 0.05% Tween-20 at 45 °C and room temperature, respectively. The last wash was carried out in 2x PBS at room temperature.

2.6.4 Microarray data analysis

Hybridized microarrays were scanned with a confocal microarray scanner (ScanArray $G_{X,}$ Perkin-Elmer, Waltham, Massachussetts, USA), using the associated software (ScanArray Express 3.0, Perkin-Elmer). Fixed parameters of laser power (52%) and the photomultipler tube (PMT) 56%, were used avoiding the differences from the acquisition processes. The dye excitation-emission wave lengths was set on 633-670 nm for Cy5. The scan was performed at a resolution of 5-µm. The dye fluorescence emission was converted into a digital output and was stored as a TIFF image (Fig. 2.3). The resulting Cy5 images were analyzed with the software Microarray Imager (CombiMatrix Corporation) in order to determine the Cy5 spot intensities.

A grid was superimposed on the image; this was built on the basis of the parameters specific for the our microarray, such as the spot spacing, the printing pins, the distances between them and the number of columns and rows of each grid. For each experiment, the left top grid was manually modified and aligned, then the alignment was automatically propagated to the rest of the grid (Fig. 2.4). The software converts the fluorescence intensity value of each spot to a numerical value.

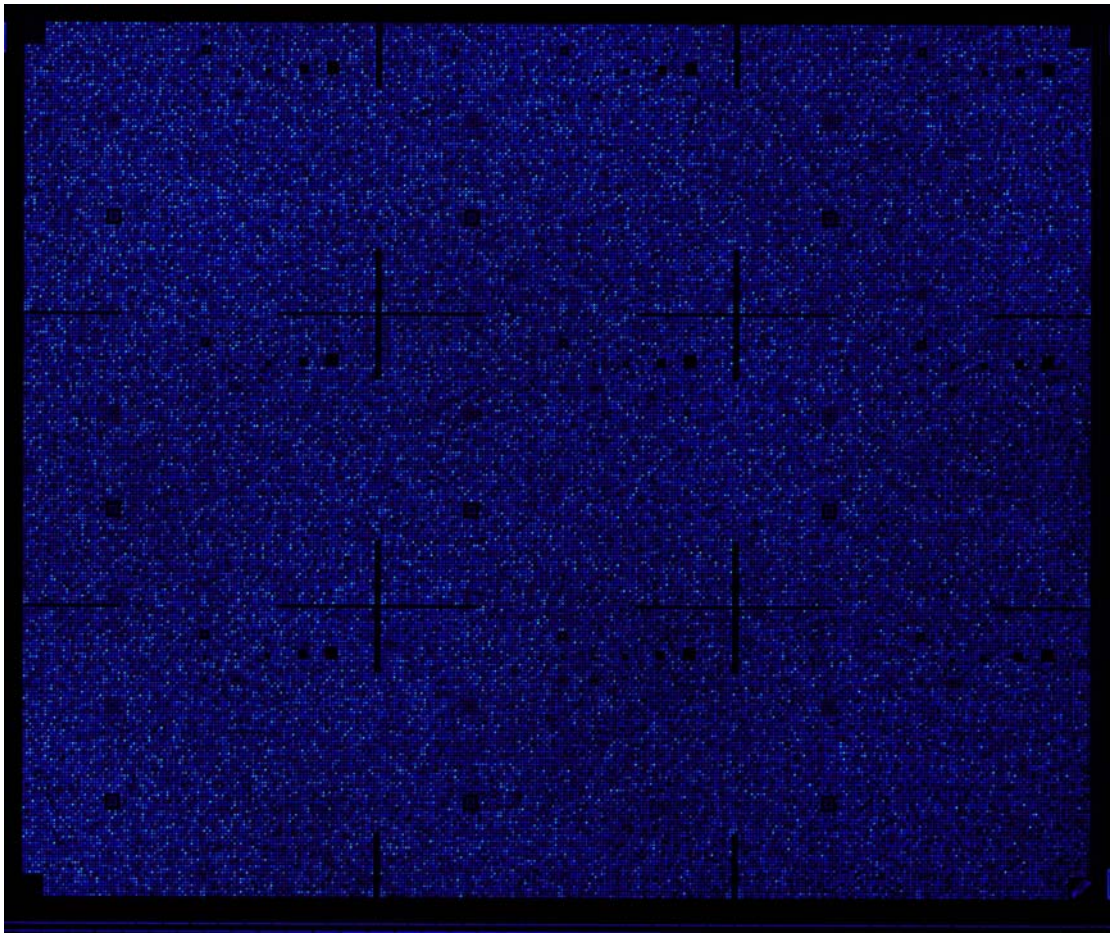


Fig. 2.3: **Fluorescent image of the Combimatrix microarray hybridised with a fluorescent cRNA sample**.

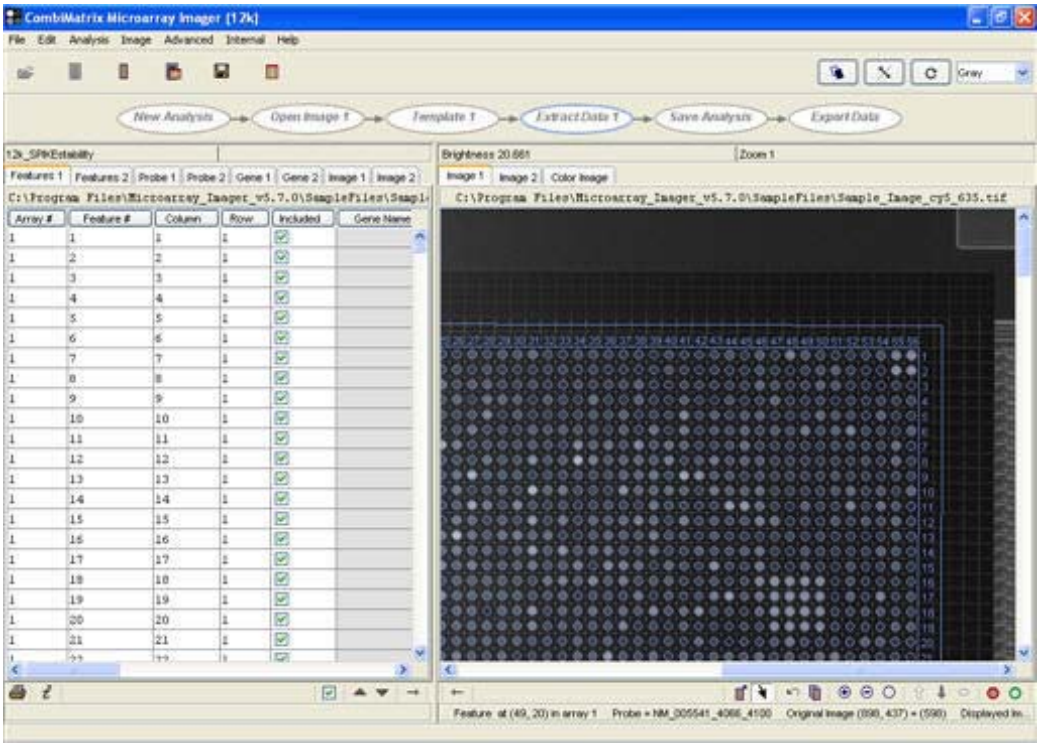


Fig. 2.4: **Microarray Imager software interface.** The grid was manually aligned with the spots on the array image.

