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Chlorogenic acid impact on Monilinia Fructicola liquid culture and cutinase production

RAPPORT DE STAGE – GAFL – INRAE

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INRAE la science pour la vie, l'humain, la terre

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GIS Fruits

Abstract :

Monilinia fructicola, a pathogenic fungus of prunus genus, is responsible for brown rot disease, one of the most important disease regarding fruit economy. This study tries to find durable way to fight against this disease by exploiting natural phenolic compounds of peach flesh. We observed the effect of chlorogenic acid (ACQ) on *M. fructicola* pathogenic capacities by adding it to a liquid culture. Fungal biomass, medium protein content and ACQ stability in the medium were explored and proteomic profiles and targeted gene expression analyses were conducted. ACQ resulted in an increase of the mycelium biomass production as well as of the protein amount. The abundance of proteins secreted by the fungus in the medium was diversely impacted by ACQ. Cutinase abundance was reduced and RT-qPCR results showed a reduction of cutinase gene expression. The expressions of three polygalacturonase genes were also decreased. We observed medium acidification along the culture and demonstrated by HPLC analysis that *M. fructicola* induced ACQ degradation. The fungus probably used ACQ derivates as carbon source. These observations indicate that ACQ or its derivates can modify pathogenetic factors by modulating gene expression resulting in a modification of enzyme secretion.

Résumé :

M. fructicola, un champignon pathogène du genre prunus, est responsable de la pourriture brune, une des maladies des arbres fruitiers les plus impactante d'un point de vue économique. Cette étude tente de trouver des moyens durables de lutter contre cette maladie en exploitant les composés phénoliques naturels présents dans la peau de la pêche. Nous avons observé l'effet de l'acide chlorogénique (ACQ) sur la pathogénicité de *M. fructicola* en l'ajoutant au milieu de culture. La biomasse fongique, le contenu en protéine du milieu et la stabilité de l'ACQ dans le milieu ont été explorés, et des profils protéomiques et analyses d'expression génique ciblées ont été menées. L'ACQ a entraîné une augmentation de la production de mycélium ainsi que de la quantité de protéines. L'abondance des protéines sécrétées par le champignon dans le milieu a été diversement impactée par l'ACQ. L'abondance de la cutinase a été réduite et les résultats de la RT-qPCR ont montré une réduction de l'expression du gène de la cutinase. Les expressions de trois gènes de polygalacturonase ont également été diminuées. Nous avons observé l'acidification du milieu le long de la culture et démontré par analyse HPLC que *M. fructicola* induisait une dégradation de l'ACQ. Le champignon a probablement utilisé des dérivés de l'ACQ comme source de carbone. Les résultats obtenus indiquent que l'ACQ ou ses dérivés peuvent modifier la pathogénicité en modulant l'expression génique, entraînant une modification de la sécrétion d'enzymes.

Ce projet a été soutenu financièrement par le GIS Fruits. Les travaux ont été menés en 2020 au GAFL à INRAE d'Avignon en partenariat avec le GRAB et le Pôle Terralia

Laboratory introduction

The GAFL Unit, located at “Saint Maurice” domain in Avignon is directed by Catherine Dogimont. The unit is dedicated to the study of Mediterranean species. Four principals research domains are explored: species diversity and domestication process; genetic and molecular resources of fruit quality; functional characterization of plant-pathogen interactions and resistance sustainable management; integration of disease resistance and fruit quality in cultivars by varietal innovation. This unit hosts the Biological Resource Center (CRB) for Solanaceae plant family (eggplant, pepper, tomato), cucurbits, lettuce, and is a branch of the INRAE CRB-Prunus. Plant amelioration, breeding and studies need to conserve a large diversity inside the species and their relatives for further research. Many greenhouses are implanted on the site and climatic chambers also for plant and insect cultures (Aphid mainly). We had access to the cellular and molecular biology platform, where the mycology lab and all related equipment were disposed.

My internship took place in this unit, in the research team named: Diversity, Adaptation, Determinants and Integration (DADI) led by Quilot-turion Bénédicte and Causse Mathilde. Team’s studies are linked to the multi-character integration of traits related to fruit quality, resistance to pests and production regularity. I mainly worked with my internship supervisor: Corre Marie-Noelle (engineer assistant, specialized in HPLC method). I also had the opportunity to work with Heurtevin Laure (a molecular biology technician) and Signoret Veronique (the peach experimental manager) and a PhD student from Italy in alternance in the team, working on *Monilinia spp.* One general objective of the work carried out by the team for many years is to contribute to the development of sustainable ways to fight against brown rot disease caused by *Monilinia spp*. This disease is a major economic problem and very few organic ways exist to fight against this disease.

My work was based on the results of the MoniBreF project (funded by Agropolis Foundation) which focused on a peach population resulting from a cross between 2 varieties, one wild (*Prunus davidiana)* and the other domesticated (Summergrand variety) and displaying a great variability in susceptibility to brown rot. The objective was to explore the effect on *Monilinia Fructicola* of the chlorogenic acid, a phenolic compound found in peach epidermis, developing liquid cultures.

Acknowledgments

I would like to express my best appreciation to all GAFL members whom have welcome me friendly and have clearly participated to my good integration in the unit.

Very special acknowledgment to Marie-Noelle Corre, my main supervisor, with whom I spent a lot of time. Thanks for your knowledge transmission's, your motivation and serenity.

A particular attention to Benedicte Quilot-Turion with your smiling organization, precious help for statistical mystery and your attentive last moments helping hand.

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Thank you for giving me all this confidence and autonomy during my internship.

A big thank you to all other GAFL members to have participate to this ambiance, and activity during work break or other. Thank you to all surface technician for your work and smile.

I'm very grateful to GIS fruits, whom have funded my internship. I express my acknowledgment to all partners, GRAB and Terralia. Thank you to Bordeaux University, Science et techno and the Mycology and phytopathology master for all you support and knowledge acquired during the formation.

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Abbreviation list

F : Monilinia. fructicola specie

ACQ : Chlorogenic acid

ANOVA : Statistical model for variance analysis

ARN : Ribonucleic Acid

BSA : Bovine Serum Albumin

CaCO3 : Calcium Carbonate

CZAPEK : Liquid medium for mushroom cultivation

Ct = Cycle threshold

DADI : Diversity, Adaptation, determinant and integration

EtOH : Ethanol

FAOSTAT : Food and Agriculture Organization Corporate Statistical Database

FeSO4.7h2O : Ferrous sulfate(II)

GAFL : Genetics and Improvement for Fruits and Vegetables

INRAE : National institute for research in agriculture, food and environment

K2HPO4 : Potassium hydrogenophosphate

KCl : Potassium Chlorure

MfCUT1 : Cutinase gene

MfPG1,3,6 : Endopolygalacturosase gene

MgSO4.7H2O : Magnesium Sulfate

PEG : Poly-Ethylene-Glycol

qPCR : Quantitative Polymerase Chain Reaction

UVProbe : Spectrophotometer software from Shimadzu

V8 : Commercial Vegetable Juice

Introduction

Prunus and peach

The Prunus genus is composed of 430 different trees and shrubs mostly cultivated for their fruits or for ornamental purpose. Many are very important in the agricultural economy, such as the almond tree (USA – 1.6Mton), peach tree (China – 14.7Mton), plum tree (China – 6.6Mton), cherry tree (Turkey 622kTon) or apricot tree (Turkey – 821kton). Fruit trees are often attacked by a large scale of diseases caused by insects, fungi or bacteria in all fruit stage development. The common response is the important use of chemical products to fight against pests. For peach fruits, 25 treatments are estimated during one season. For environmental and health reasons, other solutions are explored.

Brown rot disease

Monilinia species

Brown rot disease is caused by Monilinia fungus genus, an ascomycota belonging to sclerotinia family. This genus is composed by 35 species which attack Rosacea and Ericaceae but three are reported as most important stone fruit pathogens factor: *Monilinia fructicola*, *laxa*, and *fructigena*. (Oliveira Lino *et al,* 2016) Those species can be distinguished by morphological aspect with a trained eye (fig. 1)

Fig. 1: Peach fruit infected by three different Monilinia species.

In France, the 23 species are reported, but *M. fructicola* and *laxa* are predominant. Monilinia species infect the fruit at different stage of the fruit formation, during flowering, fruit development and after the harvest. That is why fruit brown rot is one of the most impacting disease of the cultivated fruits. The cost of losses can be huge if nothing is done to protect the culture before and after harvest. Up to 60% of the production can be destroyed by brown rot disease in 5 days at room temperature (Tosi, Spada *et al*, 1996).

