**Master thesis**

CONFIDENTIAL 3 years

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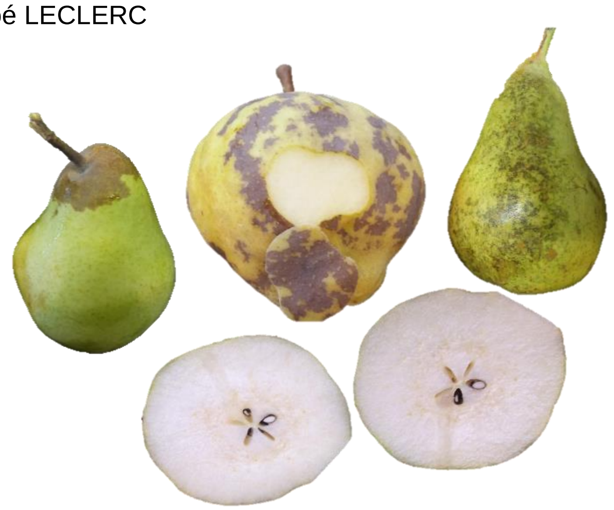
**Biochemical and statistical approaches**

By: Chloé LECLERC



Presented at Angers the 09/09/2019

Under the direction of Christian LARRIGAUDIERE

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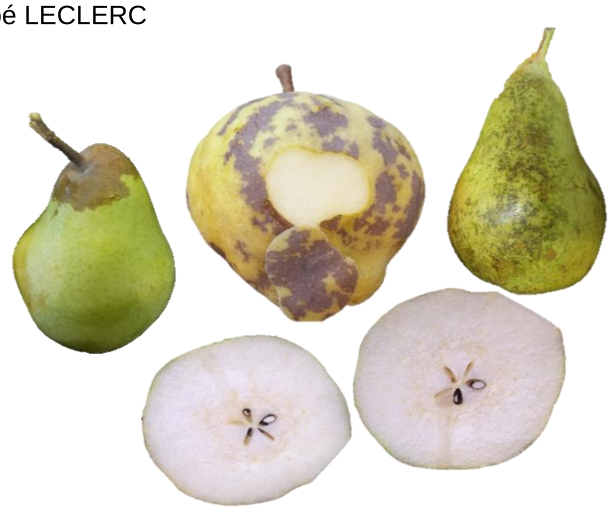

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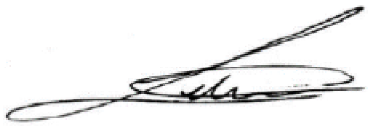

# Greetings

To begin with, I would like to thank Christian LARRIGAUDIERE, head of the Post-Harvest Physiology and Technology group in the FruitCentre of IRTA. I am immensely grateful to him not only because he gave me the chance to enter an internationally recognized Research Institute but also because he allows me to make my personal project true. Indeed, this experience brings me the opportunity to build my professional network in Spain, the country where I want to pursue my career. I would like to thank IRTA for authorizing me to attend valuable formations and meetings (Annexe XV).

I express all my gratefulness to the technicians, PhD students, engineer and researchers for my integration in the team, for the time they dedicated, their advice and invaluable support in my work. I am especially thankful to Violeta LINDO, Jordi GINÉ-BORDONABA who trusted in my capacities, shared their knowledge and taught me patiently the procedures of the experiments. Post-harvest physiology for fruit quality was indeed a topic almost unknown to me after my formation and that I wanted to study deeper.

My greetings are also addressed to the members of IRTA working on other projects, Gemma ECHEVERRIA, Tomás LAFARGA, Ingrid AGUILÓ, for raising my interest for a new sector of the plant productions, which is plant-based food innovation, processing and quality.

This fourth experience abroad would not have been possible without the strong implication of Mme TUDEAU and the support of the Department of International Student Exchanges.

I thank my professors at University of Angers for the valuable formation they dispensed. It has led me to complete my internship and my career according to my points of interest and made me discover new areas.

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# Glossary

**Climacteric fruit**: Fruits that can be harvested at physiological maturity, able to ripen off the plant and displaying a specific respiration pattern upon harvest climacteric rise (Kader, 1999).

**Controlled atmosphere (CA)**: Gas environment monitoring and adjustment (absorbers, scrubber, ventilation, adding-pump) to maintain optimum concentrations for storage. The composition is selected to increase shelf life, maintain quality and reduce disease *incidence* and *severity* (Deuchande *et al.,* 2015). Common practices include shelf life (21%$O_2$-0,4%$CO_2$), Low Oxygen LO (2.5-3% $O_2$), Ultra-LO (≈1.5% $O_2$), eXtrem-ULO (0.7-1.0% $O_2$). Dynamic Controlled Atmosphere (DCA) consists in the application of a stress followed by real-time storage monitoring of $O_2$ at 0.5%-0.8% with chlorophyll fluorescence or volatiles detection technology.

**Ethylene:** Gaseous phytohormone controlling plant growth and senescence (Reid, 1995).

**Fermentation:** Under-low or anaerobic conditions, fruit produces fermentative metabolites (acetaldehyde and ethanol) that may lead to the onset of physiological disorders, resulting in poor quality and product losses (Reid, 1995).

**Flavor**: Complex combination of *taste* and *aroma*. *Taste*: Set of sensation detected in the mouth linked to the presence of sugar and organic acid and classified by six classes of receptors: sweet, sour, salty, bitter, umami and fat-taste. *Aroma*: Usually predominant sensation surpassing tasting, given by volatile and non-volatiles compounds. Main classes are mono-, sesquiterpenes, lipids-, sugars- and amino acid-derived compounds (Gonçalves *et al.,* 2018).

**Incidence**: Number of individuals affected by a disease within a population (Moggia *et al.,* 2009).

**Indicator/index/marker:** Measurement taken as a reference associated to a particular feature. Ideal indicators in fruticulture need to be simple, inexpensive, objective, related to quality, storage life, representative and reliable of progressive changes (constant between seasons, growing areas). Technically, the measuring material should be easy to carry.

**Maturity**: *Physiological maturity*: *S*tage of development when fruit reaches its potential to ripen after harvest. *Commercial maturity:* Stage of development when a fruit shows optimal commercial characteristics. Indices of maturity are: days after full bloom or anthesis, days from harvest to onset of *ethylene* production, color, sugars, acids, flesh firmness, starch degradation, dry weight, internal ethylene concentration (Iqbal *et al.,* 2017).

**Respiration:** Physico-chemical process of conversion of sugars and oxygen into $CO_2$, $H_2O$, and heat (Saquet *et al.,* 2017b)

**Ripening:** Last phase of fruit maturation governing post-harvest physiology, shelf-life and losses. Fruit ripening behavior is characterized by 2 *respiration* patterns: *climacteric* and non-climacteric. Physiological, biochemical and developmental changes happen through a coordinated and genetically regulated program (Kader, 1999).

**Senescence:** Form of aging leading to organ/plant death. *Ethylene* signal perception lead to the onset of Programmed Cell Death, loss of cohesion between cells due to compound alterations and finally tissue de-structuration (Reid, 1995).

**Severity**: Measurement of the intensity of the damage caused by the disease (Mditshwa *et al.,* 2016).

**Shelf-life:** Time upon removal of storage during which a product remains safe and satisfactory regarding sensorial, bio-physico-chemical characteristics (Pasquariello *et al.,* 2013).

# Abbreviations

**1-MCP:** 1-Methylcyclopropene

**ACC**: Acetyl-CoA Carboxylase

**ACO:** ACC Oxidase

**ACS:** ACC Synthase

**AENOR-ISO**: Asociación Española de Normalización y Certificación - International Organization for Standardization

**AFS:** alpha-farnesene synthase

**APX :** Ascorbate Peroxidase

**AsA :** Ascorbic acid (Vitamin C)

**BH**: Brown Heart

**BOH:** Before Optimal Harvest date

**CA**: Conservation Atmosphere

**CAT**: Catalase

**CTols**: Conjugated Trienols

**DA**: Dynamic Atmosphere

**DAFB:** Days After Full Bloom

**DHA:** Dehydroascorbic Acid

**DPA:** Diphenylamine

**DW :** Dry Weight

**Eth**: Ethylene

**FID**: Flame ionization detector

**FRAP**: Ferric reducing antioxidant power

**FRU**: Fructose

**GC**: Gas Chromatograph

**GLU**: Glucose

**LO-ULO-xULO**: Low Oxygen – Ultra Low Oxygen - (eXtrem- ) Ultra Low Oxygen

**LOX:** Lypoxygenase

**MA :** Malic Acid

**MACC:** 1-(malonylamino)cyclopropane-1-carboxylic acid

**MAP**: Modified Atmosphere Package

**MAPA** *Ministerio de Agricultura,Pesca y Alimentación*

**MDA:** Malondialdehyde

**MINECO**: *Ministerio de Economía y Competitividad*

**OEVV-CPVO**: Oficina Española de Variedades Vegetales - Community Plant Variety Office

**PDO:** Protected Designation of Origin

**POX:** Peroxidase

**ppm**: Parts per million

**PPO**: PolyPhenols Oxydase

**RH**: Relative humidity

**rpm**: Revolutions per minut

**SAM**: S-Adenosyl-L-Methionine

**SDH:**Sorbitol deshydrogenase

**S6PDH:** Sorbitol-6-phosphate deshydrogenase synthase

**SOD**: Superoxide Dismutase

**SS**: Superficial Scald

**SSC :** Soluble Sugar Content

**SUC**: Sucrose

**ROS**: Reactive Oxygen Species

**TA** : Titrable Acidity

**TAA** : Total Antioxidant

**TCA:** Tricarboxylic Acid

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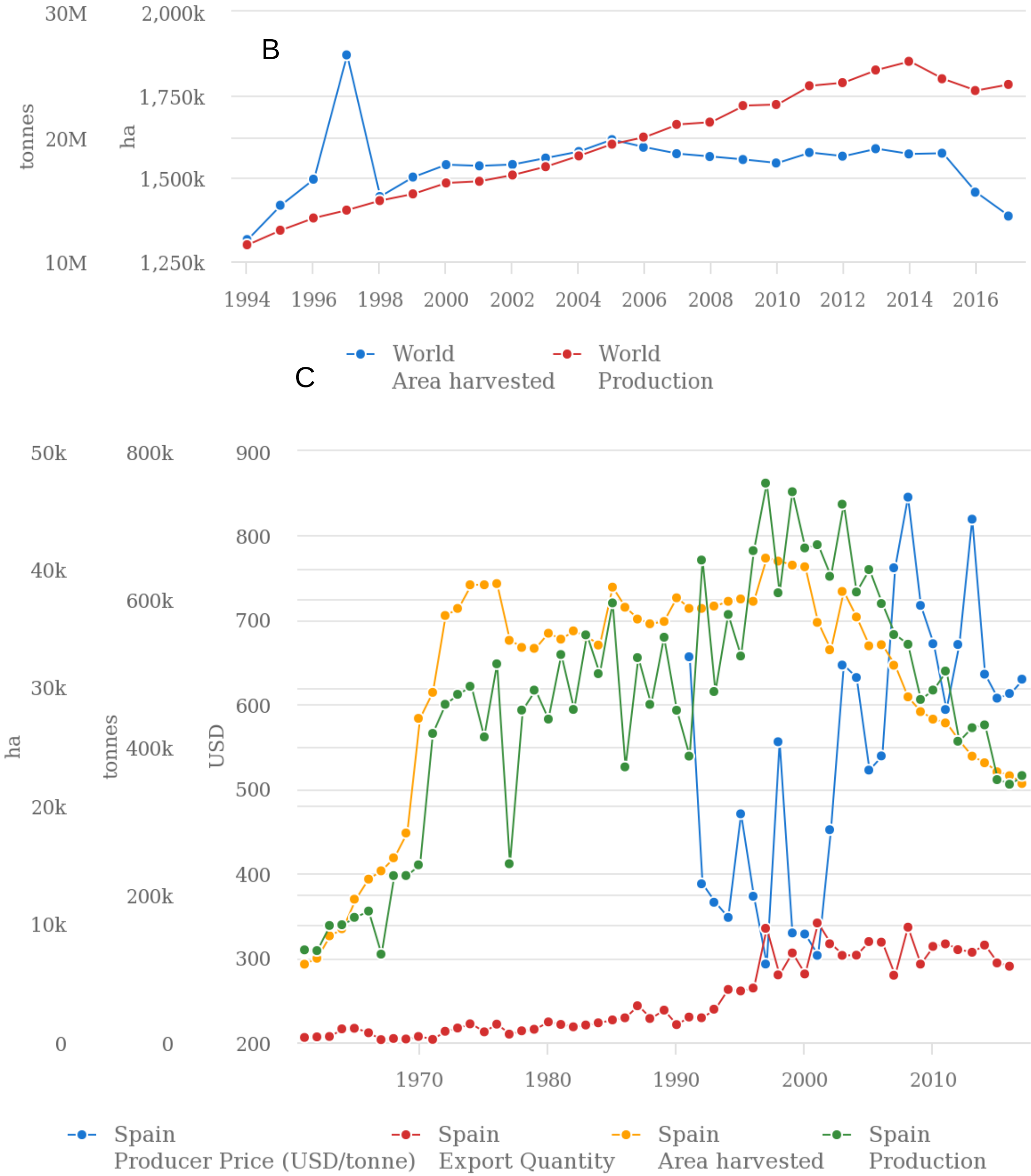


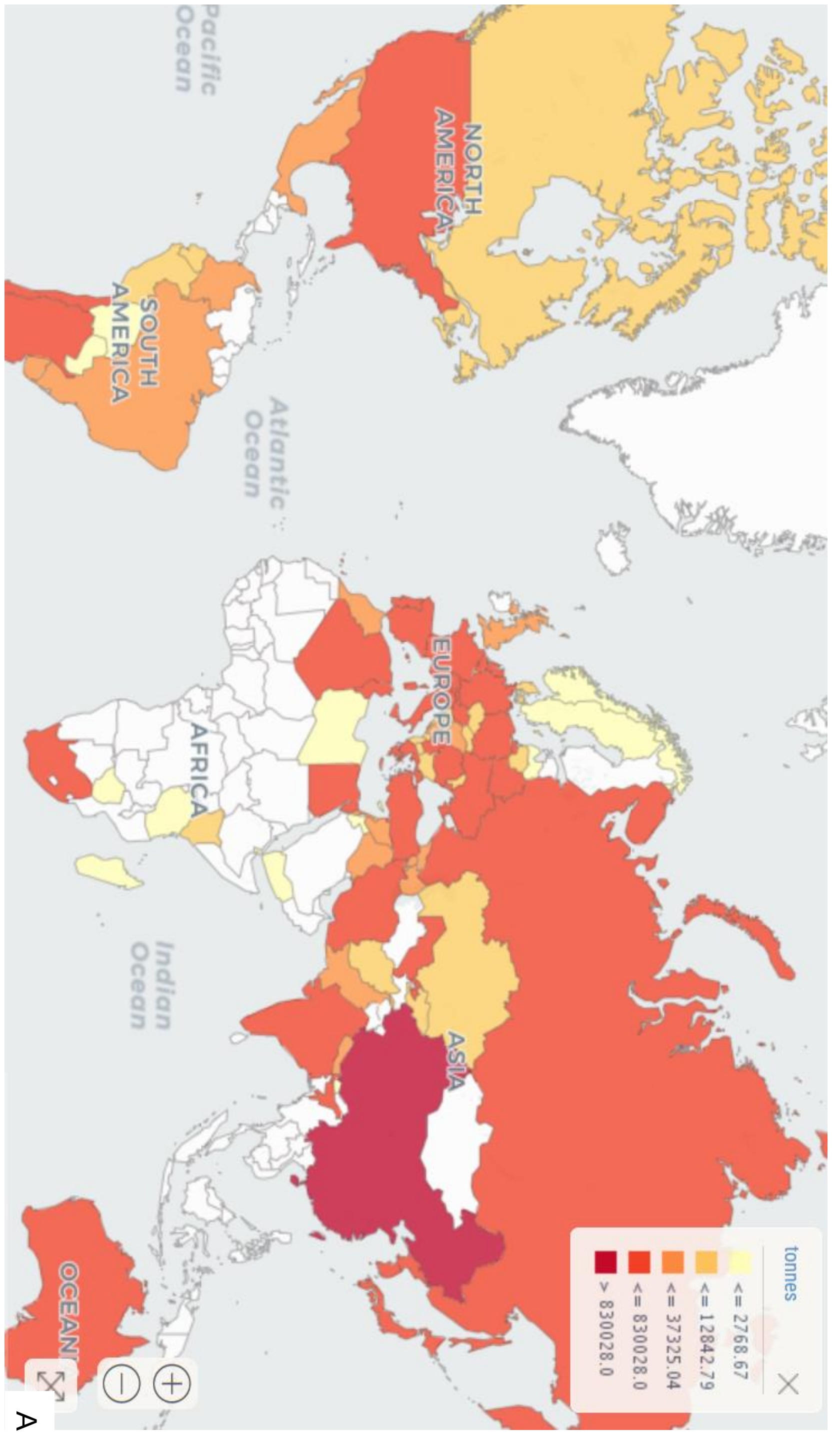




Figure 1: Pear market situation. (A) World Pear production map (B) Pear production evolution in in the world from 1994 to 2017 (C) in Spain from 1961 to 2017 (FAOSTAT 2019)

# Introduction

## *Pear production & market situation: quality improvement to re-dynamize the sector*

Pears (fam. *ROSACEAE*, subfam. *Maloidaeae,* g. *Pyrus*) are typical fruits in temperate regions (Silva *et al.,* 2014, Figure 1A). The global production totalized 22,144 million tons in 2017/18. It is expected to decrease to 19,421 in 2018/19 due to April freeze in China. The international market offers an almost all-year-round selling period thanks to germplasm diversity, climate and technology management. Pears are mainly eaten as raw fruit, processed derivatives as liquor, concentrate, juice, puree, dry, cans representing only 1/11$^{th}$ of the production (Interpera 2019). Despite of being appreciated for their multiple health benefits, flavor and texture (Vaysse *et al.,* 2005, Annex I), their consumption entered a stagnation period as reflected by a decline in terms of volumes and areas (Pablo Valenciano *et al.,* 2017, Figure 1B). The conversion of pear orchards to other crops can be explained by the strong trade competition with apple and stone fruits (price, technology of production, robustness). Numerous issues affect pear quality and conservation: the decreasing tree vigor which implicates to look for adapted rootstock, irregular yields, climate change, difficult pest and diseases management (*Psila*, pear fire blight *Erwinia amylovora*, scald) with limited active matter authorizations (European Commission and Food Safety Authority).

The implementation and integration of new techniques such as Dynamic Atmosphere (DA), Ultra Low Oxygen (ULO), 1-Methylcycloproene (1-MCP) or edible coating are expensive. Prices for producer tend to decrease (Figure 1C) and thus investments for new plantations or equipment is rare. Marketed varieties and breeding in pear sector are also limited and mainly consist in introducing new varieties in regions where they have never been planted/commercialized in before. Countries specialized in the production of small number of varieties, which favor trade exchanges (Annex II and III). The Spanish *Institute of Agrifood Research and Technology* (IRTA) characterized more than 150 varieties (1994-2017) based on their agronomic behavior, storability, gustative quality (Castellarnau and Vilardel, 2017). Breeders are recently looking for bicolor varieties (less sensitive pinkish/bronze epidermis) or hybrid accessions (European x Asian pears). Like in apples, “club varieties” are also more frequent to retain customers. Some other varieties are fire blight-resistant, do not need pre-cold storage, or are less sensitive to common physio-pathologies. **H**igh-quality fruit production is the strategy adopted to keep the pear market viable, attractive, and profitable. Still, yields, competitiveness and advances differ between countries.

In Europe, Spain gathers of the largest area dedicated to fruit production and is one of the major countries for pear production after Italy (332.309 t for 2018/19, *Ministry of Agriculture* MAPA). A decrease has been noticed since the historic pic of the 20$^{th}$ century (Figure 1C), -6% in2017/18. But pear orchards area is still important.

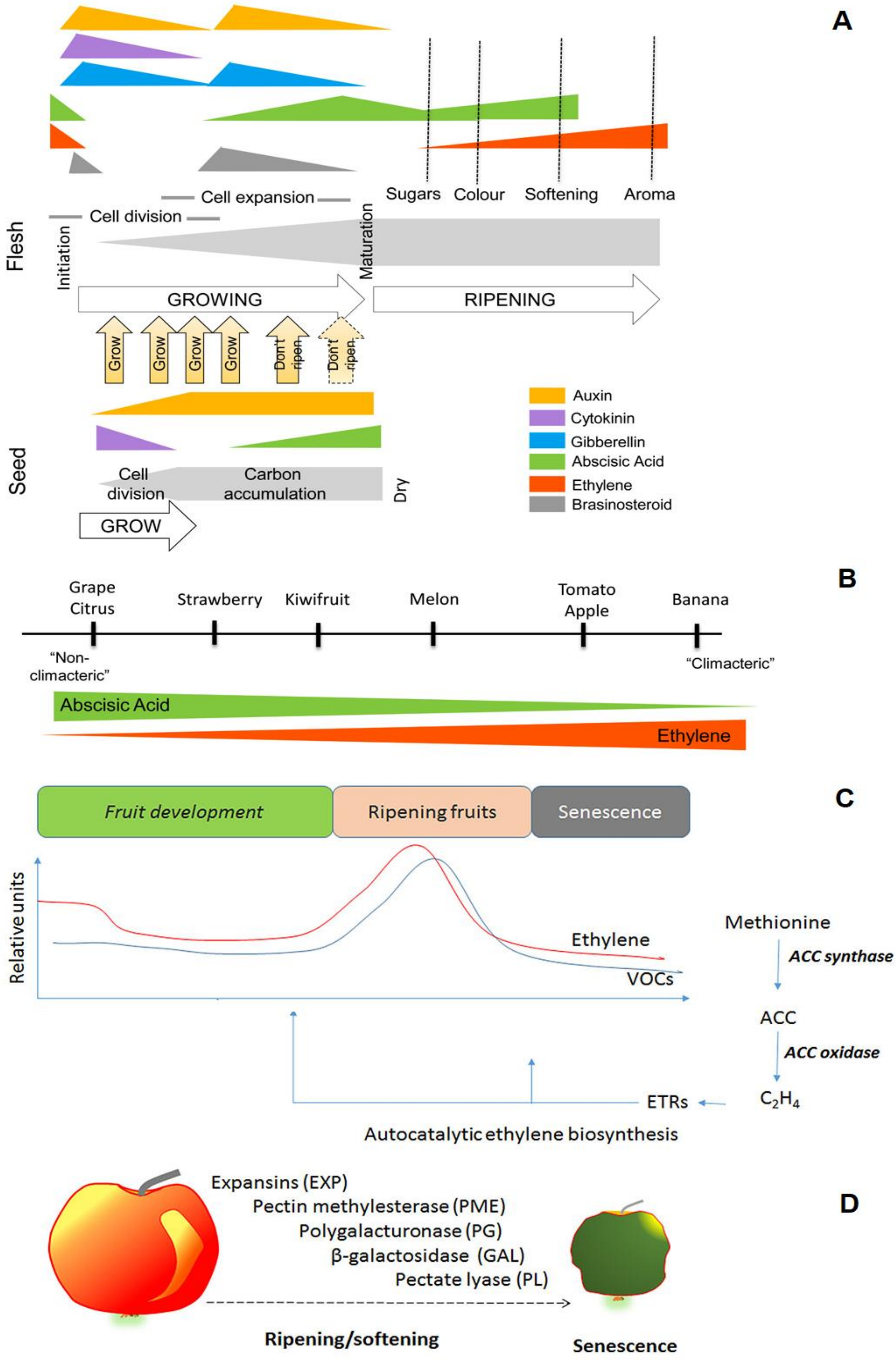



Figure 2: Simplified schemes of current knowledge on fruit maturation: Hormonal changes in flesh (A) and seed (B) adapted from McAtee et al., (2013) – Ethylene and VOC biosynthesis (C) and main enzymes involved in Fruit maturation (D) adapted from Iqbal *et al*., (2017)

Pedoclimatic conditions and the technical itinerary are essential factors influencing fruit quality. In Spain, *Rincón de Soto*, *Lleida* and *Jumilla* pears have been deserved a Protected Designation of Origin (PDO) label for their superior characteristics. This distinction enhances the value of the product and provides a reference for the consumer about the guidelines followed by the growers to reach higher quality (Botelho *et al.* 2014).

### *Pear quality management: challenges between conservation and physiological disorders*

Fruit physiology undergoes numerous and different processes according to time and tissue (Figure 2 A and B). Pears are climacteric fruit that include an off-tree maturation period (Lindo-García *et al.,* 2019). When maturing on-tree, they tend to exhibit a mealy texture, more internal disorders and poor organoleptic qualities (Lurol CTIFL, Interpera 2011). Horticultural and physiological fruit maturity thresholds are different and strongly influence fruit storability and consumer exigencies. Important economics stakes are dependent of conservation.

Fruits organoleptic characteristics can be significantly enhanced if the storage/maturation process is well managed. Exposition to cold is an important step of the post-harvest management for pears. Cold temperatures (tº) trigger ethylene (Eth) and aromas precursors synthesis which give sweet, floral and pear notes (Makkumrai *et al.,* 2014). Cold needs and response rate differ according to varieties and conditioning (El-Sharkawy et al., 2004). Maturation metabolism involving catalytic enzymes can be slowed through controlled atmosphere (CA) (Deuchande *et al.,* 2015, Figure 2C and D). Significantly less alterations senescence and pathogen-related damages are noticed.

Fruit centers and distribution platforms are commonly equipped with conservation/maturation chambers. Pear storability ranges from 3 to 5 months under regular air at -0.5ºC and up to 8 months under CA (Saquet and Almeida, 2017). But lack of knowledge about the optimal condition for each variety leads to unequal quality (PDO meeting). Storage conditions sometime trigger physiological disorders. Scald-like symptoms (SS) depreciate external appearance while Brown Heart (BH) remains invisible before the consumer cuts the fruit. The repeated disappointment of the buyers when purchasing and/or eating altered products can lead to a loss of confidence in the whole sector and impact the economy (Pablo Valenciano *et al.,* 2017). The comprehension of the factors of sensitivity would help growers in better managing their itinerary of production and their stocks. A comparison with similar disorders in other fruits like apple would be interesting to assess if some mechanisms are shared and if common solutions can be found.


Figure 3: Postharvest Research Group in The *Institute of Agrifood Research & Technology*: Organigram, Partnerships, Missions And Projects F&V Fruits and Vegetables, R&D Research and Development (C. LECLERC)

Concerned about these issues, the stakeholders of the Spanish pear sector, MAPA, *Minister of Science, Economy and Competitivity* (MINECO) and the *European Union* asked the ***FruitCentre*** of IRTA (AENOR ISO9001-OEVV-CPVO certifications, Figure 3) to find markers of early detection of different physiological disorders*.* Created to increase the sustainably, productivity and competitiveness of the Agrifood sector, IRTA applies "Science for Impact" *via* a public-private collaboration with local growers, regional market, national, European and international trade actors and research institutions. The *FruitCentre* is located in one of the most productive regions, considered as the "orchard of Spain", Lleida, Catalonia. The ***Post-harvest Working Group*** has a recognized multidisciplinary expertise in physiology, biochemistry, technology, engineering, pathology and microbiology. Its mission is to contribute to increase consumer's confidence in the fruit quality. The ***Physiology and Technology group***, headed by Dr. Christian Larrigaudière, pilots several projects on pear fruit quality and conservation.