Raw values were then normalized with quantile based method using ProbeWeaver (CombiMatrix Corporation). All hybridizations show a minimal Pearson correlation among biological replicates of 0.99 ($R \geq 0.99$) and a mean coefficient of variance (CV) intra-chip below 0.20.

The so processed data were used as input file for GeneSpring GX version 7.3 software (Agilent Tech., USA) for the gene expression analysis of the array: Spots having intensities below 800 in both treatments were excluded from further analysis and the differentially expressed genes identified. Filtered gene lists were subjected to t-tests with a false discovery rate at 0.05 ($P < 0.05$) in both ripening stages independently.

Chapter 3

Results

3.1 Ripening and susceptibility

The variation of susceptibility of strawberry fruits at different ripening stages to *C. acutatum* was examined by artificially infecting white (W) and red (R) strawberry fruits with *C. acutatum* and determining the percentage of infected fruits.

All red infected strawberries were highly susceptible to *C. acutatum*: severe anthracnose symptoms appeared already after three days of infection as brown necrotic lesions. These became darker and larger five days post infection (DPI), confirming the very high susceptibility of the strawberry CV. Alba to *C. acutatum* infection. Conversely, none of the white fruits showed anthracnose symptoms even five DPI when fruits start to turn red. (Fig. 3.1, Fig. 3.2)

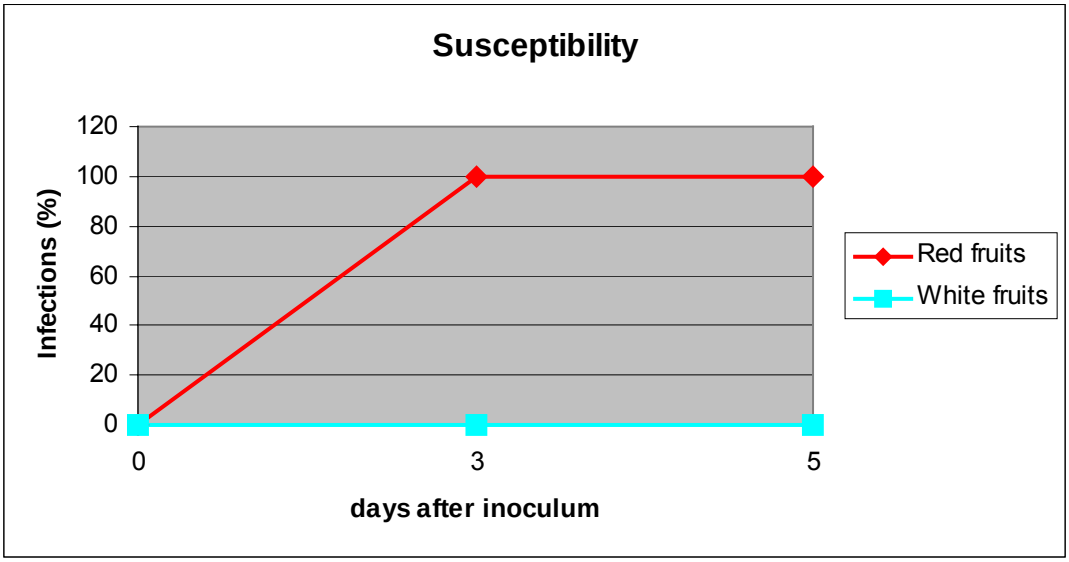


Figure 3.1: **Variation of strawberry fruit (cv. Alba) susceptibility to infection of *C. acutatum* during fruit ripening.** Three replicates of 20 fruits were inoculated with a conidia suspension ($10^6 \cdot ml^{-1}$) for 1 min or mock inoculated with water. Infections were examined after 3-5 days of incubation at 20°C, and the percentage of infected fruits was calculated.

