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Apple latent infection caused by *Neofabraea alba*: host-pathogen interaction and disease management

Presentata da: Dott.ssa Irene Cameldi

Coordinatore Dottorato **Relatore**

Prof. Giovanni Dinelli **Prof.ssa Marta Mari**

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Table of contents

Doctoral Dissertation Abstract

Apple latent infection caused by *Neofabraea alba*: host-pathogen interaction and disease management

Bull's eye rot (BER) caused by *Neofabraea alba* is one of the most frequent and damaging latent infection occurring in stored pome fruits worldwide. Fruit infection occurs in the orchard, but disease symptoms appear only 3 months after harvest, during refrigerated storage. In Italy BER is particularly serious for late harvest apple cultivar as 'Pink Lady™'. The purposes of this thesis were: i) Evaluate the influence of 'Pink Lady™' apple primary metabolites in *N. alba* quiescence ii) Evaluate the influence of pH in five different apple cultivars on BER susceptibility iii) To find out not chemical method to control *N. alba* infection iv) Identify some fungal volatile compounds in order to use them as *N. alba* infections markers. Results regarding the role of primary metabolites showed that chlorogenic, quinic and malic acid inhibit *N. alba* development. The study based on the evaluation of cultivar susceptibility, showed that Granny Smith was the most resistant apple cultivar among the varieties analyzed. Moreover, Granny Smith showed the lowest pH value from harvest until the end of storage, supporting the thesis that ambient pH could be involved in the interaction between *N. alba* and apple. In order to find out new technologies able to improve lenticel rot management, the application of a non-destructive device for the determination of chlorophyll content was applied. Results showed that fruit with higher chlorophyll content are less susceptible to BER, and molecular analyses comforted this result. Fruits with higher chlorophyll content showed up-regulation of PGIP and HCT, genes involved in plant defence. Through the application of PTR-MS and SPME GC-MS, 25 volatile organic compounds emitted by *N. alba* were identified. Among them, 16 molecules were identified as potential biomarkers.

Apple latent infection caused by *Neofabraea alba*: host-pathogen interaction and disease management

Introduction

Among the wide kinds of plant diseases caused by fungi, latent infections are a very specific category of plant syndromes causing important losses in agriculture, especially during post-harvest phase. According to Verhoeff (1974), latency is defined as *"A quiescent or dormant parasitic relationship where, pathogens spends long periods of time during the host's life in a quiescent stage, under specific circumstances, can change an active one.*" The term quiescent is designed as the period from host infection and the activation of fungal development and symptoms expression (Prusky et al., 1996). In order to gain the success, a pathogen must overcame plant defense, and to obtain nutrients required to sustain its development. Extensive studies have been carried out to determine the biochemical bases of activation of pathogen quiescence, and three hypotheses have been suggested by Prusky (1996): (i) deficiency of host nutritional resources necessary for pathogen development; (ii) presence of preformed or induced fungistatic compounds; and (iii) unsuitable environment for the activation of fungal pathogenicity factors. In support of the second hypothesis, varieties of fungistatic molecules have been shown to act as major barriers against *Colletotrichum* spp. in unripe avocado fruits (Prusky et al., 1982). In avocado also phenylpropanoid and flavonoid metabolites, such as epicatechins, have been shown to be crucial in the induction of fungal quiescence in unripe fruits (Ardi et al., 1998). In fruits and vegetables the mechanism of resistance is generally not dictated only by a single gene interaction between host and pathogen, but the interactions maintain a type of dynamic incompatibility (Prusky, 1996). This situation is triggered by the

response of the host resistance genes to the pathogens avirulent genes that prevents or retards pathogen growth, under specific host physiological conditions. During maturation and ripening, host physiological changes make the environment more suitable for pathogen colonization. This phenomenon makes the host-pathogen interaction compatible and the symptoms appear. The biological model reported above is the most common condition in postharvest pathogen-host interactions, in fact pathogens such as *Botrytis cinerea*, *Colletotrichum spp*. and *N. alba*, remaining quiescent during fruit growth and becoming virulent during senescence of host.

Neofabraea spp. and latent infections of apple fruits

Bull's eye rot (BER) of stored apples caused by *Neofabraea spp.* (E.J. Gutrie) is one of the main disease occurring in Europe and North and South America (Maxin et al., 2014, Spolti et al., 2010, Spotts et al. 1999). Fruit are infected in the orchard, but the symptoms appear on the fruits only 3-4 months after harvest, during cold storage. Fruit lesions are flat, brown and often with a lighter brown center (Snowdon, 1990), frequently centered on the lenticels, however they also occurred on wounds and around the stem or calyx. Under humid conditions, on the old lesions are present cream-colored acervuli. Rotted tissue was firm and was not readily separable from healthy tissue (Spotts et al., 2009). BER also known as lenticel rot is caused by four members of *Neofabraea* genus: *N. alba*, *N. perennans*, *N. malicorticis*, and *Cryptosporiopsis kienholzii*. The last species was found for the first time in 2001 by Jong et al. on apple in Canada and Portugal and it was subsequently described by Spotts et al. (2009). Jong's work provided a useful study on the phylogenetic relationship among *Neofabraea* species, making possible a species specific identification of the *Neofabraea* pathogen complex through a multiplex DNA amplification (Gariépy at al. 2003). The diagnostic technique developed by Gariépy et al.

(2003) incorporates five sets of specie-specific primers into a single PCR reaction, and produces distinct bands for each pathogen, making possible an accurate identification of all members of the genus *Neofabraea* to species. In Germany and UK, the presence of *N. perennans* is confirmed by Jong et al., (2001), while *N. alba* is reported as the major agent of BER in Italy and in France (Bompeix and Cholodowski-Faivre, 1998; Neri et al., 2009). The presence of *N. alba*, *N. malicorticis* and *N. perennans* was also confirmed in Pacific Northwest of U.S.A. (Gariepy et al., 2005), while in Australia were found *N. alba* and *C. kienholzii* (Cunnington, 2004). In Brasil, lenticels rot caused by *N. perennans* is one of the most important apple's postharvest disease (Spolti et al., 2010), while *N. alba* was found in apples cultivar Braeburn, Fuji, Granny Smith, Pink Lady, and Royal Gala.in Chile (Henriquez, 2005).

Biology of Pathogen

Neofabraea sp. causes canker on apple and pear trees (Jong et al. 2001, Garipey et al 2003, Gariepy et al. 2005, Henriquez et al., 2006), however it is not fully elucidated the relationship between *Neofabraea sp.* and appearance of branch cankers. In order to clarify this association Henriquez et al. (2006) induced cankers in tree branches of pear and apple by artificial inoculation of *N.alba* and *N. perennans*. All inoculation showed branch cankers, but some differences were recorded between pear and apple trees. In pear trees, cankers resulted most evident when inoculations were carried out during the autumn and winter months, while in Granny Smith trees, the inoculations successfully induced cankers in summer months. Comparing the two species analyzed *N. alba* produces smaller cankers than *N. perennans*. Cankers development began as a blackening of tissue under the inoculation point. Necrosis extended farther in length than in width. Peeling of the bark occurred at the margin of the necrotic area. Branch growth delimited the canker,

which acquired a sunken shape with cracked margins, separating from the healthy tissue. Cankers induced by both species produced conidia through most of the year, with the highest amounts at the end of summer and during autumn. These conidia are splash-dispersed to fruit. Henriquez et al. (2006) also observed natural cankers caused by *N. alba* on D'Anjou pear trees. In addition, the same study revealed as the copper sulfate treatments influenced the conidial production approximately for 1 month after treatment. In general, conidia of pathogens landed on fruit surface, germinate, produce penetration structures and penetrate into the fruit activating pathogenicity factors (Prusky, 1996), but this mechanism remains not understood for *Neofabraea* spp. fruit infections. The most important challenge of lenticel rot is to understand the mechanism that regulates the end of quiescent stage of pathogen. *Neofabraea spp.* infects fruits in the orchard, but it does not produce symptoms until a certain stage of fruit ripening, that usually happens 3-4 months after harvest.

In general, once that spores arrive on the fruit surface, the appressoria formation depends on the substances present on the cuticle. Prusky and Plumbley (1992) showed that avocado fruit wax induced the appressoria formation of *C. gloeosporioides*. According to Kolattukudy et al. (1995), plant surface lipids contain both inducers and inhibitors of spore germination and appressoria formation, and the balance between them might be responsible for the selective activation of the pathogen for initiation of parasitisation. Moreover, fruits and vegetables are provided of numerous biochemical compounds able to delay the fungal infection even if spores reach the internal part of the host. The group of substances preformed or inducible that constitutes the host biochemical barriers is very wide, and it changes depending on vegetable species, cultivar and stage of ripening etc. The preformed compounds are known as phytoanticipins while the inducible compounds

are known as phytoalexins and they are synthesized from remote precursors in response to pathogen attack. The distinction between phytoalexins and phitoanticipins is not always clear as some compounds may be phytoalexins in one species and phytoanticipins in another species. The distinction between the two compounds depend on when they are produced, either before or after infection. The preformed compounds tend to be concentrated in the outer layer of plant organs and in general, they are compartmentalized in vacuoles or organelles in healthy plant. Their quantity decreases from unripe to ripe fruit. Biotic or abiotic elicitors could induce the production of phytoalexins that are concentrated around the point of pathogen penetration or abiotic stress. The rate of phytoalexins accumulation determine the outcome of host-pathogen interaction. Susceptible host accumulate phytoalexins slower than resistant host (Kuc, 1995).

N. alba is the main BER pathogen in Italy, and very few are data about the mechanism that regulate its quiescence and the questions reported by Prusky (1996) remain unacknowledged for this pathogen.

i) What is the nature of preformed barriers to pathogen attack?

ii) How do R genes trigger defense responses and what are the physiological conditions needed for triggering defense responses?

iii) What are the roles of the various induced compounds in resistance

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Aims of the work

This work addresses latent infection caused by *Neofabraea alba* in apple trough a holistic approach. BER is very important disease for pome production, especially for the economic losses due to the product waste in post-harvest, however the information about its causal agent are very lacking. In order to find out the main factors affecting the *N. alba* quiescence, and possible alternative method of disease management, different aims were pursued:

- Evaluate the influence of "Pink Lady™" apple primary metabolites in *N. alba* quiescence
- Evaluate the influence of pH in five different apple cultivars on BER susceptibility
- To find out not chemical tool able to limit BER damages
- Identify some fungal volatile compounds in order to use them as *N. alba* infections markers. This study is still not completed, and results reported in the thesis refer to preliminary studies

Each activity will be illustrated separately, analyzing each aspect in a specific chapter.

Chapter one:

Pathogenesis of *Neofabraea alba* in 'Pink Lady™' apple and influence of the primary metabolites of fruit on disease development

1 Introduction

Bull's eye rot (BER) caused by *Neofabraea* spp. is one of the most important diseases of pome fruits, producing notable economic losses during fruit storage. The disease is particularly serious in Europe (Bompeix and Cholodowski-Faivre, 1998; Neri et al., 2009, Kellerhals et al. 2012), North-West of USA (Henriquez et al., 2004, Gariepy et al., 2005), Chile (Soto-Alvear S., 2013) and Brasil (Spolti et al., 2010), while in China BER is classified as quarantine disease (Cao et al. 2013). Fruit lesions are flat to slightly sunken, brown and often with a light brown center (Snowdon, 1990). The rotten tissues are firm, with white acervuli on old lesions. In apple (Spotts et al., 1999) and pear (Henriquez et al. 2004) trees the pathogen can induce canker formation in the branches relating to climate conditions. The fruit, contaminated in the orchard, show the symptoms only three/four months after harvest, during cold storage (Snowdon 1990). A review on pome European fruit genetic resources evaluated the resistance against some pathogens (Kellerhals et al., 2012) including *Neofabrea* spp. among the main causes of decay during apple storage and commercialization. Four members of the genus *Neofabraea* are the causal agents of BER of pome fruits (Spotts et al., 2009): *N. alba* (E. J. Gutrie) Verkley (anamorph *Phlyctema vagabunda* Desm.), *N. perennans* Kienholz [anamorph *Cryptosporiopsis perennans* (Zeller & Childs) Wollenw.], *N. malicorticis* H. S. Jacks [anamorph *Cryptosporiopsis curvispora* (Peck) Gremmen]. Recently, *Cryptosporiopsis*

kienholzii (Seifert, Spott & Lévesque) was described as being associated with BER in the Pacific Northwest of the United States (Spotts et al. 2009). In Italy, only the presence of *N. alba* (anamorph *Phlyctema vagabunda*) was reported, however there are no molecular data on the Italian strains of *N. alba*. In addition, no branch cankers were detected in Italian apple orchards and only BER symptoms on stored fruit were observed.

Despite BER is globally recognized as one of the major fungal disease of stored apple, there are very few information on its biology and host-pathogen interactions. These serious lacks are probably due also to the difficulties of pathogen culturing and conidia obtaining on artificial medium. Methods reported in literature require a very long time of incubation for conidia production on artificial medium and are not reliable. The methodology used by Neri et al. (2009) permits to produce conidia on natural substrate based on apple leaves, but does not allow perpetrating the same isolate during the time because the pathogen loses the vitality once transferred onto Malt Extract Agar (MEA) or Potato Dextrose Agar (PDA). According to Rooney-Latham et al. (2013) the sporulation of *N. alba* isolates derived from olives was observed *in vitro* on PDA after 40 days of incubation under near-UV light. Moreover, no molecular data are available for *Neofabreea* spp. species present in Italy.

The pathogen infects fruit before harvest and conidia of *N. alba* germinate on young unripe fruit, sunken into lenticels where remains quiescent for long time (Verhoeff, 1974), until fruit ripening. Among resistance mechanisms of fruit, preformed antifungal compounds present in unripe fruit are considered responsible for fruit disease resistance and their degradation during ripening process could activate the latent infections, making fruit prone to disease appearance (Swinburne, 1983). Among these preformed compounds, in the past, phenols were widely considered liable to the unripe fruit BER

resistance; in fact, when their content decreases fruit become quite susceptible to fungal attack. However, a study conducted on the influence of low temperature of fruit storage on *N. alba* infections, showed that a significant decrease in phenolic content in the skin of the fruit is not correlated with an increase of *N. alba* susceptibility (Lattanzio et al., 2001). Others mechanisms and compounds are involved in apple resistance to BER that have to be still investigated. In its review on pathogen quiescence in postharvest diseases, Prusky (1996) included an increment of nutritional factors during the ripeness process, among the factor affecting the pathogen quiescence interruption. Previous data available on the primary metabolites in apple fruit focused on a defined lapse of time. Zhang Y. et al. (2013) studied the evolution of primary metabolites in pre-harvest phase, when the fruits were still growing *in planta*, but did not analyze apple metabolism during apple storage. On the contrary, Hatoum et al. (2014), studied the primary metabolism of 'Breaburn' apple from two, until thirty-two weeks after harvest, but did not consider the pre-harvest phase. During the last decade, metabolomics has become a highly valued and widely exploited technology to explore the plant composition (Hall 2011). Among the available technology, Nuclear Magnetic Resonance (NMR) provides a powerful technique for the identification and quantification of metabolites in plant extracts. ^{1}H NMR is a robust technique, highly reproducible, non-selective able to produce structural information and quantitative data (Rolin et al 2013). This technique is the more appropriate for primary metabolites analysis (Hounsome et al., 2008), allowing accurate simultaneous quantification of many compounds in a complex mixture without need for separation. Quantitative ^{1}H NMR have been used to determine formic acid in apple juices (Berregi et al 2007) or epicatechin in apple cider (Berregi et al 2003). Recently ^{1}H high-

resolution magic angle spinning (HR-MAS) NMR have been used to explore the metabolome of three different apple cultivars (Vermathen et al 2012).

The aims of this study were to: i) develop a rapid and reliable medium to obtain *in vitro N. alba* conidia; ii) identify molecularly *Neofabraea* spp. present in Italy iii) characterize the primary metabolites in the skin of 'Pink Lady™' apple by proton-NMR analysis, during fruit growth and ripening; iv) evaluate the influence of the main primary metabolites on *N. alba* development and v) evaluate the *N. alba* pathogenesis in relationship with the changes in the primary metabolites

2. Materials and method

2.1 Fruits

'Pink Lady™' apples were obtained from an experimental orchard of University of Bologna located in Cadriano (BO), Italy. Apple trees were grafted on M9 rootstock and grown according to the integrated production guidelines. Harvest was carried out at commercial maturity which was determined using the starch iodine test scored using a 1-10 points CTIFL scale (Centre technique interprofessionnel des fruits et legumes, association Pink Lady Europe) and firmness. At harvest the starch index was 7 and the value of firmness was 63.7 $N \cdot cm^{-2}$.

2.2 Isolate collection

The isolates of *Neofabraea* spp. were obtained from apples stored in packinghouses located in Emilia Romagna region (Italy) showing the typical BER symptoms. Fruits were surface-sterilized by ethanol (70% v/v) and small pieces of rotted tissue from the edges of lesion were placed onto MEA dishes. Subsequently plates were incubated at 20°C for

15 days. The developed colonies were evaluated for a preliminary identification by colony morphology and microscopic observations of mycelium. In total, forty-five isolates were obtained. After 15 days, the colonies of *Neofabraea* spp. morphologically identified, were transferred on fresh MEA plates, and kept at 4 °C until use.

2.3 Molecular identification: DNA extraction and PCR amplification

For pathogen identification, 5 isolates of *Neofabrea* spp. were randomly selected from the collection obtained as described above. The isolates were grown on MEA at 20° C for 15 days and identified by sequencing of internal transcribed spacer (ITS) regions of ribosomal DNA. For this purpose the DNA extraction was carried out using the CTAB method described by Doyle & Doyle (1987) with slight modifications. Fungal biomass was recovered from dishes, introduced in a 2 ml-Eppendorf tube and grinded manually with blue propylene pestles. After, 1 ml of CTAB-0.04% β-mercaptoethanol solution, previously heated at 65°C for 1 h, was added and the suspension was vortexed for 30 s and 2.5 µl of proteinase-K (10 mg ml-1) were added. The suspension was then incubated at 65°C for 1 h and subsequently, 1 ml of a mixture of chloroform-octanol (24:1) was supplemented to the solution and centrifuged for 5 min at 8000 rpm. After the addition of 5 µl of RNAase (10 mg ml-1) to the supernatant, 1 ml of chloroform-octanol was added again and the solution was centrifuged as described above. DNA precipitation was achieved by adding 0.8 volumes of isopropanol to the supernatant and centrifuging for 20 min at 14000 rpm. Subsequently, the pellet was dried under vacuum, washed with 500 µl of 70% ethanol (conserved at -20°C) and centrifuged for 5 min at 12000 rpm. Finally, the pellet was air dried for 5 min and re-suspended in 50 µl of sterile double distilled water. DNA concentration and A260/A280 ratio were assessed using the Infinite 200

NanoQuant spectrophotometer (Tecan® Group Ltd., Grödig, Austria). Molecular identification of the isolates was carried out by the PCR amplification of the Internal Transcribed Spacer (ITS) regions of ribosomal DNA using the universal primers PN23 (5'-CACCGCCCGTCGCTACTACCG-3') and PN34 (5' TTGCCGCTTCACTCGCCGTT-3') described by Mouyna and Brygoo (1993), supplied by Invitrogen® (Carlsbad, California, USA) and carried out in 25 µl using 2.5 µl of TaKaRa® 10×buffer, 2 µl of 10 mM dNTPs, 1 µl of 10 mM solution of each primer, 0.2 µl of TaKaRa® Taq polymerase (5 U/ml) (Takara® Bio Inc., Otsu, Japan), 17.3 µl of double distilled water and 1 µl of genomic DNA (20-100 ng µl-1). PCR program consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 1 min with a final extension step of 5 min at 72°C. To verify amplification reactions, 7 µl of each PCR product were separated on 1.5% agarose gel in 0.5×TAE buffer, stained with GelRedTM (Biotium, Inc., California, USA) and visualized under UV light. Purification of amplification products was obtained using the Gel/PCR Extraction & Purification kit (Fisher Molecular Biology®, Pennsylvania, USA). In order to verify the DNA quality and concentration of the purified products, 5 µl were separated in a 1.5% agarose gel and compared with the MassRuler Low Range DNA ladder (Fermentas®, Vilnius, Lithuania). Purified PCR products were sequenced with both forward and reverse primers at Eurofins MWG Operon (Ebersberg, Germany). Sequences were then clipped and assembled using ContigExpress® Module for Vector NTI Advanced® software (Life Technologies®, California, USA). For each sequence, specie identification was obtained by performing a blast algorithm at the NCBI web site.