Prophylaxis technics are needed to decrease brown rot development. The orchard has to be cleaned of old infected residues during winter; old twigs and mummified fruits are potential contaminant. Air

circulation and under canopy watering have to be favorized in order to limit humidity. Fungicide are very commonly used in orchards with regular application, composed with Strobilurines (cell respiration inhibitor), triazole (ergosterol production inhibitor), Hydantoin (DNA RNA synthesis inhibitor) anilinopyrimidines (protein formation inhibitor) molecules, and sulfur. Biocontrol treatment are composed of non-toxic living organisms such as bacteria (*Bacillus suptilis, Pantoea agglomerans, Epicoccum nigrum*) can protect the fruit during development and/or post-harvest period. (Yánez-Mendizábal *et al,* 2011; Janisiewicz *et al,* 2010; Lareno *et al,* 2015) *Penicillium frequentans* and *B.amyloliquefacien* can be used for control measure as antagonistic organisms on fruit. (Guijarro *et al,* 2008; Gotor-Vila *et al,* 2017).

Life Cycle

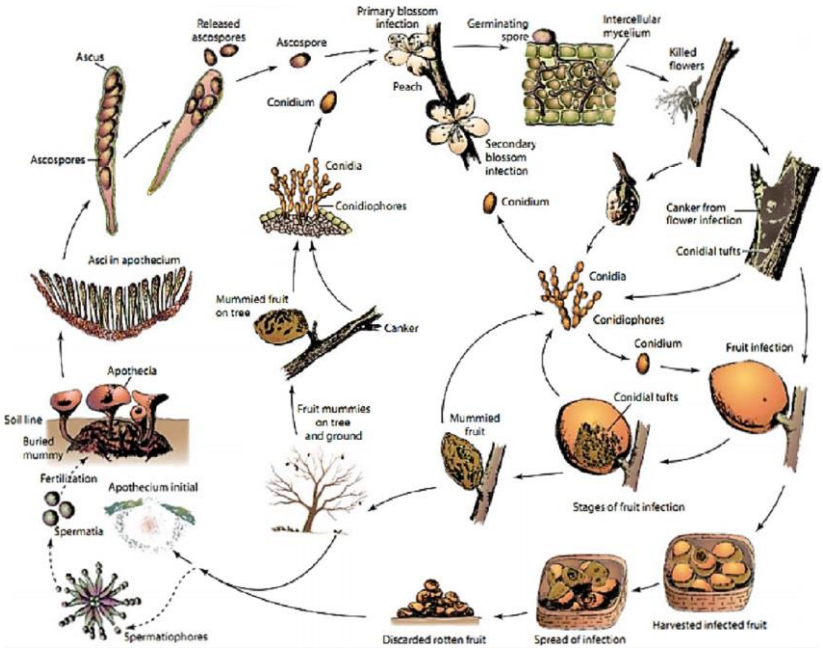

Fig 2: Monilinia spp. life cycle, from Plant Pathology

The most important way of *Monilinia* dissemination is by asexual reproduction. Sclerotia are produced at the end of the infection period when conditions are less favorable and are hidden in the mummified fruit still fixed to the tree or on the ground. When good environmental conditions appear again, asexual structures are formed on the mummified fruit and conidia are dispersed by wind or rain. Conidia can infect new formed blossoms; it is the primary infection form. Infected flowers will be killed and cankers form and produce other conidiophores which will spread conidia again for secondary infection on flowers or new formed fruits. During Monilinia development on fruits, conidia

are produced rapidly and become new sources of infection for healthy fruits. The teleomorph stage is seldom observed because of the difficult forming conditions in orchards (Holtz *et al*, 1998).

In this study, we are interested by fruit infection. *Monilinia spp.* can penetrate in many ways into the fruit; by epidermis' wounds caused by biotic and abiotic factors, by stomata, at trichrome base, or directly thought the cuticle and epidermal tissues (Bostock *et al*, 1999). The direct penetration is permitted by excretion of enzymatic material such as cutinase, polygalacturonases or pectinases (Byrde and Willetts, 1977) for degrading the cuticle and the cell wall content. *M. fructicola* is known to secrete hydrolysis pectin enzymes such as endopolygalacturosases, pectin esterase (Wade *et al*, 1992), and cutinase (Lee *et al*, 2010). It can also modify the redox state of the fruit which brings about a deregulation of the gene expression of the fruit such as genes controlling polygalacturonase enzymes. The acidification of the fruit surface also influences appressorium formation, and the fungus gene expression of cutinase and endopolygalacturosase genes (MFCUT1 and MfPG1) (De Cal *et al*, 2013; Lee and Bostock, 2007). The fruit maturity stage is a significant factor of the infection process (Mari *et al*, 200: Kreidl *et al*, 2015). During primary development steps, the fruit is photosynthetic and full of nutrients, the skin is thin and stomata used for transpiration processes could be an important entrance door for germinating conidia. Then metabolic changes occur, the pit hardens over time and secondary metabolites are produced (Biggs *et al*, 1998). An increase of terpenoids compounds is

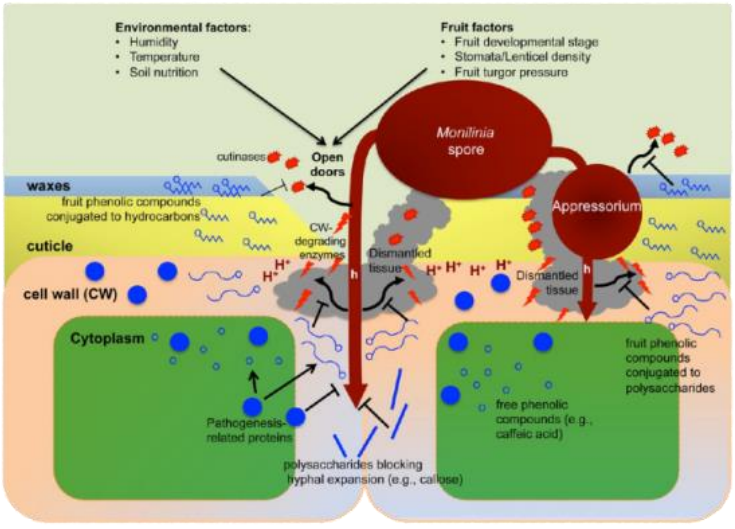

Fig 3. Monilinia fructicola strategy to penetrate into the host plant.

observed and may be related to brown rot resistance in peach fruits at this stage (Lee *et al*, 2007 Villarino *et al*, 2011; Dabbou *et al*,

2016). The susceptibility increases again during ripening period, that can be explained by sugar availability and by an important fruit radial growth that leads to skin thinning and peel cracking under favorable climatic conditions.

Role of epidermis compounds in the infection

Previous experiments in the laboratory showed an important correlation between secondary metabolites composition and fruit resistant stage (Oliveira Lino et al, 2020). At maturity stage, best resistance to Monilinia was correlated to phenolic compound presence.
Bostock et al (1999) showed that cutinase production can be reduced by chlorogenic acid. Then, they found that phenolic compounds in conidia suspension can inhibit appressorium formation (Lee and Bostock, 2007). Vilarino et al (2013) reported that contents of Neochlorogenic and Chlorogenic acids are higher in early development stage (resistant) and inhibit M. laxa melanin biosynthesis which is an important compound for appressorium formation during fungal pathogenicity. ACQ and Neochlorogenic acids are major components of fruit epidermis. These molecules and derivatives are reported to have properties, such as antioxidant and antifungal activities (Daneshtalab *et al*, 2008).

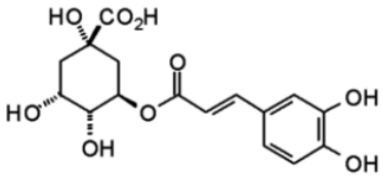

Fig 4. Acid chlorogenic molecule representation

The ACQ is a polyphenol composed by a quinic acid and a caffeic acid, link by an ester bound. In plant, this molecule is a product of phenylpropanoid pathway, and is probably involve in lignin biosynthesis (Volpi e Silva *et al*, 2019). It has antioxidant and antimicrobial properties. Other isomer of chlorogenic acid can be form during its degradation or oxidation. These compounds; as c-5CQ, c-6-CQ, t-4-CQ, c-3-CQ show anti-oxidant properties and can avoid anion radical action. (Nakatani *et al*, 2000). These results could open a selection process on peach cultivar in order to increase concentration of natural phenolic compounds in fruit exocarp to decrease peach fruit susceptibility to Monilinia infections.