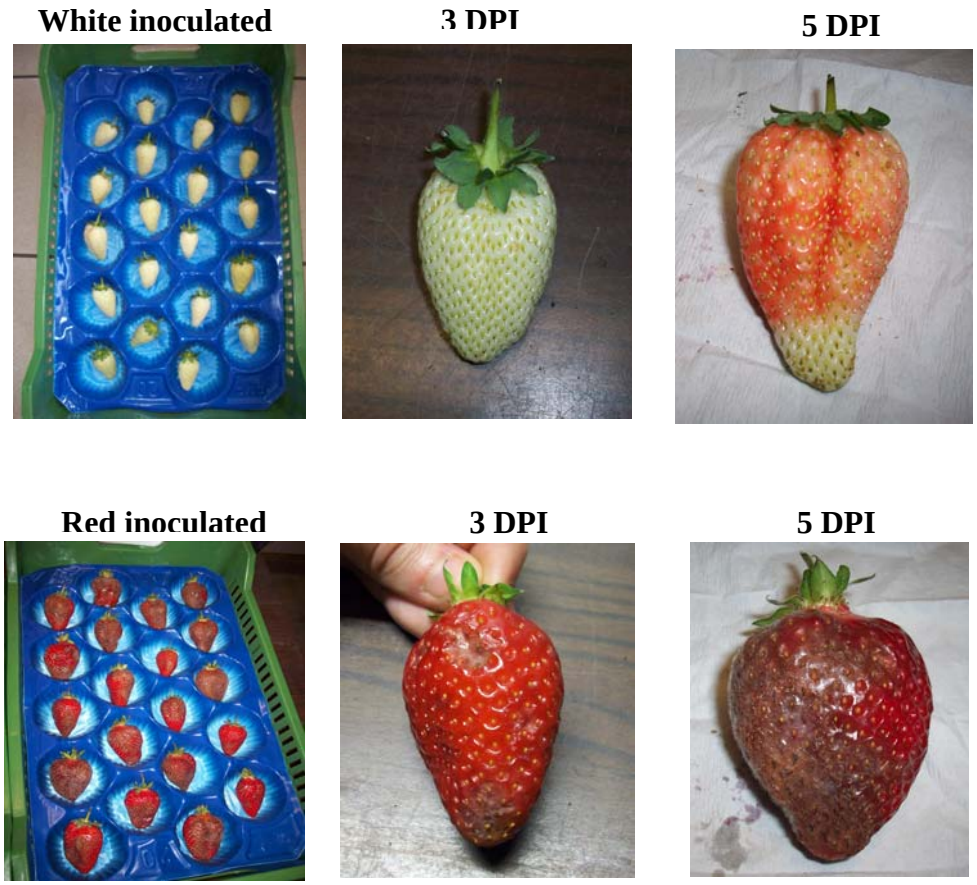


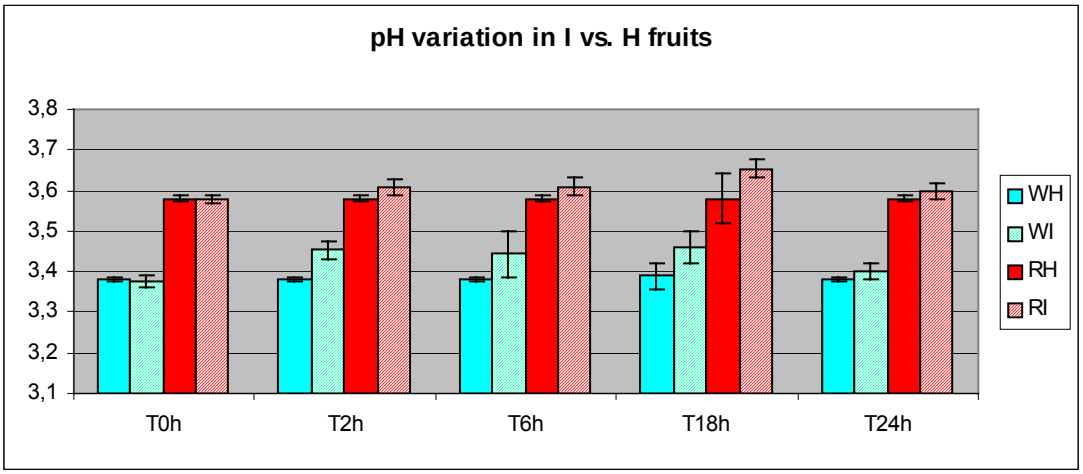
Fig. 3.2: **Susceptibility to *C. acutatum* artificial infection of strawberry during fruit ripening.** The degree of susceptibility of red and white strawberry fruits was analyzed 3-5 days post-inoculation (DPI) with a conidia suspension of $10^6 \cdot ml^{-1}$ or mock inoculated.

3.2 Analysis of fruit alkalinization

The possible correlation between fruit alkalinization and variation of susceptibility to *C. acutatum* and, in turn, the influence of *C. acutatum* infection on fruit alkalinization, was investigated by determining the concentration of ammonia ions and the associated variation of pH (generally responsible of fruit pH increase) at surface of both white and red strawberries during the first 24 HPI (hours post-inoculation).

Non-inoculated red fruits contained 22.16 μM of ammonia ions, whereas a minimal ammonia ions concentration (1.5 μM) was detectable in mock inoculated white fruits. Upon *C. acutatum* infection, in both fruits, ammonia ions slightly increased in the first two HPI, compared to mock-inoculated fruits. Upon inoculation, a drastic increase of ammonia ions is revealed at 18-24 HPI only in inoculated red fruits, indicating that the fungal infection determines high alkalinization of the surface specifically in these fruits. (Fig. 3.3).

Consistently, the pH value of non inoculated white fruits is lower than the red ones (3.38 and 3.58 respectively), indicating a higher physiological acidity of these fruits. Except for a slight increase during the first two HPI in white fruits, the inoculation with the fungal pathogen does not determine any significant variation of the pH value in both red and white fruits during the monitored 24 HPI, compared to mock-inoculated (Fig. 3.3). Therefore, the drastic increase in the ammonia ions level detected in red fruit 18-24 HPI, is not simultaneously translated to a pH increase. However, a significant increase of the pH is detected in the RI fruits later on after symptom appearance (5 DPI) to a value of 5.0 (data not shown).



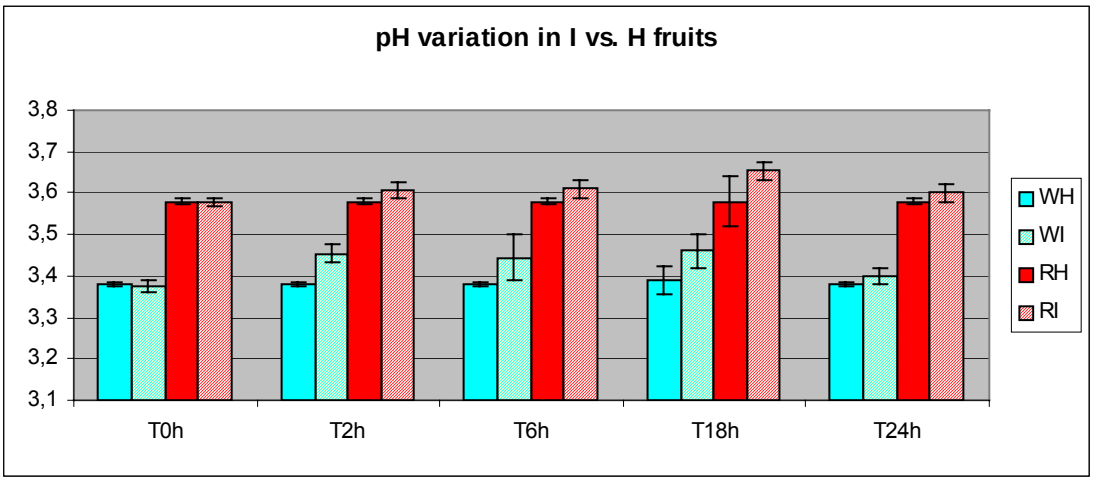


Fig. 3.3: **Ammonia ions concentration and pH of strawberry fruits inoculated with *C. acutatum*.** pH and ammonia concentration were determined in white and red strawberry fruits at different hours after inoculation (HPI). Fruits were inoculated with a suspension of $10^{6.}$ ml^{-1} *C.acutatum* conidia. Ammonia ions and pH were monitored on fruit surface in correspondence with the infection area.

3.3 Analysis of the infection timing and strategy of *C.acutatum* on strawberry fruit.

The timing of *C. acutatum* conidia germination during interaction with white and red strawberry fruits was analyzed. After 8 (HPI), the surface of both white and red fruits appeared covered of the typical septated *C. acutatum* conidia. These appear as spatially distributed in small groups of 6-10 conidia along the fruit surface, and are all un-germinated (Fig. 3.4 a; 3.4 b). On both types of fruits, most of conidia germinated after 16 HPI, showing a germinative tube of approximatively 10 un length (Fig. 3.4 c; 3.4 d); after 24 HPI all conidia are germinate and most of them develop appressoria (Fig. 3.4 e; 3.4 f).