2.4 Temperature method to produce conidia on artificial substrate

Previously studies conducted inoculating *N. alba* in different artificial substrate (data not reported), showed that tomato agar (TA) (15 g of agar technical, 500 ml of distilled water and 500 g of tomato sauce) is the best growth media for *N. alba* development. In order to find out a reliable method to produce conidia, the previously identified *N. alba* ID02 strain was inoculated on TA and stored under controlled isothermal temperatures for 14 days. The temperatures were 4, 10, 15, 20 and 25°C, selected after a preliminary study. Mycelial growth was determined as the increase in colony diameter after 14 d of incubation at different temperature. Production of conidia was evaluated by adding two mycelium plugs of 6 mm of diameter derived from a tomato agar plate incubated at 15°C for two weeks in 5 ml of distilled water agitated for 3 min by vortex. A sample (20 µl) of washing water was observed at light microscope (400 ×) and the conidial production was estimated counting the conidia with the hemocytometer. The sample unit was represented by 5 plates for each temperature. Once verified the best temperature for ID02 sporulation, five strain of *N. alba* earlier identified were evaluated for their sporulation attitude by placing TA dishes at 15°C for two weeks, ascertaining the conidia production as described above. The trial was performed in triplicate.

2.5 Study of primary metabolism of 'Pink lady™' apple by ^{1}H-NMR

Apple skin of symptoms less fruits was removed using a peeler and immediately frozen in liquid nitrogen before to be freeze dried. Apple peels were sampled from 20 weeks before harvest (unripe fruits) to 21 weeks after harvest, each 2 or 3 weeks. At each date of sampling three biological replicates were sampled and the peel of four apple fruit represented each replicate. Before ^{1}H-NMR analysis polar and non-polar primary

metabolites were extracted according to the method described by Moing et al., (2004) with slight modifications. The frozen powdered samples were lyophilised and polar metabolites were extracted from 50 mg of lyophilised powder successively with 2 ml of ethanol/water mixtures: 80/20, 50/50 (v/v) and pure water (3 ml) for 15 min at 80°C. The supernatants were combined, dried under vacuum and lyophilised. The lyophilized extracts were mixed with 500 µl of 400 mM potassium phosphate buffer pH 6.0, 1mM Ethylene diamine tetraacetic acid disodium salt (EDTA), in D_2O, titrated with KOD solution to pH 6.00 ± 0.02 when necessary, and lyophilized again. The lyophilized titrated extracts were stored in darkness under vacuum at room temperature, and ^{1}H-NMR analysis was completed within one week. For ^{1}H-NMR analysis, dried titrated extracts were solubilised in 0.5 ml D_2O, added with sodium salt of (trimethyl) propionic-2,2,3,3-d4 acid (TSP) in D_2O at a final concentration of 0.01% for chemical shift calibration and transferred into an 5 mm NMR tube. Quantitative ^{1}H-NMR spectra were recorded at 500.162 MHz and 300° K on a Bruker Avance III spectrometer (Wissembourg, France), using a 5 mm inverse probe and an electronic reference for quantification as described previously by Mounet et al. 2007.

2.7 Effect of organic acids and sugars on N. alba *conidia germination and mycelial growth*

In order to test the effect of apple primary metabolites on *N. alba* development, major fruit organic compounds were assayed on *N. alba* conidia germination and mycelial growth. Bioassays were performed using the highest concentration of organic acid and sugar contents found in apple skin by ^{1}H-NMR. Each organic acid diluted in sterile distilled water in order to obtain the desired concentration was sterilized using a filter of 45 µm and added to MEA after autoclave sterilization. For conidial germination test an

aliquot of 100 µl of conidia suspension (10^3 conidia ml^{-1}) was spread onto amended MEA as previously described. For mycelial growth evaluation a mycelial plug (6 mm Ø) taken from the periphery of actively growing pathogen culture was placed in the center of the dishes treated as reported above. The dishes were then immediately wrapped in Parafilm and incubated at 20°C for conidial germination and 15 °C for mycelial growth assays. Control was represented by unamended MEA inoculated with conidia or mycelium of pathogen as described before. The colony forming units (CFUs) and the mycelial growth were recorded after 4 and 15 days of incubation, respectively. For each compound tested five dishes were used and the assays were performed in triplicate. The percentages of the stimulation or the inhibition of conidia germination and mycelial growth caused by organic acid were calculated using the following formula: [(treatment–control)/control)] × 100. Positive values indicated stimulation and negative values indicated inhibition. For the evaluation of sugar effect, each carbohydrate was added in mineral medium agar ($NH_4H_2P0_4$ 2.0 g/l, KH_2PO_4 0.6 g/l, $MgSO_47H_2O$ 0.5 g/l, K_2HP0_4 0.4 g/l, $CaCI_2H_2O$ 0.074 g/l, Ferricitrate 0.012 g/l, $ZnSO7H_2O$ 0.0066 g/l, $MnS0_44H20$ 0.005 g/l, $COCl_26H_2O$ 0.001 g/l, Thiamine 0.0001 g/l, Technical agar 5.0 g/l) and the experiments were carried out as described above. The EC_{50} and EC_{95} values of organic acids (malic, quinic and chlorogenic) were calculated as the concentrations that inhibited mycelium growth by 50% and 95%, respectively, compared with the control. EC50 and EC95 values were calculated using the probit analysis applied to the percentage of mycelial growth inhibition (Lesaffre and Molenberghs, 1991).

2.8 In-vivo evaluation of BER pathogenesis

In order to evaluate the BER pathogenesis, apple fruits were harvested and stored at 0°C and high humidity (> 92%) in commercial condition for five months followed by 10 days of shelf-life. Every month apples were evaluated for BER incidence. The sample unit was represented by ten replicates of seventy fruits each (total 700 fruits). The experiment was conducted twice.

2.6 Statistical analysis

Data referred to biological effect of temperature and primary metabolites were subjected to one-way analysis of variance (ANOVA) using the statistical package Statistica for Windows (Statsoft Inc.). Separation of means was performed using the least significance difference (LSD) test at $P< 0.05$. Principal component analysis (PCA) was performed with absolute concentrations of 20 metabolites (centered reduced data) issued from ^{1}H-NMR analysis of apple peel harvested during apple growth and ripening, using SimcaP+ software (Umetrics, Umea, Sweden). All experiments were carried out in a completely randomized block design.

Results

3.1 Evaluation of conidia production and mycelial growth on TA substrate

The temperature of incubation influence the pathogen growth, after two weeks from inoculation, important changes in the morphology of the colonies were observed (Fig.1). At 4° and 10°C the mycelium appeared white, soft and woolly with abundant aerial hyphae. At 15°C (Fig.1 A) the mycelium remained soft, woolly, whit aerial hyphae, but the color changed into white/grey. At this temperature was also possible to observe orange acervuli in the middle of the colony. The temperature of 15°C was the optimum for

mycelial growth (Tab.2) and conidia production. The highest conidia production was found in colonies grown at 15° C (1.8 10^5 conidia mL^{-1}), while at 20°C and 10°C the conidia production was lower (1.3 10^3 and 1.2 10^2 conidia mL^{-1} respectively). At the other tested temperatures, no conidia production was observed. These data obtained with a the strain ID02 were confirmed by the other five strains previously identified as *N. alba*, and cultivated on TA at 15°C for two weeks. All isolates tested were able to produce conidia on TA at 15° C, conidia production is reported in Tab.2. The most productive strains were ID02, IC10 and ID04, that produced $1.5 \cdot 10^5$, $1.2 \cdot 10^5$ and $1.2 \cdot 10^5$ conidia mL^{-1} respectively. While the strains ID06 and CR4, appeared less productive with $1.7 \cdot 10^4$ and 3.6 10^4 conidia mL^{-1} respectively.

3.2 DNA extraction and PCR amplification

The universal primers PN23/PN34 successfully amplified a product of 800 bp. After sequencing and sequences assembly, a blast algorithm was performed and all isolates belonged to *N. alba*. Sequences A.N. for each isolate and % of identity are reported in Table 1.

3.3 Primary metabolism of 'Pink Lady™' apple

The primary metabolites of apple epicarp tissue were identified and quantified by ^{1}H-NMR spectroscopy (Figure 2, 3 and 4). The measurements were performed on apple skin sampled during apple life cycle. Sugars represent the major metabolite group in apple skin (Fig.2). Among them, sucrose and fructose were the two main sugars, while glucose

and xylose remain minor fractions. During the maturation process, fructose represents the major apple sugar from 20 weeks before harvest until 5 weeks before harvest.

Sucrose content showed a significant increase 2 weeks before harvest (130.3 mg g^{-1} DW), and a stable content until the end of the storage period (153.4 mg g^{-1} DW). On the contrary, a high content of glucose was observed from 20 (55.5 mg g^{-1} DW) to 8 (64.2 mg g^{-1} DW) weeks before harvest, followed by a decrease 1 month before harvest (40.8 mg g^{-1} DW) and a stabilization of its content until the end of the cold storage (49.3 mg g^{-1} DW). Xylose, a minor sugar (from 0.34 to 1.05 mg.g^{-1} DW) showed a gradual increase during the whole apple life cycle. Organic acids (Fig.3) represented the second major metabolite group. Among them, malic acid was the most abundant in apple fruit and its content varied from 34.8 to 12.1 mg.g^{-1} of DW (from 20 to 21 weeks after harvest). During post-harvest life, a general decrease of malic acid content was observed. The first significant reduction of malic acid happened immediately after harvest, decreasing from 24.3 to 16.9 mg.g^{-1} of DW in 2 weeks. The second decrease in malic acid content was recorded at 18 weeks after harvest, while the content remains stable until the end of the storage period (12 mg.g^{-1} DW). Quinic and chlorogenic acids showed a similar pattern. At 20 weeks before harvest, quinic acid reached the highest content at 20 weeks before harvest (9.33 mg.g^{-1} DW), and its quantity decreased gradually until 5 weeks before harvest, from this date the content remained stable (under 0.37 mg g^{-1} DW). Chlorogenic acid showed a decrease from 5.47 (20 weeks before harvest) to 1.92 (21 weeks after harvest) mg.g^{-1} DW, but no considerable changes were observed during post-harvest life. Succinic and citric acids showed some changes during the pre-harvest life. The highest content of succinic acid was observed at the harvest time (0.12 mg.g^{-1}DW), also citrate had the highest content (0.53 mg g^{-1} DW) at the beginning of the sampling time,

remaining stable during the post-harvest time (0.207 $mg.g^{-1}$ DW). Citramalic acid showed a very singular distribution pattern, in fact its content increased greatly at the harvest time passing from 0.34 to 1.62 $mg.g^{-1}$ of DW in only 2 weeks. After this increase, its content remained almost stable until the end of the storage. Amino acids were the third metabolites group. At the beginning of apple growth, asparagine showed the highest level among amino acids (1098.03 $\mu g\ g^{-1}$ DW), while from the harvest date its content was similar to aspartate.

3.*4 Principal component analysis of biochemical composition of apple during the growth and ripening*

PCA was used to integrate all identified metabolites with centered reduced data. PCA was performed with absolute concentration of 20 metabolites issued from ^{1}H-NMR analysis of sixteen dates of pericarp apple. Peel tissues were sampled from two months after full bloom, until the end of the storage period. The score plots (Fig. 5) showed that the first principal component (PC1), which explains 43.4% of the total variance, separated the developmental stages between growth before the harvest and ripening, whereas the second component (PC2), which explains 16% of the total variance, separated the samples inside each group. The group G showed a large separation on the first component corresponding to the sample harvested during the growth period. The scores plots indicated that during the apple growth, the larger amplitude of metabolic change over time. The T group corresponded to the apple sampled two and five weeks before harvest and the R group to apple sampled at the maturity stage and during cold storage period. The T group showed a similar biochemical composition while the R group showed a separation only on the second component. The corresponding loadings plot (Fig.5) amino

acids (aspartate and alanine) and organic acids (citric acid, succinic acid, quinic acid, malic acid and chlorogenic acid) was closely associated with growth phase while sugars as xylose and sucrose and choline were associated with the ripening stage. Fructose have no impact on the separation of these two groups.

3.5 Effect of organic acids and sugars on N. alba conidia germination and mycelial growth

Results on the influence of sugars and organic acids against *N. alba* growth showed that all sugars had no effect on conidia germination and the mycelial growth (data not reported). Otherwise, all organic acids at highest level, quantified in skin apple, affected *N. alba* development. Quinic (9.4 mg g^{-1} DW), chlorogenic (5,5 mg g^{-1} DW), and malic (34.7 mg g^{-1} DW) acids strongly inhibited both conidia germination and mycelial growth. In particular, malate showed an inhibition of 100 % of pathogen growth. Quinic and chlorogenic acids inhibited mycelial growth of 61.7 % and 50.0 % respectively, whereas the reduction of conidia germination was 73.3 % and 53.4 % for quinic and chlorogenic acids, respectively. Succinic and citric acids, tested at concentration 0.03 mg g^{-1} DW, stimulated the germination of conidia and mycelial growth of *N. alba*. of 44 % and 35 % for the mycelial growth and the conidial germination, respectively. In addition, the EC_{50} value of chlorogenic acid (4.6 mg g^{-1}) revealed as the acid was the most effective against mycelial growth of pathogen followed by quinic acid (6.3 mg g^{-1}) and malic acid (13.9 mg g^{-1}) (Table 3). The lowest EC_{95} value was attributed to quinic acid (14 mg g^{-1}), followed by malic acid (28 mg g^{-1}) and chlorogenic acid (29 mg g^{-1}).

3.6 Evaluation of BER pathogenesis on 'Pink Lady™' apple

In stored apple at 0°C the pathogen remained quiescent for eight week, since no BER symptoms appeared until eight weeks from harvest. After the *N. alba* incidence increased progressively reaching the 29 % at the end of storage (after 21weeks from harvest) (Fig 6)

Discussion

During last decades *N. alba* was globally recognized as one of the main pome fruit disease in the postharvest phase (Kellerhals et al., 2012; Soto-Alvear et al., 2013, Maxin et al., 2014). Our data showed that the incidence of BER in 'Pink Lady™' apple can reach high levels, after 21 weeks of storage it was 29%. BER is a serious problem for apple production however, very little is known about the mechanisms that regulates the host-microbe interaction between *N. alba* and apple fruit, and no reliable and rapid methods to produce conidia *in vitro*, on artificial medium are available, making the study of *N. alba* biology quite difficult. In this study a new based tomato substrate seemed very useful for the *in vitro* growth of pathogen and for an abundant spore production. Five *N. alba* isolates, identified by PCR and grown on this substrate, were able to produce abundant conidia with a concentration ranging from $1.5 \cdot 10^5$ conidia ml^{-1} mm^2 (IDO2) to $1.7 \cdot 10^4$ conidia ml^{-1} mm^2 (CR4). BER is a typical latent infection, characterized by a long quiescent phase that can overcome 90 days, before of the symptom manifestation. As well known, fruits and vegetables are provided of numerous biochemical compounds able to delay the fungal infection (Prusky, 1996). Among these, phenylpropanoids are usually designated as the main group of defence substances able to delay microbial infection (Naoumkina et al., 2010), nevertheless, our results showed also an important role played by the primary metabolites such as sugars, organic acids and amino acids that are found across all vegetal species within broad phylogenetic groups (Hounsome et al., 2008).

Carbohydrates represent the most important energy source for heterotrophic organisms such as fungi, for example, sugars and amino acids can contribute to zygospore formation of *Phycomyces blakesleeanus* (Leoninan et al., 1940). Organic acids are a group of organic compounds containing carboxylic groups that in solution release protons, that acidify the ambient (Hounsome et al., 2008). Plants contain citric, acetic, malic, oxalic, succinic, fumaric, quinic, tartaric, malonic, shikimic, aconitic, ascorbic, and other organic acids (Heldt 2005). From our data, organic acids can affect *N. alba* development. Chlorogenic and quinic acids inhibited conidial germination and mycelial growth, however their efficacy difficulty could be associated with postharvest latency of *N. alba*, since their concentration *in planta* significantly decreased during apple growth in the field, remained stable during postharvest phase. In addition, quinate inhibition effect was observed only at the concentration of 9.4 and 6.3 mg g^{-1}, a range concentration measured by HNMR at 20 and 17 weeks before harvest, when the fruit is completely unripe. Chlorogenic acid also inhibited *N. alba* development at concentration of 5.5 mg g^{-1}, a high concentration measured at the beginning of apple life cycle. Otherwise, the malic acid inhibited *N. alba* also at the concentrations founded in fruit at harvest and during storage. When tested at the maximum concentration of 34.4 mg g^{-1}, malic acid inhibited completely *N. alba* germination and mycelial growth. The EC_{50} value referred to malic acid (13.9 mg g^{-1}), is very close to malic content measured from 8 weeks after harvest (14.9 mg g-1) until 18 and 21 weeks after harvest (12.1 mg g-1). These results suggest that malic acid, at concentration measured during storage, does not inhibit completely the fungal infection, but can delay the host colonization by *N. alba*. In the interaction between *N. alba* and 'Pink Lady™' apple, chlorogenic and quinic acids play a defense role like feeding deterrent (Ikonen at al., 2001) in the first part of apple life cycle, but

probably their protection function is minor during postharvest phase. Not only malic acid influenced *N. alba* development, but all tested fruit organic acids affected significantly the pathogen growth. Whilst malic acid inhibited conidia germination and the mycelial growth, when tested at the detected concentration of 0.03 mg g^{-1}, succinic and citric acids significantly stimulated the pathogen development. Organic acids are essential in the metabolism of post-harvest produce; as reported by Kays & Paull (2004), some of them are important components of the respiratory tricarboxylic acid cycle and phosphoglyceric acid playing a critical role in photosynthesis. Their presence is fundamental to impart flavor, taste and odor to fruits and vegetables, but they can also represent a readily available source of stored energy (Kays & Paull, 2004). Biochemical results obtained in this work showed that during fruit postharvest, only the malic acid significantly decrease, and became stable 18 weeks after harvest, when the symptoms of BER rise considerably (Fig. 3 and Fig. 6)) . Higher plants are rich in pectic substances, high molecular mass glycosidic macromolecules. They are present in the primary cell wall as major component of the middle lamella and are the responsible for the structural integrity and cohesion of plant tissues (Pedrolli et. Al, 2009). In order to express their virulence, plant pathogens must to degrade pectic substances, and colonize the host tissue. This process requires a rich enzymatic pool displayed by fungal pathogens able to overcome this pectic barrier. Pectinase are an enzyme group that catalyzes pectic substances degradation through depolymerization and deesterification reactions. Pathogenic fungi work through depolymerization by hydrolases, and they are more active in a range of pH between 4 (Endo-PG I from *Aspergillus carbonarius*) to 5.3 (Endo PG II from *Fusarium moliniforme*) (Pedrolli et al., 2009). Recently, pH influence on the pathogenicity of fungus have been demonstrated for *Aspergillus nidulans* (Tilburn et al., 1995),

Colletotrichum gloesporoides (Alkan et al., 2013), *Botrytis cinerea* (Manteau et al., 2013) and *Penicillium digitatum* (Zhang et al., 2013), but for *N. alba* no data are provided. Our results showed that malic acid strongly affect *N. alba* conidial germination and mycelial growth, however a pH ambient reduction caused by the acid may be also considered. The outcomes described above suggest that the decreasing of malic acid can be involved in the interruption of *N. alba* latency. In fact, the optimal condition for *N. alba* growth could depend by the different organic acid balancing formed during storage that permits the recrudescence of *N. alba* pathogenicity.