General Objectives

These findings indicate a possible interference of ACQ with the production of fruit penetrating enzymes by the fungus. Thus, the objective of the study is to consolidate a fungal liquid culture experiment to analyze additional parameters in link with infection under the effect of ACQ, namely the modification of fungal biomass production, protein secretion by the fungus targeted enzymatic

activity and gene expression. The pH evolution of the culture media and the stability of the ACQ molecule in the media were also monitored.

In order to decipher the ACQ impact, different treatments were compared, with or without cutin in the media and with different concentrations of ACQ.

A proteome analysis was performed to identify the whole proteins synthesized and secreted by the fungus and to compare the amounts between treatments. On the contrary, the enzymatic and gene expression studies focused on a target: the cutinase.

Materials and methods

Experimental design

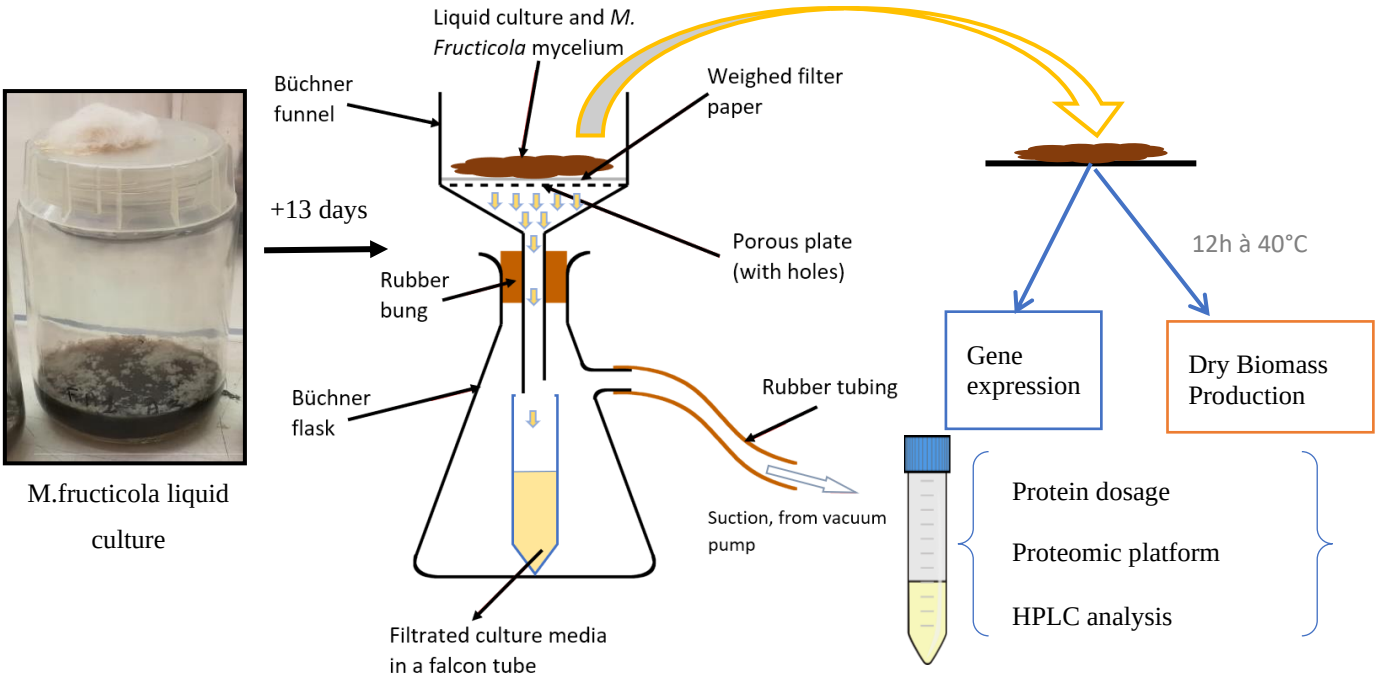

Fig. 5: Experimental design and experiences.

Liquid culture

Tests were designed in order to evaluate the impact of the chlorogenic acid on Monilinia growth and cutinase production in conditions of cutin addition. The culture was realized in autoclavable glass container with plastic pierced lids plugged with cotton for an effective respiration. Four repetitions were done, inoculated with 250µL at 10^3 spores/ml for each treatment to observe a primary effect and further experiment have been realized with 5 or 6 containers. We also tested ethanol effect on *M. fructicola* growth because during ACQ treatment solution preparation, ACQ is diluted in ethanol at 10% final concentration. Finally, 6 different treatments have been realized for ACQ experiments:

FACQ1	M. fructicola + Cutin + ACQ 1mM
FACQ2	M. fructicola + Cutin + ACQ 2mM
FA1	M. fructicola + ACQ 1mM
FCE	M. fructicola + Cutin + Ethanol 10%
FE	M. fructicola + Ethanol 10%
F	M. fructicola + 5,250ml sterile water

Table 1: Monilinia fructicola treatments composition in our experiments

Climatic chamber

Liquid cultures were placed in a culture chamber for 13 days. Growing conditions were 16h in light condition at 24°C and 8h in dark at 18°C to simulate natural cycle.

Monilinia Strains management

Monilinia fructicola conidia origin:

Monilinia strains came from previous pathogen experiment on peach fruits. These tests were done in 2018 and 2019 and conidia samples were conserved in aliquots at -20°C PEG in phosphate potassium/glycerol buffer.

M. fructicola propagation

We started cultures from these aliquots on V8 medium at 24°C on 12-12 hours light cycle. New cultures had to be cleaned because of the non-sterile aliquot preparation conditions. After few transplanting, we obtained healthy and vigorous petri dishes plates. After a pest problem (mites apparition in our incubation chamber) we moved our plates in the climatic chamber.

Conidia production

During our liquid culture preparation, we used to test the sterility of our inoculation conidia suspension by adding few microliters in a petri dishes in order to look at potential contaminations. We often observed no conidia production on these plates. When we planned new experiments, we had to produce new conidia suspensions. Transplants (up to 5) were took from conidia sterility test plates and transferred on new V8 medium plates. Conidia production in these conditions was fast and vigorous, plates were covered of conidia after 3 - 4 days.

Cutin preparation

Due to the peach fruit unavailability in spring we performed cutin extraction from apple peel following modified Bostock *et al.* (1998) protocol. 4kg Granny smith and Golden delicious apples were peeled then gently scraped with a knife to retire the maximum amount of residual pulp. Peels were dry during night at 35°C and soap in Chloroform / Methanol bath (2: 1, v/v) under extraction hood during 24h for waxes surface washing, removed and air dried under air hood flow. Peels were

soak in a Pectinase/cellulase solution (1mg/ml and 5mg/ml respectively) diluted in 0.05M acetate buffer, pH 4. A primary 6-hour incubation at 25°C was performed because of the optimal pectinase activity then, overnight at 36°C for optimal cellulase activity. Peels were washed several times with dezionized water then with ethanol 95% and dry at room conditions. Peels were powdered with a TissueLyzer several time to obtain 9.5g of fine dry powder. We stocked it under desiccation container for further experiments.

Liquid culture: preparation and composition

Composition

We used modified CZAPEK by removing glucose from normal recipe, to test ACQ impact alone. Previous experiments in the lab were done with this medium. In 2 treatments, we added some cutin (at 0.5g/L in final samples) in order to observe trigger fungal cutinase production.

Modify CZAPEK, for 1L	
Yeast extract	1g
K2HPO4	1g
MgSO4.7H2O	1,02g
KCl	0.5g
FeSO4.7H2O	0.018g
Distilled H20	1000 mL
pH adjusted to 7	

Table 2 : CZAPEK « modify » medium composition

Culture medium and treatments solution preparation:

Czapek cutin and Czapek without cutin were separately prepared in glass bottle and autoclaved during 20 minutes at 121°c, 15psi. A magnetic bar is added to Cazpek + cutin medium to be agitated during use to have the best repartition in all samples.

Chlorogenic acid (Sigma-Aldrich C3878) 5mM, 10mM solutions (protect from light) in 10% water/ethanol (9/1) were prepared. Ethanol was necessary to dilute ACQ powder. Then, solutions were filtrated in sterile tubes through 0.20µm filters under a laminar flow hood. Treatments were stocked at -20 for 1 day maximum if not used the same day.

Conidia inoculum preparation:

CZAPEK media were put under laminar flow hood, and autoclaved glass container were filled up with 20ml of corresponding media. CZAPEK + cutin media was magnetically mixed during the process. Then, for each treatment, 5ml of corresponding treatment solution was added to obtain a 1mM or 2mM solution. Finally, 250µL of conidia suspension was used for inoculation. Samples were brought to the climatic chamber for 13 days at 24°c during day and 18°C during night.