The ***Operative Group of Rincón de Soto's GOP project (03/04/2017-02/04/2020)*** was launched at the PDO's request to better understand the pre- and post-harvest factors involved the development of BH disorder. The definition of a physio-biochemical biomarker explaining BH and predicting its BH risk would lead to the development of practical and optimized operational procedures and material (non-destructive methods, storage systems).

***Pear Superficial Scald ESCALPE project (01/01/2018-31/12/2020)*** aims at characterizing the sensitivity of the 3 pear cultivars in Spain exhibiting different symptomatic SS phenotypes. The main postharvest physiological and biochemical processes are studied to find the key factors involved in the induction and the development of these disorders. A better understanding of the response of different cultivar, both at the biochemical and molecular levels, would allow to update storage guidelines for each cultivar and help in breeding less sensitive fruits.

## STATE OF THE ART

### Brown Heart

Symptoms appear after storage and affect pear core or cortex, then extending to the rest of the fruit. Generally, part of the pulp under the skin remains unaffected (Zerbini *et al.,* 2002). The tissue dries and may acquire a brownish color and form cavities. Texture and flavor are altered. Higher BH incidence is recorded under low $O_2$ - high $CO_2$ combination, the common CA conditions set up to delay ripening and senescence. Gases partial pressure and concentration were also identify as a parameter of influence (Saquet and Almeida, 2017; Wang, 2016; Wang and Sugar, 2013). At cellular and molecular level, local anoxic conditions that may occur in the fruit core may cause oxidative stress associated to production of fermentation causing the accumulation of ethanol

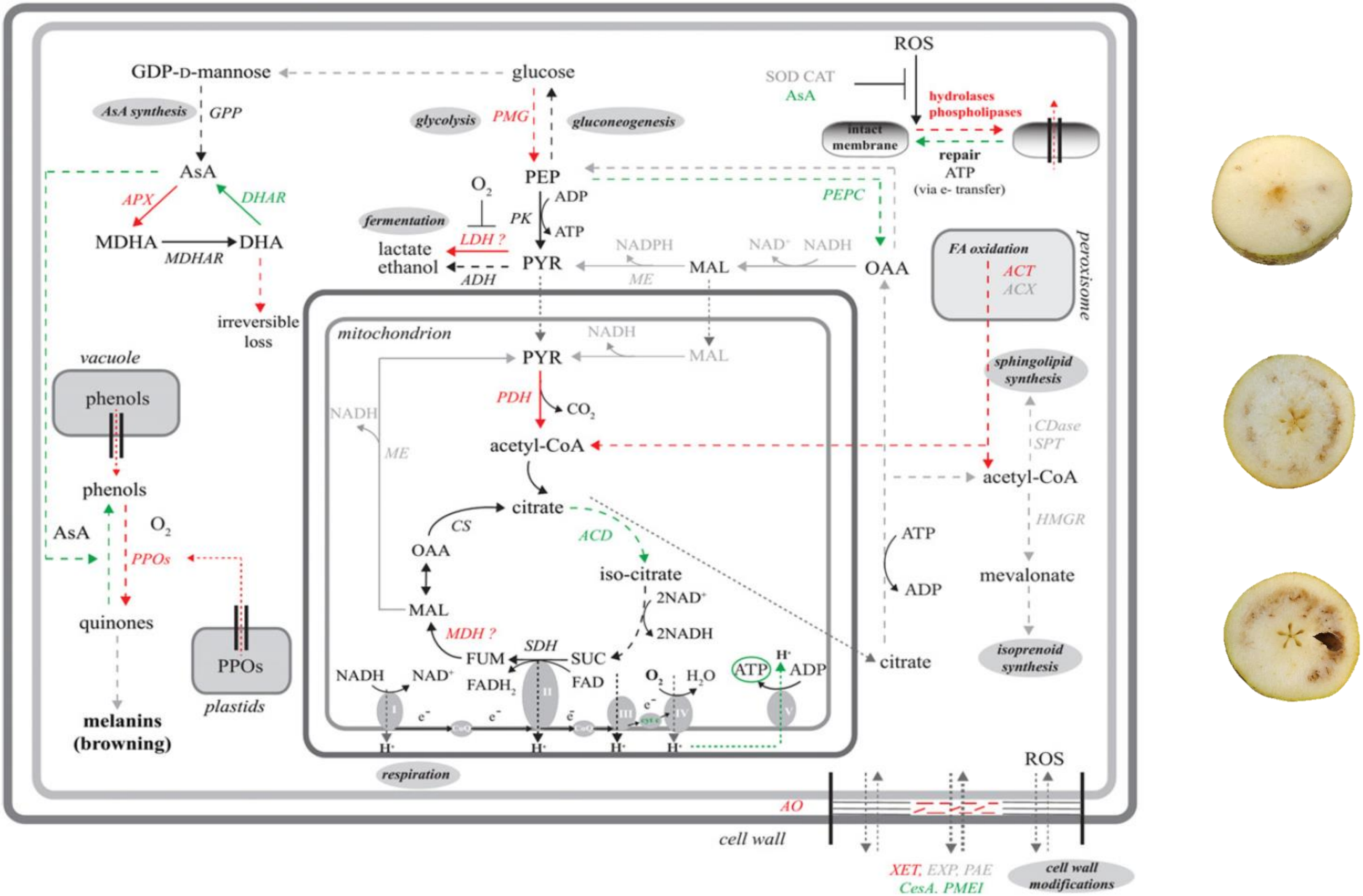



Figure 4: Browning development model during CA storage for apple Brown Heart proposed by Mellidou *et al*. 2014. Transcriptomic changes in the affected out cortex related to the gas gradient and energy potential. Induced genes in affected tissues are indicated in red, repressed genes in green, and unchanged genes in grey. Browning and cavity formation as illustration in pear (photos: C.LECLERC)

and acetaldehydes. BH disorder was also related to the endogenous antioxidant AsA (Vitamine C) levels that tends to decrease during storage (Saba and Moradi, 2016; Zerbini *et al.,* 2002). In Netherland, several authors showed that BH disorder in *'Conference'* was initiated when AsA level was lower than 2 mg/100 g Fresh Weight (Franck *et al.,* 2003; Zerbini *et al.,* 2002). This acid plays an important role in defense mechanism against free radicals action and peroxidation. Unrepaired damaged caused by the reactive oxygen species (ROS) lead to membrane breakdown and leakage of phenolics responsible for browning (Figure 4). Damages extend to the surrounding tissues and simultaneously with gases pression effect, form cavities.

Although symptoms are generally found after storage, pre-harvest factors are associated to BH development. Pears grown in Northern Europe are more affected by BH than those grown in the Mediterranean region (Deuchande *et al.,* 2016; Veltman and Plas, 2002). Fertilization practices, irrigation techniques, and genetic rootstock abilities to absorb, accumulate and distribute the minerals to the different phytoorgans are essential in the elaboration of the fruit quality. External applications of Ca, Zn, B were found to delay BH development and decrease the severity of the disorder (Khoshghalb *et al.,* 2008). Storability also depends on the maturity stage at harvest (Brandes and Zude-Sasse, 2019). The evolution of the fruit epidermis color and the differential absorbance of chlorophyll in the epidermis are related to late harvest and some physiological disorders (Khoshghalb *et al.,* 2008, Serra *et al.,* 2018). Metabolites kinetics such as starch degradation, sugar/acid conversion are other common indexes of the fruit quality. But till date, the parameters evaluated do not accurately predict BH risk. No full integrative model including minerals, biochemical and physiological data has been reported.

### *Superficial scald*

Superficial scald (SS) is a post-harvest disorder affecting apple and pears epidermis after a storage period distinguished from senescent scald, appearing after long term storage when fruit becomes senescent. Symptoms are characterized by irregular brown-black patches covering the epidermis of the fruit (Figure 5, next page)Figure 1. Although the disorder does not affect the taste or the texture, fruits are rejected by the market rules. Scald kinetics of development was described in 3 phases: 1) oxidative stress and accumulation of $\alpha$-farnesene compounds during the early cold storage period (1-2 months), 2) oxidation of $\alpha$-farnesene to conjugated trienols hydroperoxydes (CTols) and peroxide radicals' formation resulting in cellular damages 3) symptoms appearance during the shelf life period. Enzymatic polyphenol oxidase (PPO)-mediated browning involved chlorogenic acid, phenols and membrane lipids oxidation.

Pre- and post-harvest as well as orchard management causes were already mentioned by Emongor *et al.,* (1994). The tree physiology was investigated and revealed that rootstock,

Figure 5: Post storage Superficial Scald-like epidermis symptoms and cross sections in cv. 'Flor de Invierno' (photo: V.LINDO) and in cv. 'Granny Smith' apple (right Gapper et al. 2017).

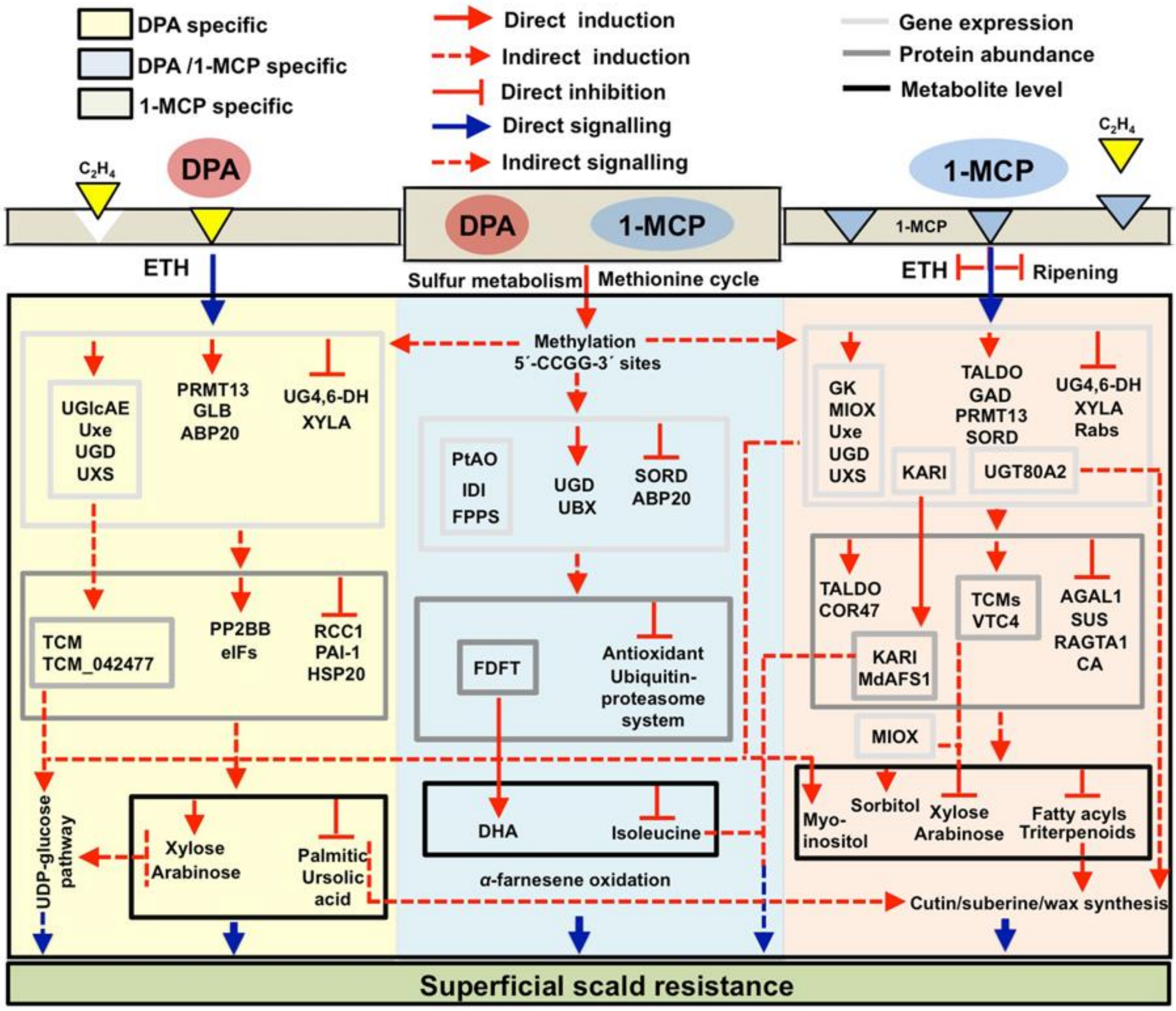



Figure 6: Proposed mechanism of regulation for Superficial Scald by DPA and 1-MCP in apple (Karagiannis *et* al. 2018)

plant age, nutrition partitioning at different stages (high N, K, $Ca^{2+}$ and low B increasing scald risks), fruit size, color and position in the tree may be determinant (Rudell *et al.,* 2017). The climate (number of hours <10°C) during the growing-season and pre-harvest weather also strongly influence the risk of scald (Blanpied *et al.,* 1991). In the same way, maturity stage at harvest date (Starch Index) and conditions of conservation: delayed storage, relative humidity, atmosphere composition and storage temperature clearly determined scald incidence (Moggia *et al.,* 2009; Saquet *et al.,* 2017a).

Till recently, SS was efficiently reduced by treating the fruit with chemicals such as etoxyquin and diphenylamine (DPA) acting as antioxidant against α-farnesene oxidation and ROS (Figure 6, Karagiannis *et al.* 2018). However, their use is now criticized by EU regulations. As a new alternative, 1-MCP ('SmartFresh™, 1996) has been widely applied to replace these chemicals. It inhibits Eth production by blocking the Eth-receptors and limiting the accumulation of α-farnesene and CTols production (Chiriboga *et al.,* 2011). Other alternatives were found in physical techniques such fast transition from normoxia 2-3% $O_2$ to hypoxia 1-1.2% (ULO- X-ULO) in CA in storage chamber or the use of DCA with very low $O_2$ levels (0.4% to 0,6%). Factors of predisposition also deals with fruit genetics: endogenous antioxidant machinery, and ethylene production. The international collaboration between the *Pear Genomics research network*, *Plant and Food Research* (PFR) in New Zealand, and INRA, France, will increase knowledge about pear physiology to improve breeding and practices. Pear genome sequence (2n=34) already allowed to identify some maturation and quality-related genes and QTLs (Busatto *et al.,* 2019). Giné-Bordonaba's recent work with the Italian *Edmund Mach Foundation* (unpublished) motivated a complementary study to V.Lindo thesis at the molecular level.

## OBJECTIVES

This work is a first trial to analyze two internal and external physiological disorders affecting pear fruits, Brown Heart and Superficial Scald, through both biochemical and statistical approaches. In both cases, it aims at finding reliable set of indicators associated to the risk of disorder occurrence and try to develop subsequently predictive models. For this purpose, a large set of data from different nature was gathered at the lab to feed statistical models.

*The GOP specific objective* is to validate controversial knowledge about BH disorder. The underlying questions are: *Is there any relationships between mineral contents in different organs and BH occurrence? Does this relationship change during the fruit formation? Is there an orchard location factor related to BH incidence in the PDO? How important are minerals and biochemical indexes in the determination of BH and fruit quality? Are these parameters*

Table 1: Characteristics of the 3 cultivars studied synthesized from (D.O.P. Peras de Rincón de Soto; Fruits de Ponent; Simões et al., 2008; Steyn et al., 2011)

| Cultivar | |  *'Conference'* |  *'Blanquilla'* (*de Aranjuez* or *Pera de agua*) |  *'Flor de Invierno'* |
|---|---|---|---|---|
| **Breeder** | Thomas Rivers | | Unknown | Unknown |
| **Origin** | England, 1994 | | supposed Spain, 18th century | Unknown- supposed Llobregat Barcelona |
| **Harvest** | Late Summer - Autumn | | Summer-Autumn: beginning of August for long conservation, 15th August if *ready to eat* | Winter |
| **Market importance** | Table/Dessert pear, fresh cut-processing | | | |
| **Caliber** | Extra and I 58-60mm | Medium | Small-medium | Medium-big, heterogeneous |
| **Shape** | Elongated (Gibberellic Acid GA) | Elongated (GA)-pyriform, regular-with short stalk | Elongated (GA)-ovoid-pyriform | irregular, round-slightly flat, strong stalk |
| **Epidermis** | Natural russeting (not induced by Cu) | Thick, matte green-brown (rusetting) to pale yellow when ripe | Fine, shiny green to slight grey, slightly red because of sun | Thick, matte pale green to yellow when ripe (slightly reddish in sunny countries) , lenticels |
| **Flesh** | firmness: [5.21-5.67]-[5.44-6.12 kg/cm$^2$] | White, fine-soft/fondant, 7-6 kg.cm$^2$, juicy | White, very juicy, soft sometimes "granulosa" in the heart, fondant | White, slightly, crispy |
| **Organoleptic attributes, smell, taste** | sweeter: 13-19°Brix, Starch 2-3C, high juiciness | Sweet-slightly acidulated, excellent taste due to high sugar/acid ratio, intense favors | Good flavor at harvest stage | Moderately sweet, acidulated, better flavor at late stage |
| **Storage capacity** | | -1 to 0ºC, 4-6 months at normal cold , 6-9 months under 3-4%$O_2$ and 1-2% $CO_2$ CA-recommended no<2kPa $O_2$ and >0.5kPa $CO_2$ (Saquet201 | Good in high RH, normal cold till December, CA till June, sensitive to $CO_2$ excess (>3%) in storage chamber, resistant to BH if harvested early but poor organoleptic attributes | Good on tree till January-February and under normal cold till April-May |
| **Consumer preferred characteristics : Ideal pear** | Medium size, pyriform shape, bright green yellow or lightly colored blush, fully ripe with better sensory quality taste is the best pear feature: sweet, juicy, and firm-slight melting, soft texture, highly flavor. Locality/Origin is valued. Less favorable features: round fruit or very elongated fruit, very green or red, mealiness and hardness | | | |

*useful for the prediction of BH? Can we identify thresholds value and develop a decision-making tool/model that could help growers during the growing season and before storage?*

*ESCALPE specific objective* is to increase the knowledge on the biochemical basis of SS in pears and determine predictive markers through transcriptomics and statistical modeling. Research questions focus on the nature and level of the differences of sensibility between 3 varieties. Conclusions are expected on the pertinence of the markers of detection and determination of threshold values. For this purpose, genetic, biochemical and physiological data was obtained through multiple methods for a multivariate analysis.

# Material and Methods

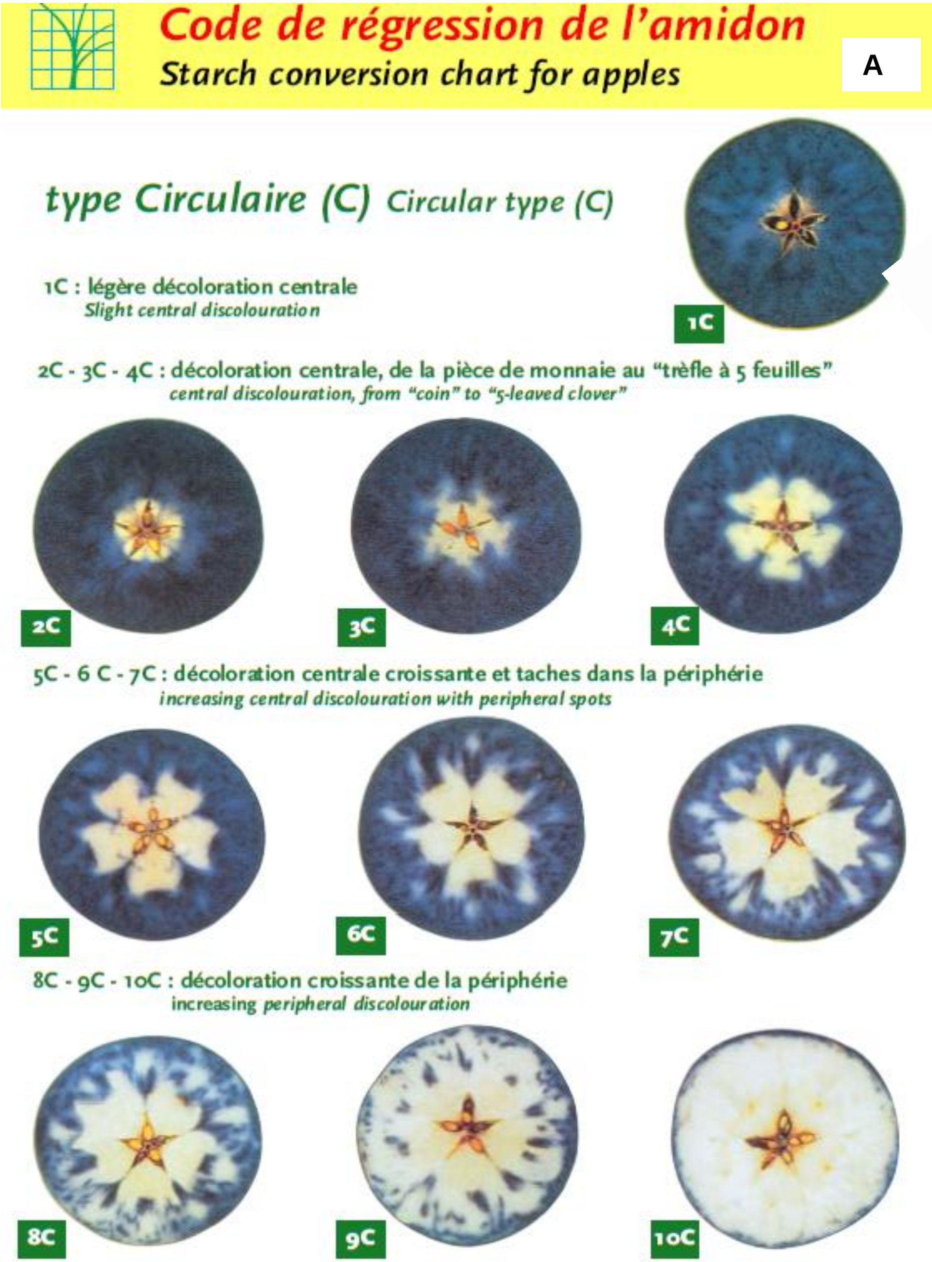
## Plant Material & Experimental design

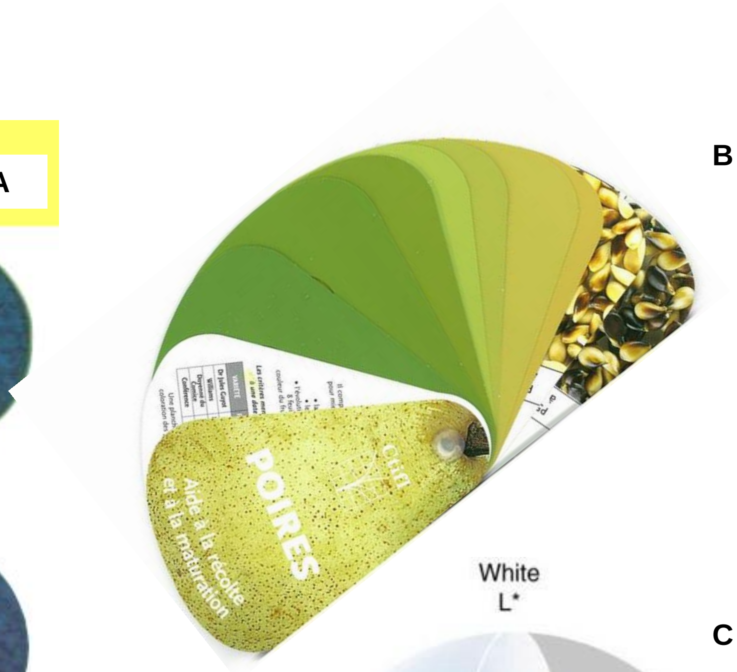
The *ESCALPE* project was designed with 3 pear cultivars (*Pyrus communis* L. cv *'Blanquilla'*, *'Conference'* and *'Flor de Invierno'*,Table 1) displaying different SS symptomatic pattern. Fruits were harvested at different dates in commercial orchards located in Lleida, Catalonia, Spain and analyzed at IRTA FruitCentre (Figure 3). Standard storage conditions were used (0 °C, 90% RH) during 4 and 6 months. Fruits were later removed for shelf life assessment at 20°C.

*GOP:* Leaves and fruits of *'Conference'* were collected on the trees of 20 commercial orchards belonging to *Rincón de Soto* PDO located in La Rioja Valley, Spain. Fruits were sampled at different dates during the growing season and transported to the laboratory. Fruits harvested at optimal harvest date were stored at 0 °C, 90% RH, under 2 different atmosphere conditions: standard storage (2.5% $O_2$ +1%$CO_2$) and at high level of $CO_2$ (1% $O_2$ + 5% $CO_2$). The samples were evaluated after 4 and 7 months of conservation. Meteorological data was collected from the 3 harvest areas (Low, Middle and High La Rioja valley) to characterize climatic factors of influence. A 2-week-delay exist in harvest time between the 1$^{rst}$ and the 3$^{rd}$ zone.

## Mineral content analysis

For GOP, the mineral content of 13 minerals were determined in leaves in spring (April) and in leaves and fruits in May, 100d after full bloom (DAFB),15 days before harvest (BOH) and at optimal harvest date (OH). DAFB was determined based on the phenological identification key of pome fruit (Meier, 2001, Figure 4). 5 sampling points of the leaf and 4 points of the fruit were analyzed. The 900 samples were analyzed hiring La Gajera laboratory and results averaged.



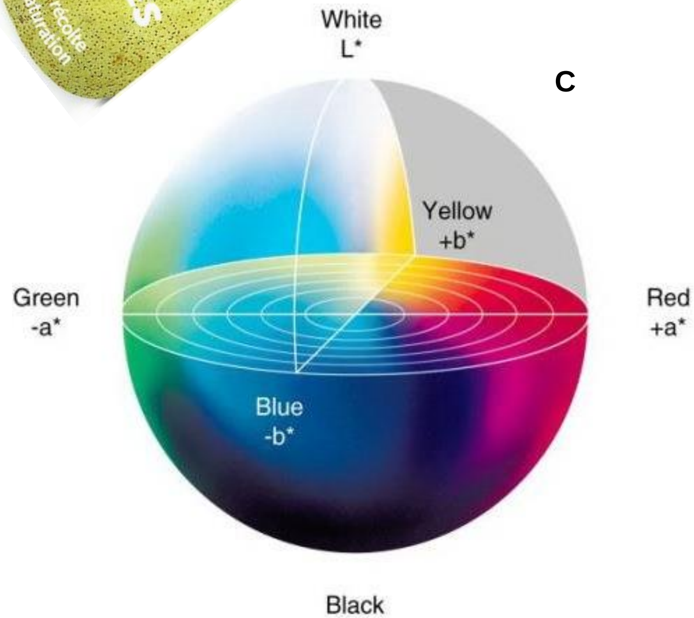




Figure 7: Quality Tests: (A) Apple-Pear starch index chart and (B) Pear Epidermis color chart (CTIFL, France) (C) CIELAB space color. The L*axis represents lightness (0: no lightness, i.e., absolute black to 100, maximum lightness i.e. absolute white).