In conclusion, our data can hypothesize that malic acid may be involved in the interaction between *N.alba* and apple fruit since BER symptoms appeared on apple only when malic content decreased, moreover results obtained on fruits are supported by *in vitro* trials. On artificial substrate *N. alba* is strongly inhibited by malic acid and EC_{95} value is very close to the concentration detected at the beginning of apple life cycle, while EC_{50} value (1.39 %) is comparable to the malic acid quantified eight weeks after harvest.

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Figures

Figure 1: Influence of temperature on *Neofabrea alba* mycelium growth at 15°C (A) and 25°C (B).

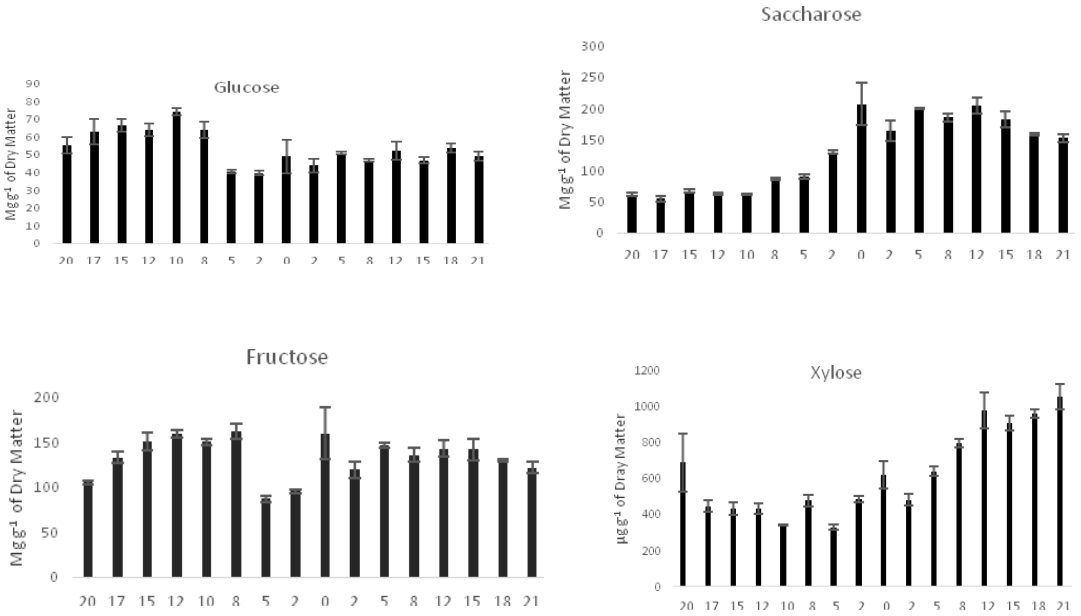

Figure 2: Sugars identified and quantified by HNMR from 20 weeks before harvest untill 21 weeks after harvest. Bars represent the standard error (SE).

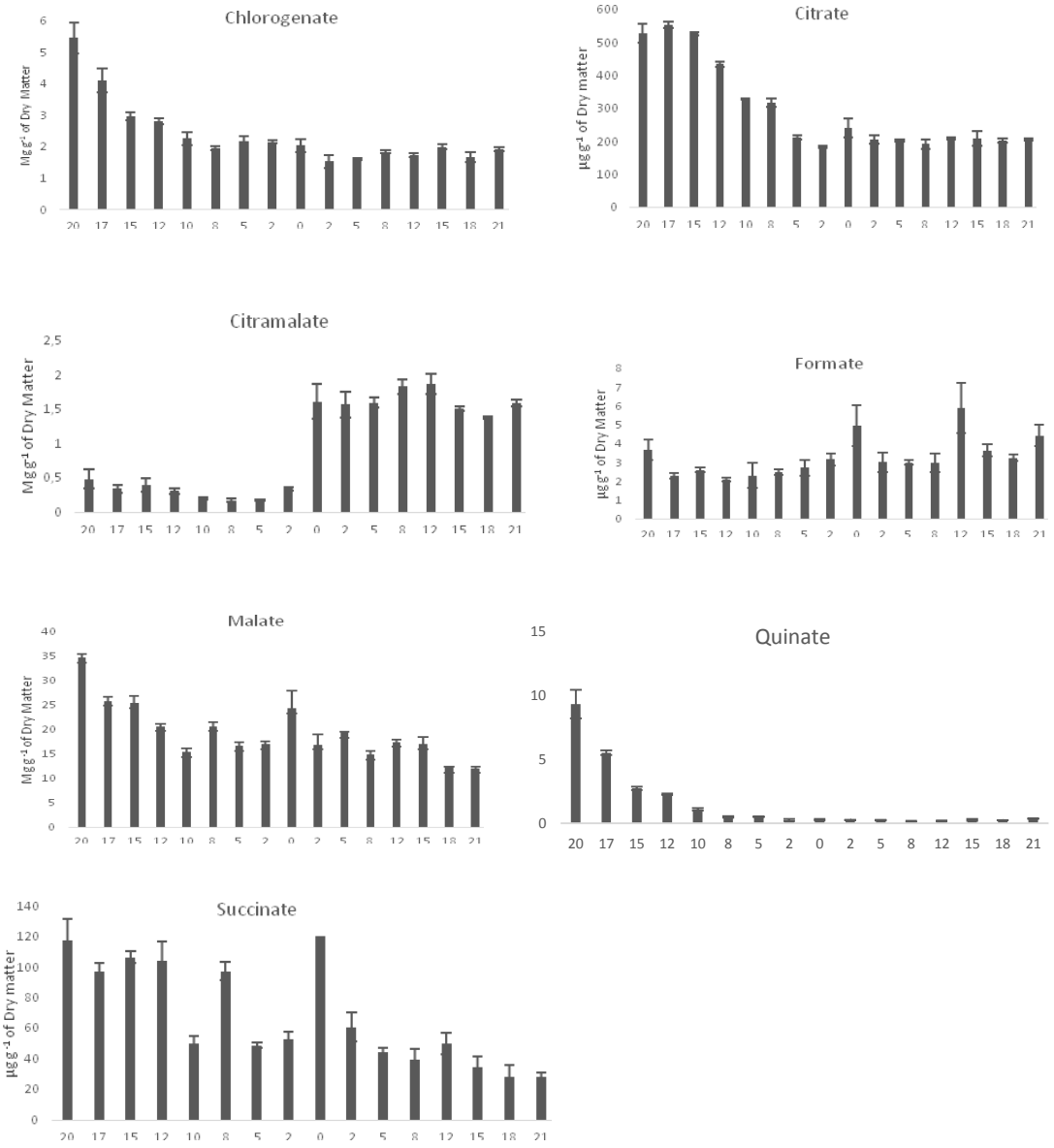

Figure 3: Organic acids identified and quantified from 20 weeks before harvest until 21 weeks after harvest. Bars represent the SE.

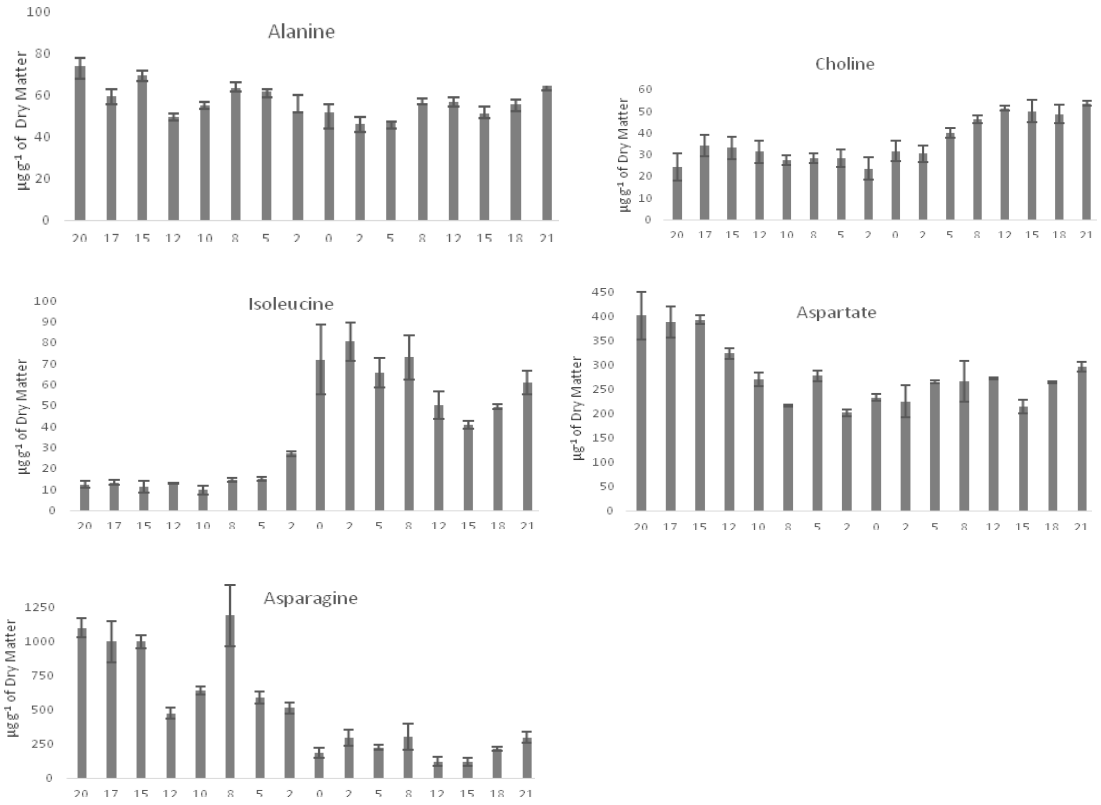

Figure 4: Amino acids identified and quantified from 20 weeks before harvest until 21 weeks after harvest. Bars represent SE.

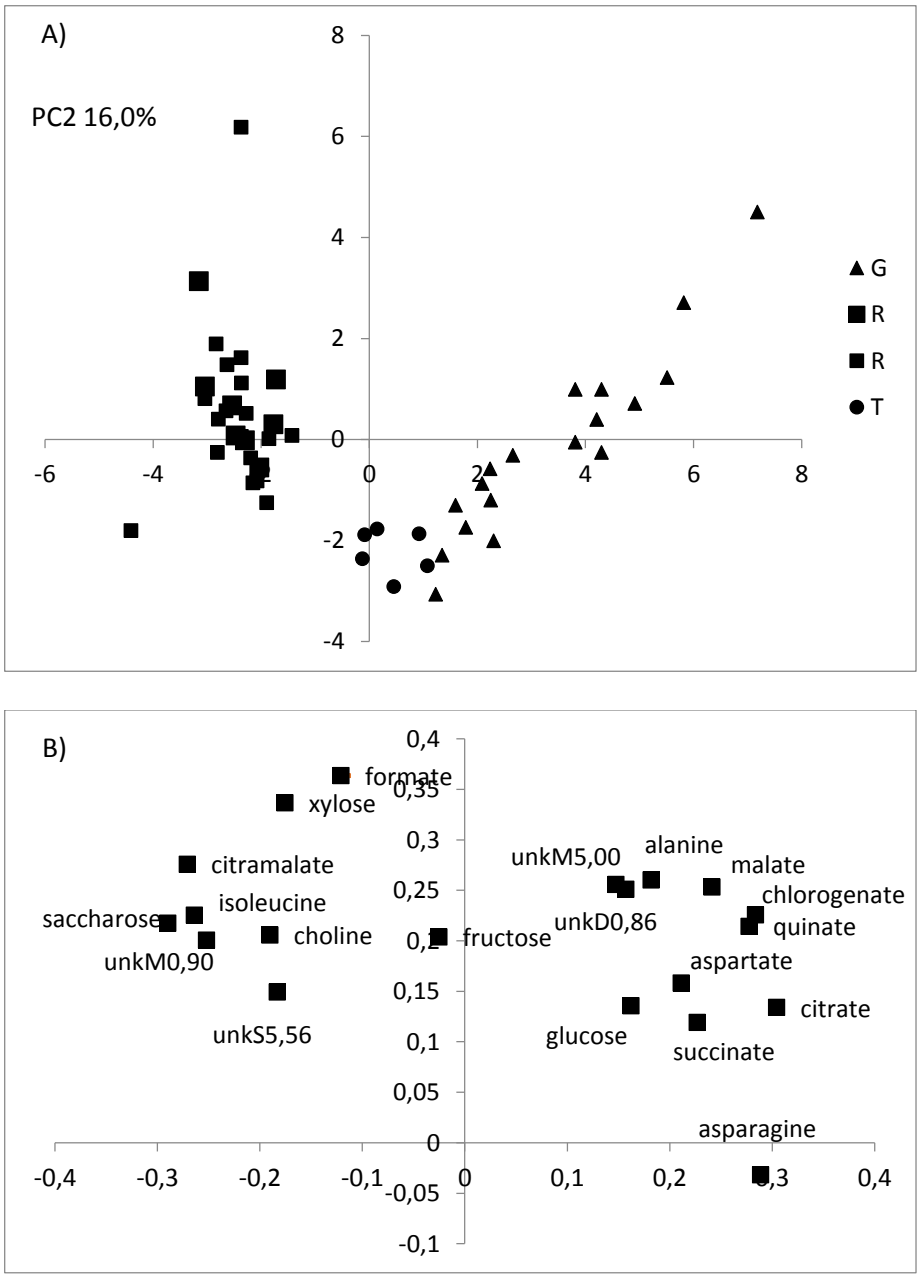

Figure 5: Principal component analysis (PCA) of 20 metabolites measured by quantitative 1H NMR spectroscopy in apple epicarp tissue at three stages of development (growth, turning and ripening). The PCA was performed with averaged data expressed on DW basis. (A) PCA scores plot of the first two principal components (PC1 and PC2) showing the distribution of the samples at three stages of development. (B) PCA loadings plot showing two sets of metabolites associated with the growth and ripening stage

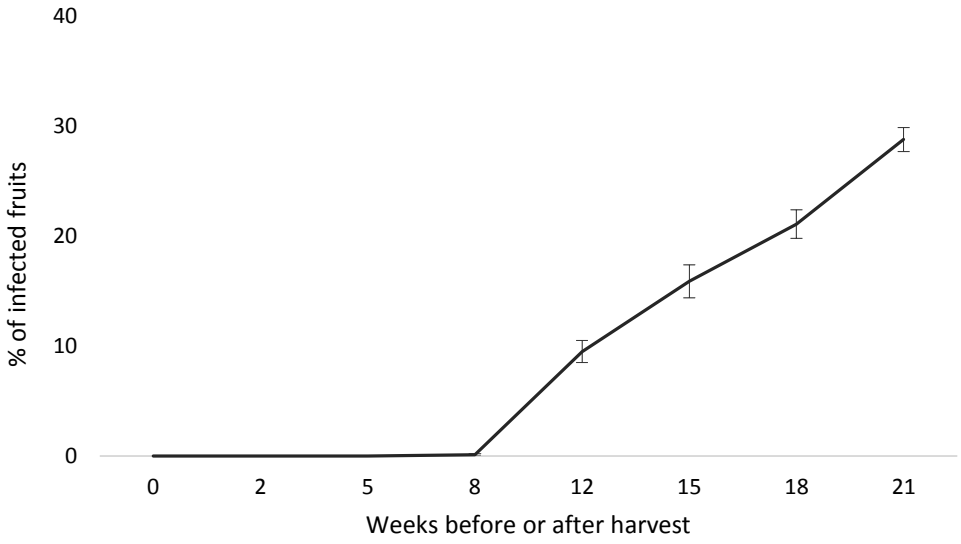

Figure 6: Evolution of Bull's eye rot in 'Pink Lady™' apple stored at 0°C for 21 weeks. Each datum is the average of 700 fruits ± SE

Tables

Table 1 Molecular identification and determination of conidial production

Strains	A.N. (GeneBank)	% identity	Conidia mm^{-2}
ID02	KJ396074	100	$1.5 \cdot 10^5$a
IC10	KJ396076	99	$1.2 \cdot 10^5$a
ID04	KJ396077	99	$1.2 \cdot 10^5$a
ID06	KJ396078	99	$1.7 \cdot 10^4$b
CR4	KJ396075	100	$3.6 \cdot 10^4$b

*Within the same column data followed by the same letters are not statistically different for LSD test ($P<0.05$).