Sample preparation

CZAPEK media are put under laminar flow hood, and autoclaved glass container are filled up with 20ml of corresponding media. CZAPEK + cutin media is magnetically mixed during the process. Then, for each treatment, 5ml of corresponding treatment solution is added to obtain a 1mM or 2mM solution. Finally, 250µL of conidia suspension is used for inoculation. Samples are brought to the climatic chamber for 13days at 24°c during day and 18°C during night.

Culture media filtration and Dry mycelium weighing

The culture was stopped at the thirteenth day and a vacuum filtration was performed on each culture medium in 50ml falcon tubes. Filters were made by blotting paper, cut into pieces corresponding to ceramic filter diameter used for filtration. Each filter was numbered and put into oven for 15 hours at 40°c. Then, filters were removed one by one from the oven and directly weight to avoid ambient humidity absorption bias between filters, to assure the more precisely weight as possible. After vacuum filtration, all residual mycelium on filters were dried into the oven during 12 hours and then weighted one by one as previously described. The culture liquid filtrate was frozen at -20°C until analyses. A test experiment was done to measure the average cutin weight in one sample. During an experiment, 6 containers were filled with CZAPEK + cutin media, as the same condition as liquid culture inoculation experiment, but without any conidia inoculation. After 13 days, we filtrated the medium at the same time of other treatments and dry weighted filters to calculate the average amount of cutin in each cutin sample.

Liquid Culture Stability

pH variation

We collected 2 samples during 16 days of culture to follow pH stability on 2 repetitions of the inoculated treatments (FCAQ1, FCE) and one repetition of non-inoculated treatments (CA1 and FE). 1ml samples were collected in 1,5ml tubes, under laminar flow hood to conserve strict sterility. Then samples were analyzed with a pH meter.

HPLC Analysis

We designed an experiment to observe how the fungi affects chlorogenic acid stability in the medium. We decided to collect 100µl on three samples of 2 treatments: FCA1 and CA1 of our liquid culture in order to follow the acid chlorogenic stability in inoculated or non-inoculated liquid culture. The medium was analyzed with HPLC at our lab. Samples were collected twice a day for 2 days and every day for 13 days. Then 900µl of methanol was added (9:1) and filtrated through a 0.45µm filter to avoid any particles which can obstruct mechanical parts in the device. 10µl were injected in an HPLC,

pair with a diode array detector (Shimadzu Prominence). Pics were integrated at 330nm for phenolic absorption visualization.

Protein extraction

A big part of the study was to find correct method to analyze efficiently our proteins samples at our lab. We tried different methods and protocols (with trichloroacetic acid (10-15%) with no interesting results). Finally, we found the glacial Acetone extraction protocol was the most effective to efficiently precipitate and harvest proteins. A volume by volume glacial acetone was added into the filtrate's tube from vacuum filtration, then, agitated and put in ice for 30min waiting for precipitation. Then, tubes were centrifugated at 15 000 RPM during 10min at 4°C and supernatant was gently discarded. Normally, protein pellet has to be rinsed and centrifugated three times. However, we had problems with pellets solubilization when adding glacial acetone and pellet formation the second time so we decided to make only once. Tubes were let under flow hood extractor during 1 hour in order to evaporate acetone residues, then, phosphate buffer (0.05M, pH 7) was added to dissolve protein pellets. Extracts were frozen at -20°c for further proteins analysis.

Protein dosage

We used a Bradford method to dose proteins. In 2ml spectrophotometric cuvettes, we added 1ml of protein samples in phosphate buffer (pH7) and 1ml of Bradford reactive, then we mixed it with pipetting and waited for 5 min. Three technical repetitions were done for each protein's samples. Absorbance was measured sample by sample with a spectrophotometer at 595nm. Concentration was determined with the "Shimadzu software" using a standard curves comparison made just before sample analysis. Standard curves were made with BSA, covering 0, 2, 4, 6, 8 and 10µg/ml of proteins. Samples were analyzed with UVprobe, a Shimadzu® software.

Proteomic analysis

During vacuum filtration process, we reserved samples (Mycelium and filtrate) of experiment D, and bring it to ProGenomix platform at Bagnols-sur-Cèze in order to determine protein composition and abundance level by UHPLCLC/MS/MS (Liquid Chromatography coupled to tandem mass spectrometry). Three conditions were analyzed and compared: Acid chlorogenic 1mM, 2mM and the control (without ACQ), also, 3 mycelium samples were analyzed, one for each condition. Samples were migrated in polyacrylamide 2D-Page, then spots were collected.

Tripsin was used for hydrolyze peptide bound in proteins and inject in UHPLC for analysis (Thermo Fisher scientific: UHPLC Ultimate – Quadripole-Orbitrap QExtractive HF). Data have been

compared with a genomic alignment on *M. fructicola* genome, and unidentified proteins have been annotated by Blast on Ascomycota genomic data.

Fresh mycelium collection and Gene Expression

In two experiments (D and E), we collected fresh mycelium just after filtration to perform mycelium gene expression. Sampling tubes were frozen in liquid nitrogen, then fresh collected mycelium was added to the tube and both conserved in -20°C freezer until use. RNA extraction was done following "NucleoSpin RNA Plant and fungi extraction kit" protocol from Macherey-Nagel. RNA concentration was verified by NanoDrop. Then a real-time quantitative polymerase chain reaction (RT-PCR) was realized with an inverse transcriptase to obtain cDNA sequences corresponding to the fungal RNA. A PCR verification was performed to detect by electrophoresis with BET intercalant fluorescent probe cDNA presence and gDNA contamination under UV. Finally, a qPCR was performed with cDNA, specific primers for *MfCUT1*, *MfPG1*, *MfPG3*, *MfPG6* and alpha tubulin genes, and Sybr Green as fluorescent agent. The alpha tubulin was used as reference gene (stable across samples) for data normalization (Chou *et al*, 2015). Three biological repetitions were analyzed for each sample and both experiments. For each sample, six technical repetitions were realized.

Statistical analysis method

Data have been analyzed with R software. Normality was tested with Shapiro wilk method, at 99% confident level. If homoscedasticity was verified, and equality of variances also (Bartlett test), Anova was realized. When a treatment effect was significant, we used a multiple comparison test (Tukey's test) to compare the treatments two by two. If normality of data was not verified, non-parametric tests were used: the Kruskal-Wallis test was applied followed by a Wilcoxon rank sum when the treatment effect was significant.

Data from RT-qPCR were analyzed with the RqPCRAnalysis R package (Hilliou and Tran, 2013). Cycle thresholds recorded were transformed into expression data normalized with the reference gene and gene relative expression levels were calculated taking one treatment as control. Because of non-normal distribution of the data (Shapiro test), comparison between treatments were performed by a Kruskal-Wallis test followed by a Wilcoxon rank sum.

Results

Chlorogenic Acid impact on Monilina biomass

During culture growth we observed that ACQ treatments look more vigorous and produced conidia aa the liquid surface more rapidly than non ACQ treatment.

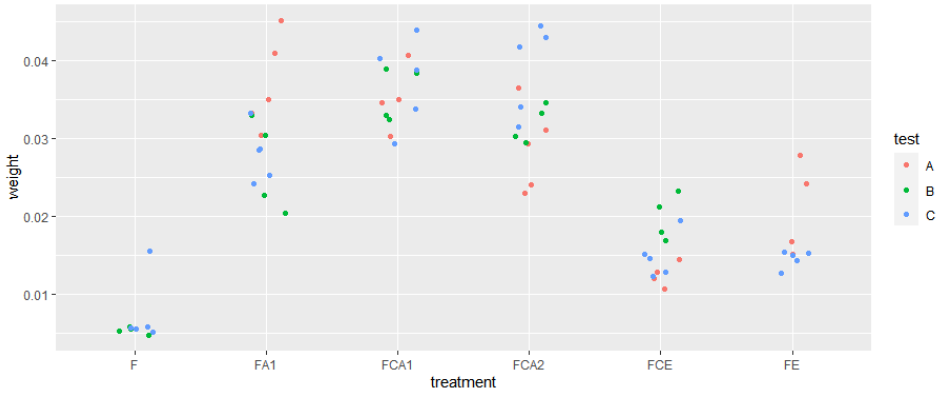

Fig. 6 : Chlorogenic acid impact on dry biomass of 3 M. fructicola liquid culture experiments; A(blue) B(green) and C (blue) based on 6 treatments; F, FA1, FCA1, FCA2, FCE, FE, (4 or 6 repetitions each) after 13 days in climatic chamber in Czapek "modify" medium.