## Brown Heart and Superficial Scald occurrence scoring

The occurrence of both disorders was monitored after 4 and 7m of storage for GOP, and 4 and 6m for ESCALPE immediately (0) and after 7d at room conditions. SS and BH disorder incidence were determined as: $Index = \frac{sum\ of\ affected\ fruits\ by\ severity\ class}{total\ of\ fruits} \times 100$. Severity classes were defined according to % of affected tissue. 60 pears were cut transversally and longitudinally and scored as following: Initiation stage, *BH1* = discrete brown spots without cavities covering <30% of the flesh, moderate *BH2* = 30-50% of the flesh showing brown color and slight cavities, severe *BH3*= more than 50% of the flesh brown with large cavities. For SS, symptomatic brown color surface was estimated: *SS1*<30%, 30%<*SS2*<50%, 50%< *SS3*<75%, *SS4*>75%.

## Initial maturity indexes

*Flesh firmness* (N) was measured on 3 replicates of 6 fruits each with a penetrometer (53200, T.R. Turoni SRL., Italy) equipped with an 8 mm probe as described by (Chiriboga *et al.,* 2011). The higher the force needed to penetrate the fruit, the more immature it is.

*Total soluble solids content* (SSC, °Brix= 1g soluble sugar on 100g) were measured on pear juice (blend of 6 fruit per replicate and 3 replicates per sampling) using a digital hand-held refractometer (PAL-1, Atago, Tokyo, Japan). Values increases with maturity stage.

*Sugars (sucrose SUC, D-glucose GLU and D-fructose FRU) and L-malic acid (MA)* were extracted dissolving 0.5 g of frozen tissue in 1.25 mL of extraction solution as described by (Giné-Bordonaba *et al.,* 2017) using commercial kits (BioSystems S.A., Barcelona, Spain). The centrifuged supernatants of each sample extraction were recovered and used for enzyme coupled spectrophotometric determination of GLU and GLU-FRU (hexokinase/phosphoglucose isomerase), SACCH (β-fructosidase) and MA (L-malate dehydrogenase) at 340 nm.

*Starch Index (SI)* was evaluated on 18 fruits samples as described by (Almeida *et al.,* 2016) with some modifications. An equatorial slice of each fruit was dipped in a solution of 0.6% (w/v) iodine in 1.5% (w/v) KI for 10 min and then SI was subjectively determined using the 10-point scale chart developed by the CTIFL (France, Figure 7A). High value indicates immaturity.

*Streif Index* was calculated as: $\frac{firmness}{SSC\ .\ Starch\ Index}$.

*Color* of the epidermis was determined with Konica Minolta CR-400 colorimeter previously calibrated (**Error! Reference source not found.**B). The results were expressed in the color space system of the *Commission International de l'Eclairage* CIE L*a*b* (Figure 7C). a*b* values (yellow-green) related to the color of the varieties were selected as indicator of maturity, yellow color being associated with ripening.

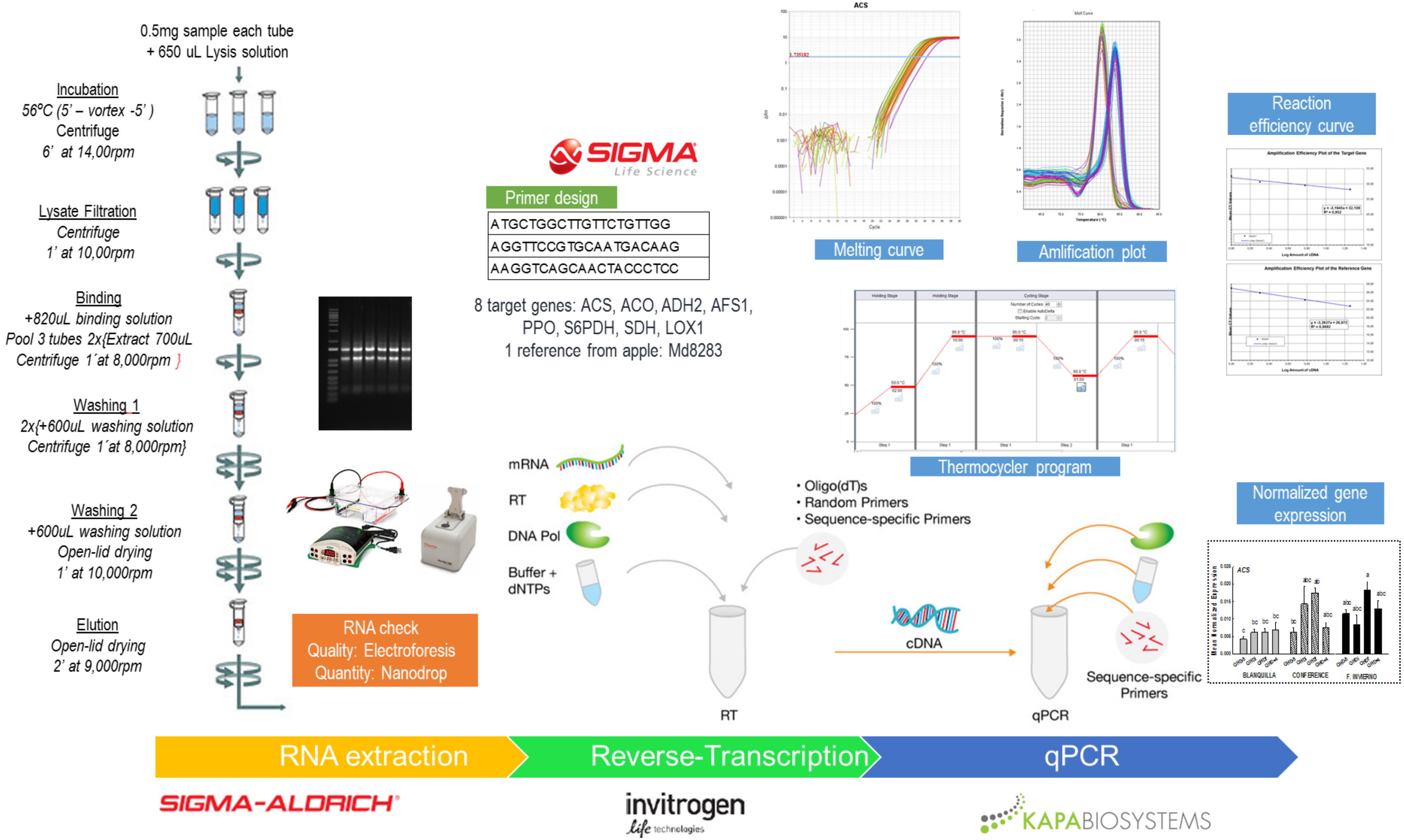



Figure 8: ESCALPE protocol for the molecular analysis of gene expression of 8 targets genes suspected involved in Superficial Scald disorder (C.LECLERC adapted from SIGMA scheme). DNA pol, Deoxyribonucleic acid polymerase; dNTP, deoxinucleotides tri-phosphate; mRNA, messenger Ribonucleic acid; RT, Reverse-Transcription; cDNA, complementary DNA; qPCR, quantitative Polymerase Chain Reaction. See abbreviation section for gene names and Figure 18 for their role in cell physiology.

*Index of Absorbance (IA)* difference ($I_{AD}=A_{670nm}–A_{720nm}$) was determined with a hand-held a DA-Meter (TR Turoni,Forli, Italy) on opposite sides of the equatorial parts of the fruit. It is correlated with the chlorophyll-α content of the fruit (absorption wavelength) and fruit ethylene production.

*Titrable/Total Acidity* (TA, g of MA. $L_{-1}$) concentrations were measured on the same juice samples and MA calculated as: $\% \text{ MA} = \frac{[\text{ml NaOH used}].[0.1 \text{ N NaOH}].[0.067 \text{ MAmilliequivalent factor}]}{\text{grams of sample}} \times 100$. Acids and TA values tend to decrease during maturation and storage.

## Physiological maturity indexes

*Ethylene production* (nmol.kg$^{-1}$) was measured as described by Giné-Bordonaba *et al.,* (2017) with some modifications. 4 replicates of 3 fruit each were placed in 2L flasks sealed with a silicon septum for sampling the gas of the headspace after 3 h of incubation in an acclimatized chamber at 20 °C. For the analysis of ethylene production, 1 mL-gas samples were taken using a syringe and injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) as previously described by (Giné Bordonaba *et al.,* 2014). Earliness of Eth production was calculated as the difference of the date for initiation of Eth production after 14d cold stress and production at harvest: $rlyEth\ (d) = initEthH - initEthS$ . Eth volumes at 7 and 15d in both conditions allow to compute activation:$act = \frac{EtH-EtS}{EtS}$.

## Biomolecular markers

*RT cDNA bank creation*: RNA extraction of the 36 samples (3 varieties, 4 sampling dates, 3 replications) was done following the *Spectrum™ Plant Total RNA* kit from Sigma-Aldrich with some modifications (Giné-Bordonaba unpublished (Figure 8). No DNAse treatment was applied. Samples were kept at -80°C for further analysis. Volumes were calculated for each sample to reach a final reaction volume of 20 µL having 2 µg RNA (100ng/µL). A 13 µL-mix containing RNA sample, RNA-free water, 1 µL oligodT and 1 µL dNTP was vortexed and spin. The thermocycling conditions were pre-set at 96°C and program as following: 65°C-5min, 4°C-2 min, 50°C-10 min, 80°C-10 min, 4°C infinite. During the first 5 min interval, a second mix of 7µL was prepared: 4µL buffer, 1 µL L-1dithiothreitol DTT, 1 µL inhibitor ribonuclease, 1µL transcriptase. The mix was vortexed, spin and added to the previous mix. After the completion of the thermocycler program, 1 µL RNase was added. Tubes were vortexed and put in a pre-set bath at 37°C for 20 min. The quality of the RNA and cDNA was check by *Nanodrop™* ND-2000 spectrophotometer.

Table 2: R Statistics and JMP Modeling details. R function in Annex VII.

| | **GOP** | **ESCALPE** |
|---|---|---|
| **Physiopathology** | ***Brown Heart*** | ***Superficial Scald*** |
| **Objective** | *Look for markers able to explain and predict the physiological disorder* | |
| **Individuals** | 1 cv. *'Conference'* | 3 cv.'Cfe', 'Bla','Finv' |
| **Origin** | 20 plots | 1 grower |
| **Tissue** | Leaf (L), Fruit (F) | Fruit |
| **Date of analysis** | 5 Sampling Dates SD during growing period till harvest (L): April, May,100 DAFB, 15BOH, OH; 4 (F): May, 100DAFB, 15BOH, OH | 4 dates close to Optimal Harvest Date OHD: $OHD_{-5}$, $OHD_i$, $OHD_f$, $OHD_{+4}$ |
| **CA conditions** | Standard (s) /high (h) $CO_2$ level | |
| **Predictor Variables** | 53: 2x13 *minerals* (N, P, K, Na, Ca, Mg, Mn, Zn, Cu, B and 3 ratios), 10 *initial maturity indexes* (weight, DW, abColor, DAmeter, SI, Firmness, TA, TSS, MA AA), *9 physiological indexes* (EtH7/15d EtStress7/15d, act7/15, iniEtH/S, earlyEt) | 26: 9 *Enzymatic markers*: ACC, MACC, ACO, ACS, LOX, PPO, POX, SOD, CAT, 12 *Biochemical markers compounds:* TAA, AsA, DHA, MA, GLU, GLU-FRU, FRU, sucrose, FRAP, phenols, α-farnesene, Ctol281, 4 *inicial maturity indexes*: Firmness, TSS, TA, SI, 8 genes (ACO, ADH2, ACS, LOX1, PPO, SDH, AFS1, S6PDH) |
| **Response variables** | Incidence of alterations after 4 and 7 months of storage of (BH4/BH7m) and total BH | Incidence of alterations at 2 removal dates: 4 & 6 months, 0 & 7 days (SS4m0d, SS6m0d, SS4m7d, SS6m7d) |
| **Data** | means - autocentered and scaled for PLS, repetitions for statistics | |
| **Statistical tests** | *Quartiles-median boxplot, Kurtosis & Skewness* : distribution and outliers<br>*ANOVA* : differences calculated according factors and group mean<br>*Student's t-test* : pairwise comparison with the group mean<br>*Tuckey s test*: multiple group discriminations compared with the group mean | |
| **Analysis & Selection of the variables** | PCA: Projection of the variables in a synthetic space to examine their relationships and the quality of their representation in relation to the response variable based on their variance.<br>*Spearman's* pairwise correlations for defining the degree of relationships with no assumption on the distribution<br>*PLS regression*: by iteration, look for a linear component of the predictors with the highest covariance with the response variable under the constraint of orthogonality. It tries to maximize the variance between predictors explained by the components and simultaneously maximize the correlation between those components and the outcomes. | |
| **Model validation** | *Voet* index: determine whether a model with a specified number of extracted factors differs significantly from a proposed optimum model<br>*PRESS*: *Predicted Residual Error Sum of Squares* measuring how well the predictive values generated by the model fit the observations.<br>*Diagnostic plots*: Quantile-to-Quantile-plot, actual vs. predicted values, residuals | |

*Quantitative Real-Time Polymerase Chain Reaction* validation of gene expression was performed with an Applied Biosystems 7500 Thermocycler the following KAPA SYBR® FAST. Targeted genes were related to Eth and sugar pathways with one reference gene *Md8283*. The cDNA templates were reverse transcribed using the total RNA previously obtained and dilutions were made assuming a 100%-RT-efficiency from the enzyme during PCR giving 100ng/µL yield. Calibration curve was drawn with 50 to 0.5 ng/µL pool of samples solution for calibration. All of the primers for selected genes were taken from Busatto *et al.,* (2019) and made by Promega. The 10 mL final mix composition includes for 1 reaction: 5µL SYBR, 0.3 µL primers each [10mM], 2 µL template DNA [3ng/mL], 0.2 µL ROX [300nM], $H_2$0. ROX passive reference is mainly used to normalize the signal. Reaction program was 40x [50°C-2 min, 95°C-10 min, 95°C-15 s, 60°C-1 min, 95°C-15 s]. Relative quantification of specific mRNA levels was performed looking at the CT values. Quality was checked with standard deviation and melting curves. PCR efficiency ranges 96%-104% and $R^2 > 0.985$ are acceptable.

## Statistical analysis

Statistical analyses were performed using statistical software RStudio v1.1.463 and R v6.0 (R core team 2018). Normality and variance equality were checked. One- and two-way ANOVA were performed and when significant differences were found, Student's *t*-test (2 groups) or Tukey's test (>2 groups) were computed with *Least Square Difference* discriminated at *p*-value<0.05 significance. Spearman's pairwise correlations were selected at α=0.05.

Basic models for both disorders were built according to multifactorial analysis *Partial Least Square* method with JMP® 14.01 PRO SAS Institute Inc. The non-linear iterative partial least squares (NIPALS) algorithm and *Leave-One-Out* validation for small sample size were used to select the number of factors that minimizes the Root Mean PRESS statistic (Table 2). As a pre-treatment, data were centered and scaled using the inverse of the standard deviation of each variable to avoid the influence of the different nature and scales of the variables (macro/micro minerals/gas/enzymes/metabolites/gene expression). Minerals' inter-relations (multi-collinearity) was ignored as in Deuchande *et al.,* (2017). The individual variable enters the algorithm to build a linear additive modeling equation. The regression models coming out were selected based on: the minimum number of factors with the highest Voet index (>0.1), the highest percentage of variance explained ($R^2X$ and $R^2Y$). For a close percentage of explanation, models with the lowest number of variables were preferred, taking care of preserving the biological sense of the data. Variable of Importance Factors (VIF) values (>1.0) were considered as representative predictors for the model. PRESS value and diagnostic plots (actual vs. predicted, residuals) were examined to judge the quality of the model.

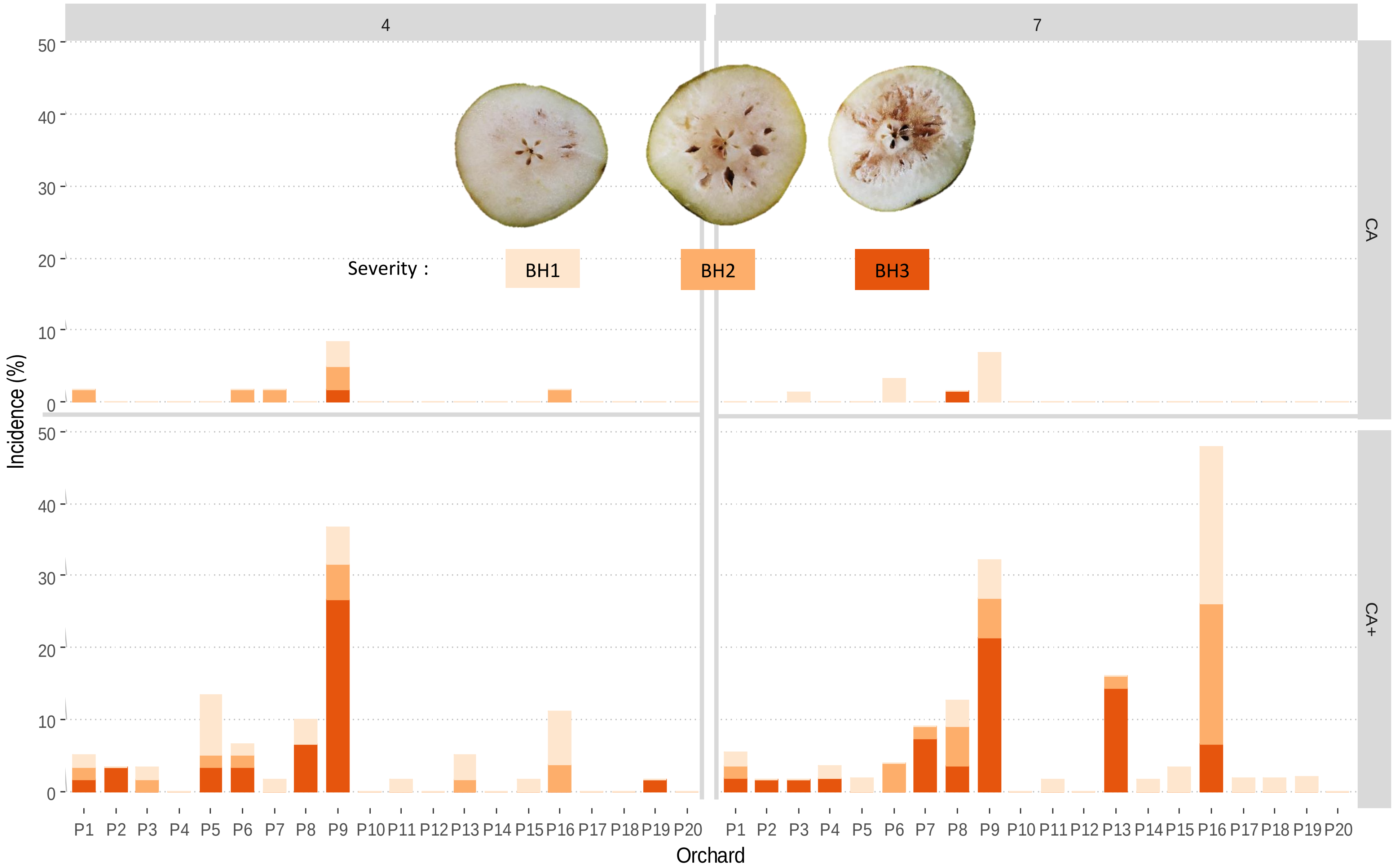



Figure 9: Brown Heart incidence (%) and severity for each orchard under standard CA and high $CO_2$ CA+ after 4 and 7 months of storage. Means of 60 fruits. Values are masked for readability (RStudio).

# Results

## GOP

BH disorder incidence: In 2018, only low BH incidence was found in La Rioja (<1% in CA, group mean) compared with alarming 2015 season (15-20%). Damage occurrence was increased in high $CO_2$ CA (CA+ >5% when CA<1%, Figure 9). BH symptoms were detected in fruits from 17 different orchards when stored in CA+ (Producer P) and only in 7 in standard CA. Maximum BH incidence level was detected in fruit from P16 (47.8%) followed by P9 (32.1%). P9 pears were found very prone to develop BH in both atmospheric conditions. High $CO_2$ level also amplifies BH damage severity: higher number of fruits had BH3 level symptoms (BH3 7m 0.08% CA to 3.00% CA+). BH disorder was also increased with storage duration (5.06% to 7.41%). Interestingly, 3 growers provided fruits batches that remained BH-free after both 4 and 7m in CA+. The characterization of these samples may help to distinguish BH related parameters.

Mineral contents*:* Individual data will not be shown for confidentiality. Leaf values respected Pomaceae Center references (Annex VI). Statistical analysis shows that means and standard deviations display great variability between the sample tissues (L/F), their origin (orchards) and sampling date (SD) (Annex VIII and Annex IX). For some microminerals, high standard deviations led to focus specifically on these parameters, either for detection of outliers or to identify variable potentially related to BH occurrence.  Leaves and fruits mineral evolution during the growing season have a distinct pattern. April leaf samples and May fruit samples that were significantly different from the rest of the samples. In both organs, dispersion of N P K content values was low suggesting relative homogenous practices in micromineral fertilization.

In leaves, Zn element did not vary much during the season. However, Zn content appeared extremely high for P18 and 20. Na level (season cumulation and by SD) were very low for P14 but high for P17 and 12. Mg element was found at high level for P18 and Cu recorded for P10, 8 and 1 reached exceptional high concentrations. Cumulative B and Ca level were the highest in the samples of the 3 producers from the highest part of La Rioja. Exceptional values were finally recorded in April and May for B, and Ca at 100DAF to the end of the sampling.

In general, K content was judged high in fruit. Na evolution during the season differed from the other mineral patterns and according to orchards. At the orchard level, differences were noticeable for minerals B (P7,11,16,18,20), Cu (P17,18), Fe, Mn, Zn (P18, 20) and especially for Na (P5,9,10), at OH in particular, (seasonal cumulation and by SD). The ANOVA test points out a significant effect of orchard location. Leaf Fe and fruit K/Mg/Ca were positively inter-correlated from 100 DAF to OH date. In May, a negative correlation between Fe-l and Cu-f was found Cross-interactions between *SD* and *Location* factors were significant for Ca,

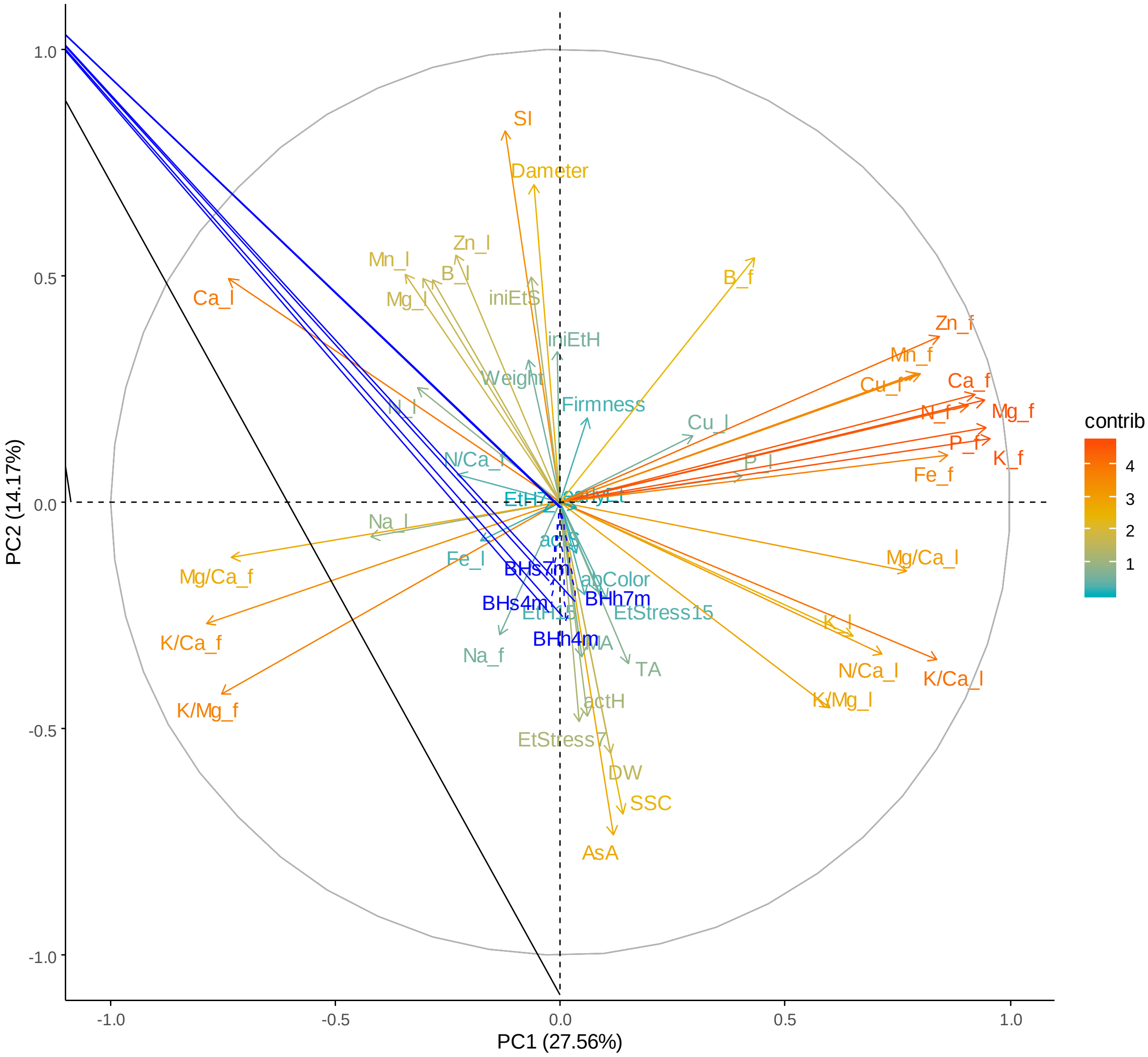



Figure 10: GOP 2018 PCA computed with May to OH data pooled together for the 20 orchards (RStudio). Minerals measured in leaves and fruits are distinguished with letter (l , f). Dashed blue arrows representing BH scoring after 4 and 7 month of storage under standard (s) and high CO2 (h) atmosphere are set as supplementary variables. Colors represent the contribution if the variable to the construction of the model

Zn, Mn and B, K/Ca in leaves and in fruit for B. Mineral evolution and levels may explain maturity and BH occurrence.

Harvest maturity indexes related to BH were analyzed only at Optimal Harvest date. PDO exigencies for SSC levels (13-19° Brix) were respected. Fruits were firmer (>6.12 $kg.cm^{-1}$) and starch regression index also higher than 2C-3C expected for more than 75% of the producers. Only 2 producers had smaller fruit than specified (Category Extra and I, >58-60mm equivalent to > 150 g). DAmeter, SSC, and SI values were found significantly different in pears coming from the highest area of La Rioja Valley (Annex IX). The fruits seemed less mature and had highest DAmeter and SI values and lowest SSC. Although not significant, fruit were also bigger (Weight and DW) and AsA and MA were among the lowest found in this experiment.

Physiological parameters: An important inter-plot variability for ethylene-related variables was found. Only post-stress Eth production at 7d (EtStress7) was found significantly lower in fruit produced in the highest part of La Rioja (Annex IX). Although not supported by ANOVA-Tukey test, these fruits presented the lowest rate of ethylene production and % of activation at 7d. Ethylene burst also started later than in fruits from the Mid and Low-La Rioja when exposed to cold-stress or not. This is consistent with our previous observations on quality indexes. More mature fruit are to be more sensitive to cold stress and consequently ethylene-burst starts earlier. Physiological variables indicate that the maturity stage at harvest is a key factor in BH disorder.