** Data are the mean of 5 plates for each medium+ SE

Table 2 Influence of temperature on mycelial growth and conidia production of *Neofabea alba* ID02 isolate

Temperature °C	**Colony diameter (mm)**	**Conidia mm^{-2}**
4	12.75 a	0
10	23.75 b	$1.6\ 10^2$ a
15	33.5 c	$1.8\ 10^5$ b
20	27.5 b	$1{,}3 \cdot 10^4$ c
25	27.25 b	0

*****Within the same column data followed by the same letters are not different for LSD test ($P<0.05$).
**Data are the mean of 5 plates for each medium+ st.er

Table 3 Determination of EC_{50} and EC_{95} values of inhibiting organic acids

	$mg\ g^{-1}$	$mg\ g^{-1}$
Malic acid	13.9	28
Chlorogenic acid	4.6	29
Quinic acid	6.3	14

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Susceptibility of five apple cultivars to Bull's eye rot and relationship with ambient pH

1. Introduction

Neofabraea spp. causes the lenticel rot of apple and pear fruits, well known as Bull's eye rot (BER). The disease is one of the main important postharvest diseases of pome fruit, producing notable economic losses during fruits storage. BER is particularly serious in Europe (Bompeix and Cholodowski-Faivre, 2000, Neri et al., 2009), North-West of United States of America (Gariepy et al., 2005, Henriquez et al., 2004) Australia (Cunnington, 2004), Chile (Soto-Alvear et al., 2013) and Brasil (Spolti et al., 2010). In China BER has classified as quarantine pest disease (Cao et al. 2013). Fruit lesions are flat to slightly sunken, brown and often with a light brown center (Snowdon, 1990), the rotten tissues are firm and white acervula can be present in old lesions. Common cultivars of apple (Spotts et al., 1999) and pear (Henriquez et al. 2004) are subjected to disease relating to climate conditions. The fruit contamination occurs in the orchard, but the symptoms appear only three/four months after harvest, during cold storage (Snowdon 1990). Four members of the genus *Neofabraea* are causal agents of lenticel rot of pome fruits: *N. alba, N. perennans, N. malicorticis* and *Cryptosporiopsis kienholzii*, (Jong et al., 2001, Spotts et al. 2009) however, in Italy only the presence of *N. alba* (E. J. Gutrie) Verkley (anamorph *Phlyctema vagabunda* Desm.) was detected. 'Golden Delicious' apple and 'Bosc' pear are highly susceptible cultivar and in certain years favorable to pathogen development high incidences of fruit infections (over 40%) can occur. Few are the scientific data on apple cultivars susceptibility to *N. alba*. The scarce information derive from packaginghouse surveys however, they are not specific on BER susceptibility and apple cultivar. A review on pome European fruit genetic resources evaluated for

disease resistance (Kellerhals et al., 2012), included BER among the main causes of decay during apple storage, but no information on cultivar resistance or tolerance to BER disease was reported. Jönsson et al. (2012) assert that also in Sweden BER is one of the most common problem during apple storage.

The latent infections produced by *Neofabrea* spp. can be influenced by many factors, such as phenolic content (Naoumkina et al., 2010), maturity stage (Guidarelli et al., 2011, Neri et al., 2014), and insufficient nutritional requirements for pathogen expression of virulence factors (Prusky, 1996). Previously, the studies of Bateman and Beer (1965) on the close relationship between pH and pathogenicity, were resumed by Prusky et al. (2001) that suggested as some fungal pathogens may enhance their virulence by locally modulating the host's ambient pH. Therefore the importance of ambient pH for the expression of some enzymes as cell-wall degrading enzymes (CWDE), represent a key factor in specific enzymes secretion (Wubben et al., 2005) allowing to pathogen the penetration in host tissue (Akimitsu et al., 2004). Each enzyme can be expressed only at a precise range of pH and for this reason the pH value modulation performed by fungi, can result crucial for host invasion and disease appearing (Akimitsu et al., 2004, Prusky et al., 2001). Molecular studies demonstrated that genes encoding CWDE are expressed, and their products secreted, only under optimal pH conditions (Eshel et al., 2002; Prusky et al., 2001). The ability to modify pH may be differentially expressed by fungi, that are able to raise (‘alkalinising fungi’) or reduce the ambient pH (‘acidifying fungi’) in view of the pathogenic process (Prusky and Lichter, 2008). Some pathogens such as *Colletotrichum gloeosporioides* (Prusky et al., 2001) and *Alternaria alternata* (Eshel et al., 2002), alkalinise their host tissues by producing significant amounts of ammonia. Other fungi, such as *Penicillium expansum, P. digitatum, P. italicum* (Prusky and

Yakoby, 2003), *Botrytis cinerea* (Manteau et al., 2003) and *Sclerotinia sclerotiorum* (Bateman and Beer, 1965), utilize tissue acidification to support their attacks via the secretion of organic acids.

The aims of the present work were to *i*) evaluate the susceptibility of five common Italian apple cultivars; *ii)* study the relationship between natural ambient pH of apple cultivar and the *N. alba* development *iv)* investigate the *N. alba* capability to affect the pH of host tissue, in order to define it as ‘alkalinising‘ ’ or ‘acidifying’fungus’.

2. Materials and methods

2.1. Fruits

'Gala Schniga', 'Golden Delicious', 'Fuji', 'Granny Smith' and 'Pink Lady™' apples were harvested at commercial maturity according to specific parameters for each ' cultivar in five different orchards located in Emilia Romagna region. Fruits were selected for uniform size, without physical injuries or apparent infections.

2.2. Quality parameters at harvest and during storage

In order to characterized the stage of maturity the following parameters: starch content, size, color, firmness, total soluble solid (TSS), I_{AD} were determined. Starch content at harvest was determined visually using the standard iodine test as described by Nyasordzi et al., 2013. The number 1 indicates maximum starch content (maximal dark stain) and index 10 represents maximum starch hydrolysis (clear stain) (CTFL starch conversion chart EURFRU). Apple size was measured at harvest by caliber and expressed in mm. Color was measured on two opposite sides of each fruit using a tristimulus colorimeter (Chromameter CR-200,Minolta, Japan) able to determine the values of lightness (L*), red-greenness (a*) and yellow-blueness (b*) parameters. Flesh firmness (FF) was evaluated on the two opposite sides of each fruit, after eliminating a thin layer of the epicarp, using an automatic pressure tester (FTA-GUSS, South Africa) fitted with an 11 mm plunger. The TSS was determined with an Atago digital refractometer (Optolab, Modena, Italy) by squeezing a part of the mesocarp in order to obtain a freshly prepared juice from each apple cultivar. The I_{AD} value was measured using a 17 portable DA-Meter (TR-Turoni, Forlì, Italy), that gives the ripening index in relation with the chlorophyll

content. The TA was determined on 20 mL of flesh juice (titration with 0.25N NaOH) using a semiautomatic instrument (Compact-S Titrator, 31 Crison, Modena, Italy). With the exception of starch content and apple size, the evaluation of each quality parameter for each cultivar was repeated at harvest, after 60, 90, 120 days of storage at 0°C and after two weeks of *shelf life* at 20°C.

2.3 Fungal cultures

N. alba ID02 isolate, deposited in GenBank and belonging to our collection, was maintained onto tomato agar (TA: 500 g of tomato sauce, 15 g of agar technical and 500 ml of distilled water) at 15°C. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 on the surface of 15-day-old cultures grown on TA and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to the desiderated concentration.

2.3 Apple inoculation by wounds

Each apple was wounded by a sterile nail, then inoculated with 20 µL of a conidia suspensions of *N. alba* 10^4 conidia/mL. Apple inoculated with distilled water were used as control. Apples were stored at 0 °C and 85% RH for 60 and 90 days. The severity and the incidence of disease were recorded by measuring the diameter of lesion and by calculating the percentage of infected wound respectively. The sample unit was represented by four replicates of five fruits each per variety. The experiment was repeated once.

2.4. pH measurement in decayed apples

In order to measure the pH of mesocarp, a micro-pH electrode was introduced into the wound (pH & Ion-Meter GLP 22+ Model 5033 pH electrode, Crison). The sample unit

was represented by four replicates of five fruits each per cultivar. The measurement of pH was performed after 60 and 90 days of storage. The experiment was repeated once.

2.5. Apple inoculation without wounds

The susceptibility to *N. alba* was tested also in unwounded fruit. For this purpose, fruit were inoculated by sprayed 8 mL/fruit of *N. alba* conidia suspension (10^5 conidia/mL). In order to allow the complete water evaporation, inoculated apples were left at room temperature overnight. BER evaluation incidence was recorded after 60, 90, 120 days of storage at 0°C, followed by 15 days of shelf life at 20°C. Fruit sprayed by 8 mL of distilled water represented control. The sample unit was represented by five replicates of 20 fruits each per *cultivar*. The experiment was repeated once. *2.6. pH measurement during storage of healthy apples*

The evaluation of the pH changes in fruit during storage was performed on healthy apples. The pH values of mesocarp were recorded as describe before, at harvest, after 60, 90, 120 days of storage followed by 15 days of *shelf life* at 20°C. In order to avoid variation due to measurement position, pH were taken always in the middle part of apple fruit. The sample unit was represented by four replicates of five fruit each per *cultivar*. The experiment was repeated once.

2.7. N. alba pH modification capability on artificial medium

The pathogen capability to modified the pH of artificial medium was evaluated in three flasks containing 30 ml of Czapek dox liquid medium modified (OXOID), acidified with natural malic acid (Sigma Aldrich), and inoculated with 2 mycelium plugs of 6 mm diameter of *N.alba*. The pH of artificial medium was adjusted until pH 3.1 and 3.4 that are the pH value determined in the less susceptible apple cultivar, at harvest and after 90

days of storage respectively. The pH was determined at the beginning of the experiment and after 10 days of incubation at 15°C placing the micro-pH electrode directly into the liquid medium . The control was represented by flasks containing 30 ml of Czapek dox liquid medium modified not inoculated. The sample unit was represented by three replicates. The experiment was performed twice.

2.8 Data Analysis

Data were subjected to one-way analysis of variance (ANOVA) using the statistical package Statistica for Windows (Statsoft Inc.). Separation of means was performed using the least significance difference (LSD) test at $P< 0.05$. All experiments were carried out in a completely randomized block design.

3. Results

3.1. Quality parameters

Data on quality parameters showed significant differences among the cultivars within the time of storage (Tab. 2). Apple cultivars with a high I_{AD} value at harvest and during storage were Granny Smith, Fuji and Golden Delicious (1.4, 1.2 and 1.5 respectively at harvest time), while Gala Schniga and 'Pink Lady™' were characterized by low I_{AD} value (0.7), at harvest and during storage. Firmness at harvest was high in Gala Schniga (73.6 N) and Granny Smith (70 N), followed by 'Pink Lady™' (65.8 N), Golden Delicious (63.4 N) and Fuji (57.3). In all apple cultivars firmness decreased considerably within storage. Total soluble solids (TSS) content at harvest is high in 'Pink Lady™' (13.3%) apple, followed by Golden Delicious (12.9 %), Fuji (12.6%), Gala Schniga (11.5 %) and Granny Smith (14%). In all cultivars, TSS increased during storage. The highest amid

content at harvest was recorded in 'Pink Lady™' (9) apple, while the lowest content was recorded in Granny Smith (4) (Tab. 1).

3.2. Artificial inoculation by wounds

The evaluation of susceptibility to *N. alba* in five commercial varieties of apple showed BER symptoms in all varieties assayed, in particular after 60 and 90 days from inoculation the 100 % of fruit were decayed (data not reported). With respect severity of disease (lesion diameter, mm) significant differences were found among varieties (Fig. 1). After 60 days from the inoculum Gala Schniga showed the widest lesion diameter (18 mm), followed by 'Pink Lady™' (17 mm), Golden Delicious (11 mm), Granny Smith and Fuji (6 mm). After further 30 days of storage, 'Pink Lady™' showed the largest lesion (25 mm) , followed by Gala Schniga (22 mm) and Golden Delicious (21 mm), and Granny Smith (14 mm) and Fuji (6 mm).

3.3. Changes in pH induced by N. alba in five apple cultivars

Data on the relationship between apple pH and *N. alba* growth are reported in Fig. 2. The pH values determined in decayed tissue ranged from 4.2 in 'Pink Lady™' and 5.6 in Fuji (Fig. 2). Significant differences between the healthy tissue and decayed tissue were found for each apple cultivar. *N. alba* decay increased the pH in all apple cultivar, from 0.38 ('Pink Lady™') units until 1.52 units ('Granny Smith') (Fig. 3).

3.4. BER incidence in five apple cultivars artificially inoculated without wounds

Data on the incidence of BER on unwounded fruits revealed the susceptibility of all varieties assayed however with different percentage of infected fruit ranging from 1% ('Granny Smith') to 27% ('Gala') (Fig. 4). All apple cultivars inoculated showed BER symptoms. However, significant differences were found among apple cultivars: at the

end of storage, ‘Gala Schniga’ showed the highest BER incidence (27% of infected fruit), while Granny Smith showed le lowest incidence (1% of infected fruit). ‘Golden Delicious’ and ‘Fuji’ resulted quite susceptible, showing a BER incidence of 17 and 9 % respectively

3.5. pH changes from harvest and within storage

The pH changes in the mesocarp of the most susceptible (‘Gala Schniga’) and the most resistant (‘Granny Smith’) apple cultivar were reported in Fig. 5. The first showed a higher pH value than the second, from harvest and during the storage. The pH of ‘Gala Schniga’ decreased from harvest (pH 4.2) until 90 days (pH 4) of storage, rising at 120 days after harvest (4.1) and at the end of shelf life *(*pH 4.2). Mesocarp pH of ‘Granny Smith’ apple at harvest was 3.2, increased gradually until 120 days after harvest to 3.6, and decreasing slightly after shelf life 3.5.

3.6 Changes in pH induced by N. alba in artificial substrate

In order to confirm the *N. alba* capability to modify the ambient pH, changes in pH induced in artificial substrate were evaluated. Starting from 3.1 and 3.4 *N. alba* was able to increase the pH of artificial substrate significantly, reaching for both initial pH a final pH around 5.

4. Discussion

During last decades, Bull’s eye rot caused by *Neofabraea* genus was recognized in Europe and in North-West America as one of the most important fungal disease during storage (Jonsson et al., 2010, Maxim et al., 2014, Spotts et al., 1999, Neri et al., 2009). In Italy, *N. alba* is recognized as the major pathogen causing BER, however, the information about this important pathogen are very few, such as the scientific data on cultivar susceptibility

are lacking. Our results showed that exists different susceptibility among the main commercial apple cultivars present on the Italian market. Artificial inoculations performed by wounds showed that the more susceptible cultivars are 'Gala Schniga' (lesion diameter of 18 mm 60 days after inoculum) and 'Pink Lady™' (lesion diameter of 25 mm 90 days after inoculum), while 'Fuji' showed the minor lesion diameter in both dates of analysis. Artificial inoculations performed without wounds, confirmed that 'Gala Schniga' and 'Pink Lady™' are the two major susceptible apple cultivar, while 'Granny Smith' resulted the less susceptible cultivar to BER, showing at the end of storage period 1% of disease incidence. All apple cultivars were harvested in the same rural area in Emilia Romagna region, but only the control of 'Gala Schinga' and 'Granny Smith' did not show BER symptoms. 'Granny Smith' can be considered one of the less susceptible cultivar to BER, while the absence of natural inoculum on 'Gala Schniga' can be due to the time of harvest, that in Italy occurs in August, when the weather conditions are not favorable to pathogen (high temperature and low humidity). Spotts et al., 1999 found that Fuji and Granny Smith apple cultivars were the more resistant among several apple cultivars evaluated for *N. malicorticis* susceptibility. Our results confirm that this two cultivars were the less susceptible among the commercial apple tested. However, Fuji, when evaluated by artificial inoculation without wounds, reproducing the natural mechanism of infection, showed a BER incidence of 10%, higher than that showed by Granny Smith (1%).

Analysis performed with purpose to evaluate the capability of *N. alba* to induce pH changes in host tissue, clearly demonstrated that the pathogen alkalinize the ambient pH of host. This feature was confirmed in all apple cultivars analyzed, whit some difference in terms of pH (Fig.3). In particular the most susceptible cultivar ('Pink lady™') and the

less susceptible cultivar ('Granny Smith') showed a pH increasing of 0.41 and 1.52 respectively. The Δ pH is a independent value respect the final pH determined in symptomatic tissues. In 'Pink lady' the pH in rotted tissue 4.05 (90 days after inoculum), while in Granny Smith pH of rotted tissue arrived at 5. In Fuji, that was found the second cultivar for BER susceptibility in natural conditions, the pH of rotted tissue arrived until 5.6, with a Δ pH of 1 unit. Previously work on *P. digitatum* and *P. expansum* (Villanova et al., 2014) evaluated the pathogen capability to modify the ambient pH at different maturity stage, but did not compare or linked ambient pH with host susceptibility. Our results indicates that in more resistant apple cultivars ('Granny Smith' and 'Fuji'), *N. alba* have to produce more alkalizing compounds in order to colonize the host tissue. Moreover the alkalization capability was confirmed also in laboratory trials, were starting from pH of 3.1 and 3.4, after 8 days of incubation, the pH reaches 5. Δ pH is not the only parameters that characterize the most and the less susceptible cultivar, also DA meter index (I_{AD}) has similar value in the resistant ('Granny Smith' and 'Fuji') and susceptible ('Gala Schniga' and 'Pink Lady™) apple cultivars. From the harvest, until the end of storage, 'Granny Smith' and 'Fuji' have the highest I_{AD}; in these two cultivars I_{AD} at harvest was 1.4 and 1.2 respectively, while in 'Gala Schniga' and 'Pink Lady™ the I_{AD} was 0.7 for both cultivars. From our knowledge, this is the first work that evaluated apple cultivar for BER susceptibility. However, more studies will be required for clarify the effect of pH on *N. alba* enzyme production, and which biochemical parameters are associated with high and low I_{AD}.

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Tables

Table 1 *Apple size ± Standard Error (SE) and starch content of five apple cultivars evaluated for BER susceptibility*

Apple Cultivar	Apple size ± SE	Starch content
Gala Schniga	74.8±0.7	6
Golden Delicious	73 ± 0.7	5
Fuji	82±1.1	8
Granny Smith	74.8±0.9	4
'Pink Lady™'	81.3±0.5	8

Table 2. *Quality parameters of Gala Schniga, Golden Delicious, Fuji, Granny Smith, Cripps Pink apples, from harvest until 120 days of cold storage at 0°C more two weeks of shelf life where apples were stored at 20°C.*

Cultivar	I_{DA} value	Firmness Ncm^{-2}	TSS %	TA %	Ground color			Blush color		
					L*	a*	b*	L*	a*	b*
Gala Schniga										
Harvest	0.7 a	73.6 a	11.5 a	0.35 a	69.3 a	-2.8 a	24.8 a	43.1 a	27.6 a	14.7 a
60 d	0.5 b	58.9 b	12.7 ab	0.12 b	70.15 a	-4.12 a	28.4 b	39.3 b	27.7 a	14.0 a
90 d	0.4 b	55 b	13.1 b	0.12 b	69.8 a	-3.6 a	29.3 b	44.4 a	25.4 b	17.5 b
120 d	0.2 c	59.1 b	13.6 b	0.12 b	40.7 c	26.2 c	15.5 c	38.8 b	27.9 a	14.7 a
Golden Delicious										
Harvest	1.5 a	63.4 a	12.9 a	0.13 a	65.8 a	-14.9 a	29.6 a			
60 d	1.18 b	47.2 b	14.1	0.13 a	67.5 b	-12.4 b	31.7 b			
90 d	1.2 b	45.5 b	14 b	0.12 a	66.5 b	-11.7 b	31.49 b			
120 d	1.2 b	40.5 c	14.7 b	0.13 a	67.5 b	-11.2 b	31.7 b			
Fuji										
Harvest	1.2 a	57.3 a	12.6 a	0.13 a	63.8 a	-11.1 a	25.4 a	40.8	14.7	13
60 d	1.3 a	55.1 b	13.3 a	0.12 a	66 b	-10.9 a	26.4 b	41.8	13.9	13.3
90 d	1.3 a	56.3 ab	13.2 a	0.12 a	65.6 b	-9.1 b	25.8 a	42.5	14.4	14.1
120 d	1.2 a	55.1 b	16 a	0.13 a	62.5 a	-8.2 b	24.8 a	43.6	12.1	15.2
Granny Smith										
Harvest	1.4 a	70.0 a	10.4 a	0.14 a	53.3 a	-16.6	25.4 a			
60 d	1.8 d	65.4 b	11.5 a	0.13 a	53.9 a	-14.1	25.4 a			
90 d	1.7 cd	61.4 cb	12.1 b	0.13 a	56.5 b	-15.3	25.8 a			
120 d	1.6 c	59.8 c	12.6 b	0.14 a	52.9 a	-13.5	25.2 a			
'Pink Lady™'										
Harvest	0.7 a	65.8 a	13.3 a	0.13 a	67.9 a	-8.2 a	27.8 a	38.7 a	30.5 a	12.3 a
60 d	0.5 b	56.4 b	14.1 b	0.13 a	64.4 b	-3.6 b	27.5 a	35,6 b	19 b	11.2 b
90 d	0.4 bc	52.9 b	14.1 b	0.13 a	66.5 bc	-5.9 c	28 a	42.4 c	27.5 c	14.1 c
120 d	0.3 c	49.3b	14.0b	0.13 a	70.48 c	-7.8	29.5 b	43.1	30.3 a	14.9 c

*Different lowercase letters indicate significant differences within the time of storage for each apple cultivar according to LSD test ($P < 0.05$).