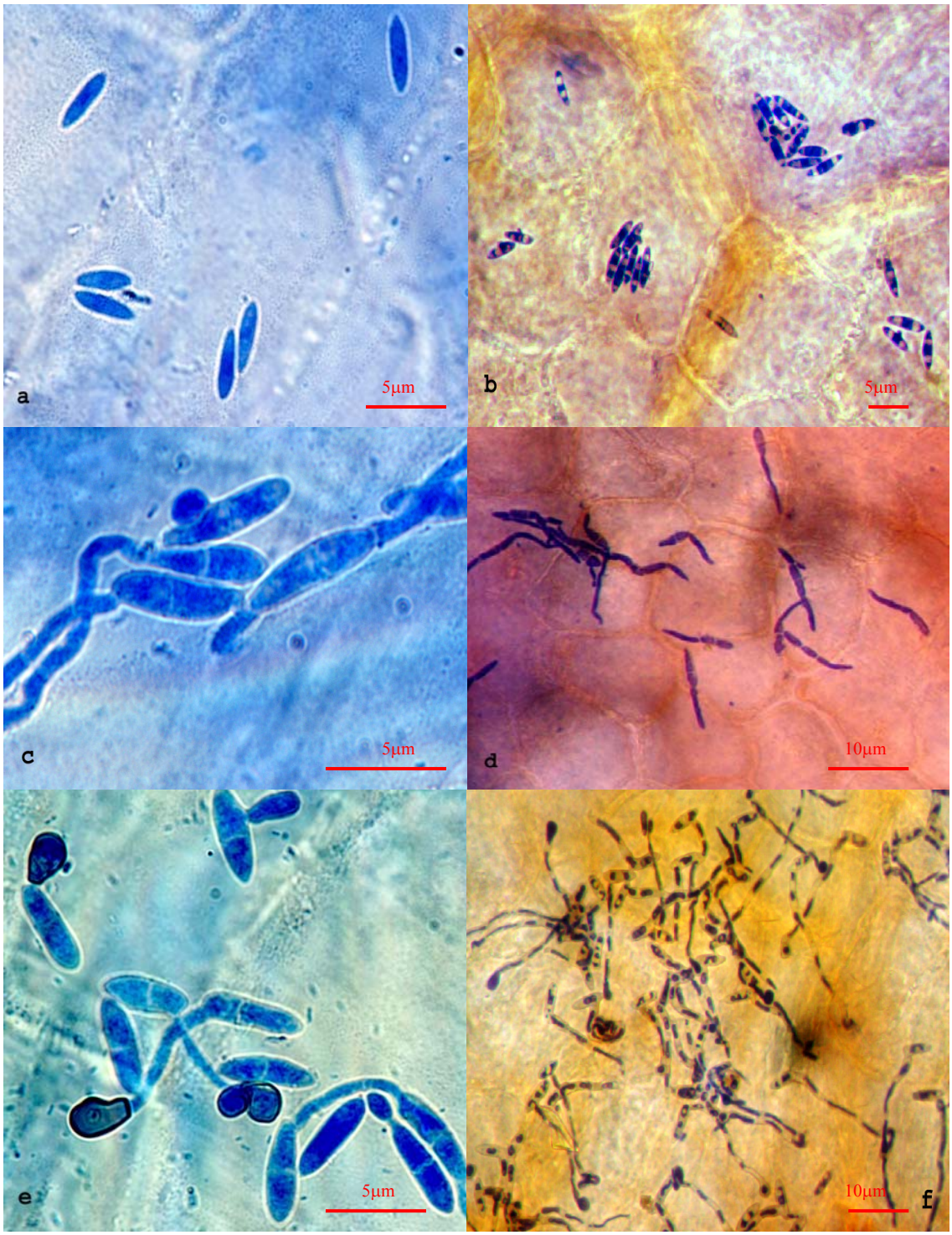


Fig. 3.4: **Surface of white (a) and red (b) strawberry fruits** inoculated with a suspension of $10^6 \cdot ml^{-1}$ conidia. Conidia were coloured with blue of lactophenol and analysed at the optical microscope after 8 hours (a and b) after 16 h (c and d) and after 24 h (e and f)

To investigate on the infection strategy adopted by of *C. acutatum* upon surface penetration of white and red strawberry fruits, strawberries at 24 and 32 HPI were sectioned longitudinally, sliced and observed at the microscope.

The infection strategy of *C. acutatum* completely varies in the two types of fruits. After 24 hours, *C.acutatum* invades red fruits intercellularly (Fig. 3.5). Conversely on white strawberries, the fungal pathogen stops growth at the stage of melanized appresorium; no hyphae are visualized underneath the fruit surface, suggesting that, on these fruits *C.acutatum,* becomes quiescent (Fig. 3.6).

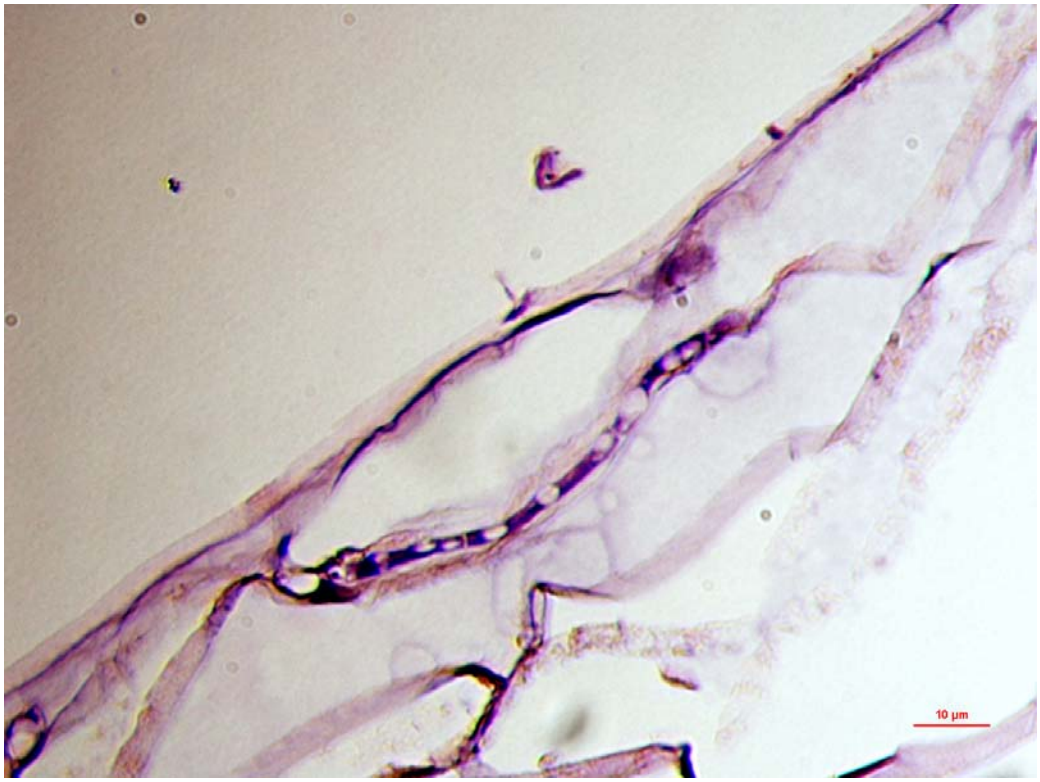


Fig. 3.5: **Section of red strawberry fruit at 24 HPI with *C.acutatum*.** *C. acutatum* is penetrated intercellularly in the epidermal layer. Sections are performed using microtome, proteins and nuclei are stained with ematossilin and eosin.