Integrative multivariable analysis: Spearman's pairwise correlations were computed between all the set of variables (minerals, physico-biochemical, physiological) for May to OH globally and at all Sampling Dates (**Annex** X). High content of Ca, Fe, Mg in fruit were related to firmer fruits while elevated K/Ca ratio had a negative effect on firmness. Foliar Ca and Cu were negatively associated to SSC and DW respectively. N/Ca relation was opposite with this parameter.

Principal Component Analysis was performed considering all the variable independent pooling May to OH data (Figure 10). The biplot shows that variance of BH incidence is poorly explained by the variables (PC1+PC2+PC3= 27.00+14.07+9.2=50.27%, arrow length). Removing the outlier P9 reduced insignificantly the total variance explained. Fruit minerals are the most contributive variables to this graphical representation. Anticorrelations between BH and leaves microminerals Mn, Zn, Mg and B are evidenced on the PCA. A putative relationship between foliar Ca content and fruit B content with BH incidence is also noticeable. Positive correlations (same orientation,

Table 3: GOP Model comparison for prediction of Brown Heart disorder (Ys) according to $CO_2$ storage atmosphere (CA/CA+), sampling date (April to OH), plant organ (F Fruit, L Leaf) and data type (raw or with calculated variables) (JMP PRO ® 14..) Hyphen indicates unsuccessful attempt of modeling

| **Test** | **Model NIPALS** | **Nº factors** | **% R² X** | **% R² Y** | **Nº VI > 1.0** | **Nº predictors** |
|---|---|---|---|---|---|---|
| Atmosphere+ SD + outliers | CA+ april | 4 | 69.67 | 91.51 | 4 | 11 |
| | CA+ may | 4 | 76.41 | 87.44 | 4 | 10 |
| | CA+ 100DAF | 2 | 37.00 | 75.42 | 4 | 11 |
| | CA+ 15BOH | 5 | 72.32 | 89.92 | 7 | 15 |
| | CA+ OH | 10 | 92.69 | 97.21 | 7 | 15 |
| | CA+ OH -P9 | 1 | 30.83 | 76.56 | 5 | 9 |
| | CA OH | 3 | 42,35 | 90,13 | 5 | 12 |
| | CA OH -P9 | 2 | 44.22 | 79.15 | 3 | 11 |
| Atmosph. + data type + outliers | CA+ OH raw | 3 | 69.54 | 64.83 | 2 | 6 |
| | CA+ OH raw - P9 | 1 | 12.93 | 76.92 | 12 | 15 |
| | **CA+ OH raw – P9,16** | **2** | **21.75** | **53.91** | **6** | **13** |
| | CA OH raw | 4 | 60.98 | 81.72 | 4 | 8 |
| | CA OH raw - P9 | 2 | 26.38 | 82.89 | 10 | 13 |
| | CA OH raw - P9,6 | 2 | 22.12 | 88.09 | 17 | 19 |
| Atmosphere+ organ + data subset | CA F OH total | 2 | 26.73 | 77.53 | 7 | 9 |
| | CA+ F OH total | 2 | 41.42 | 69.09 | 5 | 8 |
| | CA+ L+F minerals | 12 | 100 | 92.75 | 7 | 10 |
| | CA+ F minerals | - | - | - | - | - |
| | CA+ L minerals | 3 | 88.81 | 14.19 | 2 | 7 |
| | CA+ OH F quality | - | - | - | - | - |
| | CA+ OH F physio | 3 | 95.62 | 73.65 | 2 | 5 |
| | CA+ OH F minerals | - | - | - | - | - |

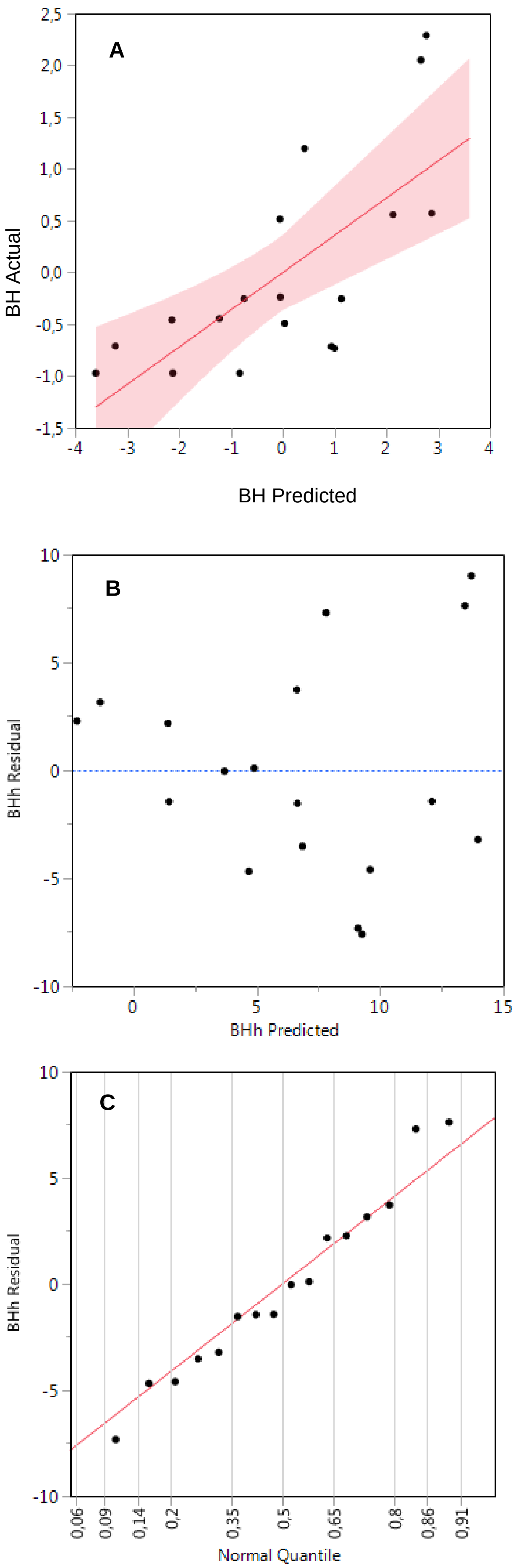



Figure 11: Selected regression model and diagnostic plots for Brown Heart incidence prediction in high CO2 storage conditions (JMP PRO ® 14).

Actual *vs* .predicted values (A) 95%-confidence zone of prediction value is colored in red. Residuals *vs* predicted values (B). Q-Q plot (C). Model computed for CA+ atmosphere condition, OH date, , raw data, removing P9 and 16 outliers. The 3 plots put in evidence elevated risk of error of prediction: large dispersion of the values from the referent lines. Enlargement pattern indicate that homoscedasticity of error (or equal variances) assumption is not exactly met.

narrow angle) between BH incidence and quality parameters SSC, DW and AsA and anti-correlations with DAmeter and SI suggest that more mature the pears are at harvest, the higher the risk of BH occurrence. Indeed, late harvest allows the fruit to accumulate sugars, increase in size and loose its immature green color. Orchards position in Annex XI.

Markers associated to higher occurrence of BH: ANOVA-Student's *t*-test was conducted to see if any parameters could be discriminant between intact and affected orchards (I/A). Some of them were found noteworthy but were not constant over the season (OH shown in Annex VIII). In April, N/Ca, K/Ca and Mg/Ca ratio were significantly different for the 2 classes in leaves, being lower in healthy fruit. In May, foliar Ca and fruit Mg content was about thrice lower in pears where BH symptoms were observed. None of the minerals in both organs were found different at 100DAF and 15BOH sampling dates. At OH, significant differences were found for the DAmeter (slight lower values in BH affected fruits) but also with time of onset of Eth burst initiation (initEtH), post stress production rates at 7d and 15d (act7 and act15) that were thrice higher. The potentially interesting minerals previously commented were not significantly discriminant at $\alpha$=0.05 level: Na (*p*-value 0.12), K/Ca-f (0.13), Mg/Ca-f (0.24), Firmness (0.19), SI (0.21), abColor (0.25).

The correlation between predictors and BH disorder varies according to storage (). Na concentration in fruit was positively correlated with BH under CA but not under CA+. In this way, N/CA ratio in fruit after 7 month of storage appeared to be a good marker for CA but only after 4m in CA+. Moreover, DAmeter strength of correlation is lower under CA+ and a weak relationships was found between BH at 7m and Color. BH occurrence is also clearly related to Eth production in CA+ or in CA, for short and long storage duration. More precisely, BH incidence was related to the date of initiation of Eth production after cold stress and to the post-stress rates at 7d. Then comparison of various sub-models was carried out according to sampling date and organ factors.

Model development: *Partial Least Square* method was used on the total dataset or subsets to correlate the mineral, physiological, physico-chemical variables to BH incidence in different atmosphere and for different duration (Ys, Table 3). Although, 2 tissues were analyzed, candidates Xs predictors were not weighted as in Structure-Function models. Y explanation for some model was high, but diagnostic plots put in evidence poor prediction abilities (residuals dispersion, Figure 11). Best fitting was obtained for CA+ at harvest removing 2 orchards detected as outliers (Voet plot, not shown). When entering all the variables (Fruits, Leaves and calculated variables act7, act15, earlyEth), the cumulative variation explained for BH incidence was higher than when using only raw data (CA+ 97.21%X-64.83%Y, CA 90,12%X-81.72%Y). However, variation between Xs was lesser. Exclusion of the outlier orchard detected with diagnostic plots reduced the number of factors needed but also cumulative variances explained.

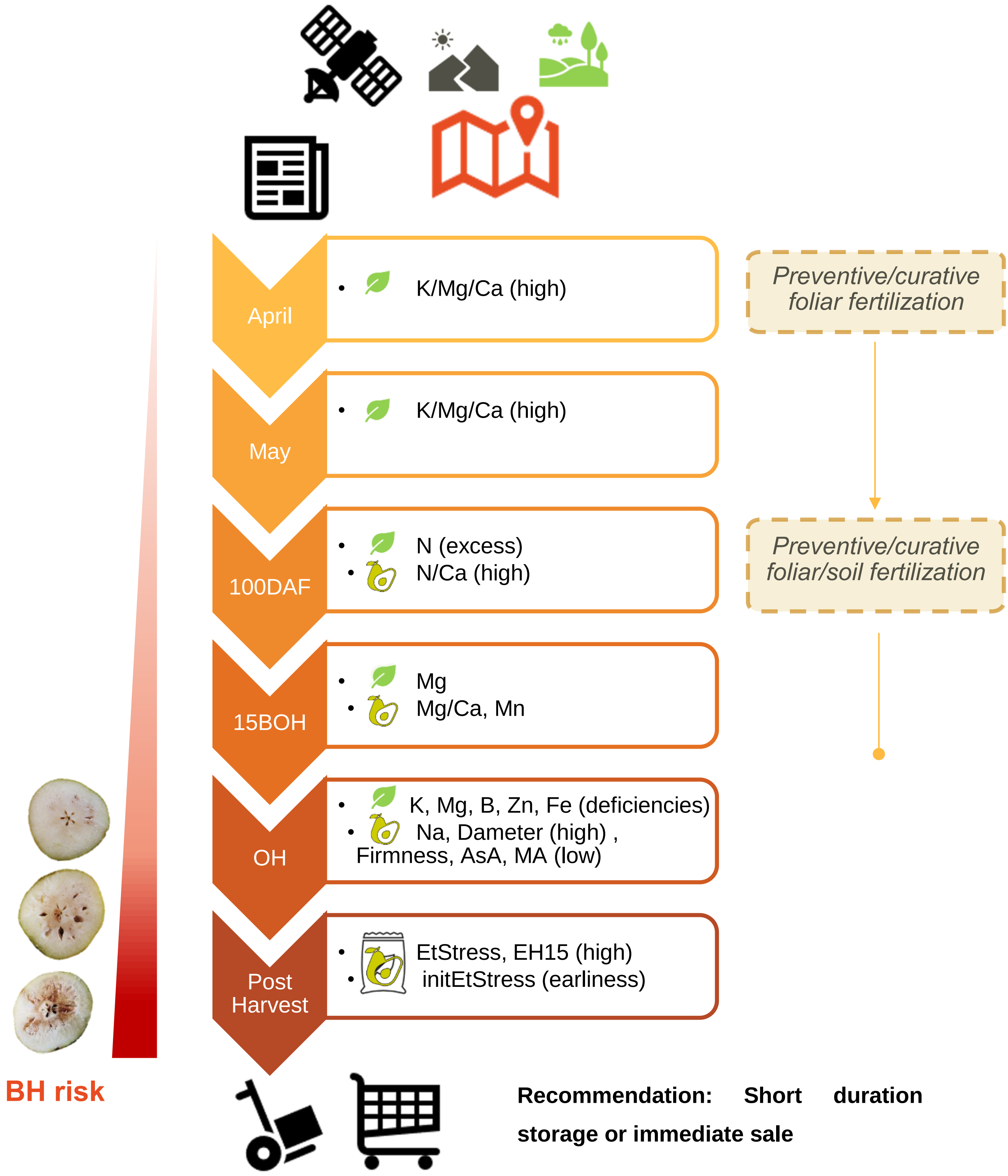



Figure 12: Proposition of checking points for parameters identified in this study related to Brown Heart disorders based on PCA, Spearman correlations and JMP equations. Considering the pedoclimatic conditions, the orchard past and practices, the indices mentioned for each date should (C.LECLERC) be monitored. If level in parenthesis is elevated (reference to be defined), the risk of Brown heart development increases. Intermediate action should be taken to avoid reaching the next warning levels. The final decision would be taken considering these checking points. Highest risk calculation would lead to the recommendation of rapid selling of the fruits.

Sub-models were computed to avoid potential interactions with other variable sets. *Variable Importance Factor* level set at >1.0 confirm the implications of the parameters displayed on the PCA biplot and equations (not shown) were in concordance with ANOVA result. In April and May, Mg/Ca (K/Ca or K) importance was verified. At 100DAF, N in both organs and N/Ca equilibrium in developing fruit enter the model. At 15BOH, Mg in leaves, (Ca, Mn, Mg/Ca, in fruits, VIF=0.9). At OH, CA+ conditions, foliar K, Mg, B content, and Na in fruit (VIF>0.9) were minerals of interest. DAmeter, EtH15, EtStress7 and initEtStress appeared as remarkable biochemical and physiological indexes. For CA conditions, minerals N, Fe *in leaves*, Ca, Zn, K/Ca, Mg/Ca in *fruits* were part of the predictive equations. Among the indexes related to quality and fruit physiology were Firmness, EtH, init, act, earlyEt. DAmeter and MA were also highlighted at VIF>0.9 level.

#### GOP CONCLUSION:

The observations confirm that BH is a $CO_2$-CA related storage disorder: incidence was higher after long storage and symptoms severity is amplified under high $CO_2$ level. Considering the pedo-climatic evolution during the growing season, a evolutive model was proposed although no clear threshold value could be determined for drawing a real tree of decision. Checking the parameters earlier than when they appear in the models for BH occurrence would allow the implementation of early preventive/corrective measures to avoid/limit BH. Results were synthesized in a guideline chart (Figure 12). Model develop here for GOP project may be valid only for *'Conference'* produce in La Rioja, in 2018. It will be verified and trained on 2019 season data.

## ESCALPE

Superficial Scald scoring: Significant differences in Superficial Scald (SS) disorder incidence were recorded between cultivars. Only *'Blanquilla'* and *'Flor de Invierno'* results are presented here. Although *'Conference'* fruit displays SS-like symptoms, they have been attributed to a still uncharacterized disorder called *Black spot. 'Conference'* alterations evaluation will thus not be exposed here. However, biochemical and molecular mechanism were kept to understand the main differences from common SS symptoms observed in *'Blanquilla'*. It is worth to notice that *'Flor de Invierno'* SS-like browning were not firmly considered as usual SS symptoms at the beginning of the study.

*'Flor de Invierno'* cultivar was dramatically more affected by SS-like disorder both in terms of incidence and severity (Figure 14 and 14, next page). The lowest level recorded was 70%. Disorder incidence was already maximum after 4m of storage regardless of harvest date and storage duration. In contrast, *'Blanquilla'* pears picked at OHD-5 exhibited only 30% of damage, a result that indicates that *'Flor de Invierno'* seems to be prone to early SS development. In general, maximum disorder occurrence (100%) and most severe damages, (85% for SS4 *'Blanquilla'* 7d evaluation) were recorded after 6m of storage.


Figure 14: Superficial Scald symptoms on sensitive cv. '*Blanquilla*' and *'Flor de Invierno*' scaled according to the surface of epidermis affected after 6 month of storage (photos: V.LINDO and C.LECLERC)

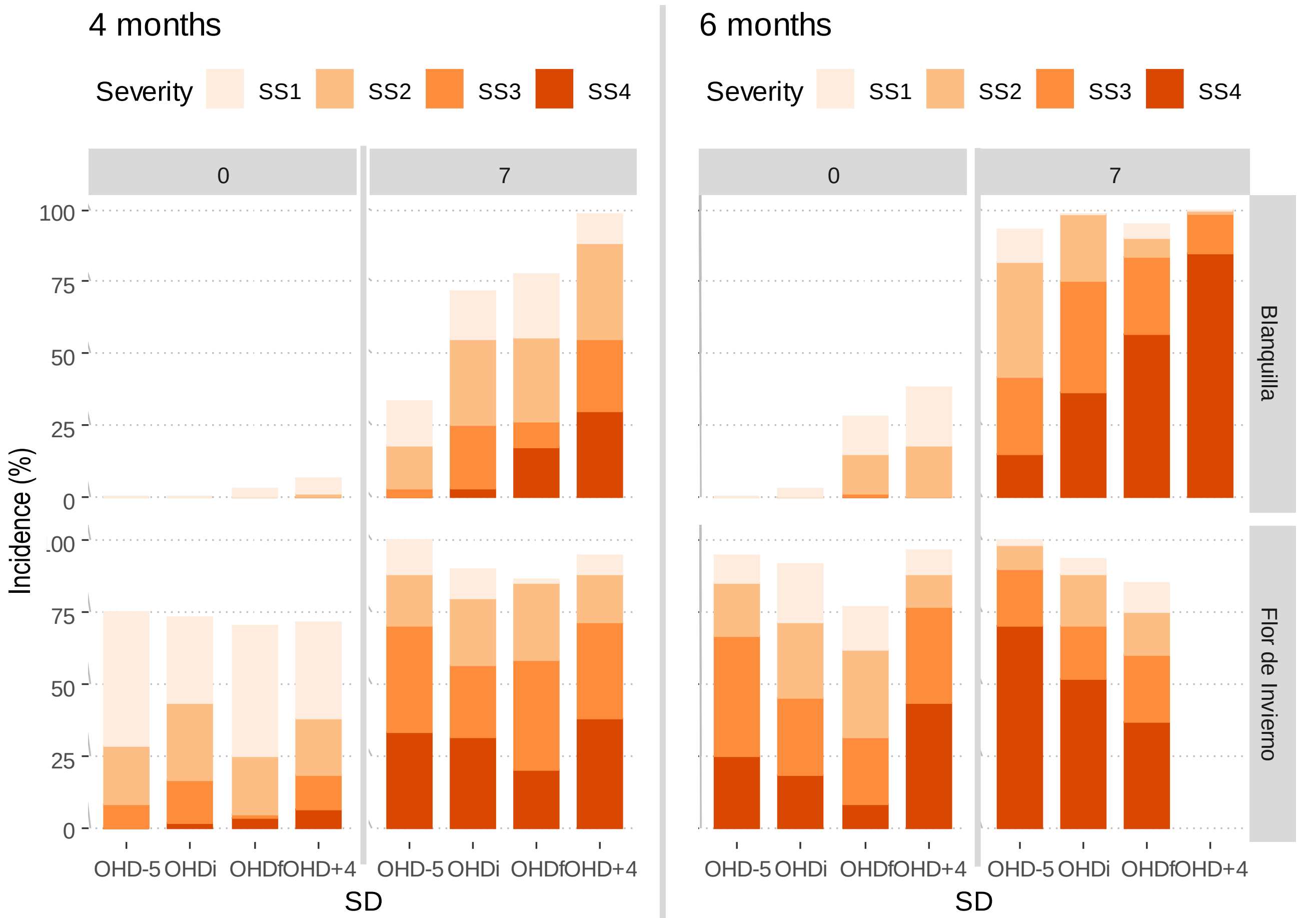



Figure 13: Superficial scald incidence and severity according to harvest date and storage duration for cv '*Blanquilla'* and '*Flor de Invierno'* 2018 season (RStudio)

Harvest date strongly influences disorder ocurrence in *'Blanquilla'* pears. Incidence increases with fruit maturity: from 0% for OHD-5 fruits to 6% and 38% at OHD+4 after 4 and 6 months of storage (0d), respectively. In contrast, *'Flor de Invierno'* being naturally highly susceptible to SS scald, no significant differences were found between harvest dates regardless of storage duration.

The storage conditions also considerably increased SS occurrence and severity in *'Blanquilla'*, especially when pears were let 7d in shelf life (normal atmosphere and room temperature). Disorder incidences in *'Flor de Invierno'* fruit in this condition after 6m were maximum.

The present analysis of pears SS disorder confirms the differences in sensibility and in rate of symptoms development between cultivars. Although damages were similar, the underlying mechanisms involved in each case needed to be clarify at the biochemical and molecular level.

### Transcriptomic analysis

The experiment was conducted to elucidate the key mechanisms involved in SS-like disorder in *'Blanquilla'*, *'Flor de Invierno'* and *'Conference'* fruit epidermis picked at different maturity stage (harvest date). The results put in evidence important differences in expression level and evolution according to the sampling dates, consistent with the development of distinct SS-like symptoms and patterns in ethylene production (not shown).

### Differences in gene expression levels between cultivars:

Compared to the 2 other varieties, *'Flor de Invierno'* exhibited the highest expression levels for the genes involved in fruit ripening-(ACS, ACO, see abbreviation section) and fermentative metabolism (ADH2) but also the lowest levels for PPO gene involved in browning and LOX gene responsible for lipid peroxidation (Figure 15, next page). Low AFS1 expression level was also found. In contrast, *'Blanquilla'*, considered very prone to SS, exhibited very low expression levels of ACO, ADH2, SDH and high for AFS1, LOX1 and PPO. '*Conference'* recorded the lowest expression level for AFS1, ADH2and SDH. For other genes, expression levels were intermediate.

### Differences in gene expression levels between to sampling date:

In *''Blanquilla''*, AFS1 expression level follows a bell-curve between $OHD_{-5}$ and $OHD_{+4}$ reaching a maximum at $OHD_i$. AFS1 is an Eth-sensitive enzyme, this may explain the down regulation observed in slightly over mature fruit. ACO expression level was minimal for

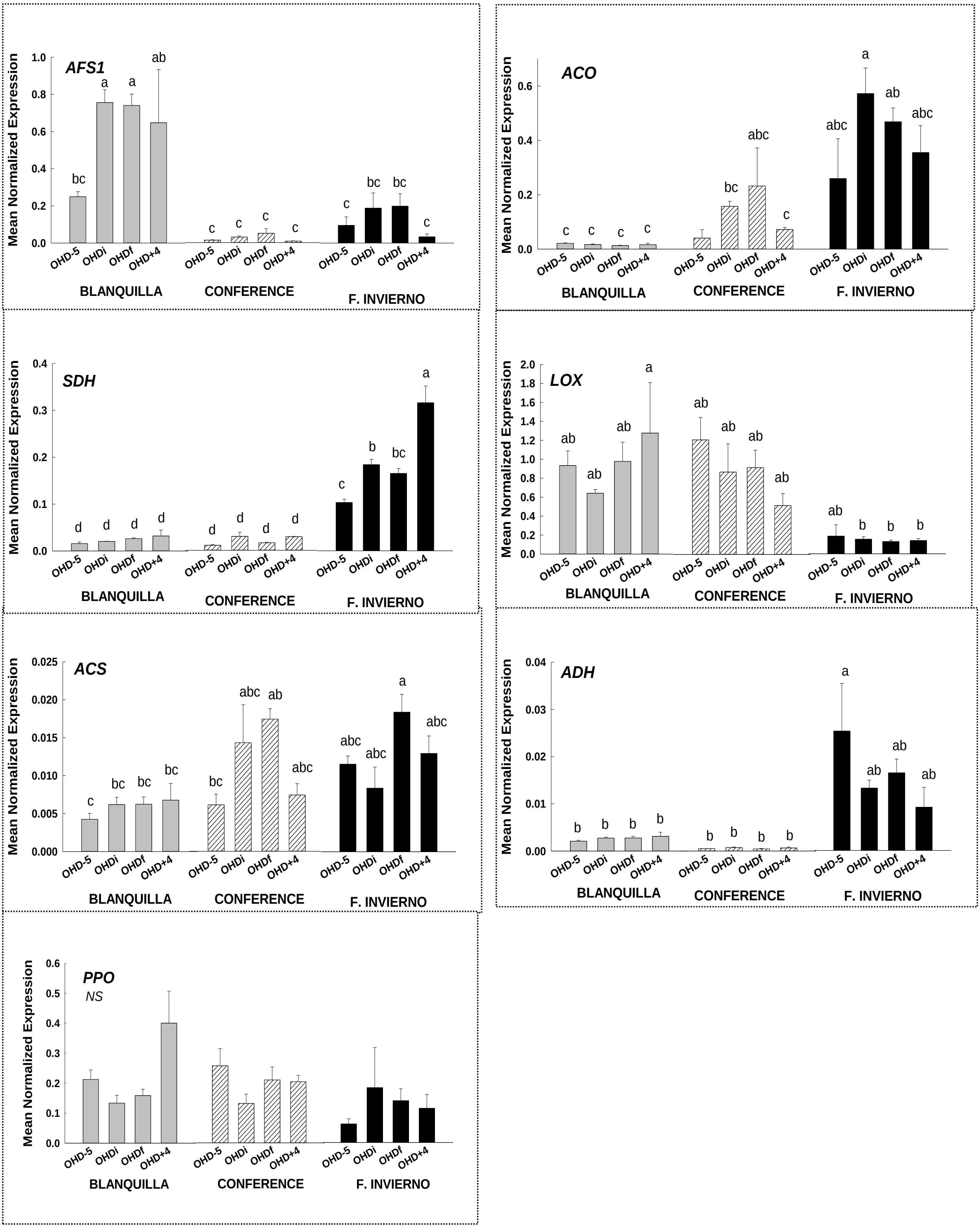



Figure 15: Normalized Gene Expression for the 8 genes of interest selected for ESCALPE project. Statistical results from ANOVA-Tukey test JMP® 14 PRO and graphics from SIGMAplot

*"Blanquilla"* and decrease in more mature samples, while the evolution for the two other cultivars draw a bell-curve. Maximum expression level in 'Flor de Invirerno' was at $OHD_{i.}$. Similar bell-curve shape evolution was noticed for ACS in *'Conference'* and *"Flor de Invierno"* which is consistent with the metabolic relationships existing between the two enzymes.

LOX1 and ADH2 expression level tend to decrease in *"Flor de Invierno"* during fruit maturation but increases in *"Blanquilla"*. *'Conference'* pattern was more difficult to interpret, results showing up-down variations for ADH2.