The experiment was very reproductible and no difference between our independent tests was observed (*p-value* = 0.746). Fig. 1 shows a clear impact of ACQ on *M. fructicola* biomass production after 13 days of culture. ACQ does not inhibit *M. fructicola* growth but on contrary strengthens the growth. Indeed, ACQ treatments are significatively different from other treatments (*p-value* < 0.001), and double the biomass when added to the culture but there is no difference between ACQ1 and ACQ2 treatments (*p-value* < 0.05). Presence of cutin (FCE) seems to have no effect on biomass compare to ethanol alone (FE) (*p-value* = 0.36). We can observe a significant difference between F and FE biomass (*p-value* < 0.01) which makes FE a bad control treatment. It will not be considered anymore in the following.

Protein content

We made many protein extractions and dosages and we observed an important disparity between the independent experiments, in particularly with FA1 and FCE treatments. Anyway, it appeared that FCA2 protein content is significatively higher than FCA1 and FCE and FE (Table 3). It is also slightly higher than FA1 protein level. FA1 protein level is significatively higher than the ones of FCE and FE.

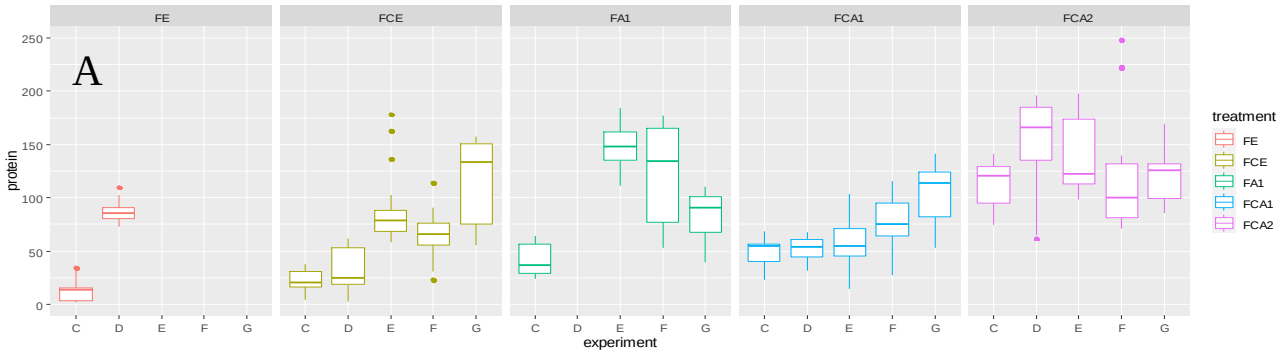

Fig. 7: Protein dosage of liquid cultures samples from M. fructicola cultivation. C, D, E, F and G experiments are represented (A) after 13days in growing chamber. B is same data but all experiments have been pool. 4

B

treatments were measured; FCA1, FCA2, FA1, FCE and FE. Proteins were extracted by using glacial acetone method.

It is also slightly higher than FA1 protein level. FA1 protein level is significatively higher than the ones of FCE and FE. FCA1 and FA1 average protein contents are also significatively different. On the contrary, protein content of FCA1, FCE and FE are not different. These results can be misinterpreted because of important measure variation among different experiments.

Table 3: pvalues of Pairwise comparisons using Wilcoxon rank sum test for protein data.

	FE	**FCE**	**FA1**	**FCA1**
FCE	1.00000	-	-	-
FA1	0.00025	1.5e-5	-	-
FCA1	1.00	1.00	0.0027	-
FCA2	7.5e-11	9.0e-16	0.302061	< 2e-16

pH stability

All culture samples inoculated with *M. fructicola* showed an acidification of the medium. At Day 0 we observed a 0.2 difference on basal pH level between ACQ and non-ACQ treatments, non-ACQ treatment being less acid than ACQ. This difference is stable and clearly visible between CA1 and CE along all the culture (0.3 at maximum point). Inoculated treatments began around 6.6 (6.45 and 6.73 for FCA1 and FCE respectively), then pH decreases immediately from the conidia germination until the end of the experiment (4.9 and 5.23 for FCA1 and FCE respectively). An interesting result can be observed with slope of average pH curves. The slope is quite equal, -0.325 for FCA1 and -0.334 for FCE. pH is decreasing on a similar way in both treatments. CE lost 0.08 pH point and CA1 decreased by 0.2 pH point. This experiment shows a very important effect of the *M. fructicola* inoculation on pH stability in the liquid culture.

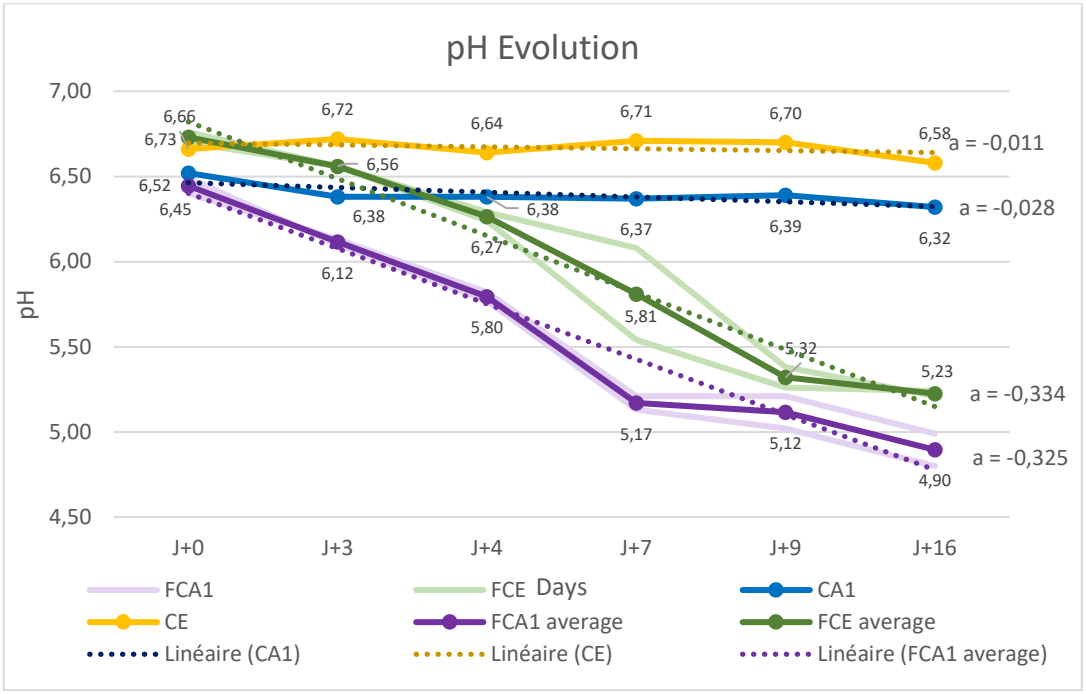

Fig. 8: pH evolution of liquid culture inoculated with M. fructicola conidia suspension (FCA1 and FCE) and non-inoculated (CA1 and CE) treatments during 16 days of culture in culture chamber.

Chlorogenic acid stability in liquid culture

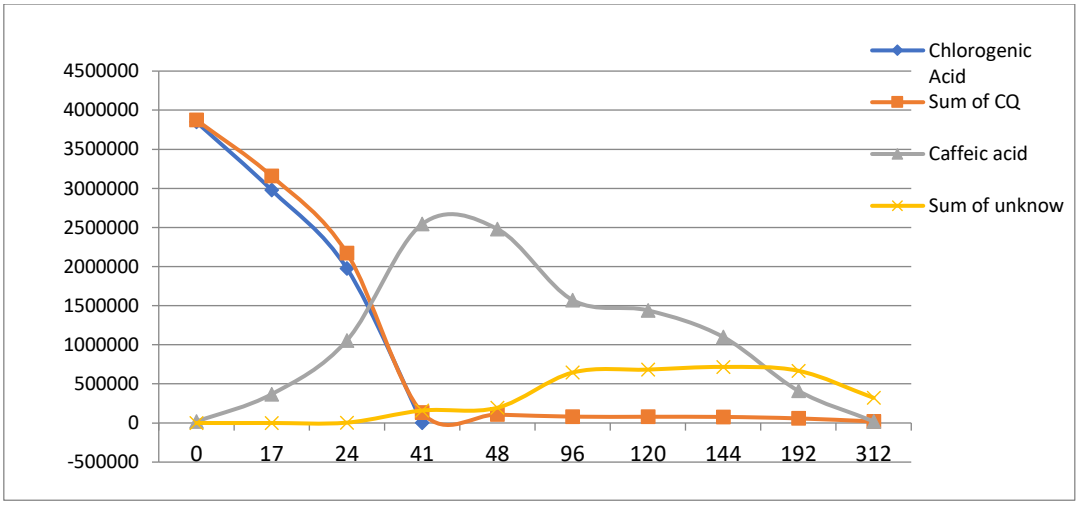

Fig. 9: Chlorogenic acid stability in FCA1 (M. fructicola conidia +Cutin + chlorogenic acid 1mM) treatment, (FCA1) during 13 days of culture. Analyzed with HPLC and integrated at 330nm.