Figures

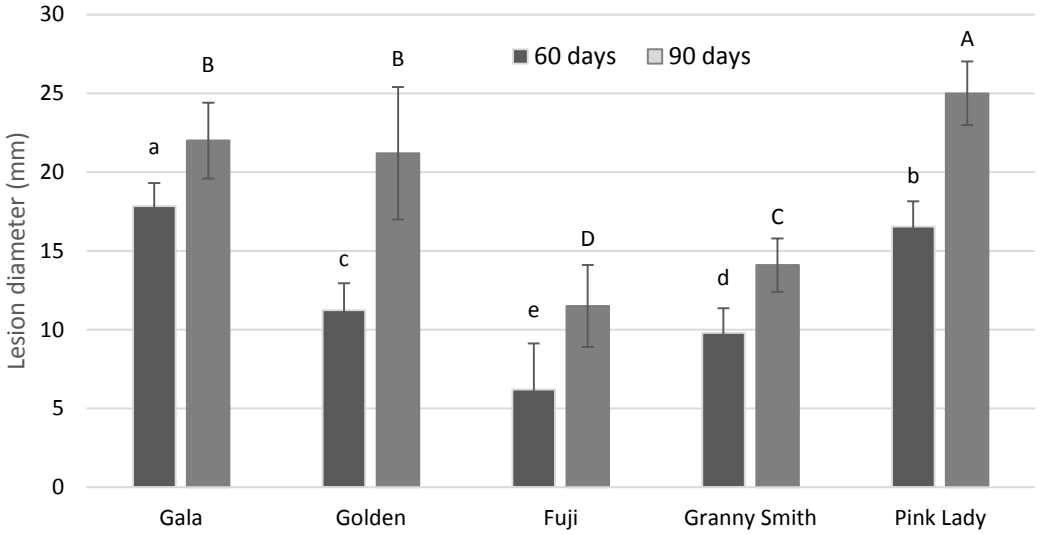

Figure 1. *Susceptibility evaluation to BER in 'Gala Schniga', 'Golden Delicous', 'Fuji', 'Granny Smith' and 'Cripps Pink' apple after 60 and 90 days from inoculum. Fruits were wounded, inoculated with Neofabrea alba (10 conidia per ml) and stored at 0°C. Each data is the average of 100 fruits.Within 60 days after inoculation (lower case) and 90 days after inoculation (upper case) different letters, represent significant differences among apple cultivars according to LSD test (P < 0.05).*

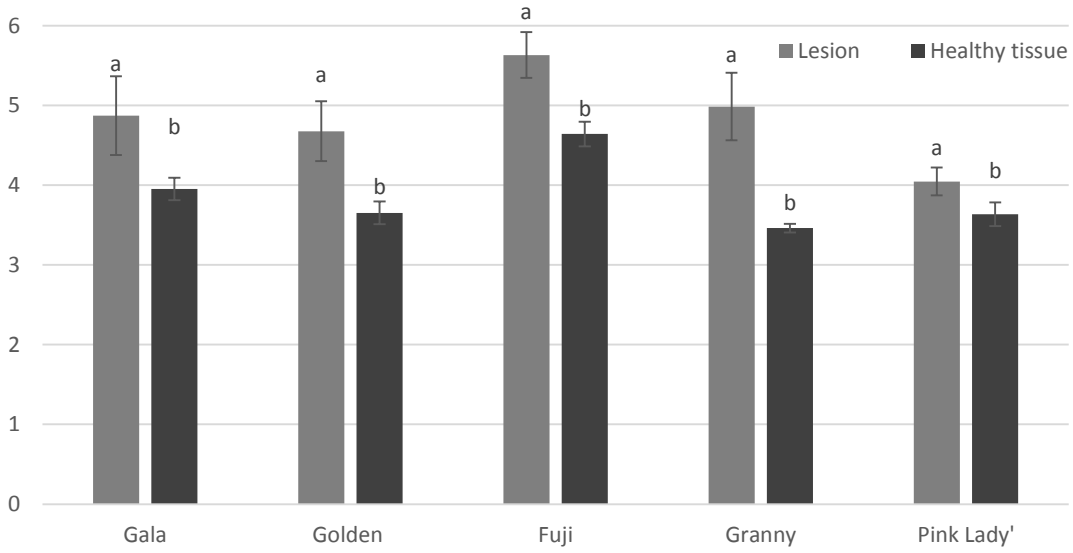

Figure 2. *pH in lesion and in healthy tissue of both apple side 1 cm distant from decayed tissue inoculated by N. alba. Gala Schniga, Golden delicious, Fuji, Granny Smith and Pink Lady™, were inoculated with N. alba at 10^5 conidia/mL, harvested at commercial maturity and stored at 0°C for 90 (B). For each apple cultivar, different lowercase letters indicate significant differences of pH in decayed and healthy tissue according to the LSD test (P value< 0.05). Each pH value represents the mean of 20 apples after 60 days of conservation.*

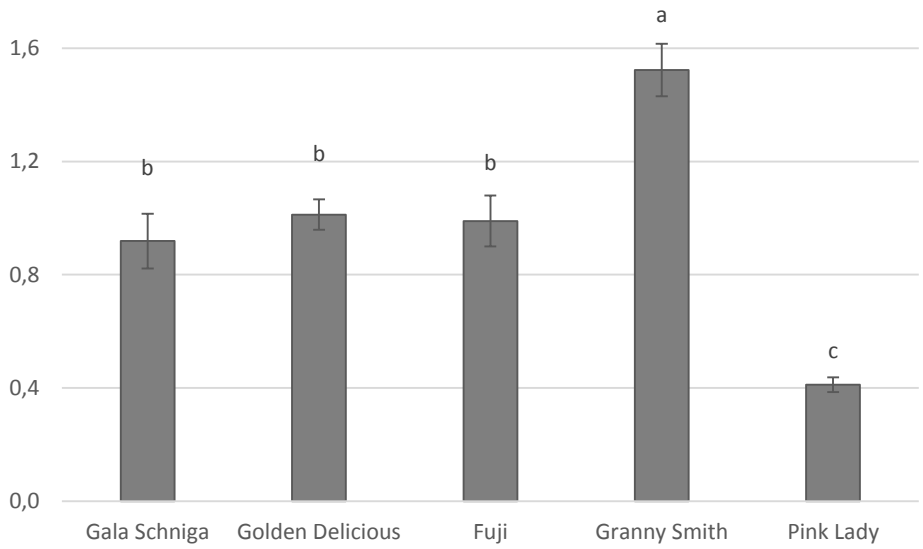

***Figure 3**. Increasing of pH value (Δ pH) in decayed tissue inoculated by N. alba 1*10^4* compared with healthy tissue of the same apple*. Different letters, represent significant differences between healthy and decayed tissue according to LSD test (P < 0.05).*

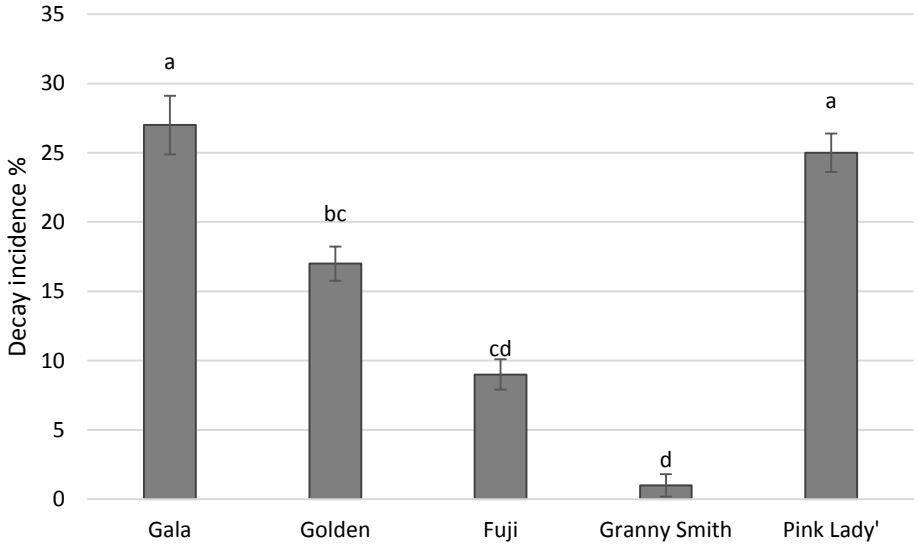

***Figure 4.** Disease incidence in Gala Schniga, Golden, Fuji, Granny Smith and Cripps Pink apple cultivars, after 120 days of cold storage (0°) and 15 days of shelf life (20°C). Different letters represent significant differences among apple cultivars according to LSD test (P < 0.05)*

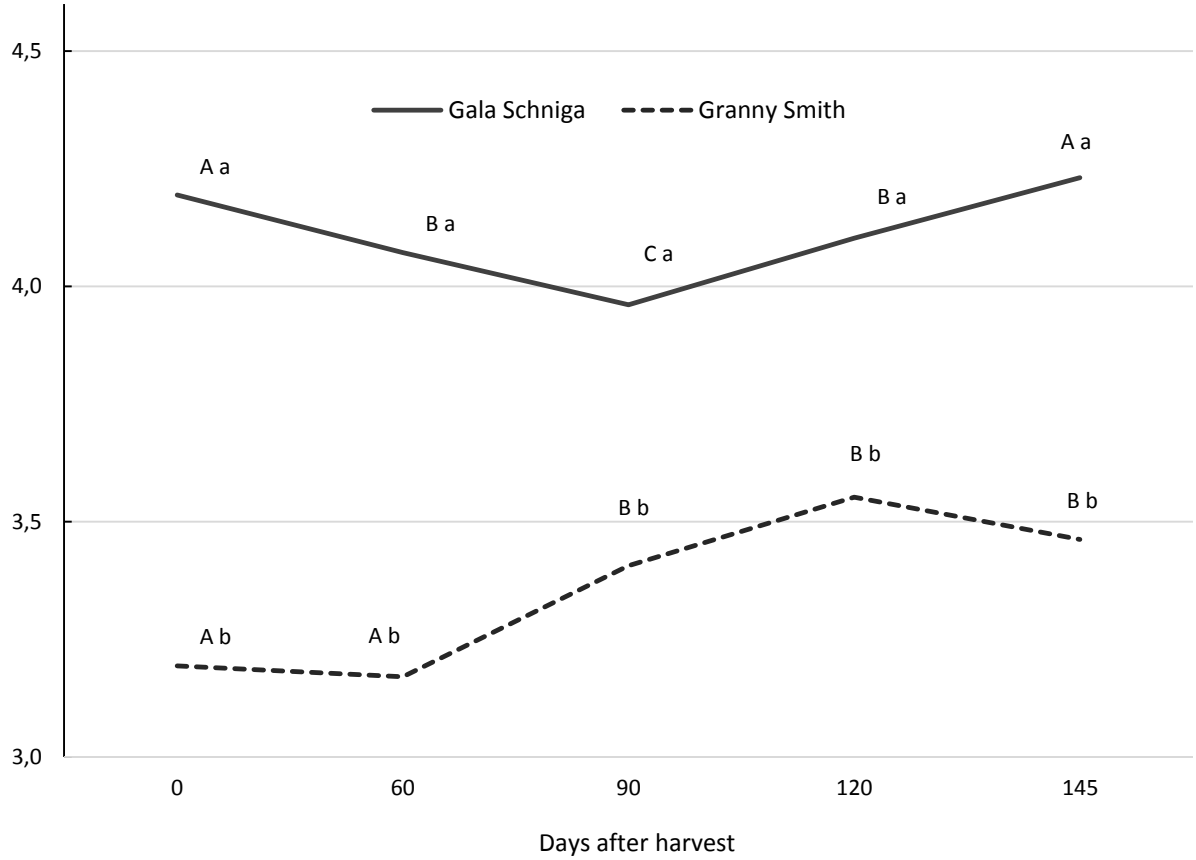

Figure *5.Changes in pH values from harvest until the end of cold storage and shelf life in Gala Schniga and Granny Smith apple. Values represent the mean of 20 fruits for each cultivar for each date of analysis. Lowercase letters refer to pH differences among the apple cultivars in each data of analysis. Uppercase letters refer to pH changes within the time course comparing each apple cultivar with itself. Different letters represent significant differences according to LSD tes (P<0.05).*

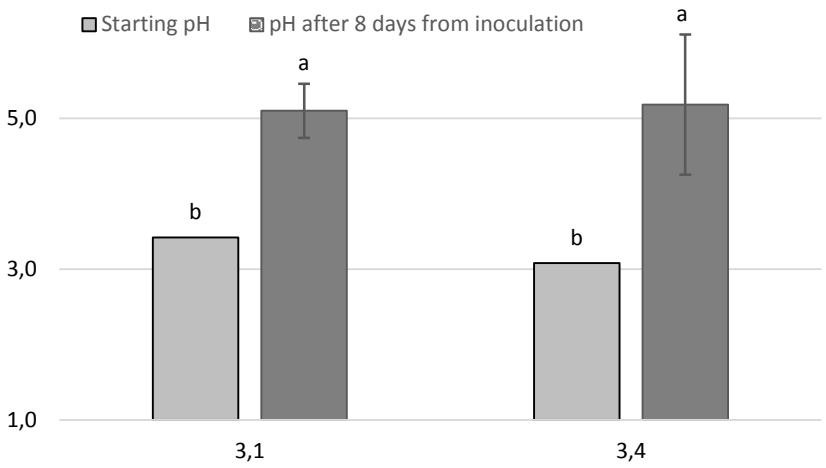

Fig.6. *Induction of pH change on artificial medium inoculated by N. alba 1 10^4 conidi/mL. Flasks inoculated were incubated at 15°C. Different letters represent significant pH changes from initial pH of substrate and pH of substrate after 8 days from inoculation according to LSD test (P<0.05).*

Bull's eye rot of 'Pink Lady™' management by DA-meter application: practical implications and transcriptional level of ripening, defence involved genes

1. Introduction

'Pink Lady™', is an extensively cultivated apple cultivar. Since 1990, this variety is present in the main apple-producing areas of the World, because of its excellent sensory attributes like firmness and fine dense flesh, crisp, juicy, excellent flavor, and high sugar-acid balance (Lopez et al., 2007). 'Pink Lady™' apples originated from a cross between 'Lady Williams' and 'Golden Delicious' combine the sweet of 'Golden Delicious' with the firm, long-storing fruit of 'Lady Williams' (Cripps et al., 1993). In Europe, this apple variety is harvested in November, and can be stored before commercialization until 25 weeks (Lopez et al., 2007). Chile a primary producer of 'Pink Lady™' exports the apple in Asia, Europe and United States; after 15-40 days of maritime transportation (Soto-Alvear et al., 2013), fruits are stored in refrigerated rooms for other 3-4 months. The attitude of this apple cultivar to maintain the quality parameters during storage and market distribution is very appreciated by fruit industry and has contributed to an intense diffusion of this variety in the world. However, physiological disorders as superficial scald or flavor alteration and fungal diseases can cause dramatic product losses. Among the fungal diseases causing apple rotting during cold storage, Bull's eye rot (BER) is one of the most important and in favorable years, the disease incidence can exceed 30% of apple harvested (Neri et al, 2009). Four species belonging to *Neofabraea* genus: *N. alba*, *N. perennans*, *N. malicorticis* and*Cryptosporiopsis kienholzii* are considered the casual

agents of apple BER (Bompeix and Cholodowski-Faivre, 2000; Gariepy et al., 2005, Jönsson et al. 2012). The fruit symptoms consist of circular necrotic lesions that are usually flat or slightly concave and generally dark and firm; under humid conditions, conidia and acervuli are produced in a waxy matrix (Verkley, G. J. 1999). In Italy *N. alba.* is the main specie responsible of BER in late harvest apple varieties (Neri et al., 2009).

The maturity stage at harvest can influences postharvest life of many fruit and vegetables (Özgen et al., 2002, Sandoval Chávez et al., 2014, Vilanova et al., 2013). 'Pink Lady™' apple appear completely asymptomatic at harvest, and BER symptoms emerge from fruits that completed their ripening process during storage at 0°C, starting from three months after harvest. Since apple at harvest time appear completely symptoms less, it should be crucial to evaluate their propensity to show BER symptoms before selling. In the last years, the use of a non-destructive device, the DA-meter (DA-meter, Sinteleia, Bologna, Italy) was applied on apple fruits on tree to determine the optimal harvest time (De Long et al., 2014, Costamagna et al., 2013). DA-meter provides an index (index of Absorbance Difference = I_{AD}) (Ziosi et al., 2008) able to indirectly quantify the chlorophyll content. Closer is the I_{AD} value to 0, less chlorophyll is present in fruit peel, and more advanced is the fruit maturity. In this study the use of DA-meter was applied at the harvest time on 'Pink Lady™' apples grown in Emilia-Romagna, the most important Italian region for 'Pink Lady™' production. The aim of this work was to find or a relationship between I_{AD} and BER symptoms appearance. Moreover, transcript levels of ripening and phenylpropanoid biosynthesis related genes were assessed in each major I_{AD} class at harvest and during shelf life.

2. Materials and methods

2.1 Fruit

Apple fruits were harvested in orchards located in Emilia Romagna region (Italy). The orchards were under conventional management, but no fungicide treatments against *N. alba* were performed. Fruits harvested at commercial maturity, free of visible wounds and rots and homogeneous in size.