According to ANOVA-Tukey test, *"Blanquilla"* and *'Conference'* results were not different for SDH gene. In both cases, expression followed a linear increase pattern. Significant highest levels were found for *"Flor de Invierno"* at $OHD_{+4}$ in particular. Although expression levels were different, a similar pattern was observed in ADH2 for *'Conference'* and *'Blanquilla',* but an opposite one for *"Flor de Invierno"*.

PPO enzyme-related gene expression was not found statistically different. *'Blanquilla´* and *"Flor de Invierno"* show an opposite pattern. Expression levels first increase from $OHD_i$ to $OHD_{+4}$ in *"Blanquilla"*. *"Flor de Invierno"* PPO expression levels first increases and then very slightly decrease at the same dates. PPO levels of expression were almost constant in case of *'Conference'* across the growing season.

Fruit transcriptomic profile differs between maturation stage and varieties. It mainly concerns activation of the stress/defense responses. Over-regulated '*Flor de Invierno'* ethylene-pathways provides interesting clues and may suggest differences with '*"Blanquilla"* for ethylene sensitivity.

## Multivariate statistical approach

### *Principal component analysis*

The biplot was generated based on all the variables collected from quality, biochemical and molecular characterization of the fruits. It was first done without molecular data (PC1+PC2= 44.5+21=65.5) and then including normalized gene expressions (PC1+PC2= 44.89+20.81=65.76). Results are clearly separated by cultivars (Figure 16, next page). This confirms the different profiles observed in relation to SS-like symptoms**.** Conference data was intentionally included, although it biases the construction of the representation. For this cultivar brown epidermis symptoms were not associated to SS but likely to *Black Spot*. Nevertheless, it seemed interesting to visualize which common variables could be associated to this disorder. Except for *'Conference'*, variables related to SS seem to change according to the maturity stage of the fruit ($OHD_{-5,\ i,\ f,\ +4}$, bottom-up migration on the plot).

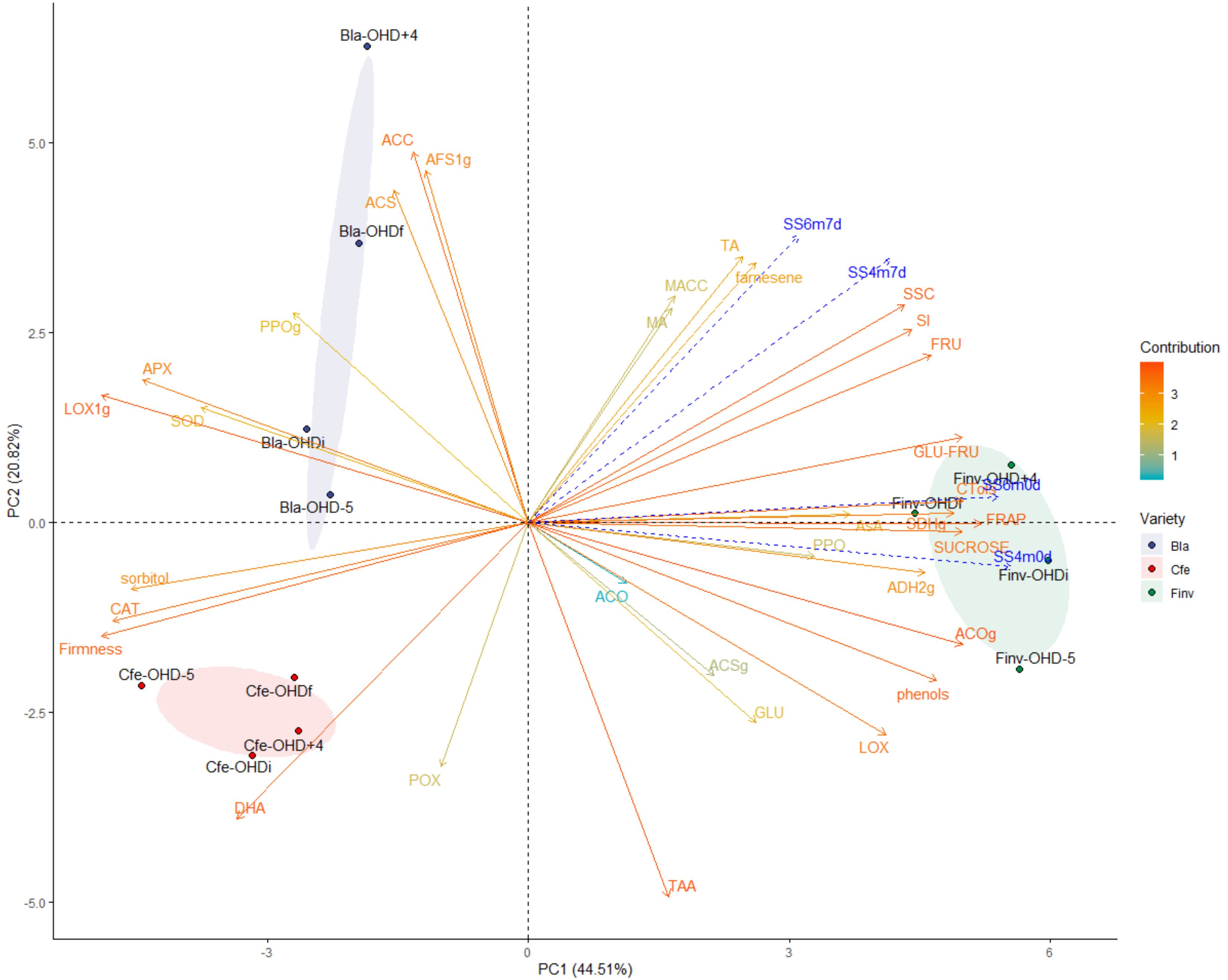



Figure 16: ESCALPE PCA Biplot for the 3 varieties (RStudio). Confidence ellipses are drawn around the 3 groups corresponding to the 3 cultivars '*Conference* (Cfe), *Blanquilla* (Bla), *Flor de Invierno* (Finv)' and Harvest Date results ($OHD_{-5,\ i,\ f,\ +4}$). Variable color indicates their importance of contribution in the construction of the graph. Biochemical data is distinguished from molecular gene expression (g). Superficial Scald incidence (SS) recorded immediately (0d) and 7 days (7d) after 4 and 6 months (4m/6m) are set as supplementary (dash blue arrows). See abbreviation section for enzyme names.

Immediate post-storage alteration scoring data (SS4m0d and SS6m0d) pointed toward *'Flor de Invierno'*. This is coherent with the field and laboratory observations: this cultivar being more sensitive to SS and the symptoms appeared earlier, even during storage. Variables highlighted were also related to sugar, antioxidant and ethylene pathways.

SS7d arrow position indicate changes when fruits are let in shelf life conditions. Angle of the arrows increases toward a more intermediate position with *'Blanquilla'* cluster. This is because SS incidence was enhanced for this cultivar in these conditions. SS-like disorder seems to be more related to firmness loss, and antioxidant potential. Starch arrow confirm that more mature fruits are more prone to early SS-like disorder development.

For a more specifically orientated SS characterization, the entire analysis (statistics and PCA biplot) were also computed with only *'Blanquilla'*(PC1+PC2= 51.20+17.57=66.77%), *'Flor de Invierno'* (PC1+PC2= 45.30+33.65=78.95%) and *'Conference'* dataset (PC1+PC2= 41.36+37.13=78.49%, Annex XII, Annex XIII Annex XIV).

*Partial least Square Regression Model:* Because only *'Blanquilla'* symptoms were considered to be representative of a standard SS disorder, PLS model was built with the dataset of this cultivar as an indicative tool for the next campaign. Predictors included biochemical, physiological and transcriptomic data to model SS incidence (Xs). Y response was SS incidence after 4m of storage and 7d of shelf-life because of higher variances between sampling dates. One iteration only was needed. The examination of the VIP table was in concordance with the PCA, Figure 16).

Briefly, most important biochemical predictors (VIF>1) were those linked to ethylene maturation/stress metabolism (ACC, ACO, ACS), ROS stress/detoxication pathways (CAT, farnesene, CTols), sugar related metabolism (sucrose, sorbitol, Starch Index SI) and phenols oxidation (PPO). TA and Firmness parameters were also involved. Inclusion of molecular data reveals that the gene expression associated to predictors above mentioned (SDH, AFS1, ADH2 and ACS) are important factors. Employment of automatic statistical methods allowed to reduce the number of variables from 34 to 14. Still, it worth take note of the variables close to the exigent threshold imposed: ACO (both, enzyme and gene expression level), APX, SOD, DHA, phenols.

Table 4. ESCALPE '*Blanquilla'* models comparison for Superficial Scald prediction: full model (all data) and subset models regardless to storage duration, specifically for 4 months, immediately after of removal (0d) or after 1 week of shelf-life (7d) (JMP PRO ® 14).

| **Test** | **Model NIPALS** | **Nº factors** | **% R² X** | **% R² Y** | **Nº VIF > 1.0** | **Nº predictors** |
|---|---|---|---|---|---|---|
| | BlaSS | 1 | 62.006054 | 81.170033 | 14 | 29 |
| Shelf-life | BlaSS0d | 2 | 86.759878 | 98.354445 | 17 | 27 |
| | BlaSS7d | 1 | 61.906876 | 82.005479 | 17 | 25 |
| Shelf-life + storage duration | BlaSS4m7d | 1 | 62.213782 | 98.354440 | 17 | 26 |
| | BlaSS4m7d - moldecular | 1 | 61.407543 | 98.229355 | 14 | 21 |

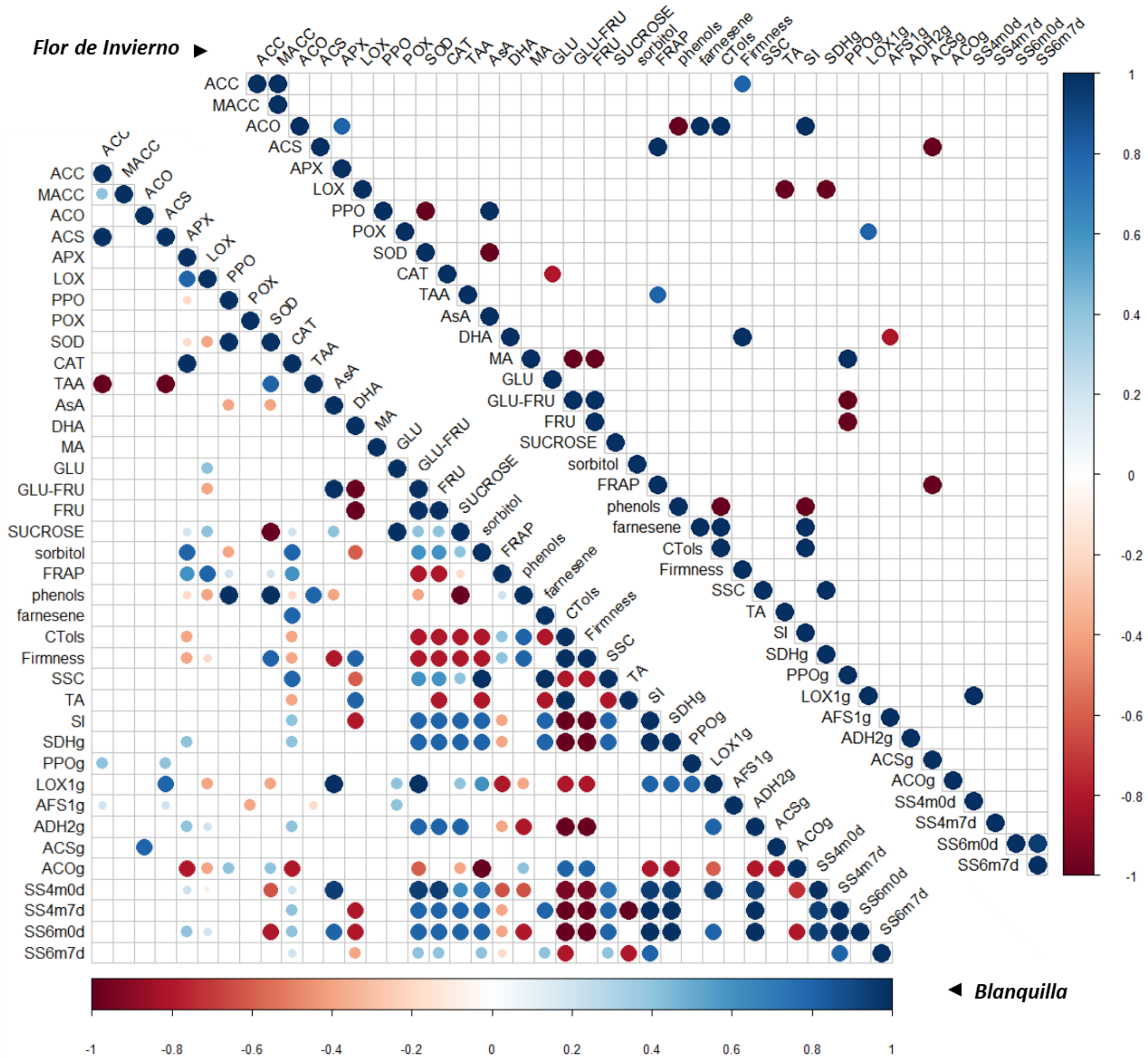



Figure 17: Comparative correlograms between '*Blanquilla'* and '*Flor de Invierno'* computed (RStudio). Blank cell were insignificant correlation according to Spearman's method with p<0.05. Color intensity and circle size correspond to the correlation coefficients. A value of ± 1 indicates a perfect degree of association between the two variables. + sign indicates a positive relationship and a – sign indicates a negative one.

The full model including all storage and shelf life data was able to cumulate 61% of Xs variance due sampling date and 82% of variance observed in SS symptoms (first model Table 4). Models build with 4 and 6m storage data after immediate removal of the fruit from storage chamber (SS4m0d) better explains both variables (Xs 86 and Y 98%) but requires 2 factors. For this reason, either the model using only 4m data after 1 week of shelf-life (SS4m7d) with or without molecular data would be preferred.

*Correlogram*

Spearman's pairwise correlation computed separately put light on some co-regulations between the important factors revealed in the PLS equations (Figure 17). Only these ones are briefly highlighted here. For '*Blanquilla*', in a first instance, ADH2, ACS, and SDH were strongly negatively correlated to α-farnesene, this parameter being associated to CTols, Firmness and TA. α-farnesene was also positively correlated to SSC, starch, ACO content but negatively to ACOg. SSC was positively related to CAT and negatively with ACOg. Finally, this last enzyme was positively associated with sorbitol a negatively correlated with POX.

*'Flor de Invierno'* SS symptoms were positively associated to LOX1 gene expression according to Spearman's test (Figure 17). LOX1g was related to POX enzymes and firmness which is positively associated with ACC, α-farnesene and DHA. LOX enzyme is related to SDHg, APX enzyme and negatively with TA and Starch Index. α-farnesene exhibits a positive correlation with CTols and this one in turn had a negative relationship with phenols content.

## ESCALPE Conclusion

This analysis put in evidence different type of alterations associated with SS-like disorders in *''Blanquilla''*, *''Flor de Invierno''* and*'Conference'*: they differ by their precocity, intensity and rate of symptoms development. Together, biochemical and molecular analysis assisted by statistical approach provide explanations to these observations and will be discussed. In *''Flor de Invierno''*, high expression levels of senescence-related pathways (ethylene production, sugar degradation, fermentative metabolism) lead to the rapid and severe browning. *'Blanquilla'* disorder is strongly associated to ethylene and α-farnesene/CTol

Table 5: Mineral roles in plant physiology synthesized from Faust (2019), Adams *et al.* and Brunetto *et al*. (2015), LEPE - University of Talca and de Freitas *et al.* (2014), Whitaker *et al*, (2009), Khoshghalb *et al*., (2008).

| | Characteristics | Deficiency | Excess |
|---|---|---|---|
| **N** | Depends on grafting combination (cultivar/rootstock), environment and orchard management, higher during the first fruit stage, due to post winter dormancy, N availability important for new shoot and fruits, spring growth, depends on past year accumulation | small size, lack of flavor, high firmness, early maturation | large fruit, low firmness, increased breakdown susceptibility, in summer delay maturation - high susceptibility to pest and decay |
| **P** | Low requirement, decrease toward fruit center | Fruit softening, high susceptibility to internal breakdown and chilling injury (influenced by low t° and senescence, small size, poor flavor, high acidity, high juiciness | Less aroma and sweetness |
| **K** | Source-sink translocation of minerals, sugars, MA accumulation | Low fruit size | Low storage suitability, cork spot |
| **Ca** | Phloem immobile element reliant on transpiration water flow-aquaporin interaction and cell wall, competition with Mg and K in the soil, and vessels CEC (xylem), pH important, strongly related to postharvest disorders. Promoted by K fertilization | membrane breakdown and/or cell wall failure; in fruit related to blossom end rot, Brown Heart, Cork spot (bitter pit Ca/Mg as indicator), black end, internal breakdown, senescent breakdown, water core, alfalfa greening, high respiration, earlier maturation and peel yellowing | cellular toxicity, over rigid cell walls, developmental abnormalities, delay in ethylene evolution |
| **Mg** | Carbohydrate formation and partitioning, protein biosynthesis, mobile ion, associated with fruit firmness, juice, TA, pH, TSS | Due to imbalances with K/Ca and Mn: antagonism with K, competition with Ca | Firm fruit, greening effect (delayed chlorophyll degradation storage disorder in cv. *d'Anjou*', increase decay occurrence |
| **Fe** | Poor transport. Related to redox system, chlorophyll, and photosynthesis/respiration. Associated DW, size, SSC, AsA | Unavailability due to alkaline calcareous soil/Mn or P excess, weak tree, poor production, small fruits, reddish, hard and prone to cracking | |
| **Mn** | Chlorophyll enzyme system | More frequent on organic and high-pH sandy soils. Plant uptake can be reduce by high K, Fe, Cu and Zn | |
| **Zn** | Enzyme system, require for Trp-IAA precursor, important in starch metabolism, co-factor in photosynthesis, nucleic acid metabolism, protein biosynthesis, SSC, combined application with Mn | Reduced growth and yield, spring problems relation sandy soil and soil t°. BH, low size, fruits deformation, sourness, early ripening. | |
| **Cu** | Enzyme system (PPO) | Accentuated by excessive N | Due to soil treatment, Cu-based pesticides, Bordeaux mixture. Can induce Fe deficiency |
| **B** | Plant growth and development, carbohydrate chemistry and reproductive system, cell division, sugar transport, GABA synthesis, associated with Ca | Breakdown and disorganization of tissues, reduce flowering, malformation, BH, low size, deformation, cracking, Can be due to excessive humidity corking, pitting, black end | early maturation, lower storage suitability |

# Discussion

## GOP

Brown Heart has usually a low incidence in South countries compared to cold-humid zones. But after 2015 damages, *Rincón de Soto* PDO members expressed great expectations for founding pre- and postharvest indicators to preserve the high quality and credibility associated with their label and to limit economic losses and consumer disappointment. CA composition and pressure are usually set based on fruit parameters measured at harvest. To avoid storage disorders, the standard European recommendations are 2-2.5 kPa $O_2$ and 0.7 kPa $CO_2$ (Rizzolo and Zerbini, 2012).The experimental design of this work intentionally included a high-$CO_2$ stress modality to “amplify/accelerate” symptoms occurrence. Indeed, BH is not always observed as it is is season-location dependent. Post-storage BH alteration evaluation was consistent with Eccher-Zerbini *et al.,* 2002. This work verifies the definition of BH given by Larrigaudière *et al.* who tries to clarify the current confusion in the nomenclature about physiological disorders in conservation.

In this study, *‘Conference’* pear cultivar was grafted on BA-29 and Franco, the most common rootstocks so the genetic factor of the plant material was assumed to be minor. Furthermore, the practices of the producers of *Rincón de Soto* PDO were also presumed to be homogenous as they must follow the chart rules and are regularly controlled by accredited evaluators.

Plant nutrition depends of *Genotype x Environment x Management* factors interactions. Disequilibrium during the growing season negatively affect yields, fruit quality and storability (Table 5, Eccher-Zerbini *et al.,* 2002). Tree micronutrient unbalance seems to create predisposition for BH disorder development later in storage. The high variance noticed in April for some minerals contents may be the result of the tree nutrition and mineral reserves constituted during the previous year. Consequently, all trees were not starting with the same reserves and capacities, which highlight the importance of early mineral analysis. Global decreasing trend was coherent with the tree physiology and minerals mobilization and transport. The extreme Cu values could be attributed likely to a local treatment. Pears *russettting,* induction or prevention of some disease use Cu-based treatments. Zn augmentation in fruit for some plots of the high PDO zone started after April. Interestingly, same scenario occurs for Mn. Although La Rioja PDO is globally described as a terroir, our results suggest a zoning pattern. Orchards map was in accordance with the experimental observations. Plots recording high Na level are those situated in the characterized alluvial soil-at the eastern part of ‘La Rioja’ along the Ebro River. he others are mostly located in clay soil (ferrous or calcareous). Fe values could be related to Fe-chelates

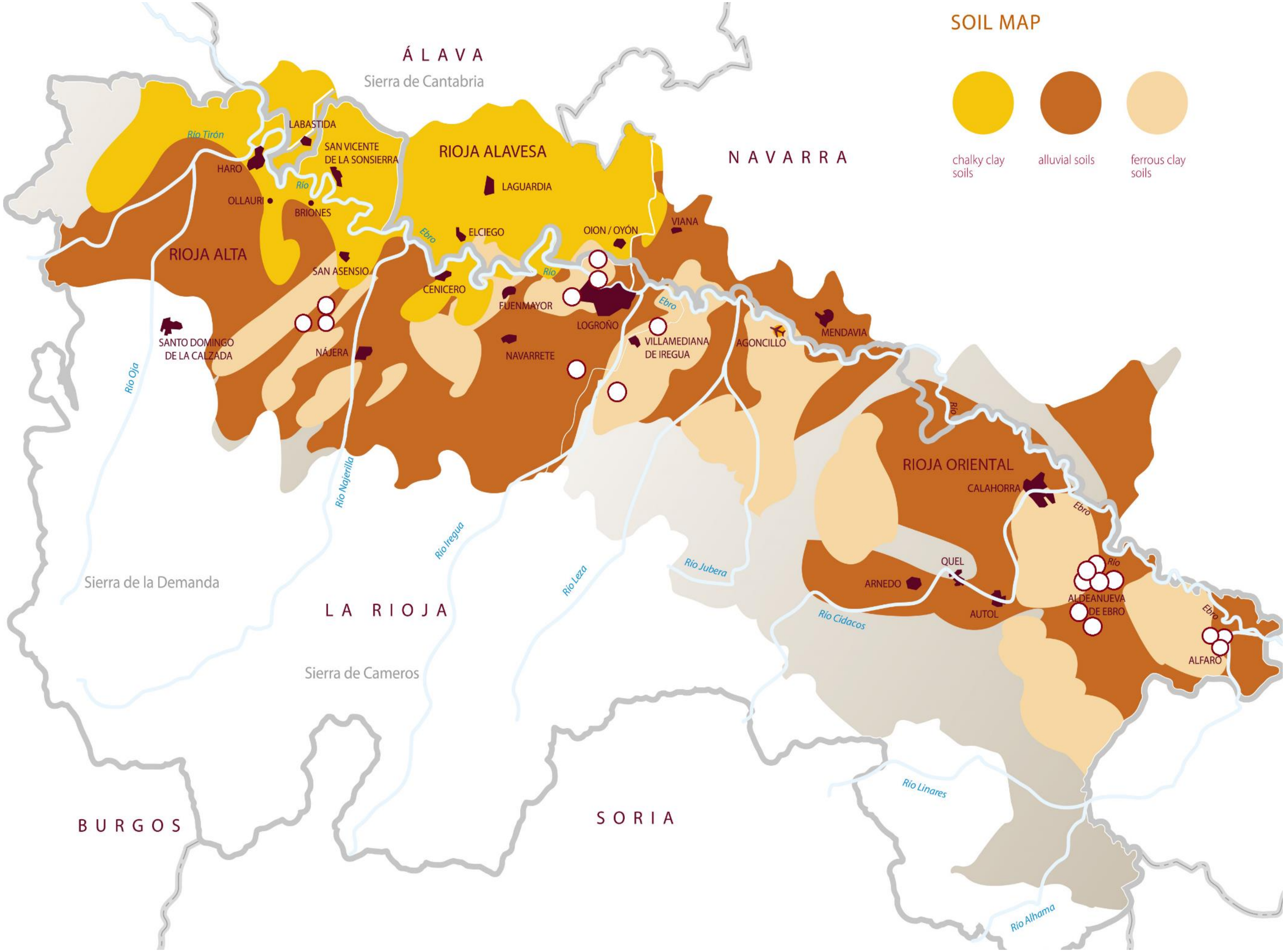



Figure 18: Soil Map adapted from La Rioja Wine. Orchards are represented by the red circles. Note the 3 orchards located in the Highest part of la Rioja (Alta)).

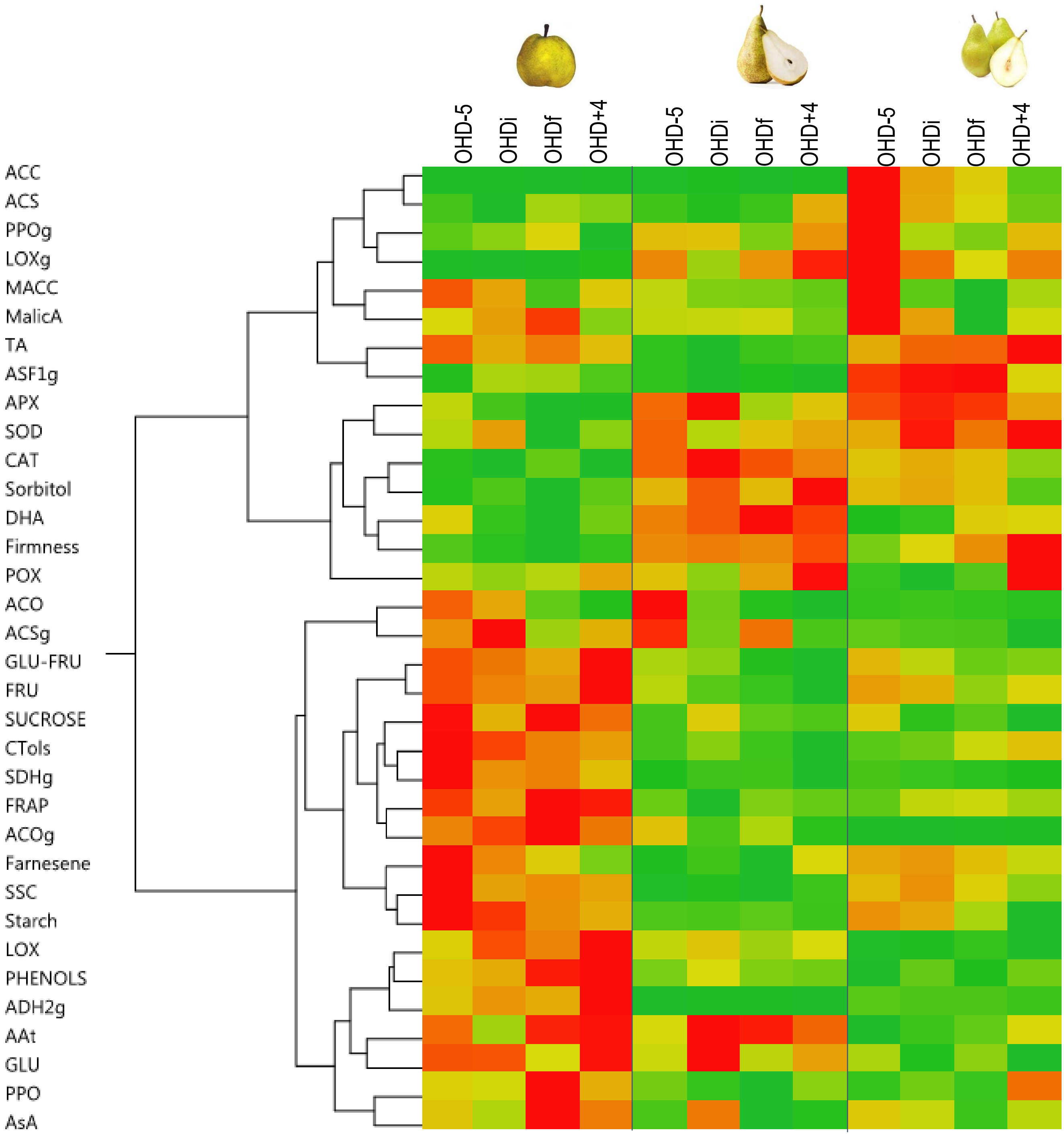
application against the common pear tree chlorosis in the Ebro Valley (PDO meeting). The expected implication of B and Ca deficiency in BH development are now recognized, especially at harvest date for B (Xuan et al., 2003). Conclusions about Ca variability were more difficult to attribute only to soil nutrition as some of the plots located in a deficient zone recorded very high levels in both leaves and fruits. These minerals are essential in cell walls equilibrium and stability responsible for fruit firmness. They likely play a determinant role in browning and cavity formation Khoshghalb *et al.,* (2008), recommended application of these elements for prevention of physiological disorder of on-tree growing fruit or before storage. N/K/Mg:Ca indexes were studied by Gastol *et al.* 2009. K:Ca especially was found reliable for internal browning prediction in apple.