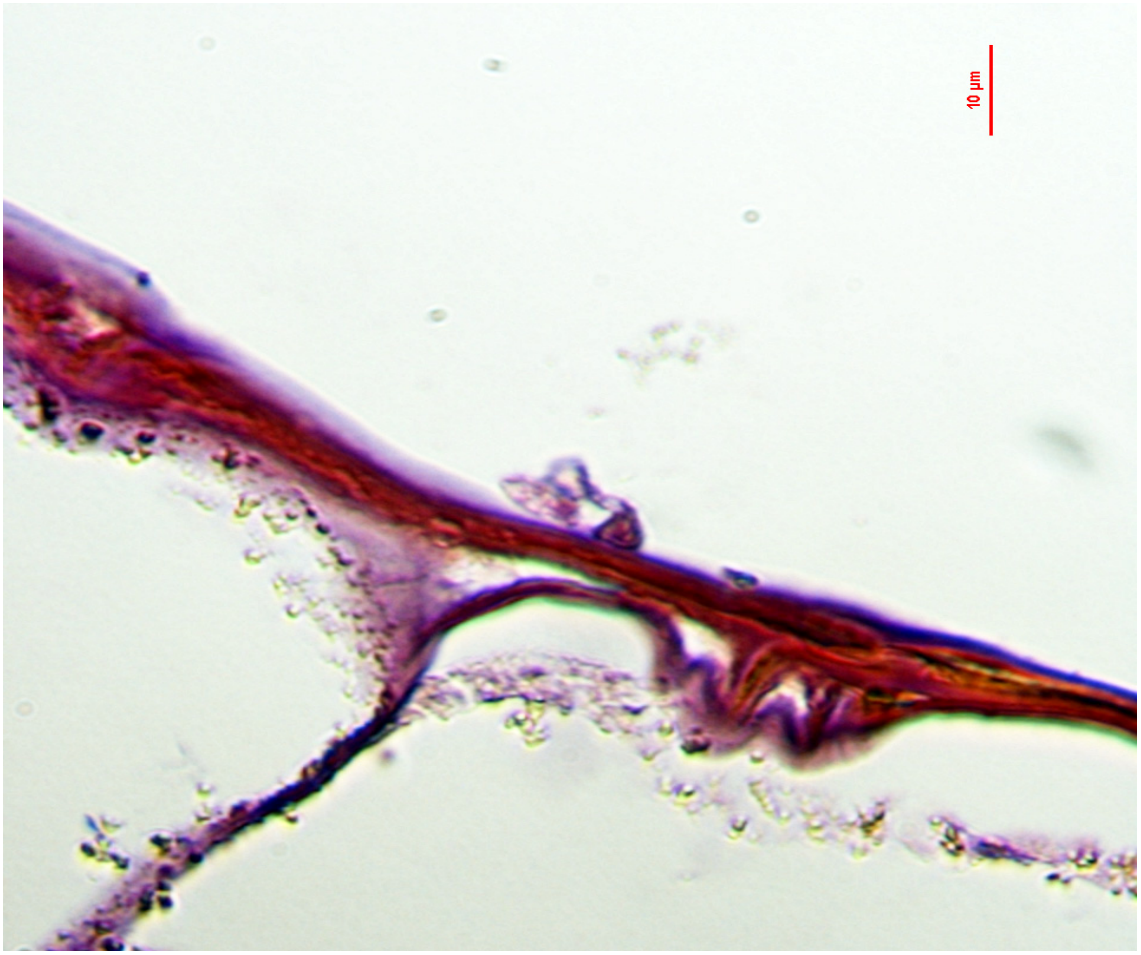


Fig. 3.6: **Section of white strawberry fruit at 24 HPI with *C.acutatum*.** *C.acutatum* becomes quiescent at the stage of melanized appressorium. Proteins and nuclei are stained with ematossilin and eosin

On red fruits at 32 HPI, hyphae of *C.acutatum* invade the fruit tissue more deeply both inter- and intracellularly (3.7 a), whereas, on white fruits, the fungus appears still quiescent as melanized appressorium (3.7 b).