2.2. Apple selection according to I_{AD} and determination of quality parameters

At harvest, in order to obtain two I_{AD} classes, apple fruits were analyzed using a 17 portable DA-Meter (TR-Turoni, Forlì, Italy) for chlorophyll degradation. Apple fruits with an $I_{AD} > 0.8$ were classified in the high (H) class, corresponding to the less ripe apples than fruit with an $I_{AD} < 0.79$ that were classified in the low (L) class, corresponding to the riper apples. In order to avoid an overlapping of samples, apples with the I_{AD} of 0.79 and 0.80 were not selected for the evaluation of BER pathogenesis in different I_{AD} classes. The following destructive analysis were performed in each class: firmness (FF), starch, total soluble solids (TSS) and ethylene production. FF was evaluated on the two opposite sides of each fruit, after eliminating a thin layer of the epicarp, using an automatic pressure tester (FTA-GUSS, South Africa) fitted with an 11 mm plunger. Starch content at harvest was determined visually using the CTIFL scale (Centre technique interprofessionnel des fruits et legumes, association 'Pink Lady™' Europe) by staining an equatorial sliced half of the apple with Lugol's solution composed of (2.5 g) iodine (I2) and (10 g) potassium iodide (KI) in 1 L of distilled water. The staining pattern was scored against the CTIFL table of photographs with a range of 1–10 starch content, the number 1 indicates maximum starch content (maximal dark stain) and index 10 represents

maximum starch hydrolysis (clear stain). The TSS was determined with an Atago digital refractometer (Optolab, Modena, Italy) by squeezing a part of the mesocarp in order to obtain a freshly prepared juice from each I_{AD} class. For quality traits 10 apple fruits for each IAD class/trial represented sample unit.

2.3. BER evaluation during post-harvest life and evolution of I_{AD} index and

Naturally infected fruits, previously classified by I_{AD} as described above, were stored at 0°C for 150 days and BER symptoms appearance were detected during apple post-harvest life. Sample unit was represented by three replicates of 75 fruit for each I_{AD} class. Additional 'Pink Lady™' samples, 3 replicates of 20 fruits for each I_{AD} class, were stored at 0°C and monitored for I_{AD} changes for 120 days of storage. Monitoring activity was carried out bordering with a permanent pencil a 1 cm^2 area on both apple equatorial side and measuring the I_{AD} value into the marked areas after 0, 60, 90 and 120 days of storage. At the same time, an additional plot of 300 fruits randomly selected, (not divided according to I_{AD}) were stored at 0°C and once BER symptoms emerged I_{AD} value was recorded at both lateral points of rotted lenticel. Each experiment was repeated once.

2.4. RNA extraction and qRT-PCR analysis

For molecular analysis, apple peel belonging to the two I_{AD} classes as described above was samples at harvest time and after 3 and 5 months of storage. Apple tissue were collected using a peeler, immediately placed in liquid nitrogen and stored at -80°C until the day of analysis.Total RNA was extracted from the two I_{AD} classes of apple fruit as described by Vilanova et al., 2014. DNA was removed using TURBO DNase (Ambion). The concentration and purity of total RNA was determined as the 260/280 nm and

260/230 nm ratio using a ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, USA) and the integrity was checked by electrophoresis in 1.5% agarose gel. Reverse transcription was performed using 1ug total RNA with oligo-d(T)18 and ImProM-II ReverseTranscriptase (Promega, Italy) following manufacterer's instructions. The qRT-PCR assays were carried out within the Mx3000PTM QRTPCR System thermocycler including the associated software program from Stratagene. Each 12,5ul reaction was performed by mixing 50ng cDNA with Maxima® SYBR Green/ROX qPCR Master Mix (2X; Fermentas) and 200nM of specific forward and reverse primers. Negative control reactions replacing the cDNA template contained either sterile water. Each reaction was repeated twice. PCR cycling parameters were 10 min at 95°C, 45 cycles of 30 s at 95°C, 1 min at 57°C or 59°C, 1 min at 72°C. All primers in this study were designed using the online software Primer-Blast (National Center for Biotechnology Information, Bethesda, MD; available at Primer-Blast) and are listed in Table 1 whit the appropriate melting temperature used. The actin housekeeping gene was used to normalize raw data and calculate relative transcript levels by standard curve method.

2.7 Data Analysis

Data were subjected to one-way analysis of variance (ANOVA) using the statistical package Statistica for Windows (Statsoft Inc.). Separation of means was performed using the least significance difference (LSD) test at $P< 0.05$. All experiments were carried out in a complete randomized block design.

3. Results

3.1. Determination of quality parameters and apple separation according to I_{AD}

Fruit were divided in two I_{AD} classes: apple with the I_{AD} value < 0.79 (low class) and apples with I_{AD} > 0.8 (high class). In both trails the high I_{AD} class displayed the highest mean value of firmness (85 and 90 N cm^{-2}) and TSS content (13.8% and 14.3%), and the lower starch content (7). however, no significant differences were found between the 2 I_{AD} classes (data not reported).

3.2 Incidence of BER on different I_{AD} classes

'Pink Lady™' apples are highly susceptible to BER, after 150 days of storage at 0°C the incidence of disease was on average 47% and 68.4 % in the trial 1 and 2 respectively (Fig. 1). In addition, in both trials, the fruits of L class were significantly more rotted than fruit of H class. In particular, fruits belonging to H class showed a BER incidence of 23.5% and 55.6% versus a BER incidence of 71.83% and 81.2% in the L class, respectively for the trial 1 and 2.

3.3 Evolution of I_{AD} and evaluation of correspondence value between BER appearing and I_{AD}

The I_{AD} evolution in 'Pink Lady™' apple was accurately monitored every 30 d, for 120 d of storage at 0°C in the same area, previously marked. Results showed that during storage, the I_{AD} value, decreased significantly in both I_{AD} classes (Fig. 2). In the H class the I_{AD} value ranged from 1.05 at harvest to 0.72 after 120 d of storage, while for the L class the I_{AD} values ranged from 0.79 at harvest to 0.52 after 120 d of storage.

Results reported in Fig.3 showed that the 80% of rotted lenticels appeared in apple with a I_{AD} ranging from 0.4 to 0.79 . The highest percentage of BER symptoms appear in correspondence of I_{AD} value of 0.4, where is located the 19% of rotted fruits, followed by I_{AD} value of 0.7 and 0.6, with 16% and 15% of rotted apple respectively. Increasing the I_{AD} value decreases considerably the emergence of BER disease, in fact in apple fruit with I_{AD} value > 0.8 the percentage of symptomatic fruit is very low (from 2.5% at I_{AD} value of 0.9 until 0.5 at I_{AD} value of 1.3), disappearing with I_{AD} value $\geq$1.4.

3.4 Influence of I_{AD} on ripening related genes

Results reported in Fig.4 showed the relative expression of polygalacturonase-inhibiting protein (PGIP) and 1-Aminocyclopropane-1-carboxylate oxidase (ACO). Both analysed genes were differentially expressed from harvest until the end of the storage. In both I_{AD} classes the maximum PGIP expression level was registered three months after harvest (87.5-fold and 29.4-fold for H class and L class respectively, when compared to harvest time) while the minimum expression level was found at harvest time. Five months after harvest the PGIP expression level decreased, especially in the H class where the expression level decreases 2.2-fold, also in the L class the expression level decreased but only 1.3-fold. Between the I_{AD} classes significant difference was found only three months after harvest, where I_{AD} class > 0.8 was found 1.7-fold higher than I_{AD} class <0.79. The ACO expression level showed a similar pattern, also in ACO the maximum expression level was found for both I_{AD} classes three months after harvest, and the minimum expression level was found at harvest time. The difference expression level between the I_{AD} classes were found after three and five months from harvest. In both case the H class showed an up-regulation of ACO relative expression, that was 2 and 1.4-fold higher than L class at three and five months after harvest respectively.

3.5 Influence of I_{AD} on *phenylpropanoid metabolism related gene*

The expression of HCT, PAL, C4H and AMP-dependent synthetase and ligase family protein was also analyzed by qRT-PCR in two I_{AD} classes (Fig.5). The HCT gene relative expression level showed significant differences between the time storage studied and the I_{AD} classes after three months from harvest, where the H class showed an up-regulation (1.4-fold). PAL relative expression level was significantly different between the two I_{AD} classes from harvest until five months of storage. In each date of sampling, the L class (I_{AD} value < 0.79) showed a high relative expression of 1.7, 1.8 and 2-fold at harvest, three and five months after harvest respectively. On the other hand C4H relative expression level did not show significant difference between I_{AD} classes. However, C4H shows significant difference if relative expression within the time storage, in the higher class the maximum expression level appears at three months after harvest. In AMP-dependent synthetase and ligase family protein the relative gene expression between the I_{AD} was found significantly different at five months after harvest only, where I_{AD} class > 0.8 showed an up-regulation (1.3-fold), while within the time storage there are significant differences in both I_{AD} classes.

4. Discussion

'Pink Lady™' apple is very susceptible to BER. In the present study after 150 d of storage at 0°C, the incidence of disease was over 50% (Fig. 1), confirming the cultivar susceptibility found also by others authors in Chile (Soto Alvear and De los Angeles 2012). The fruit infection occurs in the field and since fruit appear asymptomatic at harvest, it could be very important to know what could be the incidence of disease in

order to manage the BER in a profitable way, using no-destructive tool. In general, the I_{AD} developed by Ziosi et al. (2008), can be considered among the more sensitive and confident non destructive method to determine the progression of ripening in peach, nectarine and apple fruits (Bonora et al., 2014, De Long at al., 2014, Lurie et al, 2013). In the present work BER development was evaluated on 'Pink Lady™' apple in relation to $I_{AD.}$ Before storage, fruit were classified in two classes of I_{AD}, one with low values (<0.79) and second with high value of I_{AD} (>0.8). Results showed that apples with low I_{AD} value are significantly more susceptible to *N. alba* than apple with high I_{AD} value. After five months at 0°C, the percentage of BER in the L and H classes was 71.8% and 23.15 % respectively in the trial 1, and 55.6% and 81.2% in the trial 2. I_{AD} is easy to obtain since not require statistical processing data, but only the determination of cultivar-specific relationship between the ripeness stage and I_{AD} value (Ziosi et al., 2008) therefore it could spread for both practical and scientific application (Nyasordzi et al., 2013, Toiven and Hampspn, 2014). The I_{AD} values determined on the same fruits of two I_{AD} classes changed during storage, decreasing around 0.7-0.8 points for month (Fig. 2). The I_{AD} decreasing during storage was found also in Honeycrisp™ (De Long et al., 2014), while during *shelf life* at 20°C similar trend was found in peaches (Lurie et al., 2013). In addition, results derived from I_{AD} measurements on fruit showing symptoms of BER revealed that the 80% rots appeared in apple with I_{AD} value < 0.8 (Fig.3). The highest percentage of rotted lenticel occurred in fruit with an I_{AD} value of 0.4. While, apple with I_{AD} value ≥ of 0.8 showed low percentage of BER symptoms (20 % totally), and no BER symptoms were found in apple with I_{AD} values higher than 1.3. From our knowledge, this is the first work that suggests a relationship between the I_{AD} value and the susceptibility to *N. alba*. All results obtained in this study confirm the hypothesis that BER is related with apple

ripening and maturity stage. Previously, other authors have found similar results for other latent infections. Guidarelli et al. (2011) found that green strawberry were less susceptible than red strawberry to the infection of *Collototrichum acutatum*. Neri et al. (2014) found an increasing of strawberry susceptibility to *Botrytis cinerea* in relationship to the progress of ripening process. The reasons of latent infection interruption can be various, but many authors sustain a crucial role of phenylpropanoid pathway in the mechanisms that regulate the pathogen host interaction (Dixon et al., 2002, Guidarelli et al., 2011, Prusky et al., 1982). Phenylpropanoid pathway is initiated from deamination of phenylalanine to form cinnamic acid followed by hydroxylation and methylation of the aromatic ring to generate a variety of phenolic compounds that are often involved in plant defence (Dixon et al., 2002). In this study the relative expression level of three genes involved in this pathway was evaluated: PAL, C4H and HCT. Phenylpropanoids have been commonly identified in many defensive reactions, including constitutive phytoanticipins, inducible phytoalexins, and the signaling molecules, which regulate the plant defence system (Naoumkina et al., 2010, VanEtten et al., 1994).

Another group of molecules involved in plant defences is represented by Polygalacturonase-inhibiting protein (PGIPs), extracellular plant proteins capable of inhibiting fungal endopolygalacturonases (PGs). Plants have evolved different PGIPs with specific recognition abilities against the many polygalacturonase (PGs) produced by fungi, and are wide spread in vegetal kingdom (De Lorenzo et al., 2001). Gregori et al. (2007) showed that PGIPs extracted by 'Cripps Pink' apples, could have an inhibition effect against *C. acutatum,* and in order to evaluate a possible implication also in *N. alba* defence mechanism, PGIPs expression level of both I_{AD} classes, was evaluated.

The application of qRT-PCR analysis was able to find out significant differences between the I_{AD} classes in 6 out of 7 marker genes evaluated (Fig. 4 and Fig. 5).With respect to PAL relative expression levels, differences between the two fruit classes were found from the harvest until five months of storage. PGIP showed a significant up regulation of the H class only at three months after harvest, while ACO and HCT show a significant up regulation of the H class at five months after harvest also. AMP-dependent synthetase and ligase family protein relative expression, show significant difference between the classes only five months after harvest. Our results display a higher relative expression of PGIP in the I_{AD} class > 0.8 than class < 0.79 (1.7-fold), suggesting that PGIPs could be involved in apple defence against *N. alba*. HCT was highly expressed in apple with I_{AD} value > 0.8, suggesting that also phenylpropanoid pathway derived compounds can play an important role in the apple defence mechanism. Studies conducted on *Nicotiana tabacum* phenylpropanoid metabolism showed that HCT gene is involved lignin production (Hoffman et al., 2004). As a major branch of phenylpropanoid metabolism, lignin has been shown to be part of chemical plant defence against plant pathogens (Naoumkina, et al., 2010). In mango fruit a higher lignin content induced by nitric oxide (NO) treatment, can be included among the factors that inhibited *C. gloeosporioides* colonization. From our knowledge, this is the first work that analyzed the relative expression of marker genes in different I_{AD} classes of apple fruit, at harvest and during storage. With respect to analyzed genes expression levels during fruit storage, results in this work revealed that with exception of C4H gene, all genes showed a higher expression level during storage than at harvest time. This result indicate that the maturity level at harvest, don't influence only the quality attributes during storage (De Long et al., 2014, Nyasordzi et al., 2013), but also some factors involved in apple defence. From our data,

DA-Meter is confirmed as a robust tool, able to evaluate the fruit ripening stage and the results were supported by molecular analysis. In addition, for the first time it has been possible classified asymptomatic fruit in two classes of I_{AD} that showed a significant difference in BER incidence. In particular apple with $I_{AD} < 0.8$ at harvest, are more subjected to BER appearance in storage. For this reason, the I_{AD} application seems a useful tool in apple *N. alba* rot management able to limit chemical treatments for BER control. If automatized, this technology should make be possible to evaluate asymptomatic fruits after harvest, before storage, for their propensity to show BER after 150 d of storage, helping to manage the postharvest phase of 'Pink Lady™' apple.

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***Table 1**. Primers used in qRT-PCR reactions*

Gene name	Gene acronym	Accession No.	Primer sequence (5'-3')	Annealing temperature (°C)
Actin		AB638619	F: CTATGTTCCCTGGTATTGCAGACC R: GCCACAACCTTGTTTTTCATGC	57
Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase	HCT	AT5G48930	F: AACCTGGTGCTGCAGATACT R: TAGCTGACTGTATTCCCGGC	57
Phenylalanine ammonia-lyase	PAL	AF494403	F: GGAGCTTCACCCCTCAAGAT R: TCAATGGATAAGTGGCGCTG	57
Cinnamate-4-hydroxylase	C4H	AT2G30490	F: TGGTGAATGCTTGGTGGTTG R: GTTAGCCTCCACCTTCGACT	57
AMP-dependent synthetase and ligase family protein		AT4G05160	F: CAGCTGAACTTGAAGGGCTG R: GTACACAATATGCAGCGGGG	59
Polygalacturonase inhibiting protein	PGIP	DQ185063	F: ATCTCTCCCACAACCAGCTC R: TATCACGGATGCGTCACCTT	57
1-aminocyclopropane-1-carboxylate oxidase	ACO	Q00985	F: GAGCTGGTGAGTCATGGGAT R: GCTCCTTGAACCTTTGCTCC	59

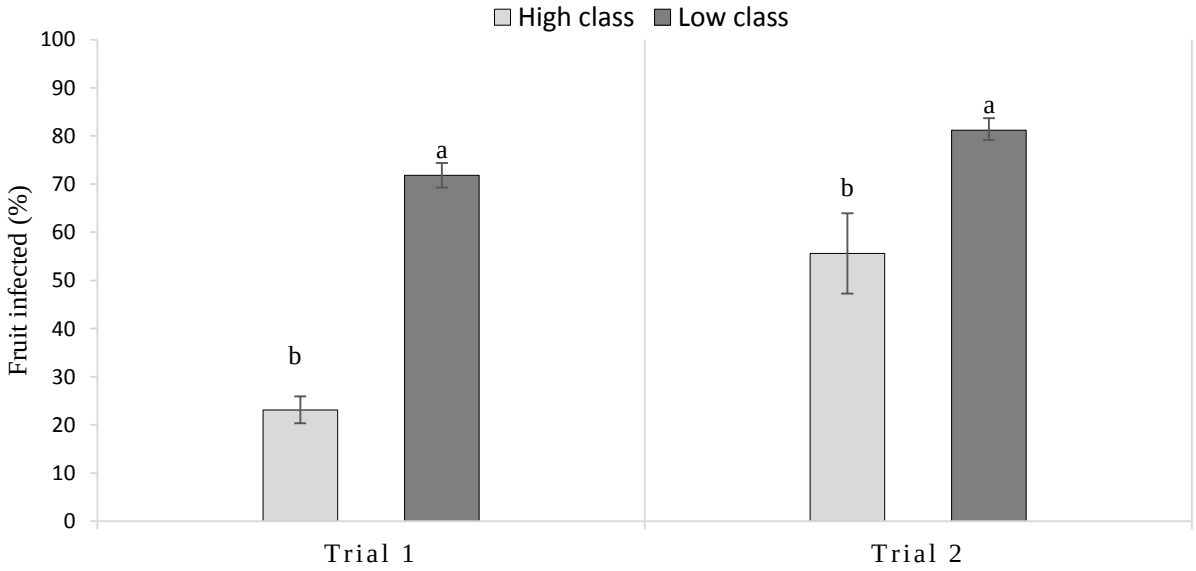

Figure 1 *BER incidence in Pink Lady™ apples after five months of storage at 0°C divided according to I_{AD} value. BER incidence values are the means of three replicates of 75 fruits ± standard error. Within each orchard according to LSD test columns with different letters are significantly different at P < 0.05.*

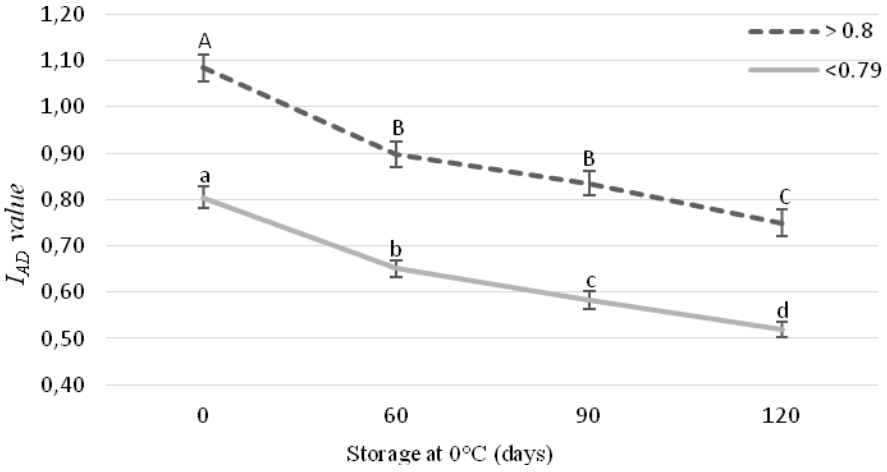

Figure 2 *Changes of Index AD (I_{AD}) during storage at 0°C. Each value is the mean of 3 replicates of 20 fruits each ± SE and within the same I_{AD} class different letters represent significant differences according to LSD test (P<0.5).*