Seasonal variations (hot, dry Mediterranean summer) and quality of the irrigation water are known to influence the plant hydro-nutrition. Watering source and application mode were also mentioned as potential explanation of Na variability (Ebro River as a sink). Interestingly, plots recording high BH incidence are those situated in a characterized alluvial soil, eastern La Rioja (Figure 18).

Orchard plantation rate, pruning and thinning practices are also known to influence mineral uptake and distribution. Indeed, farmers of the low and mid-zone of la Rioja used to conduct the orchard with a lower fruit charge than farmers of the highest zone, which is known to favor BH. The different individual practices mentioned can explain the inter-orchard variability found in minerals. All these observations reinforce the importance of early analysis for pre-symptomatic detection of micronutrient deficiencies to apply corrective measures. For the growers, markers based on leaves samples are more interesting as the fruits remain safe from destructive analysis (PDO meeting). Unfortunately, models developed in this work demonstrated that BH occurrence prediction always included fruits indexes.

Correlations between mineral, biochemical and physiological data were evidenced in this study. Main factors responsible for BH risk were associated to fruit maturity stage at harvest and to Eth. For instance, earliness of Eth burst, and rate of production appeared to be two important predictors. SSC evolution rate and starch degradation index in contrast, seemed less reliable as they depend from the respiratory pattern at harvest and are affected by storage atmosphere. Thus, another parameter is needed to assess the climacteric stage of the fruit at harvest.

Firmness and sugars, are the main indexes for commercial maturity but does not always agree with physiological maturity. Charoenchongsuk *et al.,* (2018), demonstrated that chlorophyll degradation is regulated by both ethylene-dependent and independent mechanisms and that *russetting* did not interfere. Late picked *'Conference'* pear had a pronounced decrease of




| Role | Predictor | Role | Predictor | Role | Predictor |
|---|---|---|---|---|---|
| **Ethylen formation** | ACSg<br>ACS<br>ACC<br>MACC<br>ACOg<br>ACO | **Glu/AsA antioxidant pathway** | DHA<br>TAA<br>APX<br>AsA<br>FRAP | **Sugar metabolism: degradation** | SI<br>sorbitol<br>SDHg |
| **Non-enzymatic browning** | AFS1g<br>farnesene<br>CTols | **Enzymatic browning** | PPOg<br>PPO<br>phenols | **osmo/ cryoprotection** | SUCROSE<br>FRU<br>GLU<br>GLU-FRU<br>SSC |
| **ROS-scavenging** | POX<br>SOD<br>CAT | **Membrane damage: phospholipid peroxidation** | LOX1g<br>LOX<br>Firmness | **Cytosolic acidification - fermentation** | ADH2g<br>MA<br>TA |

Figure 19: Heatmap drawn with all predictors (JMP14 ® PRO) and their role associated in cell physiology

chlorophyll content in shelf-life (Błaszczyk, 2012). Although this chlorophyll-related index is anexternal measure, this study demonstrated that pre-storage DAmeter value were associated BH health/damaged fruit distinction and thus could be useful for BH prediction.

## ESCALPE

Pear Superficial Scald (SS) sensitivity is cultivar-specific and have been studied in the pedoclimatic conditions of different countries of production: '*Rocha'*, (Saquet and Almeida, 2017), '*Bartlett (Williams)*', (Wang and Sugar, 2013; Whitaker et al., 2009), '*Packham's Triumph'*, and '*Beurrée d'Anjou'*, (Calvo et al., 2018), '*Abate Fetel'*, (Rizzolo et al., 2018), *'Conference'* (Rizzolo 2005), 'The 'omics' approach questions the accepted knowledge and goes deeper in the comprehension of plant physiology. It brings the opportunity to clarify some misidentified disorder like pear skin browning symptoms. Biochemical approach enabled V.Lindo to characterize different profile related to SS-like symptoms of 3 cultivars produced in Spain. Confidential data will not be shown but comments related to her main findings will help in this discussion. Figure 19 will help in visualizing cultivar differences.

### SS-like symptoms develop during maturation:

In contrast to Brown Heart and others common storage disorder, early-harvested apple were reported more susceptible to SS (Jemric *et al.,* 2006). In this study on pears, it was not the case: incidence and severity increased with harvest maturity stage in *'Blanquilla',* as in '*d'Anjou'* pears (Zhi and Dong, 2018). Here, SS-like symptoms observed for the 3 cultivars were associated with molecular and biochemical parameters that should now be described them as following.

### 'Blanquilla', an example of the generally accepted model of SS disorder:

Immediate post-storage (0d, PCA) damages were related to sugar metabolism which is solicited for osmotic adjustment (Bustamante *et al.,* 2016). Symptoms after 7days of shelf life were related to AFS1, α-farnesene and CTols. This is consistent with the generally accepted model of SS-development in apple stating that SS is basically due to α-farnesene oxidation.

Strong correlations were found between the accumulations of ACC and ACS enzyme activity and gene expression in accordance with the accepted scenario giving to Eth a prominent role in SS development. Eth signal up-regulates AFS1 which leads to an accumulation of α-farnesene. ACS, AFS1 genes expressions and ACS, AFS1 enzyme activity were in concordance, indicating that fruit responsiveness to Eth increases during maturation (receptors), explaining then the highest SS incidence and severity observed in more mature fruits. Eth sensitivity increases with maturity which explains the rising SS incidence. ACO gene and enzyme behavior are also coherent and does not seem to be a limiting factor to Eth production nor play a major role in SS.

Sugars are involved in respiration and plant defense mechanisms, limiting cellular damages by acting as osmo/cryoprotectant in response to chilling stress like in SS (Mellidou *et al.,* 2014). Sorbitol metabolism in particular was found to be sensitive to high temperature and related to fruit development stage (Liu *et al.,* 2013). Sorbitol-6-phosphate dehydrogenase synthase S6PDH is negatively affected by high temperatures, while SDH activity is stimulated. In this work, S6PDH was not analyzed so its possible implication in the control of SS in *'Blanquilla'* is uncertain. However, a clear down regulation of SDH, consistent with sorbitol accumulation, may lead to reduce SS damage. Still, high SS incidence was recorded in late-picked '*Blanquilla'* pears. Sorbitol protection may not be the principal defense mechanism of this cultivar, suggesting that SS is mostly determined by the oxidation of α-farnesene. PPO enzyme also appeared as an important variable in *'Blanquilla'*. PPO gene was upregulated when fruit acquire their maturity stage while antioxidant level diminished (V.Lindo data). Thus, enzymatic browning due to phenol oxidation by PPO may have aggravated SS symptoms in the most mature fruits.

'Flor de Invierno': a chilling injury disorder rather than a scald-like disorder:

The first evident difference observed between *'Flor de Invierno''* and *'Blanquilla'* was found in their rate of ethylene production: *''Flor de Invierno''* pears produced very low levels of ethylene. Besides Eth peak upon harvest was not always clearly identified or came very late (20d). This behavior is characteristic of winter pear ('*Blanquilla'* being considered as a summertype), which need low temperatures to start autocatalytic production of ethylene (El-Sharkawy et al., 2004). *'Flor de Invierno'* pears also exhibited very low levels of ACC (V. Lindo data), a result that explains the fruit incapacity to produce ethylene. A totally opposite pattern was also found in ethylene and ACC gene expression: *'Flor de Invierno'* ACO and ACS genes were clearly up-regulated but the fruit remain incapable of producing ACC and subsequent ethylene. Deficiencies of the two compounds may have a role in the ACO-ACS regulation: their concentration may be a signal determining the activity of both enzymes (feed-back control).

*'Flor de Invierno'* sugar metabolism also differs from *'Blanquilla'*: despite of having dramatically higher expression levels of SDH gene, sorbitol content were lower. This could be consistent with chilling injury. This hypothesis is supported by low expression levels of the AFS1 gene, found to be cold-inhibited, and a subsequently poor $\alpha$-farnesne accumulation. For these divergences with the standard SS mode (ethylene and $\alpha$-farnesene), browning symptoms should be attributed to another damage. LOX, ADH2 and phenols also appeared to be related to '*Flor de Invierno'*. Specific high phenols content in '*'Flor de Invierno''* could explain the early intense browning symptoms due to abundantsubstrate available for PPO enzyme reaction.

Interestingly, high level of ADH2 expression could be interpreted in the same way as a factor increasing ethanol production that contributes to the protection of the cell. Ethanol should be measured to assess this idea. Besides, histological analysis will be of interest to confirm the hypothesis of typical chilling disorder.

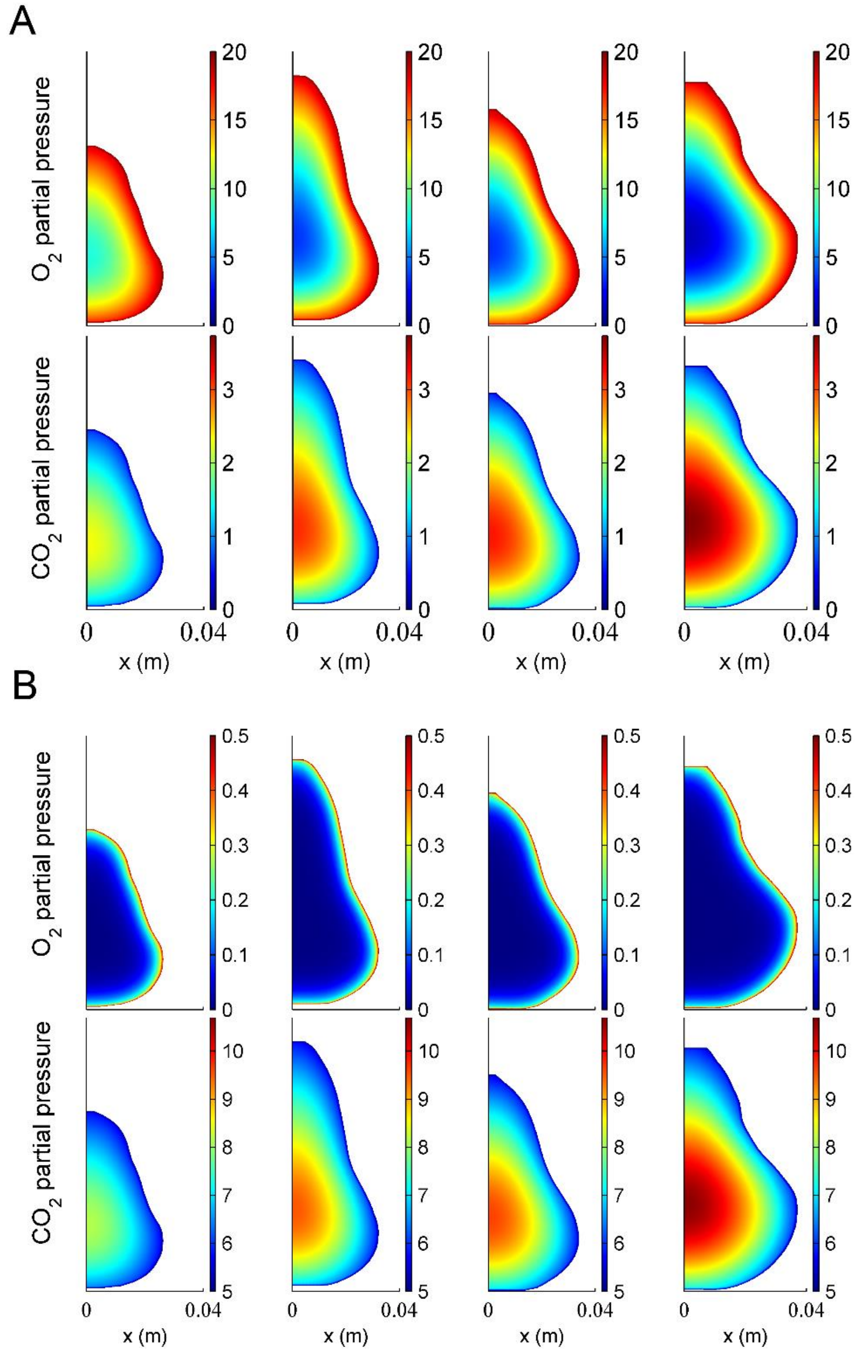
### *'Conference'*, Black spot

α-farnesene and PPO seem having less importance in *'Conference'* black spot. The cultivar was associated to less parameters, mainly related to sugars and loss of antioxidant capacity related to maturity. High levels of sorbitol compared with the other cultivars were found but with a decreasing trend over the season similarly to SSC. High DHA level may suggest a decrease in the antioxidant system capacity to control acidification and oxidative stress in late harvested fruit. This is consistent with other results (Veltman *et al.,* 1999).

In *'Conference'*, AFS1 gene expression remain low irrespectively to harvest date. As for *'Flor de Invierno',* the rate of ethylene and ACC production were low and were likely involved as before in the up-regulation of ACS and ACO gene expression (Chiriboga *et al.,* 2012). Although these mechanisms are shared with *'Flor de Invierno'*, it is unlikely that disorder in *'Conference'* were also due to chilling injury: SDH gene was not upregulated and remained at low level of expression as observed for '*'Blanquilla'*. These observations could explain why symptoms incidence did not changed during shelf life (data not shown). Available data of this study does not allow to clearly explain Black spot occurrence. As is an emerging problem, additional studies on the conditions in which appear the disorder in *'Conference'* are needed. Histological analysis and "omics" technique could provide valuable knowledge on the disorder.

### *Discussion about modeling:*

Lack of data and unequal number of observations both for GOP and ESCALPE considerably reduced the power of the model (means, repetitions, data pooling). When repetitions data were available, imputation would have been needed due to missing data. However, it will be possible to train the model with a validation set from related studies and with the next harvest season data. It is difficult to model any relation for the years with poor disorder incidence. Furthermore, the use of all set of variables simultaneously and without weighting them according to physiological important event may reduce the capacity of explanation of the model. The response variables used for GOP were the average of pools of fruits coming from the different plot. Consequently, the artificial statistic separation (Affected/Intact) at 5%BH incidence did not truly reflected the characteristics of symptomatic or healthy fruits. For ESCALPE tissues analyzed were not exactly damaged skin but mix. This dilution of some potentially interesting values might explain our unsuccessful attempt of defining thresholds. Several iterations were necessary to find a valid statistical model according to Voet criterion. The inclusion of large number of variables does not always increase accuracy of the prediction. The selection of the most adequate model requires expert



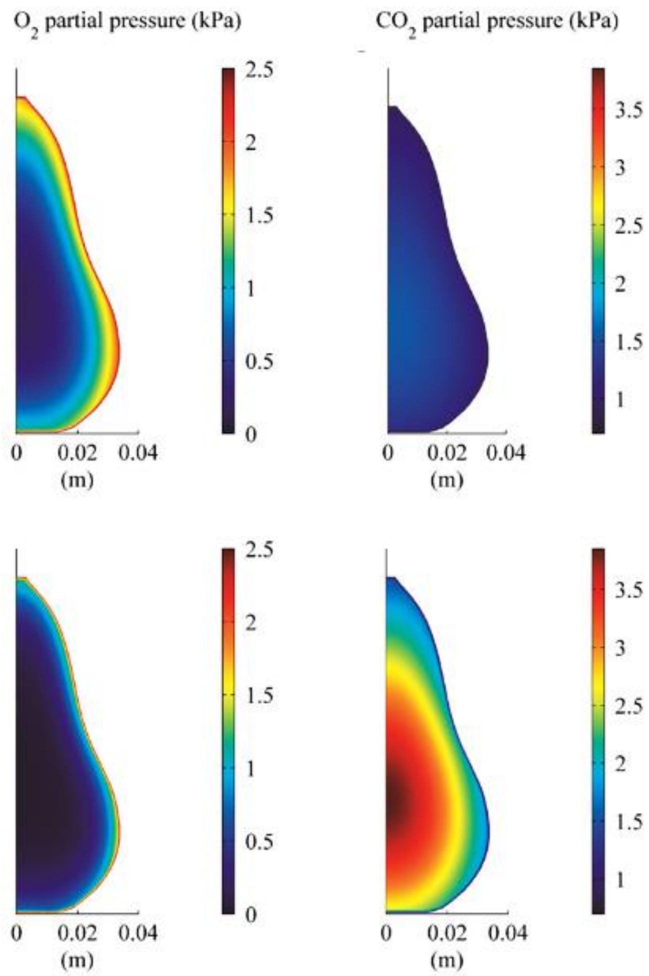




Figure 20: Model of Pear $O_2$ and $CO_2$ internal pressure according to their shape. The last conditions are known to induce Core breakdown (Ho et al., 2010)

judgment's, several years and locations for validation as Dan *et al.,* 2015 did and in other climatic conditions than Spanish ones. Dan *et al.,* 2015 procedure first included the design of a model for each variable. The construction of a global model by splitting and then merging several sub-models is possible (Structure Function L-Peach model).

Models have been developed in other crops such as roses, wheat, apple, avocado, peach, and mango. In pears, simulations of $CO_2/O_2$ partial pressure in cold stored fruit allowed to explain core-browning and the influence of fruit size and maturity stage (Figure 20, Ho *et al.,* 2010). As an example of predictive model, Dan *et al.,* 2015 offered an equation for optimizing harvest picking for long-storage (accuracy of 24 h, $R^2$= 0.9925). The predictors include weight, firmness, seed color and SI. Deuchande *et al.,* 2017 also attained a 78% variation explanation with mineral predictors of BH disorder in '*Rocha'* pear.

Our models are complex in term of diversity of nature of the predictors, time of sampling (pre-postharvest and storage monitoring). Capacities of prediction could be improved by collecting fruit data pre- and post- conservation. Evolution of the predictors would allow to understand in more detail the development of the disorders and the interactions between these variables. Besides, when scoring the alterations, damaged and healthy fruit should be separated for analysis. Additional enzymatic and molecular information could complement the sets of data: some specific metabolite or gene expression might be specifically associated to BH. These basic modeling trials were computed for description, explication and prediction of the disorder but for each one specific methods and criteria exist (Mallow's CP, AIC, BIC). RIDGE, LASSO and Elastic Net with penalization might be more appropriate for prediction. The results of our simple linear regression relationships were used for verifying factors' role in both disorders.

# Conclusion

A major challenge in fruit evaluation is the ability to detect physiological disorders at a pre-symptomatic stage. Traditional determination with maturity indexes (firmness, SSC, TA…) only allows an estimation of commercial quality. To assess the physiological maturity of the fruit, the identification of pertinent markers is needed to optimize fruit management in centrals and guarantee fruit quality. In this study, the number of indicators was high and diverse, justifying a Multivariate and Model-assisted statistical approaches. Thanks to both modeling trials, it was possible to reduce significantly the number of parameters to the most highly correlated variables with the disorder. Although, regression model needs to be reinforced with more data, predictors retained here will help in defining new biomarkers of interest and develop further guidelines.

Table 6: GOP Black spot modeling under different storage atmosphere and duration

| Model NIPALS | Nºfactors | % R² X | % R² Y | Nº VIP > 1.0 | Nº predictors | |
|---|---|---|---|---|---|---|
| IPreCA4m | 2 | 46.02002 | 70.471103 | 7 | 11 | B, DAmeter, EtH7, EtH15, SI, IniEtH, DW, Mg, Na, SSC , Zn |
| $CA^{+}4m$ | 3 | 47.545665 | 66.148657 | 9 | 11 | AsA, Color, EtH15, EtS15, Cu, K, Fe, iniEtH, iniEtS, DW, Zn |
| CA7m | 1 | 45.52917 | 56.480983 | 3 | 5 | AA, B, Na, Peso, Zn |
| $CA^{+}7m$ | 3 | 50.573179 | 82.60976 | 6 | 9 | AM, EtStress7, EtH7, EtStress15 EtH15, K, iniEtH, DW, N, SSC, Zn |

Achievements:

For Brown Heart disorder, pre-harvest tree nutrition was highlighted for early prevention. In addition to NPK-routinely-monitored, foliar Mg, Ca in and fruit Mg, Ca, Mn should be checked at the beginning of season. When harvest date is expected, B, Zn and Fe in leaves, Na in fruits should complement the mineral report. Before the entrance in storage chamber, firmness, DAmeter, should alert the grower to label the batch for early selling of already mature fruit. During conservation, a fruit sample could be used to check the level of ethylene production at 7 and 15d, recording the initiation of ethylene burst. Superficial Scald disorder in the 3 cultivars studied could be distinguished to other scald-like symptoms *via* biochemical data reflecting molecular regulation and emphasizing on AFS1, ACS, PPO, farnesene and CTols role. Quality parameters Firmness, SSC, and Starch ratio, refers as the Streif Index ratio and SSC/TA ratio, already use for organoleptic characterization, are still meaningful. Pear and apple involve similar parameters but are regulated differently i.e. CTols (Giné et al., 2013) and response vary with initial maturity stage.

Link between the 2 projects and perspectives:

Modeling is becoming incredibly more common and could become an answer to prevent food loss and waste. This work demonstrated that explicative and predictive model have promising application to study the occurrence of either internal or external disorder during storage. Models may help to adapt logistic chain for higher fruit quality and enhance client confidence, favoring the Fruit & Vegetables consumption as recommended by the OMS. Some variable used in these two projects were common, reflecting interconnection in the metabolism responses. Complementary study could analyze the molecular determinism of Brown Heart in different varieties. On the other hand, pre-harvest nutrition and environment impact on Superficial Scald have to be studied in more details. Black Spot in '*Conference'* pears was evaluated at the same time as Brown Heart disorders for GOP. The 2 projects analyzed fruits from distinct areas (La Rioja/Lleida) and practices and climate may also differ, still some minerals appeared to be related to Black Spot in CA (Zn, B, Cu). Interestingly, physiological and quality parameters highlighted were the same as in ESCALPE project (ethylene-related maturity stage at harvest and antioxidant, Table 6). These remarks should be taken cautiously as the 2 trials were not initially design for cross-information. However, the idea of merging data to feed a new integrative model would help to better understand pear fruit physiology, test virtually practices effect and evaluate the disorder-associated risks. Dynamic Controlled Atmosphere control is a promising integrative system (chlorophyll fluorescence and gas measurements) and target-gene checks are emerging (Nsure®).