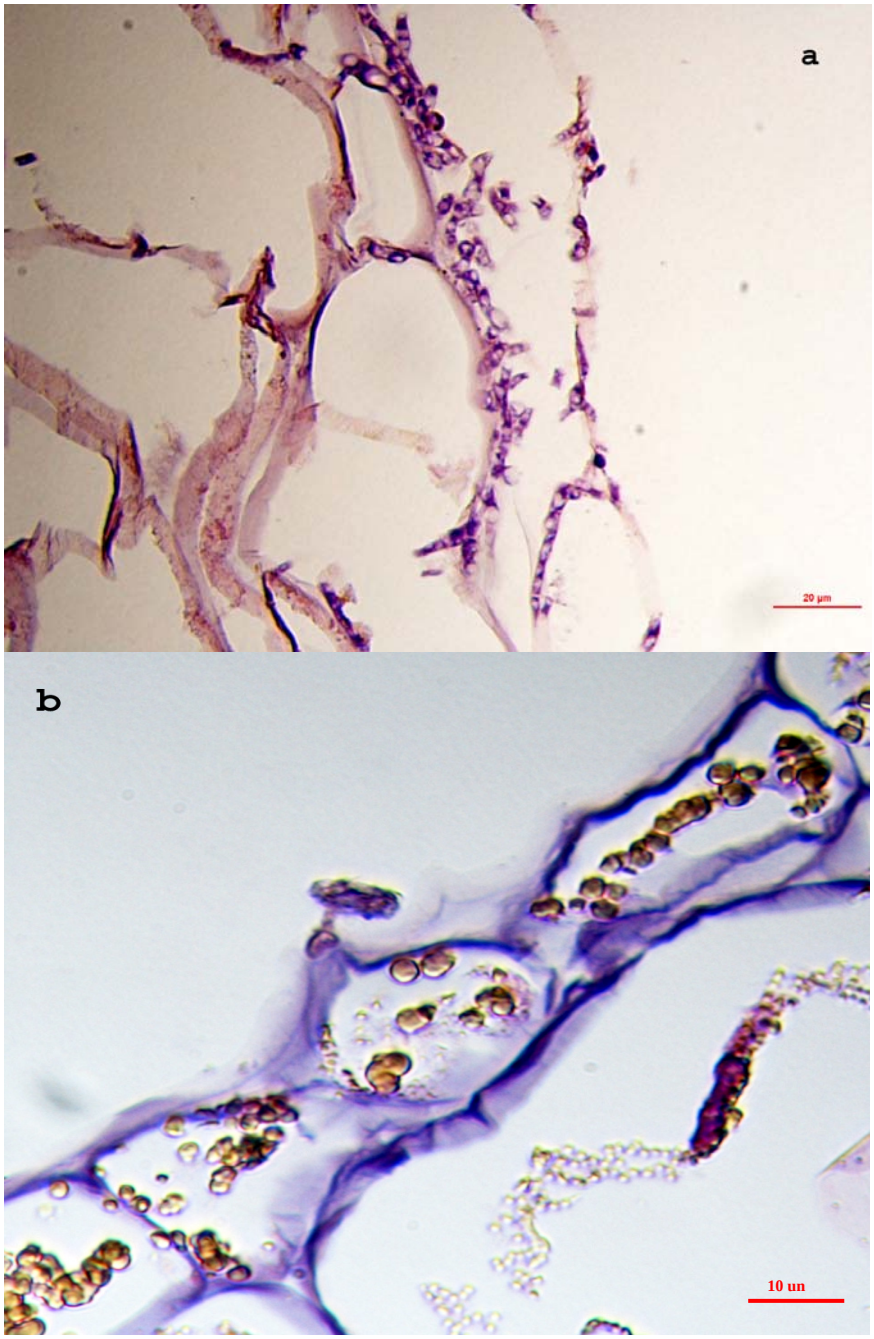


Fig. 3.7: **Sections of red (a) and white (b) strawberry fruits at 32 HPI with *C.acutatum*.** Proteins and nuclei are stained with ematossilin and eosin

3.4 Expression of genes of the phenylpropanoid and flavonoid pathway

In order to investigate on the a possible role the of phenylpropanoid and flavonoid metabolisms in determining the quiescence of *C. acutatum*, a gene expression analysis was undertaken on white and red strawberry fruits.

The variation of expression of genes encoding for the phenylpropanoid enzymes phenylalanine ammonia lyase (PAL) cinnamate 4-hydroxylase (C4H), and flavonoid enzymes (Fig. 3.8), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3β-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), flavonoid glycosyltransferase (FGT), leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), known to be involved in the biosynthesis of several antifungal compounds, was monitored by Real Time qPCR analysis in white and red fruit samples at 24 HPI with *C. acutatum* (WI and RI respectively) or mock-inoculated (WH and RH). (Fig. 3.9).

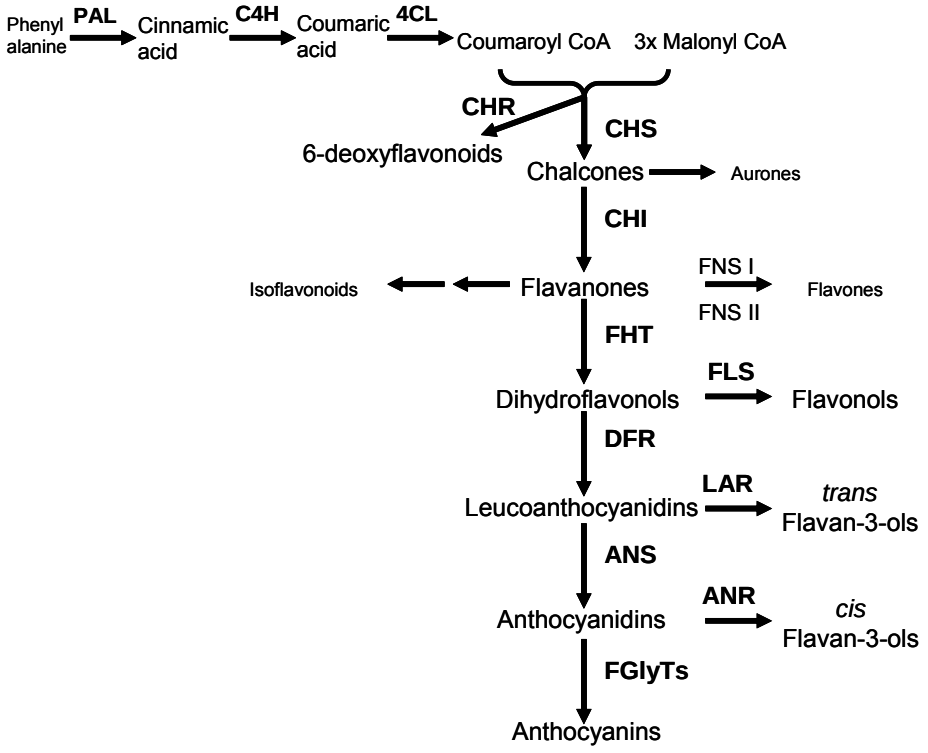


Fig. 3.8**: Phenylpropanoid and Flavonoid pathways**

No difference in the expression of these genes was detected between mock inoculated and inoculated fruits, except for *chs* and *ans*: in particular *chs*, involved in the synthesis of chalcones, decreases of about 2,4 times in the white inoculated sample. On the other hand *ans* transcript level involved in the synthesis of anthocyanidins decreases of about 2,3 times in white inoculated samples (Fig. 3.9).

Considering the expression in white and red strawberry fruits,the transcript level of most of the phenylpropanoid and flavonoid genes, is higher in the red fruits with respect to the white ones, indicating an up-regulation of these genes during ripening. Specifically, the mRNA level of *pal* and *c4h*, which encode for the enzyme responsible for the branching from phenylalanine aminoacid and cinnamic acid respectively, increase respectively of about 4,3 and 2,1 times in the red fruits.

Furthermore, the mRNA level of *chs*, the first enzyme of flavonoid pathway and *fht*, responsible for dyhidroflavonols production, increase respectively of about 3,4 and 2,7 times in red fruits. On the other hand the transcript level of *ans* and *fgt* responsible for anthocyanidins and anthocyanins biosynthesis at the downstream of flavonoid pathway, increase respectively of about 2,1 and 3,1 times in red fruits.

Conversely, the mRNA level of *chi* and *dfr* does not show any change either upon inoculation or with fruit ripening, since no significant difference is detected in their expression between red and white fruits.

Only the genes encoding for LAR and ANR, the enzymes involved in the proanthocyanidin (PA) biosynthesis, particularly in the epicatechin production, appear up-regulated in white fruits; indeed their mRNA level increase of about 5,6 and 1,8 times respectively (Fig. 3.9).