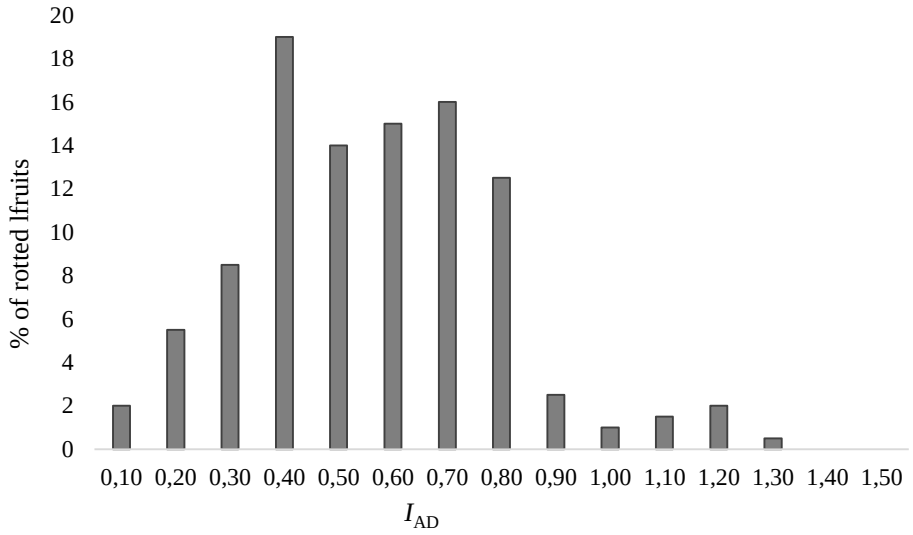

Figure 3. *DA-index (I_{AD}) in correspondence to BER symptoms appearing. Bars represent the percentage of 200 symptomatic fruits in each DA-index value from 0.1 to 1.4.*

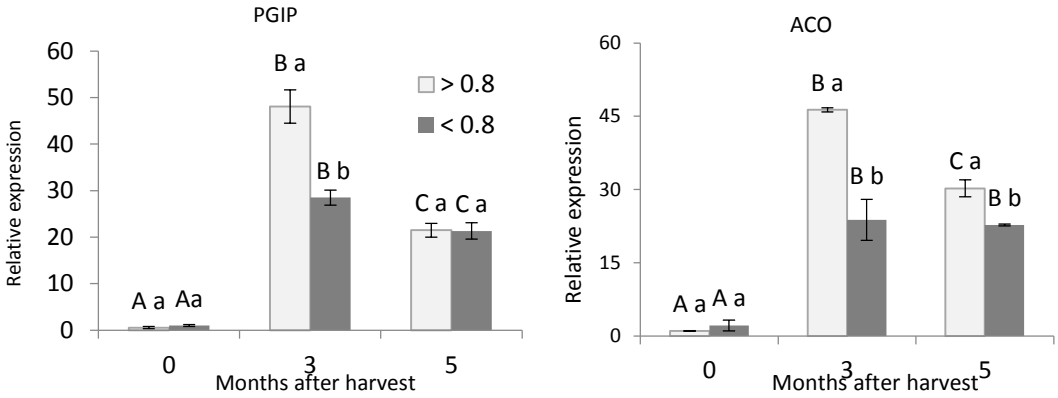

Figure 4. *Relative expression levels of genes of Polygalacturonase-inhibiting protein (PGIP) and 1-Aminocyclopropane-1-carboxylate oxidase (ACO) in 'Cripps Pink' apple belonging to DA classes selected. Apple fruit were harvested and stored in semi-commercial condition at 0°C. Sample fruit were analyzed at harvest time, after 3 and 5 months of storing period. All values were normalized using actin housekeeping gene. Each value is the mean of e replicates of 3 fruit each ± SD. Within the same point (lower case) and within the time storage course (upper case) different letters represent significant differences according to LSD test ($P < 0.05$).*

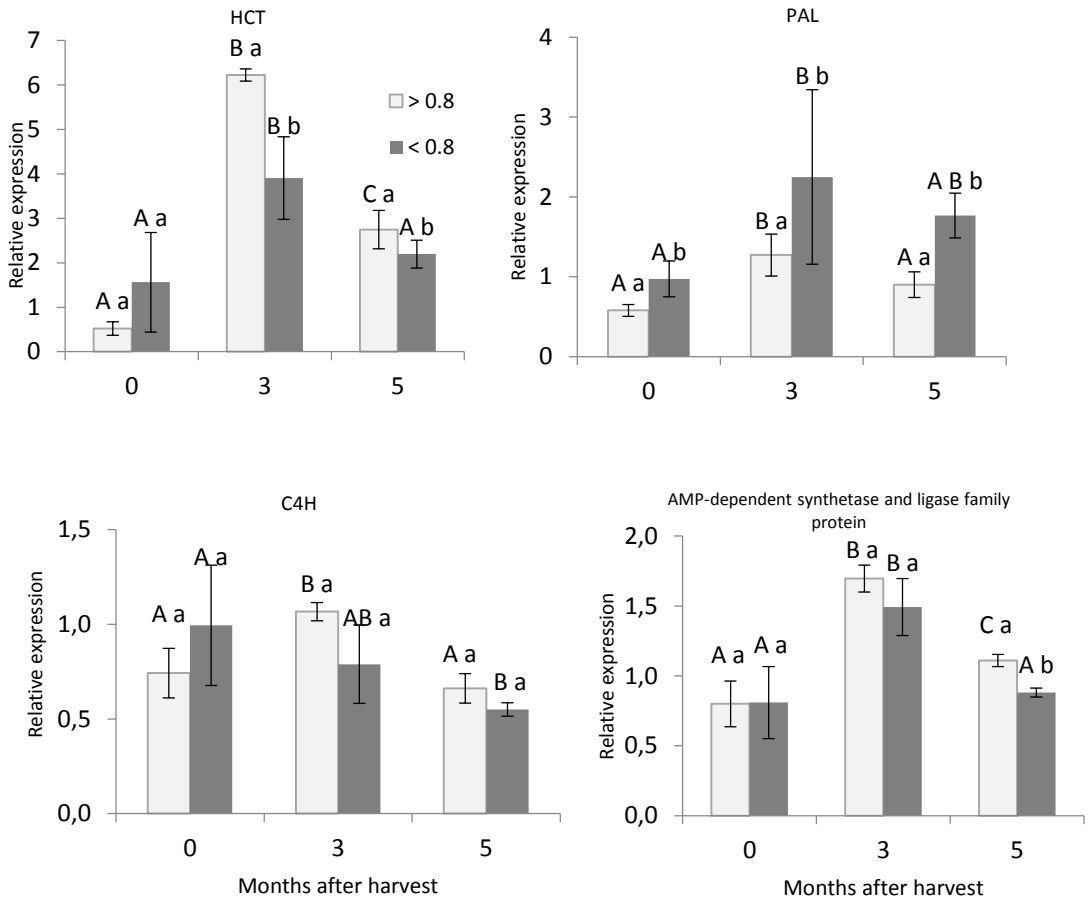

Figure 5. *Relative expression levels of genes of Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), Phenylalanine ammonia-lyase (PAL), Cinnamate-4-hydroxylase (C4H), AMP-dependent synthetase and ligase family protein, in 'Cripps Pink' apple belonging to DA classes selected. Apple fruit were harvested and stored in semi-commercial condition at 0°C. Sample fruit were analyzed at harvest time, after 3 and 5 months of storing period. All values were normalized using actin housekeeping gene. Each value is the mean of e replicates of 3 fruit each ± SD. Within the same point (lower case) and within the time storage course (upper case) different letters represent significant differences according to LSD test (P < 0.05).*

Characterization of *Neofabrea alba* volatile profile: preliminary study for an early detection of Bull's eye rot

Introduction

Microorganisms, such as bacteria and fungi emit a large spectrum of volatile organic compounds (VOCs) (Mc Neal and Herbert, 2009). VOCs are carbon-based solids and liquids that readily enter the gas phase by vaporizing at 0.01 kPa at a temperature of approximately of 20 °C (Pagans et al., 2006). About 250 VOCs have been identified from fungi where they occur as mixtures of simple hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including, among others, benzene derivatives, and cyclohexanes (Chiron and Michelot, 2005; Korpi et al., 2009; Ortiz-Castro et al., 2009). Fungal VOCs are derived from both primary and secondary metabolism pathways (Korpi et al., 2009) and because VOCs can diffuse through the atmosphere and soil, are ideal "infochemicals". Recently they were used as ecological biomarker to identify different fungal groups (Müller et al., 2013), moreover a their practical use for the detection of many fruit disease was investigated. Blasioli et al. (2014) found specific volatile disease markers for two important potato diseases: 1-hepten-3-ol, 3,6- dimethyl-3-octanone, 3-ethyl-3-methylpentane, 1-chloroctane, and benzothiazole were detected in potato affected by brown rot (*Ralstonia solanacearum*), whereas 2 propanol and toluene were markers of potato ring rot (*Clavibacter michiganensis* subsp. *sepedonicus*). Moalemiyan et al. (2007) identified two molecules:1-pentanol and thujol, produced only in infected in mango inoculated with *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides* respecetvely. Others potential

biomarkers were found for *Botrytis cinerea* strawberry infection (Vandendriessche et al., 2012), however, 2-methyl-1-butanol, 2-methyl-1-propanol, 1-octen-3-one and 1-octen-3-ol were detected only in high infection stage of strawberries.

Neofabraea alba is the causal agent of Bull's eye rot (BER), one of the most important fungal pathogen for apple production during post-harvest phase. BER is a latent infection, which has the characteristic to develop symptoms after 90 days from harvest, during commercial storage at 0°C. BER symptoms consist of circular necrotic lesions that are usually flat or slightly concave and generally dark and firm, under humid conditions, conidia and acervuli are produced in a waxy matrix (Verkley, G. J., 1999). This disease is particularly serious for 'Pink Lady™' apples, because this cultivar is characterized by a long time of storage (Lopez et al., 2007), and in favorable years the product losses can overcome 40% of stored apple (Cameldi et al., 2014). The economic damages, due to the wastage of apple within storage can be dramatic. Since fruits appear completely symptomless at harvest, it could be very useful to identify a volatile biomarker able to detect BER before symptoms appearing. The aim of the present work was to define the volatile profile of *N. alba* on two different media, in order to identify volatile markers for the detection of this important plant disease. To reach this goal the application of combined GC-MS and PTR-MS was applied. Among techniques currently available, solid phase micro extraction coupled to gas chromatography–mass spectrometry (SPME/GC-MS) is routinely applied as a solvent less method for collecting and analysing volatiles in static headspace, and proton transfer reaction–time of flight–mass spectrometry (PTR-ToF-MS) is a new technology that allows real-time analysis without pre-concentration and chromatography (Blake et al., 2009; Biasioli et al., 2011). Two different growth media were used in this study to test the effect of the substrate

composition on the VOCs produced by *N. alba*: PDA and tomato agar (TA). PDA was chosen because is a high nutrient media with dextrose and potato starch as carbon sources, commonly uses for many fungi. TA was chosen because previously test (data not reported) showed that this medium improve *N. alba* sporulation capability and mycelial growth.

2. Materials and methods

2.1. Pathogen and samples preparation

N. alba strain ID02, was grown on tomato agar (500 g of tomato sauce, 15 g of agar technical and 500 ml of distilled water) at 15°C for two weeks. In order to obtain a conidia suspension dishes were washed by sterilized distilled water containing Tween 80 0.05% (V/V) and the suspension was adjusted to a final concentration of $1 \cdot 10^4$ conidia per mL. Aliquot of 100 µl of the conidia suspension was transferred into 20-mL vials (Supelco, Bellefonte, PA), containing 4.5 mL of PDA or tomato agar and incubated at 15°C for different time with respect the type of analysis, in particular 3, 20 and 30 days for GC-MS analysis and 5, 7 and 14 days for PTR-MS. The control was represented by not inoculated PDA and tomato agar vials. Sample unit was represented by 4 vials.

2.2. SPME GC-MS analysis

Headspace volatile compounds were collected by a 2 cm Solid Phase Microextration fibre coated with divinylbenzene/carboxen/polydimethylsiloxane 50/30 lm (DBV/CAR/PDMS, Supelco, Bellefonte, PA, USA), inserted through a Teflon/silicone septum using a manual holder (Supelco, Bellefonte, PA, USA). The fibre was exposed to the *N. alba* VOCs emission for 30 min. Volatile compounds adsorbed on the SPME fibre

were desorbed at 250°C in the injector port of a GC interfaced with a mass detector operating in electron ionization mode (EI, internal ionization source; 70 eV) with a scan range from *m/z* 35–300 (GC Clarus 500, PerkinElmer, Norwalk CT, USA). Separation was achieved on an HP-Innowax fused silica capillary column (30 m, 0·32 mm ID, 0·5 lm film thickness; Agilent Technologies, Palo Alto, CA, USA). The GC oven temperature program consisted in 40°C for 3 min, then 40–220°C at 4°C min-1, stable at 220°C for 1 min, and then 220–250 at 10°C min-1, and finally 250°C for 1 min. Helium was used as the carrier gas with a constant column flow rate of 2 mL min-1. Compounds identification was based on mass spectra matching with the standard NIST05/Wiley98 libraries and retention indices (RI) of authentic reference standards.

2.3. PTR-TOF-MS analysis

Measurements of the headspace of *N. alba* sample vials were performed with a commercial PTR-TOF 8000 apparatus from Ionicon Analytik GmbH (Innsbruck, Austria). During measurements 120 sccm (Standard Cubic Centimeters per Minute) of zero air was continuously injected into the vial through a needle and the outflow going through a second heated needle was delivered via Teflon fittings to the PTR-ToF-MS. Each measurements lasted for 3 minutes and automatic switching of vials was done using an adapted GC autosampler (MPS Multipurpose Sampler, GERSTEL). The ionization conditions in the PTR-ToF-MS drift tube were the following: 110 °C drift tube temperature, 2.30-mbar drift pressure and 480-V drift voltage. This led to an E/N ratio of about 135 Townsend (1 Td = 10_17 cm2 V_1 s_1). The inlet line consisted of a PEEK capillary tube (internal diameter 0.04 inches) heated at 110 °C. The inlet flow was set at

90 sccm and 50 sccm of argon was added to the gas mixture from the sample headspace. The ions exiting the drift tube were detected using a time-of-fight (ToF) mass analyzer operated in its standard configuration (V mode). The sampling time per channel of ToF acquisition was 0.1 ns, amounting to 350,000 channels for a mass spectrum ranging up to m/z = 400. Every single spectrum is the sum of about 28,600 acquisitions lasting 35 □s each, resulting in a time resolution of 1s.

2.4. Data analysis

Dead time correction, internal calibration of mass spectral data and peak extraction were performed according to a procedure described by Cappellin et al. (2010 and 2011), using a modified Gaussian peak shape. Peak intensity in ppbv was estimated using the formula described in literature (Lindinger et al., 1998), using a constant value for the reaction rate constant coefficient (k = 2 × 10_9 cm3 s_1). This introduces a systematic error for the absolute concentration for each compound that is in most cases below 30% and could be accounted for if the actual rate constant coefficient is available (Cappellin et al., 2012). All data detected and recorded by the PTR-TOF-MS were processed and analyzed using MATLAB (MathWorks, Natick, MA) and R (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1 SPME-GC/MS analysis

N. alba on TA favored the development of a more complex volatile blend composed by 25 compounds (Tab. 1) respect to the volatile blend obtained on PDA composed by 17 and 14 VOCs after 20 and 30 days respectively (Tab.2). After 30 days of incubation, 3-methyl-1-butanol (2.6E+10) and phenylethyl alcohol (1.8E+10) were the main VOCs

emitted by *N. alba* grown on TA, followed by 2-methyl-1-propanol. The compounds 1-propanol, 2,3-dihydro-benzofuran, 2-ethyl-1-hexanol, 2-furanmethanol, benzyl alcohol, 2,3-dihydro-benzofuran, styrene, 1-hexanol were also emitted by pathogen grown on TA (Tab.1), while ethanol (1.09E+10) and phenylethyl alcohol (3.2E+9) followed by 2-methyl-butanal and ethyl acetate were the main VOCs emitted by pathogen grown on PDA (Tab. 2). The concentration of 6-methyl-5-hepten-2-one, typically detected in headspace from uncultured TA, showed a strong decrease in vials inoculated with *N. alba*. In *N. alba* grown on TA, the concentration of the compounds 3-methyl-1-butanol, phenylethyl alcohol, 2-methyl-1-propanol and 2-ethyl-1-hexanol increased during the whole incubation time and 3-methyl-1-butanol was the main compound emitted from *N. alba* after 23 and 32 days of incubation at 15°C (Figure 1). The concentrations of the compounds styrene (the main compound emitted after 3 days of incubation) and 2-methoxy-phenol showed decreasing pattern after 20 and 30 days of incubation. Among the all VOCs identified by GC-MS, 16 compounds were detected only in cultured media, 15 compounds were emitted in TA and 1 in PDA (Tab.3).

3.2 PTR-MS analysis

PTR-MS allowed to quantify the VOCs emitted by *N. alba* showing that the abundance of each organic compounds produced changed according to the length of incubation time, and cultural medium (Fig.5. and Fig 6.). Thirty-two masses were detected by PTR-MS in volatile emission from *N. alba*, however only 7 compounds were identified. After five days of incubation the major compounds produced in TA were methanol (53.7 ppbv), followed by mass 44.99 (unknown) (22.3 ppbv) and 1-butanol (9.9 ppbv), while in PDA the major compounds produced after 5 days of incubation were propanal (18.6 ppbv), mass 44.99 (12.8 ppbv) and acetaldehyde (12.0 ppbv). After 7 days of incubation, the

most abundant compounds produced in TA were acetaldehyde (284.9 ppbv), ethanol (118.8 ppbv) and methanol (75.8 ppbv). In PDA after 7 days of incubation, the main VOCs produced were ethanol (84.3 ppbv), acetaldehyde (79.9 ppbv) and mass 44.99 (75.0 ppb). After 14 days of incubation the most abundant VOC produced in TA was ethanol (252.3 ppbv), followed by acetaldehyde (249.7 ppbv) and methanol (161.5 ppbv), while the most abundant VOCs emitted by *N. alba* grown in PDA were acetaldehyde (46.8 ppbv), mass 44.99 (42.2 ppbv) and ethanol (23.1 ppbv). Changes in VOCs abundance were observed also during the *N. alba* development, for some compounds the highest production was observed after 7 days of incubation (acetaldehyde in TA) (Fig.5.) and the all three most abundant VOCs emitted in PDA (Fig. 6.). Others important for their abundance VOCs were 1-butanol in both cultural media and propanal in PDA only.