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# ANNEXES

Annex I: Pear Nutrition Fact (ANSES-CIQUAL APRIFEL 2017) per 100g

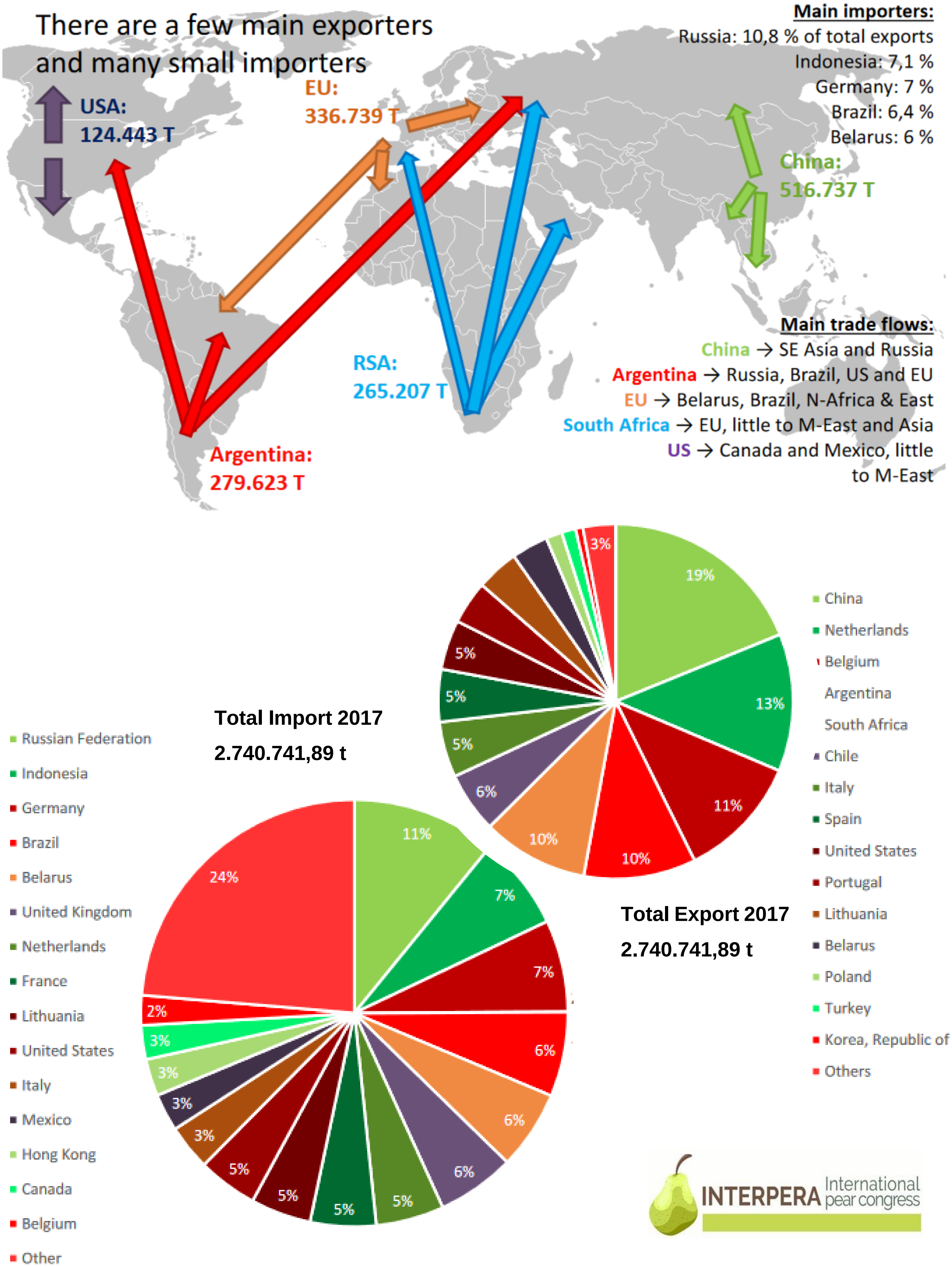
| Elements | Min - Max | Mean* Qty. | %Daily Values** |
|---|---|---|---|
| Energy | | 57 | |
| Water | 81.6 - 88.4 g | 83.5 g | - |
| Proteins | 0 - 0.63 g | 0.49 g | 0.98 |
| Lipids | 0.01 - 0.4 g | 0.27 g | 0.39 |
| Saturated fat | 0.022 - 0.07 g | 0.067 g | 0.34 |
| Glucids | - | 10.9 g | 4.19 |
| Sugars | 8.47 - 11.8 g | 9.01 g | 10.01 |
| Fibers | 2.5 - 4.8 g | 2.9 g | - |
| **Vitamines** | **Min - Max** | **Mean Qty.** | **%Daily Values** |
| Provitamin A (β-carotene) | 6 - 27 µg | 14 µg | - |
| Equivalent Vitamin A (Retinol) | 1 - 4.5 µg | 2.33 µg | 0.29 |
| B1 (Thiamin) | 0.007 - 0.023 mg | 0.015 mg | 1.36 |
| B2 (Riboflavin) | 0.011 - 0.049 mg | 0.021 mg | 1.5 |
| B3 (Niacin) | 0.097 - 0.5 mg | 0.23 mg | 1.44 |
| B5 (Pantothenic Acid) | 0.03 - 0.08 mg | 0.06 mg | 1 |
| B6 (Pyridoxine) | 0.006 - 0.043 mg | 0.022 mg | 1.57 |
| B9 (Total Folate) | 4 - 22 µg | 11.5 µg | 5.75 |
| C (Ascorbic Acid) | 1.5 - 5.7 mg | 4.62 mg | 5.78 |
| E (Tocopherol) | 0.12 - 1.28 mg | 0.41 mg | 3.42 |

| Minerals & oligo-elements | Min - Max | Mean Qty. | %Daily Value |
|---|---|---|---|
| Ca | 3.53 - 14 mg | 6.46 mg | 0.81 |
| Cu | 0.028 - 0.13 mg | 0.071 mg | 7.1 |
| Fe | 0.044 - 0.43 mg | 0.072 mg | 0.51 |
| I | 0.15 - 0.6 µg | 0.4 µg | 0.27 |
| Mg | 3.65 - 11.3 mg | 8.23 mg | 2.19 |
| Mn | 0.019 - 0.074 mg | 0.03 mg | 1.5 |
| P | 8 - 22.2 mg | 15.4 mg | 2.2 |
| K | 79 - 165 mg | 132 mg | 6.6 |
| S | 0.21 - NC µg | - | - |
| Na | 0 - 3.54 mg | 1.8 mg | - |
| Zn | 0.03 - 0.27 mg | 0.097 mg | 0.97 |
| **Polyphenols** | **Min - Max** | **Mean Qty.** | **%Daily Value** |
| Flavonoids | - | 4.82 mg | - |
| Phenolic acids | - | 11.81 mg | - |
| Stilbenes | - | 0.05 mg | - |
| Total Polyphenols procyanidins (96%), hydroxicinnamic acids (2%), arbutine (0,8%), catechins (0,7%) | - | 16.68 mg | - |

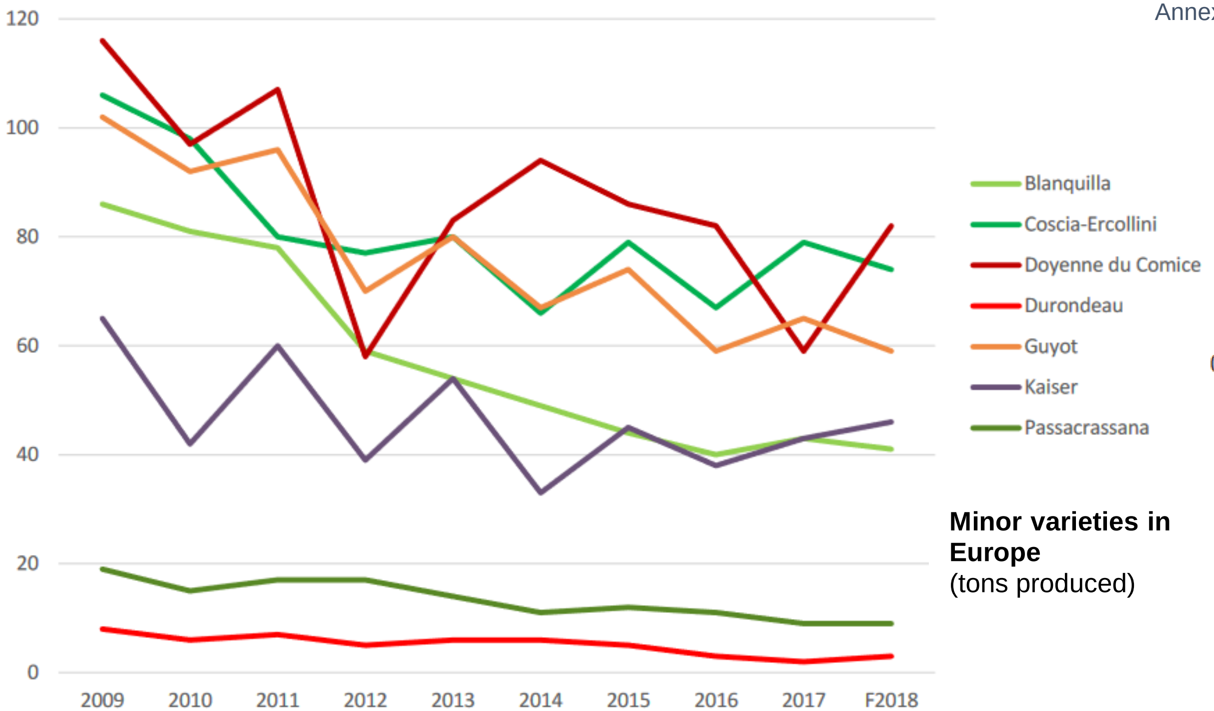
**Mean composition given as infomation : values are rough estimates being likely change according to variety, maturity, growing conditions etc. Polyphenols data come from, Phenol-Explorer 3.0. Database. All the other information is from « Table de composition nutritionnelle des aliments Ciqual (2017) - ANSES, except Vitamin A equivalent corresponding to Béta-carotene divided by 6* **Percent Daily Values are based on a 2,000 calory- diet.

Health allegation: Source of fibers (>3g for 100g FW) and source of S (>15% Daily Value)

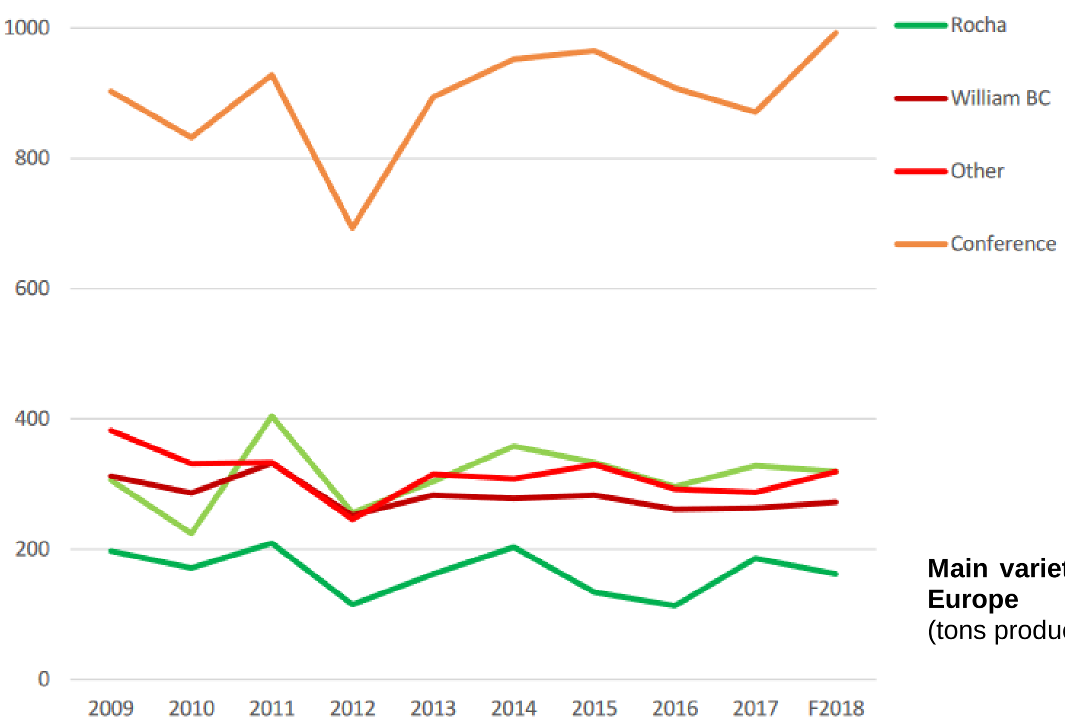
Annex II: Word trade exchanges of pears (Deruwe, Interpera 2019, data 2017)



Annex III: Pear variety market (Deruwe, Interpera 2019, data 2017)

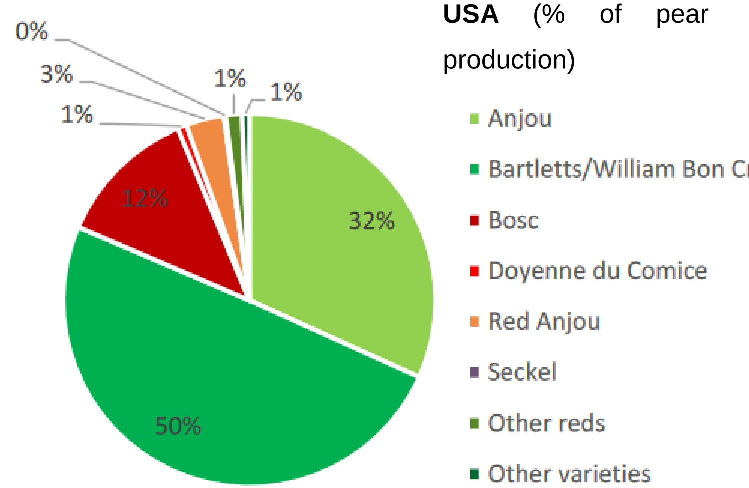



**Minor varieties in Europe**
(tons produced)

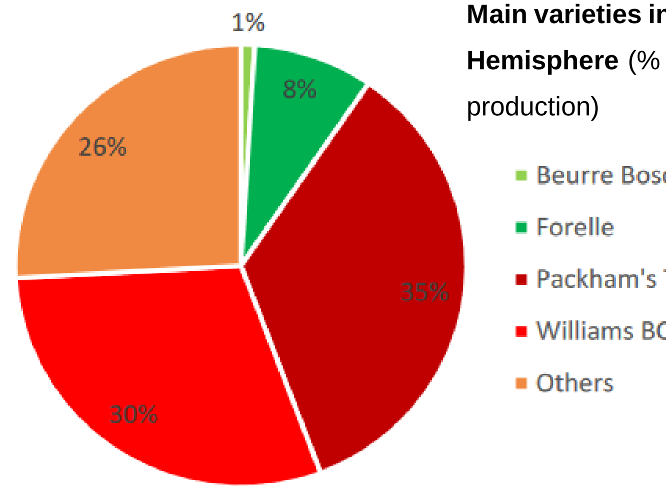



**Main varieties in Europe**
(tons produced)

**Main varieties in the USA** (% of pear production)




**Main varieties in South Hemisphere** (% of pear production)



Annex IV: Plot Design in ESCALPE grower properties (Google Map)


Annex V: *'Conference'* floral phenology for full bloom determination (E) and next sampling dates according to Meier 2001

B C D E F G H

Annex VI: *Pomaceae Center of Talca* recommendations for nutrients in pear tree leaves

| N (%) | P (%) | K (%) | Ca (%) | Mg (%) | Fe (ppm) | Mn (ppm) | Zn (ppm) | Cu (ppm) | B (ppm) |
|---|---|---|---|---|---|---|---|---|---|
| **2,3-2,8** | >0.25 | 1,0-2,6 | >1.0 | >0.35 | 100-200 | 20-300 | 30-50 | 5-12 | 30-70 |

**DATAFRAME PREPARATION**

library("xlsx2", "readxl","tidyrm", "tidyselect")

Import files: *read_excel()*

Set factors: *factor()*

Create identifier and meaningful subset: *paste(), row.names()*

Select data: *subset()*

**UNIVARIATE STATISTIC ANALYSIS**

library("stats", "agricolae", "Matrix")

Means, standard deviation, standard error : *ddply(summarise)*

Kurtosis and skewness: *kurtosis(), skew()*

Normality and Variance Homocedasticity: *shapiro.test(), bartlett.test()*

ANOVA- post hoc Tukey and Student's tests: *aov(), TukeyHSD*

Correlations: *cor(), cor.mtest*

**MULTIVARIATE ANALYSIS**

PCA: *PCA()*

**MODELING**

library("pls","plsdepot", "FactoMiner", "factoextra", "lattice")

plsr()

**PLOT**

library ("ggplot2", "ggpubr", "GGally", "ggiraph","ggiraphExtra", "corrplot")

ggboxplot (), ggdensity() gghistogram(), ggbarplot(), ggline(), ggscatter(), ggsave()

fviz_eig(), fviz_pca_var(), fviz_pca_ind(), fviz_nbclust(), fviz_contrib

corrplot

Annex VIII: Means, standard Deviations and ANOVA-TUKEY tests for Sampling Date and Affected/Intact orchards at OH based on leaves and fruits sampled in the 20 PDO La Rioja plots (Rstudio)

| | SD | N (mg/100 g DW) | P (mg/100 g DW) | K (mg/100 g DW) | Ca (mg/100 g DW) | Mg (mg/100 g DW) | Fe (ppm) | Mn (ppm) | Zn (ppm) | Cu (ppm) | B (ppm) | Na (ppm) | N/Ca | K/Ca | K/Mg | Mg/Ca |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leaf | April | 3,03 ± 0,45 a | 0,38±0,08 a | 1,65± 0,15 a | 0,78± 0,16 c | 0,26± 0,03 c | 80,5± 47,21 ns | 33,05± 27,26 b | 46,95± 19,98 b | 64,75+± 109,33 | 28,75± 15,04 a | 120,45± 75,53 a,b | 4,13± 1,32 a | 2.22±0.54 a | 6.30±0.76 a | 0.35±0.06 a |
| | May | 2.01±0.22 b | 0.20±0.04 b | 1.63±0.20 a,b | 1.11±0.21 b | 0.32±0.04 b | 67.30±23.63 ns | 31.45±23.99 b | 41.75±35.43 b | 16.20±5.40 | 20.4±10.95 b | 74.05±55.27 a | 1.87±0.41 b | 1.52±0.35 b | 5.24±1.03 b | 0.29±0.04 b |
| | 100DAF | 2.23±0.19 b | 0.20±0.04 b | 1.49±0.22 b,c | 1.66±0.39 a | 0.37±0.05 a | 65.30±14.72 ns | 48.20±38.50 a,b | 47.10±59.78 a,b | 15.05±4.05 | 20.9±4.41 b | 112.45±67.54 a | 1.40±0.32 b,c | 0.96±0.32 c | 4.10±1.00 c | 0.23±0.04 c |
| | 15BOH | 2.16±0.17 b | 0.18±0.03 b | 1.36±0.23 c,d | 1.77±0.43 a | 0.35±0.06 a,b | 70.90±23.03 ns | 59.35±61.17 a | 62.80±97.53 a | 14.15±3.36 | 22.75±2.69 b | 168.90±100.80 a,b | 1.26±0.23 b,c | 0.81±0.25 c | 4.05±1.14 c | 0.20±0.03 c |
| | OH | 2.08±0.16 b | 0.17±0.03 b | 1.27±0.21 d | 1.79±0.36 a | 0.33±0.05 a,b | 73.45±20.16 ns | 54.85±53.22 a | 57.95±89.79 a,b | 13.50±2.91 | 24.15±2.62 b | 216.1±128.20 a | 1.19±0.19 c | 0.74±0.20 c | 3.95±1.06 c | 0.19±0.03 c |
| Fruit | May | 336.58±46.61 a | 49.54±5.29 a | 367.05±21.38 a | 49.6±12.01 a | 29.41±3.73 a | 3.58±0.78 a | 1.30±0.39 a | 4.63±0.68 a | 2.60±0.63 | 4.70±2.15 a | 9.56±8.76 ns | 7.23±2.22 ns | 7.82±1.92 c | 12.62±1.30 c | 0.61±0.09 |
| | 100DAF | 92.75±22.13 b | 17.10±2.07 b | 175.35±14.06 b | 14.42±3.19 b | 10.99±1.05 b | 1.47±0.38 b | 0.54±0.23 b | 1.83±0.47 b | 1.26±0.34 | 2.30±0.76 | 13.75±13.87 ns | 6.72±2.00 ns | 12.66±2.61 b | 16.03±1.24 b | 0.78±0.11 |
| | 15BOH | 73.08±17.91 b | 16.25±2.48 b | 163.05±16.76 b,c | 11.21±2.26 b | 9.91±0.90 b,c | 2.00±0.42 b,c | 0.62±0.13 b,c | 2.50±0.45 c | 1.53±0.31 | 3.39±0.60 | 15.69±12.92 ns | 6.80±2.22 ns | 14.95±2.54 a,b | 16.49±1.39 b | 0.91±0.14 |
| | OH | 76.05±16.35 b | 16.09±1.94 b | 152.40±16.36 c | 9.11±2.10 b | 8.19±0.83 c | 1.31±0.34 c | 0.46±0.14 c | 1.26±0.51 d | 1.12±0.24 | 2.80±0.82 | 14.23±14.15 ns | 8.87±3.28 ns | 17.37±3.59 a | 18.67±1.68 a | 0.93±0.17 |

| | BH | N (mg/100 g DW) | P (mg/100 g DW) | K (mg/100 g DW) | Ca (mg/100 g DW) | Mg (mg/100 g DW) | Fe (ppm) | Mn (ppm) | Zn (ppm) | Cu (ppm) | B (ppm) | Na (ppm) | N/Ca |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leaf | A | 2,06±0,21 | 0,17±0,03 | 1,33±0,16 | 1,75±0,25 | 0,34±0,06 | 71,63±17,74 | 42,88±20,27 | 36,50±32,91 | 13,00±2,45 | 25,25±2,60 | 234,00±104,71 | 1,20±0,20 |
| | I | 2,09±0,11 | 0,17±0,03 | 1,23±0,23 | 1,82±0,42 | 0,33±0,05 | 74,67±19,69 | 62,83±57,23 | 72,25±91,57 | 13,83±3,16 | 23,42±2,30 | 204,17±151,35 | 1,19±0,19 |
| | | N (mg/100 g DW) | P (mg/100 g DW) | K (mg/100 g DW) | Ca (mg/100 g DW) | Mg (mg/100 g DW) | Fe (ppm) | Mn (ppm) | Zn (ppm) | Cu (ppm) | B (ppm) | Na (ppm) | N/Ca |
| Fruit | A | 74,94±16,82 | 16,48±1,80 | 151,63±10,85 | 8,29±1,47 | 7,96±0,52 | 1,26±0,41 | 0,45±0,09 | 1,13±0,42 | 1,20±0,21 | 2,91±0,88 | 20,30±16,26 | 9,64±4,08 |
| | I | 76,78±16,81 | 15,83±2,03 | 152,92±20,62 | 9,66±2,42 | 8,34±1,02 | 1,34±0,21 | 0,46±0,18 | 1,35±0,50 | 1,07±0,26 | 2,73±0,65 | 10,19±11,86 | 8,35±2,72 |
| | | Weight (g) | DW (g) | Firmness (N) | Dameter | abColor | SI | SSC (°Brix) | TA | AsA (mg/g FW) | MA (mg/g FW) | | |
| | A | 196,61±16,86 | 11,71±2,22 | 63,36±5,39 | 2,00±0,05 | 23,38±2,38 | 3,53±1,52 | 14,10±0,93 | 2,39±0,71 | 5,19±1,13 | 2,98±0,75 | | |
| | I | 194,56±36,32 | 12,19±1,89 | 66,37±4,43 | 2,09±0,09 * | 22,4±1,42 | 4,36±1,19 | 13,78±0,86 | 2,23±0,45 | 4,60±1,23 | 2,74±0,49 | | |
| | | EtH7 | EtStress7 | act7 | EtH15 | EtSress15 | act15 | iniEtH | iniEtS | earlyEt | | | |
| | A | 0,67±0,62 | 2,80±2,81 | 8,11±11,43 | 3,24±3,70 | 9,29±5,33 | 20,59±32,17 | 11,75±4,92 | 4,96±3,88 | 6,79±6,97 | | | |
| | I | 0,23±0,08 * | 0,62±0,48 * | 1,75±1,72 | 1,70±1,54 * | 9,53±5,10 | 11,66±16,89 | 14,83±5,07 | 8,87±2,37 * | 5,96±4,03 | | | |

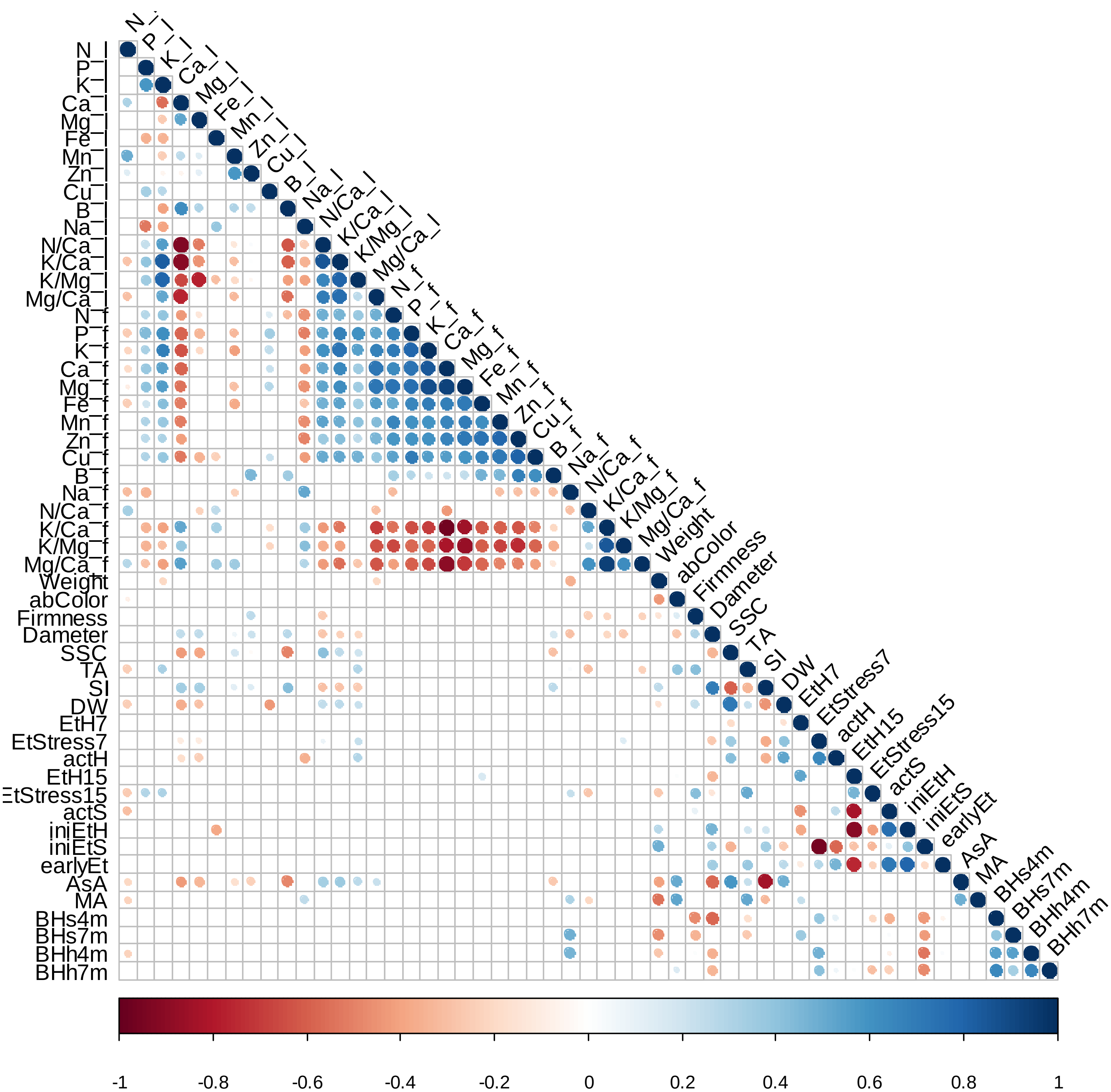
Annex IX: Means, standard Deviations and ANOVA-TUKEY tests for Geolocalization factor based on leaves and fruits sampled in 20 PDO La Rioja plots (RStudio)

| | Geo | N (mg/100 g DW) | P (mg/100 g DW) | K (mg/100 g DW) | Ca (mg/100 g DW) | Mg (mg/100 g DW) | Fe (ppm) | Mn (ppm) | Zn (ppm) | Cu (ppm) | B (ppm) | Na (ppm) | N/Ca | K/Ca | K/Mg | Mg/Ca |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leaf | H | 2.38±0.34 ns | 0.22±+0.09 a,b | 1.31±0.31 b | 1.95±0.75 a | 0.37±0.08 a | 61.07±24.24 ns | 96.27±88.36 a | 144.53±132.74 a | 18.67±15.06 ns | 34.6±16.51 a | 113.87±77.02 ns | 1.56±1.05 b | 0.89±0.65 c | 3.89±1.62 | 0.21±0.07 c |
| | M | 2.31±0.46 ns | 0.25±0.09 a | 1.53±0.19 a | 1.39±0.38 b | 0.32±0.05 b | 72.54±30.33 ns | 36.71±15.82 b | 36.97±18.99 b | 19.14±10.95 ns | 22.31±5.63 b | 134.09±126.15 ns | 1.87±0.91 a,b | 1.21±0.45 b | 4.92±1.08 | 0.24±0.06 b |
| | L | 2.27±0.48 ns | 0.21±0.09 b | 1.49±0.25 a | 1.29±0.43 b | 0.32±0.06 b | 73.88±21.20 ns | 36.18±23.64 b | 33.38±23.35 b | 30.46±72.55 ns | 20.78±4.39 b | 148.76±85.98 ns | 2.17±1.52 b | 1.38±0.74 a | 4.84±1.36 | 0.27±0.08 a |
| | H | 154.73±101.76 ns | 24.20±16.95 a,b | 209.67±101.26 ns | 23.14±22.22 ns | 15.22±10.05 ns | 1.81±0.97 b | 0.88±0.50 a* | 3.21±1.38 a | 1.74±0.92 ns | 5.03±2.49 a | 4.20±2.18 b | 8.26±2.46 ns | 12.25±4.18 ns | 14.93±2.50 b | 0.80±0.18 ns |
| | M | 147.53±125.03 ns | 26.19±15.56 a | 215.00±89.49 ns | 21.96±18.62 ns | 14.73±9.11 ns | 2.30±1.22 a | 0.72±0.47 a | 2.67±1.43 b | 1.72±0.80 ns | 3.18±1.21 b | 6.47±3.85 b | 7.11±2.32 ns | 12.97±4.67 ns | 16.03±2.73 a,b | 0.79±0.19 ns |
| | L | 139.54±114.40 ns | 23.89±13.78 b | 215.55±90.54 ns | 19.85±16.14 ns | 14.37±8.57 ns | 2.03±0.89 b | 0.69±0.34 a | 2.28±1.31 c | 1.53±0.56 ns | 2.86±0.89 b | 20.83±13.85 a | 7.35±2.79 ns | 13.65±4.41 ns | 16.21±2.48 a | 0.83±0.18 ns |