At first step of the experiment, chlorogenic acid concentration decreased rapidly, no trace of it is found after 48 hours in an inoculated liquid culture (Fig. 9). The same trend is observed for ACQ derivate molecules (c-5CQ, c-6-CQ, t-4-CQ, c-3-CQ combined in 'sum of CQ' in Fig. 9. Caffeic acid appeared simultaneously with the ACQ degradation, its maximum concentration was at 41 hours, then it decreased while other unknow molecules appeared from 48 hours. In comparison, in

non-inoculated liquid culture (Fig. 10), after 13 days (312 hours), ACQ integration is still at 40% of the initial measure (0).

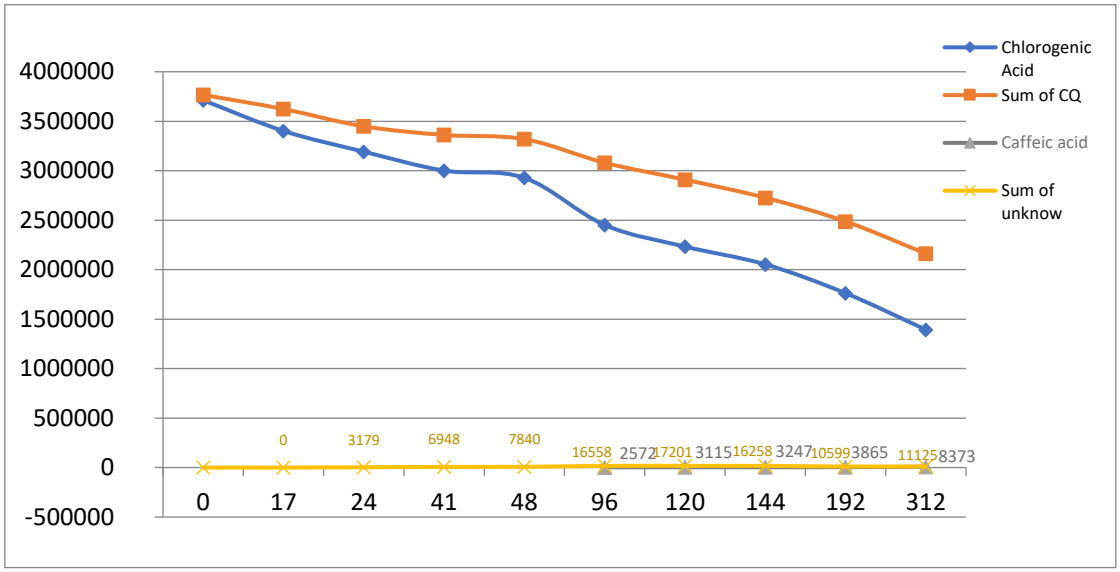

Fig. 10 : Chlorogenic acid stability in CA1 (Cutin + chlorogenic acid 1mM) treatment during 13 days of culture, without M. fructicola. Pics were integrated at 330nm, Analyzed with HPLC and integrated at 330nm.

A few unknow molecules appeared but ACQ is probably mainly converted into derivates: c-5CQ, t-4-CQ t-3-CQ, under the impact of water, light or oxygen oxidation. Only a very little amount of Caffeic acid appeared in the medium after 96 hours. These results strongly suggest *M. fructicola* is greatly involved in chlorogenic acid degradation in our liquid culture experiences

Protein identification

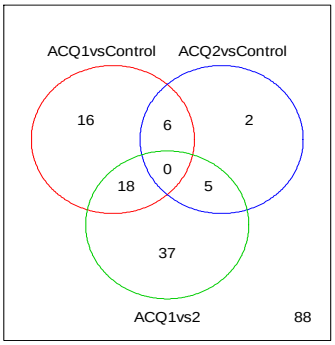

Fig. 11: Venn diagram of the presence of the 172 proteins secreted in the medium by M. fructicola in the 3 different treatments.

The proteomic platform returned a list of 3079 proteins but only 2062 were certified with at least 2 peptides. The comparison between filtrate and mycelial samples allowed to identify 172 proteins secreted (largely predominant in the filtrate sample) by the fungus in the medium. In the following, we the control (Fig. 11). We also looked at proteins with pathogenicity properties or with a function that may be related to phenolic compounds degradation.

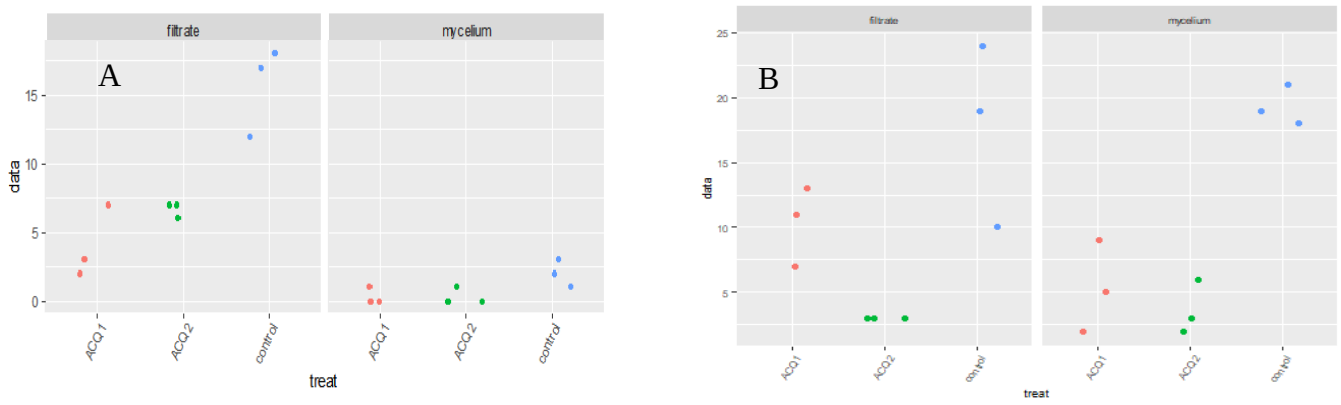

Fig. 12 : Monilinia and Massarina eburnean orthologue cutinase abundance in the 3 treatments; ACQ1 (orange), ACQ2 (green) and control, FCE (blue) from M. fructicola liquid culture samples, determinated by UHPLCLC/MS/MS analysis at Progenomix platform.

In the filtrate samples, 2 cutinases and 6 other enzymes (protease, lyases, hydrolases) were found to be significantly different in ACQ1 et ACQ2 treatments compared to the control condition FCE (p =0.002 and *p-value* =0.009 respectively). Cutinase was less abundant when ACQ was present in the medium and a very low cutinase abundance was found in mycelium samples (Fig. 12). In contrast, a second cutinase, ortholog of a cutinase described in *Massarina eburnea*, was not specific to the secretome. profiles were comparable between Mycelium and Filtrate samples with an impact of ACQ reducing the abundance. This reduction was significant only in MYCELIUM samples (p = 0.0012 and 0.00065 between ACQ1 and ACQ2 and versus control respectively)

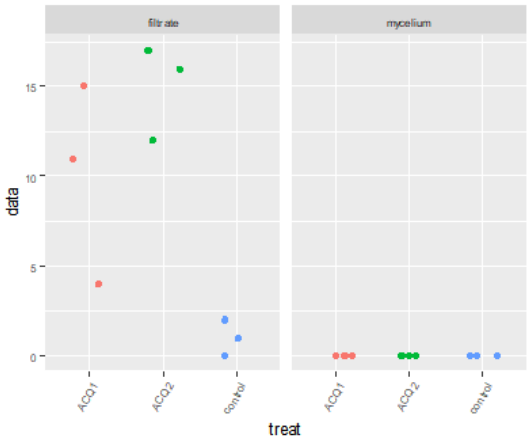

Fig. 13: Laccase protein abundance in the 3 treatments; ACQ1 (orange), ACQ2 (green) and the control FCE (blue), from M. fructicola liquid culture samples, determinated by UHPLCLC/MS/MS analysis at Progenomix platform.