Finally, the expression of *Famyb*, a myb transcription factor down-regulating the expression of flavonoids metabolism genes, was also investigated (Fig. 3.10). Consistently to the down regulation of the flavonoid genes detected in the white fruits, the *Famyb* gene is found upregulated is in the mock inoculated white fruits vs. red ones. Interestingly such a difference in expression is not detectable in the inoculated white vs. red fruits, suggesting that the fungal infection interferes with the physiological regulation of this metabolism at the transcriptional level (Fig. 3.10).

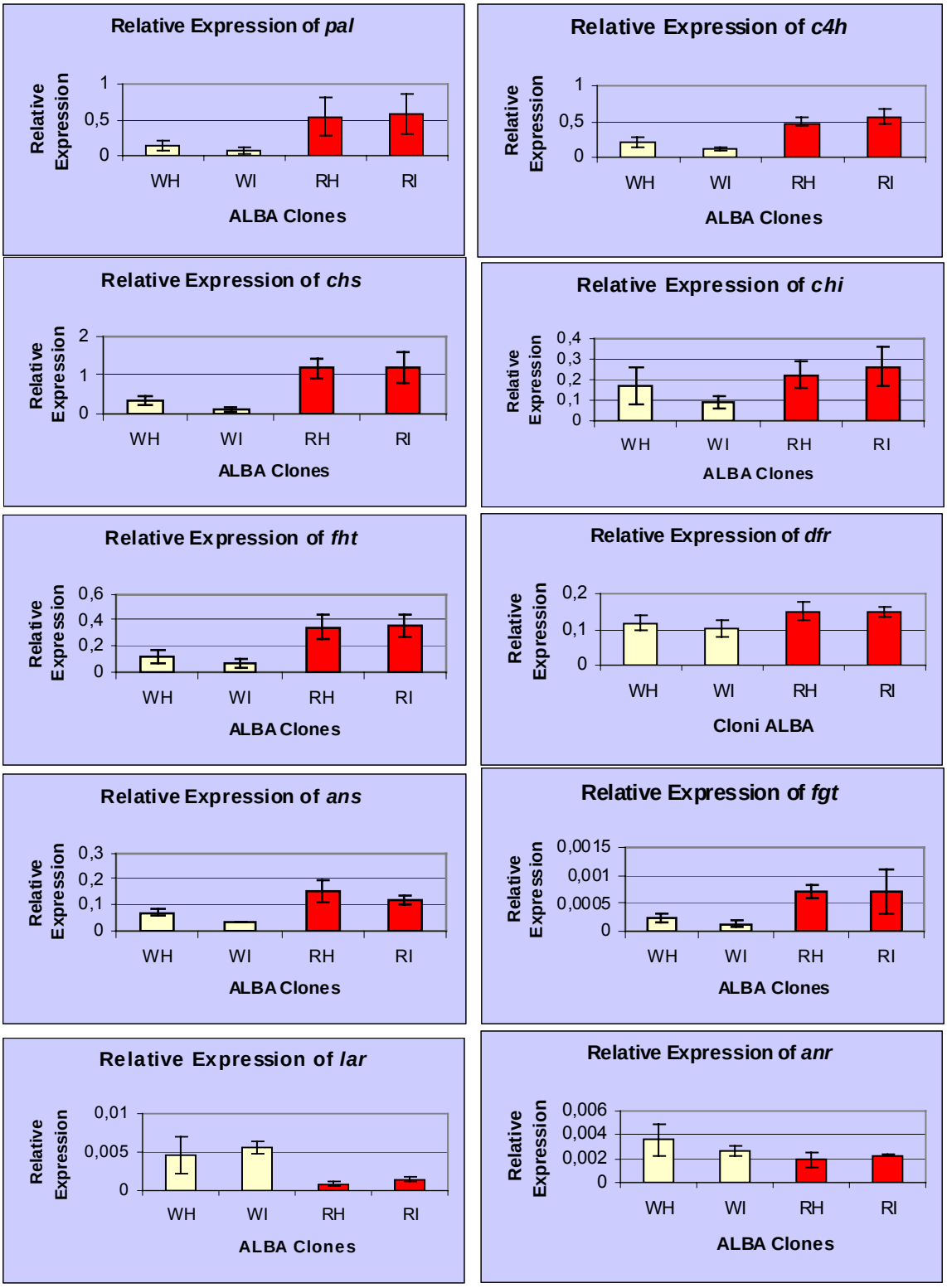


Fig. 3.9**: Expression levels of genes of the phenylpropanoid and flavonoid pathways in W and R strawberry fruits at 24 HPI.** Three replicates of cDNA were synthesized from three separated RNA preparations. Concentration of each target cDNA was normalized to the amount of elongation factor 1-alpha cDNA.

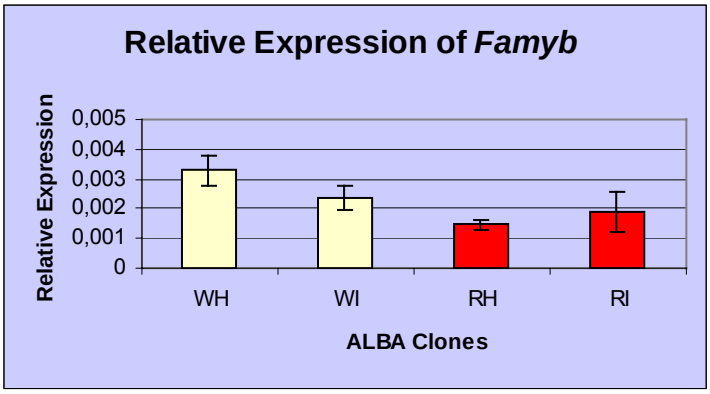


Fig. 3.10: **Expression levels of *Fa myb* genes in white and red strawberry fruits at 24 HPI.** Concentration of each target cDNA was normalized to the amount of elongation factor 1-alpha cDNA. Three replicates of cDNA were synthesized from three separated RNA preparations.

Chapter 4

Discussion

For many postharvest fungal diseases, pathogens infecting unripe fruits stop their growth and enter in a quiescent stage until fruit ripens. Then, the pathogen can restart its colonization, invades tissues and causes visible symptoms. For this reason, quiescence can cause extensive economic losses in fruit production at the postharvest stage.