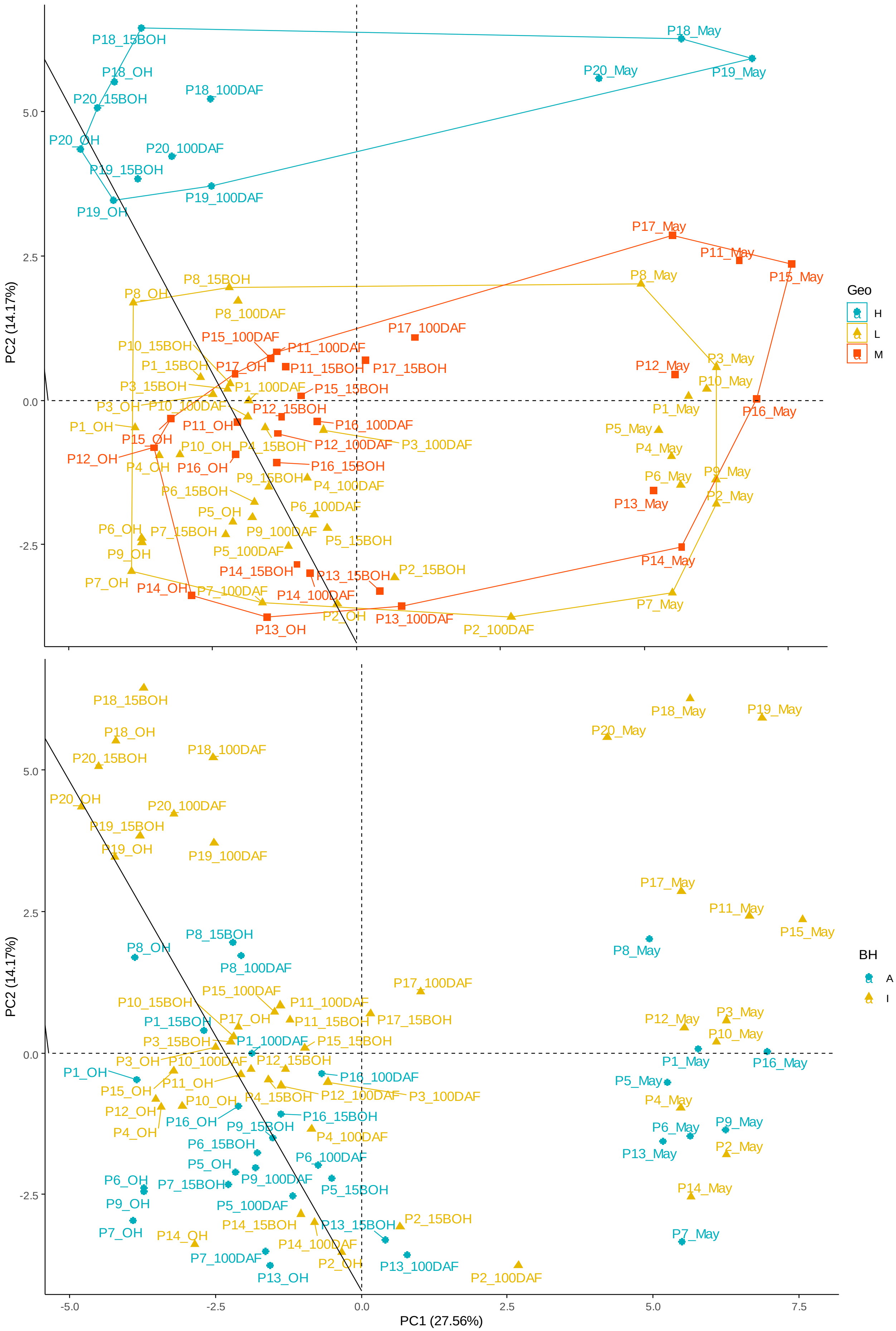
| | Geo | Weight (g) | DW (g) | Firmness (N) | DAmeter | abColor | SI | SSC (°Brix) | TA | AsA (mg/g FW) | MA (mg/g FW) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fruit | H | 206.792±17.07 ns | 10.72±1.44 ns | 69.37±2.43 ns | 2.18±0.02 a | 22.23±0.34 ns | 5.89±0.41 a | 12.59±0.28 b | 2.08±0.30 ns | 3.39±0.47 ns | 2.62±0.55 ns |
| | M | 206.85±20.00 ns | 12.27±1.26 ns | 64.81±3.90 ns | 2.04±0.11 b | 23.17±2.66 ns | 3.72±1.25 a,b | 14.35±0.85 a | 2.43±0.43 ns | 4.83±1.05 ns | 2.65±0.48 ns |
| | L | 183.93±32.27 ns | 12.19±2.36 ns | 64.15±5.78 ns | 2.02±0.05 b | 22.69±1.43 ns | 3.67±1.32 b | 13.99±0.72 a | 2.23±0.68 ns | 5.28±1.18 ns | 3.03±0.67 ns |

| | Geo | EtH7 | EtH15 | act7 | EtS7 | EtS15 | act15 | iniEtH | iniEtS | earlyEt |
|---|---|---|---|---|---|---|---|---|---|---|
| | H | 0.30±0.05 ns | 0.42±0.09 b | 0.39±0.10 ns | 0.41±0.09 ns | 5.49±4.35 ns | 10.80±7.24 ns | 19.00±2.94 ns | 10.00±1.63 ns | 9.00±2.16 ns |
| | M | 0.25±0.20 ns | 1.75±1.97 a | 6.74±9.70 ns | 3.05±3.94 ns | 11.99±3.52 ns | 20.43±24.79 ns | 14.14±5.81 ns | 6.64±2.93 ns | 7.50±5.80 ns |
| | L | 0.54±0.58 ns | 1.63±2.43 a | 3.76±7.33 ns | 2.34±1.65 ns | 8.83±5.13 ns | 12.92±26.73 ns | 11.60±3.72 ns | 6.97±4.09 ns | 4.63±5.25 ns |

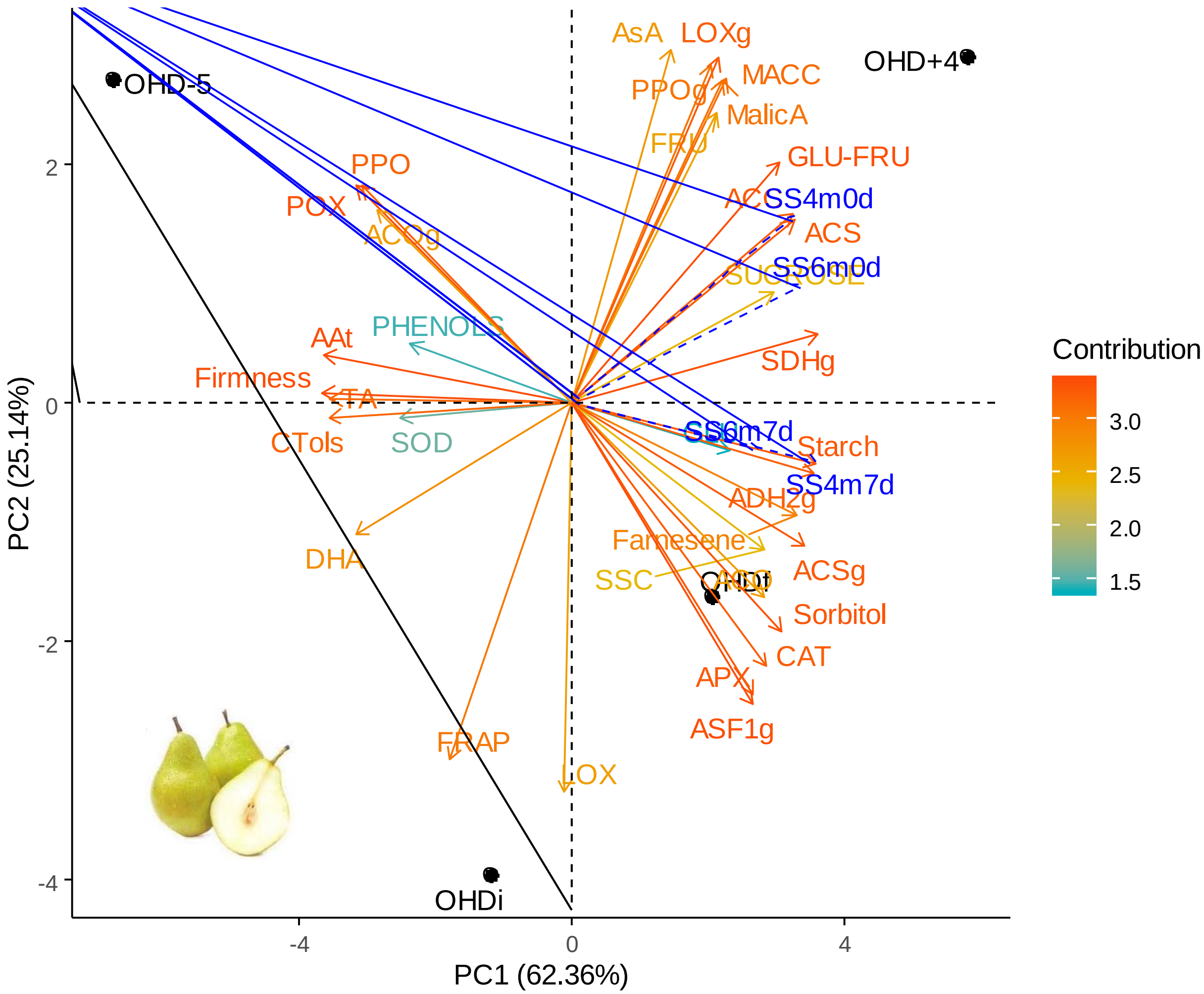
Annex X: GOP2018 Correlogram based on Spearman's computed correlation α=0.05 for May to Harvest together (RStudio).



Annex XI: PCA individuals plot evidencing clustering by geographical location (up) and Superficial Scald disorder occurrence (bottom) in orchards classified as Affected (A) or Intact (I). Outlier included (RStudio)



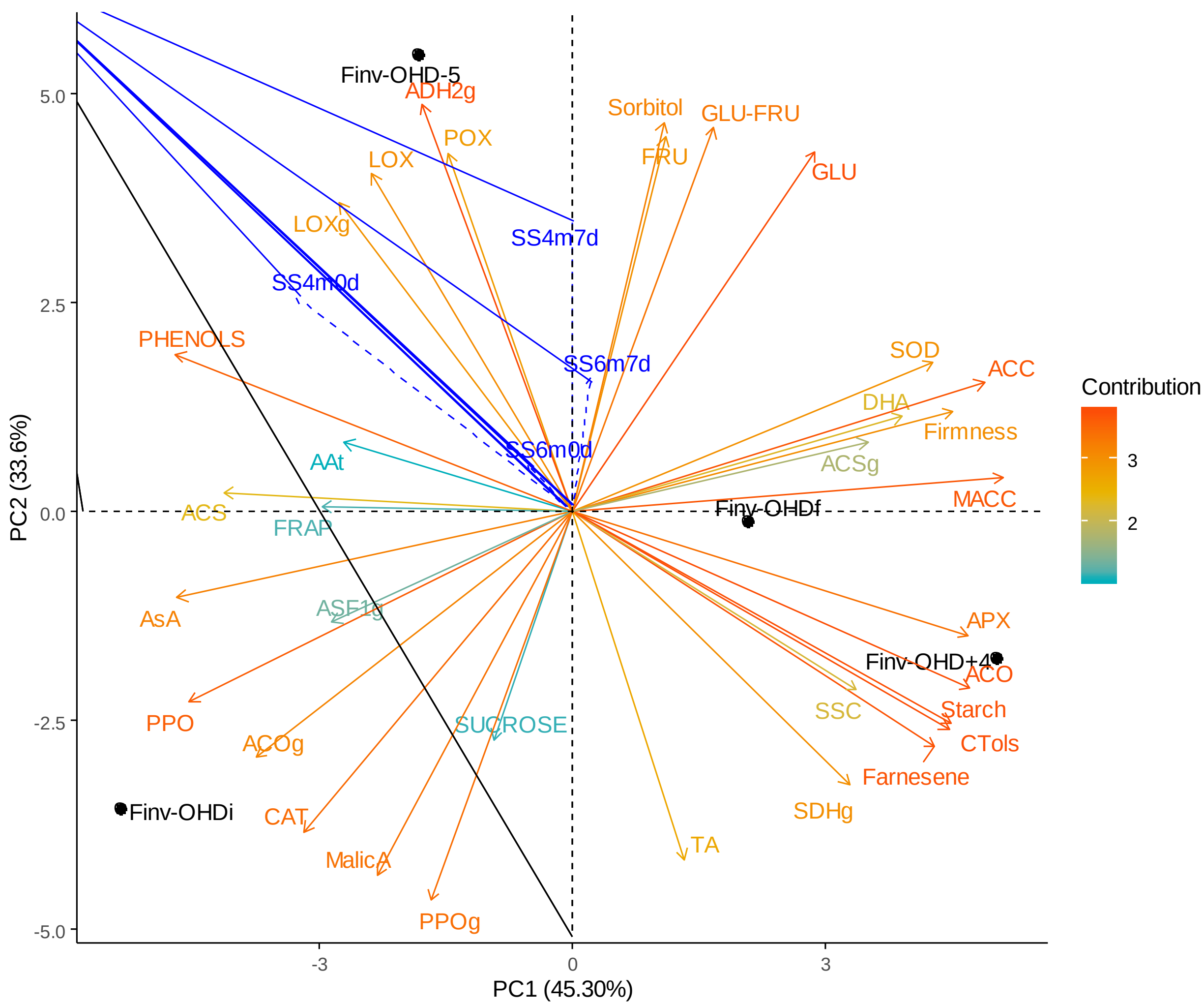
Annex XII: ESCALPE '*Blanquilla'* PCA (RStudio) and Variable importance table of the SS4md7 model (JMP14®PRO). Model presented in the result section.




**Variable Importance Table**

| X | VIP |
|---|---|
| ACC | 1,2110 |
| MACC | 0,9384 |
| ACO | 0,8478 |
| ACS | 1,2178 |
| APX | 0,7878 |
| LOX | 0,1347 |
| PPO | 1,0016 |
| POX | 0,9935 |
| SOD | 0,9600 |
| CAT | 0,8556 |
| AAt | 1,2356 |
| AsA | 0,5879 |
| DHA | 1,0989 |
| MalicA | 0,8571 |
| GLU | 0,8759 |
| GLU-FRU | 1,1353 |
| FRU | 0,7935 |
| SUCROSE | 1,1259 |
| Sorbitol | 0,9617 |
| FRAP | 0,7904 |
| PHENOLS | 0,8818 |
| Farnesene | 1,0497 |
| CTols | 1,2089 |
| Firmness | 1,2531 |
| SSC | 0,8507 |
| TA | 1,2499 |
| Starch | 1,1944 |
| SDHg | 1,2643 |
| PPOg | 0,8725 |
| LOXg | 0,8573 |
| ASF1g | 0,7946 |
| ADH2g | 1,2168 |
| ACSg | 1,1388 |
| ACOg | 0,8507 |

Annex XIII: ESCALPE '*Flor de Inieno* PCA (RStudio) and Variable importance table of the SS4md7 model (JMP14®PRO)

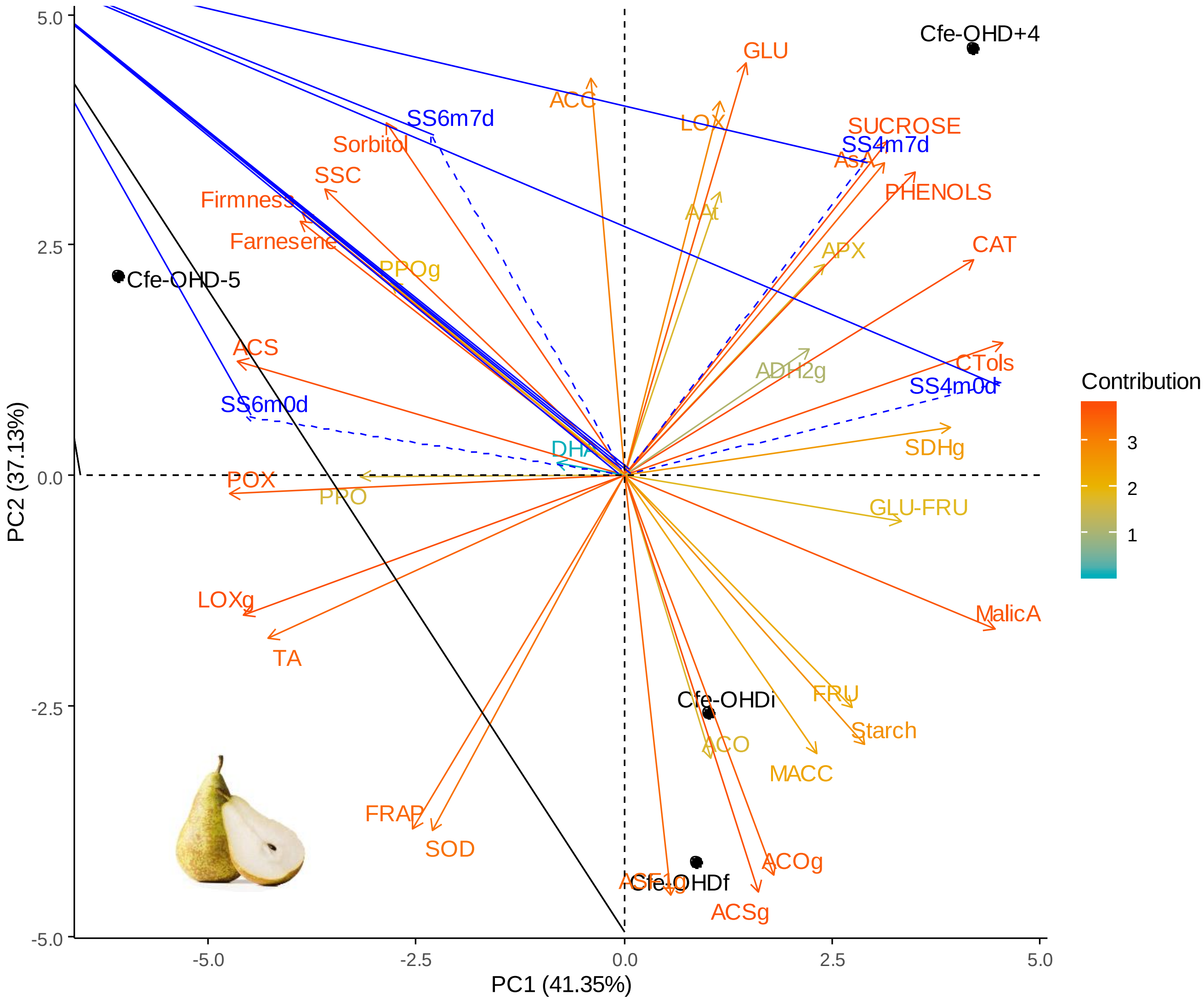



**Variable Importance Table**

| X | VIP |
|---|---|
| POX | 1,1829 |
| AAt | 1,0365 |
| DHA | 0,7767 |
| MalicA | 1,0447 |
| GLU | 0,8097 |
| GLU-FRU | 1,0976 |
| FRU | 1,1840 |
| FRAP | 0,9294 |
| PPOg | 1,0762 |
| LOXg | 1,0666 |
| ASF1g | 0,9796 |
| ADH2g | 0,8110 |
| ACOg | 0,8901 |

| Model NIPALS | Nºfactors | % Var. Expl for Cumulative X | % Var Expl for Cumulative Y | Nº VIP > 1.0 | Nº predictors |
|---|---|---|---|---|---|
| FinvSS4m7d | 1 | 84.555686 | 99.651586 | 7 | 13 |

Annex XIV: ESCALPE '*Conference* PCA (RStudio) and Variable importance table of the SS4md7 model (JMP14®PRO)




**Variable Importance Table**

| X | VIP |
|---|---|
| ACC | 0,1179 |
| MACC | 0,7970 |
| ACO | 0,4227 |
| ACS | 1,4318 |
| APX | 1,0248 |
| LOX | 0,6207 |
| PPO | 0,8212 |
| POX | 1,5632 |
| SOD | 0,7697 |
| CAT | 1,4090 |
| AAt | 0,2782 |
| AsA | 1,1797 |
| DHA | 0,5052 |
| MalicA | 1,3302 |
| GLU | 0,6619 |
| GLU-FRU | 1,2264 |
| FRU | 0,9558 |
| SUCROSE | 1,1146 |
| Sorbitol | 0,7601 |
| FRAP | 1,0079 |
| PHENOLS | 1,2347 |
| Farnesene | 1,1180 |
| CTols | 1,5327 |
| Firmness | 1,1288 |
| SSC | 1,0100 |
| TA | 1,4916 |
| Starch | 0,6998 |
| SDHg | 1,1285 |
| PPOg | 0,6552 |
| LOXg | 1,5072 |
| ASF1g | 0,1065 |
| ADH2g | 0,5424 |
| ACSg | 0,3911 |
| ACOg | 0,4905 |

| **Model NIPALS** | **Nºfactors** | **% Var. Expl for Cumulative X** | **% Var Expl for Cumulative Y** | **Nº VIP > 1.0** | **Nº predictors** |
|---|---|---|---|---|---|
| CfeSS4m7d | 1 | 41.174122 | 61.280459 | 17 | 34 |

Annexe XV: Other activities during the internship at IRTA FruitCentre

| | |
|---|---|
| **XVIII Jornada tècnica de postcollita**<br><br><br><br><br>**I Jornada tècnica Valorizatció d'excedents hotyofrutícoles i subproductes de la transformació de vegetals** | Analysis of 2018 harvest season and predictions, emergent diseases at harvest, use of volatile compounds for detection of fungi pressure and other fruit default, post-harvest fruit maturation control, social trends in fruit packaging, commercials and demonstrations of innovative solution.<br><br>Analysis and strategies to fight against food losses and waist at different levels: postharvest conservation, processing industries, consumer awareness. European project AGRIMAX presentation. Phytochemicals, microorganisms, and peptides from plant sub-products for functional and technological use. |
| **GOP Project meeting** | Discussion of the current fruit harvest season, presentation of the 1rst results of the project with PDO stakeholders, re-orientation and definition of the next steps of the project |
| **Technical demonstrations and formation**<br>METLER TOLEDO<br><br>PROMEGA<br><br>IRTA: Good Practice in Temperature/Controlled Atmosphere Storage Chamber<br>Good practices in the Field<br>Good practices in the Lab | <br><br>Pipettes and Spectrophotometer: principles and correct handling<br><br>Maxwell ® RSC 48RNA/DNA extraction with integrated quantification<br><br>Visit of the facilities, demonstration of Employer´s tool kit when working alone and presentation of protection equipment, explanation of the new symbols on chemicals |
| **Participation to a study on Bread and Cracker enriched with microalgae** | Baking according to previous protocols based on sensorial panelist test results<br>Gas Chromatography analysis of the samples |
| **Participation to panel test** | Taste (strawberries, prunes, nectarines, peaches, microalgae enriched-bread/ cracker/cookies)<br>Visual assessment of fresh cut potatoes and strawberry for minimal process test, edibility and willingness to purchase |
| **Participation to field experimental** | Help in experiment set up |

| | |
|---|---|
| | **Degree**: Master<br>**Option** : Plant Biology<br>**Specialization** : Quality of Specialized Plant Productions |
| **Authors(s)** : Chloé LECLERC<br>**Date of birth** : 23/04/1996 | **Host organization** : FruitCentre IRTA<br>**Adress**: Parc Científic i Tecnològic Agroalimentari de Lleida (PCiTAL).<br>Parc de Gardeny, Edifici Fruitcentre<br>25003 Lleida, Catalonia,Spain<br>**Tutor** : Christian LARRIGAUDIERE |
| **Page number** : 29 Annexe(s) :13 | |
| **Year of the presentation** : 2019 | |

**French Title:** Facteurs pré-récoltes impliqués dans le développement des maladies physiologiques post-récolte du brunissement interne et de l'échaudure superficielle de la poire. Approches biochimiques et statistiques.

**English Title:** Pre-harvest factors involved in Superficial Scald and Brown Heart Post-harvest Physiological Disorders in Pear. Biochemical and statistical approaches

**Résumé**: Les désordres physiologiques comme le brunissements interne et l'échaudure sont un véritable challenge pour la commercialisation des poires. Depuis l'interdiction des produits chimiques de contrôle, le développement de nouvelles alternatives de contrôle est devenu une nécessité cruciale pour la pérennisation du secteur et la production de fruit de qualité. Pour répondre à ce problème, différents modèles de prédictions ont été développé dans ce travail. Dans le cas du brunissement interne, la nutrition de la plante (K/Mg/Ca, B et Zn) et le stade de maturité à la récolte (Indice de Streif et paramètres liés à la production d'éthylène) se sont révélés les facteurs les plus déterminants. L'analyse menée sur l'échaudure a permis de distinguer les désordres survenant chez 3 cultivars. Combinée aux études transcriptomiques, les bases biochimiques ont pu être précisées aboutissant à l'identification de marqueurs de prédiction pour chaque variété. Ces modèles préliminaires seront améliorés au cours des prochaines campagnes de récolte, mais ces tentatives montrent qu'une prédiction statistique du brunissement interne et de l'échaudure de la poire est possible et prometteuse pour l'obtention de fruit de qualité.

**Abstract:** Physiological disorders such as internal browning and superficial scald are a real challenge for pear marketing. Since the prohibition of chemicals, the development of new control alternatives has become a crucial necessity for the maintenance of the productive sector and for fruit quality. To answer this problem, different models of prediction were developed in this work. In the case of internal browning, plant nutrition (K/Mg/Ca, B and Zn) and stage of maturity at harvest (Streif index and parameters related to ethylene production) were found to be the most important factors. The analysis performed on superficial scald allowed us to differentiate the disorders affecting 3 varieties. Combined with transcriptomic studies it made possible the establishment of the biochemical basis associated and the identification of predictive markers for each variety. The preliminary models will be improved with next season data but demonstrate that a statistical prediction of internal browning and superficial scald in pear is possible and promising for the maintenance of fruit quality.

**Mots-clés:** Poire, désordre physiologique, conservation, atmosphère contrôlée, échaudure superficielle, brunissement interne, indices, modélisation

**Key-words**: Pear, physiological disorder, storage, controlled atmosphere, superficial scald, brown heart, indexes, modeling