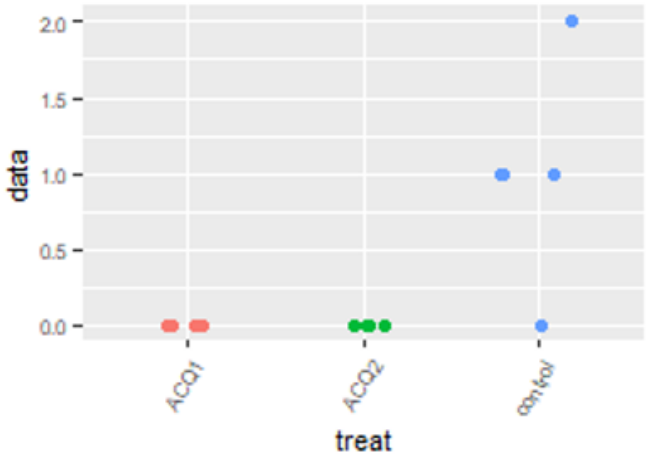

Fig. 14:

Cellobiodeshydrogenase protein abundance in mycelium samples in the 3 treatments; ACQ1 (orange), ACQ2 (green) and the control FCE (blue). Measured by UHPLCLC/MS/MS analysis at Progenomix plateform

Laccase enzyme is statistically more secreted in ACQ2 (*p-value* <0.01) (8 times more) and ACQ1 (*p-value* <0.05) (5 times more) treatments than in control. The enzyme is not detected in mycelium samples. We also observed that the 40S ribosomal protein, in mycelium is less represented in ACQ1 and ACQ2

treatments (1.5 time less). A very low abundance of this protein is detected in filtrate. In mycelium, cellobiodehydrogenase abundance level (Fig.14) is significantly higher in control condition than in ACQ 1 et 2 treatment (*p-value* <0.01)

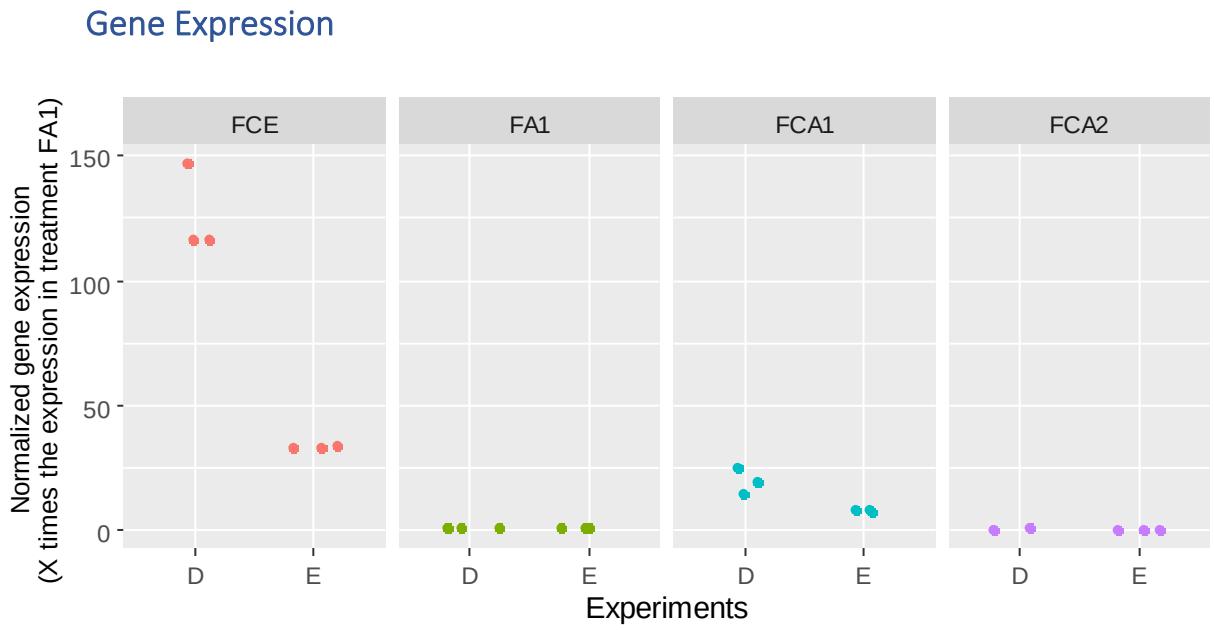

Fig. 15 : Mfcut1 gene relative expression (expression in FA1 treatment taken as reference) in two independent experiments (D and E) with 4 different treatments : FCA1, FCA2, FA1, FCE.

The qPCR analysis, performed on samples from 2 experiments (D and E), displayed interesting results on cutinase and 3 polygalacturonases gene expression. We can notice a very good reproducibility between the three biological repetitions and similar results from the 2 experiments, although expression of cutinase in FCE treatments was higher in experiment D.

As for cutinase gene expression, it was clearly impacted by the presence of ACQ in the medium *(p-value* ≤ 0.0028). Chlorogenic acid also seems to have a dose impact on MfCUT1 gene expression: it was lower in FCA2 than FCA1 treatments (*p-value* = 0.0022). Also, the comparison between FA1 and FCA1, showed that cutinase was less expressed without cutin in the medium (*p-value* = 0.0028).

Concerning expression of polygalacturonase genes, quite similar levels of expression were observed between the 3 genes. The FCA2 treatment reduced MfPG gene expression compared to the 3 other treatments for the 3 genes (differences are significant apart in the case of *MfPG1* for treatments FCE and FA). Thus, there is a dose level of chlorogenic acid impact on MfPG gene expression: ACQ concentration in FA1 and FCA1 was not enough to reduce gene expression of *MfPG1* and *MfPG3*. However, in the case of *MfPG6*, the expression was significantly reduced in presence of ACQ at 1mM as well (*p-value* ≤ 0.0028).

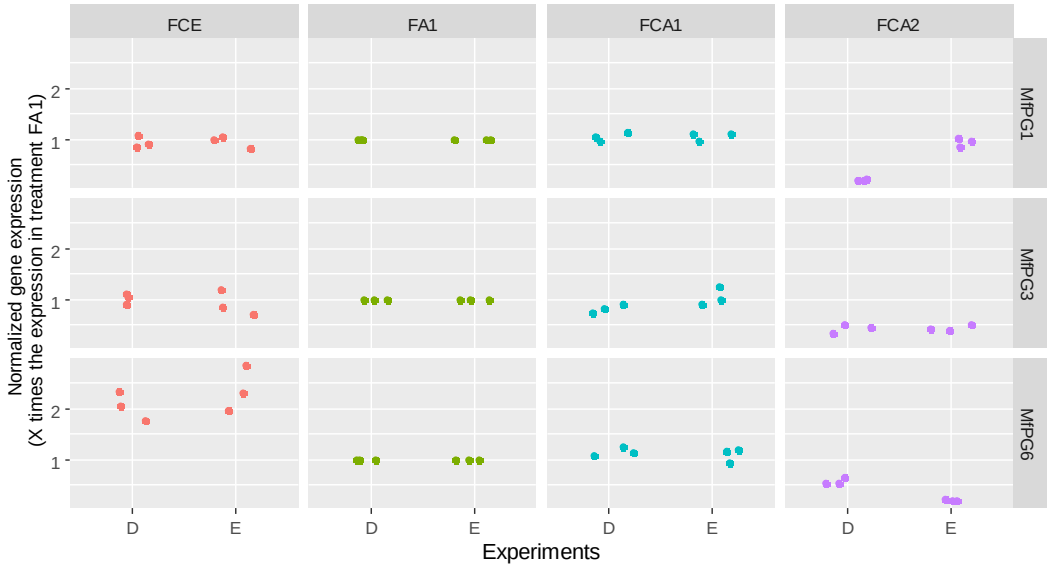

Fig. 16: Cycle threshold of MfPg1 (red), MfPg3 (green) and MfPg6 (blue) gene expression of 4 different treatments: FCA1 (and FCA1B), FCA2, FA1, FCE and their corresponding experience

Discussion and Perspective

A big part of my work was to design a way to easily culture Monilinia in liquid medium. The evolution of the protocols and experimental design I proposed, compared to past experiments, represent a significant improvement. Contaminations were very rare and repetitions can be done, allowing robust statistical tests to be performed.