Colletotrichum ssp. are severe pathogens causing fruit quiescent infection (Jeffries et al., 1990). First investigations on *Colletotrichum* infection were carried out in banana and avocado fruits (Muirhead & Deverall, 1984; Prusky et al. 1991a; Rappel et al. 1989; Prusky and Plumbey, 1992) and few hypothesis have been proposed to explain the physiological mechanisms inducing fungal quiescence on unripe fruits. These take into consideration i) the mechanical resistance of unripe fruits, ii) the presence of pre-formed inhibitory substances during this period, iii) the presence of an unsuitable environment to satisfy the requirements of the pathogen, and iv) phytoalexin production in unripe fruit (Jeffries et al. 1990, Prusky et al. 1996, Prusky and Lichter, 2007). Limited studies have been carried out on the variation of

susceptibility of strawberry fruits to *C. acutatum* during fruit development (Howard et al. 1992). The results described here reveal that only fully ripe strawberry fruits develop anthracnose symptoms, typical of *C. acutatum* disease, whereas, unripe white fruits are not susceptible to *C. acutatum* invasion. Despite its great economic importance, the reason of this drastic variation of strawberry susceptibility is not yet clear.

Works carried out on *C. gleosporoides* – avocado fruit interaction, have shown that the environmental pH at which the pathogen grows, plays crucial role in determining the exit from quiescence and infection activation. Differently from infection of unripe fruits, where the disease becomes quiescent, increases in ammonia ions concentration and pH values, have been detected after seven days of *C. gleopsoroides* infection in ripe avocado fruits, concomitantly to the appearance of anthracnose symptoms (Yakoby et al. 2000; Prusky et al. 2001; Prusky and Yakoby, 2003). For this reason, the increase in ammonia ions and pH is associated to the exit from *Colletotrichum* quiescence.

So far, reports on *in vivo* measurements of ammonia ions concentration and pH during the first hours of *Colletotrichum*-fruit interaction are missing. Therefore, our measurements made during the first 24 hours of interaction between strawberry fruits and *C.acutatum* provide important clues on the exact timing of fruit sensing mechanisms.

Our data reveal that ammonia ions concentration drastically increases from 18 to 24 HPI with *C. acutatum* inoculation only in R strawberry fruits. Such increase of ammonia ions could well determine the susceptibility of red fruits to pathogen invasion. On the other hand, during the same time period, no increase in fruit pH is detected in these fruits, suggesting that the variation in ammonia ions is an early event with respect to the increase in pH, which occurs later in correspondence with symptom appearance.

The histological analysis of the infection process of *C.acutatum* on W and R strawberry fruits from 8 to 32 HPI reveals, for the first time, the early histological events underneath the *C. acutatum* – strawberry fruit interaction.

The different colonization strategy shown by *C. acutatum* on W or R fruits, with melanized quiescent appressoria on white fruits and penetrated intercellular hyphae on red ones at 24 h after inoculation, suggests that, only red fruits provide the fungus with the suitable environment for penetration and invasion of the host tissue. Consistently to the previous reports on the *C.acutatum* infection of strawberry stolons and petiols (Curry et all, 2002), the early intercellular penetration of *C. acutatum* hyphae on red strawberry fruits suggests that this fungus does not establish the intimate cytoplasmic interaction typical of hemibiotrophs, and indicates the absence (or a very brief presence) of a biotrophic phase in this type of interaction. The subsequent necrotrotrophic colonization of red fruits by *C. acutatum* is typical of a subcuticular intramural infection strategy, previously reported for stolon and petiols (Curry et al., 2002).

The brief, or absent, biotrophic phase shown in our results for *C. acutatum* infections of fruits, extends the uncertainty about calling these fungi hemibiotrophs,.

At the same time, the environmental conditions found by the fungus on unripe strawberry fruits, are limiting for the fungal growth and colonization, as demonstrated both by the lack of ammonia increase and the fungal growth arrest in these fruits. Our results show that the element preferred by *C.acutaum* on white strawberries for quiescence is the melanized appressorium. Furthermore, our data indicate a correspondence between the fungal penetration in R fruits and the increase in ammonia ions concentration at 24 HPI, supporting the hypothesis that ammonia ions play a crucial role for pathogen growth.

Several phenylpropanoids compounds are involved in plant defence, either as preformed compounds or as inducible products after pathogen attack, both in local and systemic resistance (Treutter, 2006). It is know that the activity of the phenylpropanoid enzymes is regulated at the transcriptional level (Davies et al.

2003). The promoter sequences of the key genes of this pathway have been extensively studied, revealing regions regulated during development and after biotic and abiotic environmental stimuli (Dixon et al. 2002). Therefore, difference in gene expression of these genes during fruit development could associate with a difference in the enzyme activity and, as a consequence, with a change in phenylpropanoid endogenous level.

During ripening, strawberry fruit requires the production of several antioxidant compounds necessary for achenes lignification process (Aharoni et al., 2002); according to this, in red fruits the expression increase of *pal* and *c4h* genes, regulating the metabolic branches leading to production of chlorogenic acid and lignin, could be correlated to the lignification process of achenes during fruits ripening. On the other hand, increase in the transcript levels of the flavoinoids genes, as *chs*, *fht*, *ans*, and *fgt* detected in R fruits with respect to the W ones, is probably correlated to the anthcyanins accumulation during fruits ripening. In R fruits, the decrease in the transcript level encoding FaMYB, a transcription factor involved in negative regulation of flavoind genes expression (Fornale et al. 2006), supports the hypothesis of an important role for these compounds on the ripening process.

With respect to quiescence, is noteworthy the upregulation in W fruits of the genes encoding for LAR and ANR. Indeed, LAR and ANR are downstream enzymes in the flavonoid pathway, specifically involved in the synthesis of conjugated tannins from catechin and epicatechin molecules. The physiological reason underneath the production of such compounds is still unknown, however, it is known that tannins have an established role in defence against different type of parasite (Dixon et al 2002). In particular, the role of flavan-3-ol-epicatechin was largely discussed by Prusky (Prusky et al. 1982, 1988). He reported that among the natural inhibitors of fungal growth, the unripe avocado fruit contains antifungal diene compounds. In ripe fruits, these are degraded by the lipoxygenase (LOX) enzymes, which, is negatively regulated by the flavan-3-ol-epicatechin compounds. Therefore, the high level of flavan-3-ol-epicatechin in unripe fruits ensures a high level of antifungal diene compounds and inhibition of fungal growth.

Accordingly, up-regulation of *lar* and *anr* genes found in white strawberries, could lead to an increase of epicatechin levels in unripe fruits with respect to the red ones, suggesting that a similar mechanism of epicatechin regulation can induce *C. acutatum* quiescence. Further biochemical measurements of the actual concentration of these compounds in W and R fruits are needed to support this hypothesis.

Remarkably, *chs* and *ans*, are the only genes found significantly regulated upon infection exclusively in WI strawberry fruits. Downregulation of these flavonoid genes is supported also by the microarray analysis results. It could be interesting to explore whether such a downregulation is associated to a shut down of the main flavonoid pathway, in favour of activation of secondary branching pathways, possibly leading to antifungal compound synthesis.

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