4. Discussion

N. alba is one of the most important apple pathogens causing latent infections and such as other fungal pathogens, growing produced VOCs. Similarly, *Colletotrichum gleosporioides*, *Lasiodiplodia theobromae* of mango, agents of anthracnose and stem-end rot respectively (Moalemiyan et al., 2007) and *Botrytis cinerea* in strawberry (Vandendriessche et al., 2012) induced emission of chemical biomarkers. However, the disease detection performed by identification of volatile emissions by fungi was possible only at high level of infection, while no pathogen VOCs markers were recorded at low disease severity. In the present study, the preliminary data referred the VOCs production by *N. alba* grown onto two artificial medium: PDA and TA. Cultural media and time of incubation influenced *N.alba* volatile emission. The VOCs production by microorganisms was mainly investigated as mechanism of action of some antagonists. For example, *Muscodor albus* was recognized as fungal antagonist that produced a wide

number of VOCs as Propanoic acid, 2-methyl-,methyl ester, Methyl isobutylketone , Propanoic acid, 2-methyl-,methyl ester (Ezra and Strobel, 2003). In addition, it showed the capability to change volatile blend relating to substrate of growth emitting five different volatile blend when inoculated in five different growth media (Ezra and Strobel, 2003) with a different biocontrol effect. *M. albus* VOCs in fact, have a fungistatic effect on many seeds fungal pathogens, and this effect changed the intensity according to different volatile blends emitted (Ezra and Strobel, 2003). Another biological control agent *Aureobasidium pullulans* produced VOCs such as (1-Butanol,2-methyl, 1-Butanol, 3-methyl, 1-Propanol, 2-methyl, Phenethyl alcohol) active *in vivo* and *in vitro* trials against some important fruit postharvest pathogens (Di Francesco et al., 2015). From our data, the *N. alba* growth on TA favored the development of a more complex blend, with respect to growth on PDA. Alcohols were the main compounds emitted by *N. alba* both in TA and PDA, in particular 3-methyl-butanol in TA and ethanol in PDA; while the phenylethyl alcohol was emitted by *N. alba* in both cultural media. Few compounds of other chemical classes were emitted: a furan (2,3-dihydro-benzofuran) and an aromatic hydrocarbon (styrene) in TA, and an aldehyde (2-methyl-butanal) and an ester (ethyl acetate) in PDA. The analysis in PTR-MS detected more volatile masses (32 masses) than SPME-GC-MS (25 compounds on TA and 14 compounds on PDA, however only 6 compounds were identified by PTR-MS. PTR-MS technique permits a reliable quantification in ppbv of VOCs. Results obtained showed that the most abundant compounds produced by *N. alba* were ethanol, acetaldehyde, mass 44.99, and 1-butanol in tomato agar (Fig. 5), and acetaldehyde, mass 44.99, ethanol, propanal and 1-butanol in PDA. Among VOCs identified by PTR-MS, butyl propanoate was emitted by *N. alba* grown on TA only, while propanal was emitted by *N. alba* cultured only on PDA. In this

work 16 VOCs were identified as potential biomarkers compounds and some of them were identified also as potential biomarker for others plant disease. Among the potential biomarker for *B. cinerea* detection, Vandendriessche et al. (2012), include 2-methyl-1-propanol. The same molecule was detected in cultured and uncultured of both PDA and TA, however, comparing the areas of controls and cultured media, the production resulted higher in cultured media. Also styrene can be included among the potential biomarker of BER; previously it was detected in mango fruits infected by *C. gleosporioides*, *L. theobromae* (Moalemiyan et al., 2007) and in PDA inoculated by *Ralstonia solanacearum* (Blasioli et al., 2013). The new technologies as PTR-MS coupled with SPME GC-MS seem useful to detect and analyze qualitatively and quantitatively VOCs emission. Their application aimed to identify pathogen volatile markers, for a finding of plant diseases seems of increasing interest. Further studies, based on pathogen VOCs recognizing, should be addressed to develop a strategic tool for an early detection of BER and other latent infections.

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Table 1 *VOCs emitted by Neofabrea. alba grown onto tomato agar for 3, 20 and 30 days at 15°C*

Volatile organic compound identified By SPME GC-MS	Area at different length of incubation (days)		
	3	20	30
1-propanol	547460384,8	95263886,29	854134188,9
2-methyl-1-propanol	482047793,8	958115124,4	540106834
3-methyl-1-butanol	3711862200	13789667263	26251918870
Styrene	110073816231	555902741,7	325785703,2
Acetoin	81891894,85	52373293,34	22229249,7
4-Penten-1-ol	63731023,15	30836215,23	40662446,07
4-methyl-1-Pentanol	141361954,1	17152186,4	40279316 5,4
3-methyl-1-pentanol	306879766,7	77087029,1	41733260 9,1
6-methyl-5-hepten-2-one	80822061 8,5	73980101 7,5	15965525 4,9
1-hexanol	510604 80,19	89596012,41	32027587 1,6
4-methyl-3-penten-1-ol	246674404,2	15926117 0,1	35047282 5,2
2-isobutylthiazole	549965 38,08	0	19276566,39
6-methyl-5-hepten-2-ol	576520367,7	102656 1060	19884180 2,2
2-ethyl-1-hexanol	744640 27,79	244627 28,73	647869 913,1

1-(2-furanyl)-ethanone	7360043 1,41	2749534 9,93	7135410 8,22
2-(methylthio)-ethanol	9428230 4,02	1049246 70,5	1087838 49
2-furanmethanol	1561308 53,9	3046669 79,9	3737763 68,4
(E)-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	6238965 37,2	1670719 5,52	2101224 2,16
2-methoxy-phenol	1914316 723	1213693 65	6922693 7,64
1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester 2-methyl-propanoic acid	1456661 91,4	1596311 04,3	1556636 46,3
Benzyl alcohol	1805616 63,7	3977161 01,4	3878254 87,5
Phenylethyl alcohol	1156988 601	5182244 380	1871170 8970
6-pentyl-2H-Pyran-2-one	4426084 029	3353673 854	4065649 174
2-methoxy-4-vinylphenol	2388672 11,6	4083578 31,1	6699523 3,62
2,3-dihydro-benzofuran	5684157 63,4	1461000 421	2985078 14,6

***Table 2**. VOCs emitted by* N. alba *grown onto PDA for 20 and 30 days at 15°*

Volatile organic compound identified by SPME GC-MS	Area at different length of incubation (days)	
	20	30
2-methyl-propanal	49454462,9	0
Acetone	128199124,2	0
Hexamethyl-cyclotrisiloxane	204634218	222913434
Ethyl acetate	528974297	611289895,8
2-butanone	37244380,6	19765432,15
2-methyl-butanal	183566635,9	71130249,43
3-methyl-butanal	230140125,6	120389858,7
Ethanol	6623213575	10955043106
Octamethyl-cyclotetrasiloxane	58784112,98	69025547
2-methyl-1-propanol	1022108964	1414378211
Tetradecamethyl-hexasiloxane	245413531,2	344050421,5
1-ethenyl-4-methoxy-benzene	451291989,6	270866162,8
1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester 2-methyl-propanoic acid	174276073,2	158368963,1
Benzyl alcohol	61016346,91	40143619,3
Phenylethyl alcohol	2721760001	3215582266
6-pentyl-2H-Pyran-2-one	1949403081	1561482114
2-methoxy-4-vinylphenol	7717198,8	0

***Table 3**. VOCs detected by GC-MS only in cultured media PDA and Tomato Agar*

	Retention time (minutes)	PDA	Tomato agar	Probability %
1-Propanol	**6.1**		**x**	**96.8**
Styrene	12.56		x	61.3
4-Penten-1-ol	13.91		x	75.6
4-methyl-1-pentanol	**14.25**		**x**	**80.4**
1-hexanol	15.33		x	59.4
4-methyl-3-penten-1-ol	16.31		x	73.4
6-methyl-5-hepten-2-ol	18.28		x	73.8
2-ethyl-1-hexanol	18.97		x	62
2-(methylthio)-ethanol	**19.96**		**x**	**97.5**
2-furanmethanol	23.14		x	76.9
2-methoxy-phenol	27.6		x	76
1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester 2-methyl-propanoic acid	27.9	x		77.2
Benzyl alcohol	27.92		x	65.4
2,3-dihydro-benzofuran	37.78		x	49.7

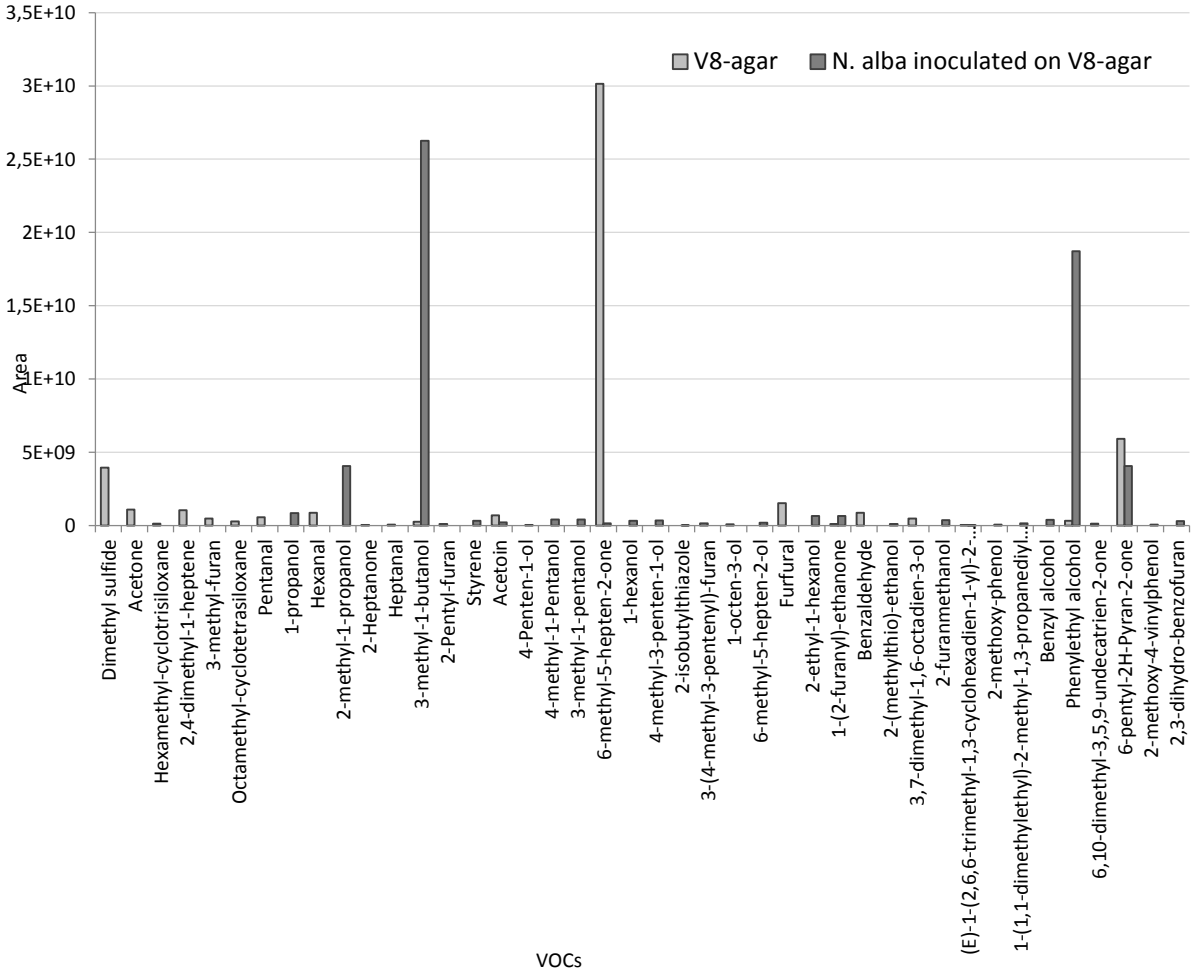

Figure 1. *VOCs emitted after 30 days of incubation at 15°C by tomato agar and tomato agar inoculated with N. alba*

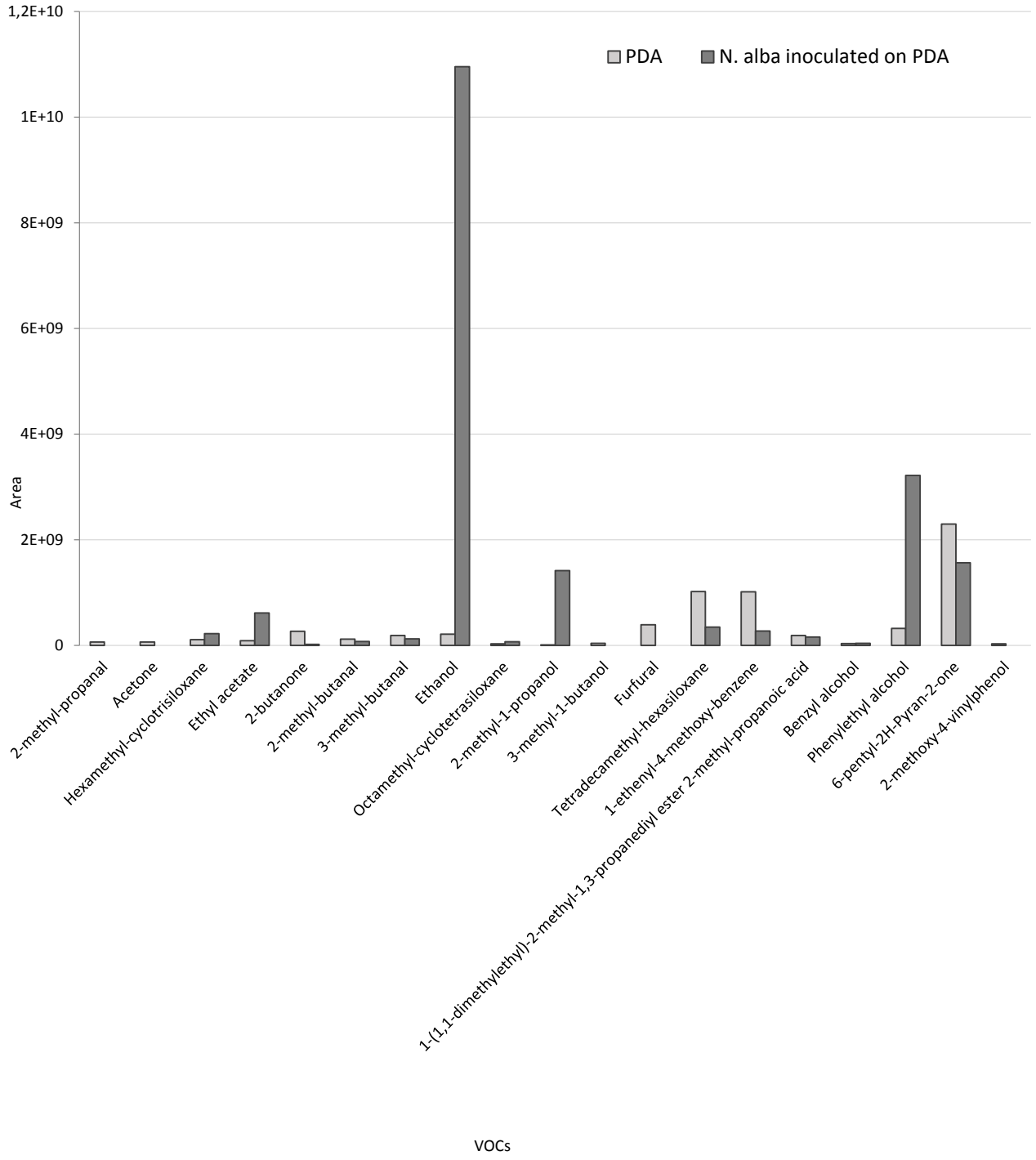

Figure 2. *VOCs emitted after 30 days of incubation at 15°C by PDA and PDA inoculated with N. alba*

General Conclusions

The present study focused on lenticel rot of pome fruits, known as Bull's eye rot (BER). The research activity evaluated different aspect of this disease, two aspects connected to the interaction between the host and the pathogen, and two aspects connected to the disease management and detection.

Findings obtained showed that in 'Pink Lady™', *N. alba* infection is delayed by malic acid, the most abundant primary metabolite after sugars. Malic acid content decreased during the hall life cycle of apple, from two months after full bloom, until the end of post-harvest life. During post-harvest, the malic decreasing raises especially from 4 months after harvest, when BER symptoms reach 30%. Bioassays performed on artificial medium amended with malic acid, confirmed the inhibition effect on *N. alba*.

The study conducted on five commercial apple cultivar showed that also the pH value of apple could be considered as factor affecting BER development. Granny Smith resulted as the less susceptible cultivar, while Gala Schniga as one of the most susceptible. The pH during storage of these two varieties have different values, and pH of Granny Smith remain always lower than Gala Schniga pH. Moreover, in this study, the capability of *N. alba* to modify the ambient pH was investigated. Results showed that the pathogen was able to increase the pH in all tissues infected. The magnitude of alkalizing effect was higher in less susceptible cultivar as Granny Smith and Fuji, where *N. alba* increased the host pH of 1.52 points.

With the purpose to find out alternative method for BER management, a non-destructive device able to detect the chlorophyll content in apple fruit was applied at harvest. 'Pink Lady™' apple were divided in two groups according to their chlorophyll content index,

expressed as I_{AD}. Results showed that apples with low I_{AD} (low chlorophyll content) value are significantly more susceptible to *N. alba* than apple with high I_{AD} value. After five months at 0°C, the percentage of BER in the low and high classes was 71.8% and 23.15 % respectively in the trial 1, and 55.6% and 81.2% in the trial 2. Relative gene expression level of seven genes involved in plant defence were studied through qRT-PCR, and results obtained showed that PGIP and HCT genes are up-regulated in the apple with high chlorophyll content. This two genes regulate two important pathway of plant defence, the production of protein that inhibit fungal enzymes, and the production of phenylpropanoids, phenolic compound involved in many plant-microbe interaction.

The last part of the research work performed aimed to identify volatile organic compounds (VOCs) marker able to detect the presence of *N. alba* infection. As potential biomarkers compounds, 16 VOCs were identified: propanol, styrene, 4-penten-1-ol, 1-hexanol, 4-methyl-3-penten-1-ol, 6-methyl-5-hepten-2-ol, 2-ethyl-1-hexanol, 2-(methylthio)-ethanol, 2-furanmethanol, 2-methoxy-phenol, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester 2-methyl-propanoic acid, benzyl alcohol and 2,3-dihydro-benzofuran. However, this study is not completed, and VOCs emitted by inoculated fruits have to be still investigate.

In conclusion we can assert that during the PhD activity several factor affecting BER development were verified:

- Chlorogenic, quinic, and malic acid inhibithion effect
- Ambient pH affecting *N. alba* virulence
- Involvement of PGIP and HCT genes in apple defence mechanism

Moreover, new insight on biological characteristic of the pathogen were clarify.