Biomass production

Our treatments had significant effects on *M. fructicola* dry biomass. First, ethanol used to dilute Chlorogenic acid appeared to be a nutrient source for *M. fructicola* (FE differed from F results). In natural environment, many fruits ferment and ethanol is produced during fermentation. At high concentration, ethanol is toxic to fungi and other organisms but at lower concentrations, it may be an advantage to metabolize it in order to detoxify the environment and use it as a nutrient source. As for ACQ, it has shown antifungal properties against phytopathogenic fungi (Martinez *et al*., 2017). Thus, we expected a toxic effect of ACQ on our colony but unexpectedly the biomass production showed opposite results. Dry biomass was statistically higher in treatments with ACQ than in non-ACQ ones but there was no difference between ACQ 1mM and ACQ 2mM. The biomass increase can be related to ACQ and ethanol metabolization. Indeed, as there was no direct sugar source in the medium, *M. fructicola* had to find carbon elsewhere. Peptides from yeast extract can be used or ethanol, as mentioned before, but we suggest that M. fructicola is also able to degrade chlorogenic acid to use derivate compounds. Indeed, medium with ACQ 2mM resulted in twice more ACQ derivate molecules from its degradation than medium with ACQ 1mM. We could expect a significant biomass difference between FAC1 and FAC2 treatments but final biomass was similar. The comparison of

fungal biomass between FA1 and FCA1 treatments showed that, surprisingly, the presence of cutin in the medium had no effect on *M. fructicola* growth.

Protein Extraction

We performed many tests on protein extraction and tried different dosage protocols. We encountered some obstacles during protocol execution: it was impossible to make 3 washes with acetone, the pellets didn't dissolve and centrifugate well. This result could have resulted in a loss of protein when acetone was discarded. We also had difficulty to restore a *M. fructicola* clean culture after the experiment stop imposed by the sanitary situation (confinement) and results obtained (E F and G experiments) were somehow heterogeneous. Bostock *et al.* (1999) showed that ACQ decreases cutinase content, so we expected to find less protein in cultures with ACQ. Surprisingly FCA2 treatment resulted in higher protein content compared to other treatments. This is quite opposite to what we expected. It is probable that, in presence of ACQ, *M. fructicola* secreted other unexpected proteins to modify its growth environment. Anyway, these results have to be confirmed, in particular because of the important experiment effect. Probably data were also influenced by the technical problems that occurred during the dosage.

Evolution of the liquid medium along the culture

After biomass observations we followed ACQ stability and pH variation in order to have a better understanding of what happened during the culture. HPLC analysis of inoculated or non-inoculated medium displayed very interesting results. ACQ decreased very rapidly and was completely absent of the culture after only 2 days after germination. At the same time Caffeic acid proportionally increased. When ACQ totally disappeared, caffeic acid content stopped increasing and began to decrease. In parallel, new unknow compounds appeared. This suggests that ACQ is cut at the ester bound between quinic acid and caffeic acid. But we were not able to observe quinic acid because of non-similar wavelength detection with chlorogenic compounds family. Non-inoculated samples showed spontaneous degradation of ACQ as well, but to a much lower degree. ACQ was still found (40%) in the liquid culture after 13 days. We detected some traces of unknow molecules (24 hours) and caffeic acid (96 hours) suggesting natural degradation effects by light, water, or oxygen oxidation. All these results clearly indicated that *M. fructicola* metabolized chlorogenic acid and other derivate compounds produced during its degradation. This observation raises questions about our experimental design. Are we observing chlorogenic acid effect or derivate compounds effect? Other experiments have to be set up to test directly the effect of derivates compounds from chlorogenic acid, particularly caffeic acid and quinic acid impact on biomass and cutinase production. Monilinia is known for its capacity to acidify the host tissue mediated by gluconic acid. Our results confirmed this fact since pH clearly decreased with time in inoculated medium only. The pH has an effect on

fungal radial growth and this effect is optimal in acidic media (pH = 2.4) but sporulating capacity is optimal at pH = 5.3 (Obi et al, 2017). Acidification also impacts fungal pathogenicity, increasing gene expression, in particular expression of MfPG2 and MfPG3 (De Cal et al, 2013). A future experiment can be set up to follow ACQ stability in a non-inoculated acidic media in order to see if pH have an impact on normal chlorogenic acid degradation.

Proteomic analysis

Proteomic results showed an important impact of chlorogenic acid on cutinase production. We found two different cutinases, one was specific of the secretome, *M. fructicola* cutinase MfCUT1, and the other appeared to be described in *Massarina eburnean*. For the latter, the profiles were comparable between Mycelium and Filtrate samples with an impact of ACQ reducing the abundance. The MfCUT1 cutinase was less abundant in the filtrate for treatments with ACQ than without. This confirms Bostock *et al*.'s conclusions. Same observations were done for the other protein annotated as a cutinase ortholog, which showed a significant difference between FCA2 and FCE cultures, both in Filtrate and Mycelium samples. For both cutinases, results with ACQ1 and ACQ2 were not statistically different. A dose effect of ACQ on cutinase abundance cannot be statistically underlined for the moment and other repetitions have to be done to reinforce these results. Laccase protein is found to significantly more abundant in ACQ treatments. This enzyme as is known as a polyphenol reductase with a low substrate specificity and a high catalytic activity against polyphenol in acidic media. In Demkiv *et al*. (2020), *M. fucticola* has been used for laccase production and some studies show how laccase is related with chlorogenic acid oxidation (Ma *et al*., 2009) or polyphenol degradation using oxygen in grape fruit (Zimdars *et al*., 2017). A perfect candidate protein to test how ACQ is degrade by M. fructicola. In mycelium, cellobiohydrolase gene, an enzyme involve in fungal pathogenicity and cell wall degradation, is found to be very significantly higher in control than ACQ treatment (Fig. 14). ACQ and derivates can have undescribed effect on various plant pathogenic enzymes. Some peptidase, protease or hydrolase enzyme were found affect by treatments. Due to the lack of nutrient, *M. fructicola* certainly change its metabolic behavior and is more focus on protein degradation. Test have to be done with nutritive media. These proteomics results show the variety of compound impact by ACQ and derivates.

Gene expression

With the q-PCR analysis we observed how related pathogenic genes like *MfCUT1*, *MfPg1*, *MfPG3*, *MfPG6* were expressed in the different treatments. The presence of cutin in the medium induced *MfCUT1*gene expression. Again, we confirmed the negative impact of ACQ on the expression of the *MfCUT1* gene and an effect of dose. This is in adequation with the abundance of the corresponding protein in the different cultures and corroborates Bostock *et al*. (1999) findings. These analyses also

concluded on the effect of ACQ on MfPGs genes. ACQ2 significantly modified the expression of all tested polygalacturonase genes and *MfPG6* is the only one significantly impacted by ACQ1 as well (FA1 or FCA1). This decrease may result in a reduction of abundance of the corresponding enzymes which may hamper the fungus efficiency during the infection.

Conclusion

The experimental design and filtration method have been well improved. Thus, we were able to compile concomitant evidences at different biological levels, that ACQ impacts the expression of cutinase genes and the secretion of cutinase enzymes. These observations are in adequation with reference works of the literature (Bostock *et al*., 1999) and offer new results concerning proteomic profiles and gene expression. As for biomass production, we found that medium with ACQ resulted in higher biomass production. This is not surprising since it was shown that ACQ has no effect on mycelial growth and thus no direct toxicity to the pathogen. On the contrary, the presence of ACQ may contribute to the brown rot resistance by interference with the production of pathogenic factors such as enzymes degrading host polymers. The proteomic analysis revealed a very large diversity of proteins secreted by the fungus in the culture medium and important modifications of the secretom between treatments. This implies that the total protein content, without any purification, is not a direct synonym of cutinase secretion. To complete the panorama, it would have been interesting to compare cutinase activity between treatments. We have devoted many efforts to performing enzymatic analyses, by measuring absorbance with a spectrophotometer, to record esterase activity in the filtrates. Unfortunately, results were not robust enough to be presenting here. More tuning is needed. Bostock *et al*. (1999) showed that cutinase activity was not directly inhibited by ACQ. In addition, in our experiments we observed that *M. fructicola* clearly degraded chlorogenic acid after only 2 days of culture. This suggests that ACQ derivates compounds may also act on pathogenic factors of *M. fructicola*. Thus, more experiments have to be done with ACQ degradation products, such as quinic or caffeic acids. It could be interesting to find which residue is used by the fungi during ACQ metabolization and which enzymes are needed to metabolize ACQ. In another way, the fungi seem to be able to modify its environment to either decrease negative effects of some molecules or produce usable carbon sources by rapid metabolization.

In conclusion, the experimental design developed proved to be robust and interesting. It can be used in future experiments to i) increase repetitions and improve the power of the statistical analyses, ii) to explore other genes by RT-qPCR, and iii) test the effect of other phenolic compounds or ACQ derivates. Finally, we can also set up such experiments on other *Monilinia* candidates, such as *M. laxa*.

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