# Dissecting the influence of the orchard location and the maturity at harvest on apple quality, physiology and susceptibility to major postharvest pathogens

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## Abstract

‘Golden Reinders’ apple quality parameters, fruit physiology, biochemical composition and susceptibility to *Penicillium expansum* and *Rhizopus stolonifer* were analysed in fruit harvested from four different locations (two valley and two mountain orchards) and from the same valley orchard at six different maturity stages. Growing location strongly influenced the taste- and health-related fruit composition whereas the fruit maturity at harvest mainly affected the ethylene biosynthetic pathway and ethylene-dependent quality traits such as the fruit firmness and starch index. The fruit maturity at harvest, but not the growing location, also affected the severity of the infection caused by *P. expansum* and *R. stolonifer*, with mature fruit showing higher susceptibility to pathogen infection. Besides, by employing a Partial Least Square (PLS) regression model, our data showed that the severity of the lesions caused by *R. stolonifer* were intimately related to the fruit ethylene production. Overall, the results from this study demonstrate that differences in environmental conditions between orchards (mountain vs valley) strongly influenced the composition of ‘Golden Reinders’ apples without affecting the susceptibility of the fruit to two major postharvest pathogens.



**Keywords**: Antioxidants, ethylene metabolism, *Penicillium expansum, Rhizopus stolonifer*, sugars

## 1. Introduction

Although apple production in the European Union has remained stable over the past two decades, apple production for the same period in Spain has decreased by 20% (FAOSTAT, 2019). Such decline is due, in part, to the fact that more than half of the Spanish apple production is located in the Ebro Valley, an area characterized by dry and warm weather conditions, that detrimentally affect certain apple quality attributes, such as colour and firmness (Iglesias et al., 2008) as well as the fruit storability (Emongor et al., 1994). It is already well known that climatic differences between cultivation areas play an important role in the fruit physiology and, therefore, in its final quality (Corelli-Grappadelli and Lakso, 2004; Karagiannis et al., 2020) as well as its nutritional value (Crespo et al., 2010). Indeed, apples obtained in mountain areas are expected to have a better organoleptic quality (Faust, 2000) and enhanced content of antioxidants (Karagiannis et al., 2020). The main environmental differences between orchards located at the same latitude but at different altitude are generally temperature and solar radiation incidence (Körner, 2007). Temperatures reached on the field weeks before harvest strongly influence the taste-related composition of the fruit (Woolf and Ferguson, 2000), as well as its aroma (Dixon and Hewett, 2000). In particular, apples grown under warm temperatures tend to accumulate higher soluble sugars (Seo et al., 2003) and lower malic acid (Tomana and Yamada, 1988). However, an excess of high temperature may inhibit starch metabolism in apples (Smith et al., 1979) as well as ripening and ethylene production in some plants (Biggs and Handa, 1988). Enhanced solar radiation, on the other hand, is also known to enhance the synthesis of antioxidants in fruit (Wang, 2006; Karagiannis et al., 2020).

In addition to the influence of environmental conditions, the fruit maturity stage at harvest clearly affects the final fruit quality and its market value in a wide range of pome fruit

(Ingle et al., 2000; Lindo-García et al., 2020). Fruit picked immature may have a suboptimal organoleptic quality (Echeverría et al., 2004) whereas over-mature fruit will exhibit a limited storability (Guerra and Casquero, 2010), poor firmness (Harker et al., 2010) and, to some extent, lower nutritional value (Huang et al., 2007). In this sense, it is feasible to speculate that differences in the fruit composition and physiology associated to orchard location and/or harvest date may lead to differences in the fruit susceptibility to postharvest diseases (Baró-Montel et al., 2019; Sun et al., 2017; Torregrosa et al., 2020; Vilanova et al., 2012). *Penicillium expansum* and *Rhizopus stolonifer* are considered two of the main apple postharvest pathogens due its wide incidence during storage (López et al., 2015). This said, few studies are currently available comparing the susceptibility to postharvest pathogens in apples grown in mountain and valley areas. Accordingly, our study was focused on comparing the fruit quality, physiology and susceptibility to major postharvest pathogens in 'Golden Reinders' apples grown in the Ebro valley and the Pyrenees Mountains, two areas very close to each other but with very different climatic conditions. Besides, on-tree ripening was monitored to further assess if differences observed between locations were caused by environmental conditions or by maturity differences.

## 2. **Materials and Methods**

### 2.1. *Plant material and experimental design*

The trial focused on assessing the influence of the growing location was carried out with 'Golden Reinders' apples harvested from four different orchards located in the province of Lleida (Catalonia, North-East Spain): two orchards from valley areas, Alcanó (214 meters above sea level, masl) and Vilanova (195 masl), and two from mountain areas, Llesp (989 masl) and Gotarta (1191 masl) were used. Fruit were harvested at the Optimal Harvest Date (OHD) from trees of similar age and grown on the same rootstock (M9) as well as similar agronomical practices. The trial assessing the influence of the fruit maturity was carried out with fruit picked from the Gimenells IRTA Experimental Station (260 masl) at 6 different harvest dates starting 7 days before the OHD ($OHD_{-7}$) and picking up the fruit weekly up to 28 days after the OHD ($OHD_{+28}$). In both experiments, the OHD was based on local grower recommendations mainly based on flesh firmness and starch index values for this specific cultivar. Upon harvest, 150 fruit per location or harvest date were randomly harvested from 10 trees and immediately transported to the laboratory. From those 150 harvested a set of 30 fruits for each sampling point or location were used for quality evaluations while another set of 6 fruit were used for monitoring the dynamics of the fruit ethylene production. Postharvest pathogens inoculation was done on 80 fruit (40 apples for each of the pathogens tested). Finally, an additional set of 15 fruit (3 replicates of a pool of 5 fruit each) fruit per location or harvest date were peeled and the pulp was grinded and frozen in liquid nitrogen and kept at -80 ºC until further biochemical analysis. The 19 remaining harvested fruits were discarded.

### *2.2. Instrumental Quality parameters*

On arrival to the laboratory, flesh firmness was measured on two opposite sides of 30 fruit per orchard and harvest date, after cutting a slice of peel, using a GÜSS FTA

penetrometer (FR Turoni, Forly, Italy) equipped with an 11mm plunger tip as described by Johnston et al. (2002).

Starch index was evaluated using equatorial slices from 15 out of 30 fruit used for firmness measurements by dipping them in an iodine solution (I2-KI) for ten minutes. The starch index was assigned to each fruit using the starch scale from 1 to 10 developed by the CTIFL (France).

The same fruit used in the firmness determination was also used for making the juices required to measure the Soluble Solids Content (SSC) and Total Titratable Acidity (TTA). In this case, six juices were made using a pool of 5 apples each (6 replicates from the pool of 5 fruit each). Soluble Solids Content (SSC) was determined from the juice with a PAL-1 Pocket refractometer (ATAGO, Tokyo, Japan). Total Titratable Acidity (TTA) was measured by diluting 5 mL of juice with 10 mL of deionized water and titrating with NaOH 0.1 N until phenolphthalein colour change as described by Giné-Bordonaba et al. (2016).

### *2.3. Ethylene production and ethylene-related precursors and enzymes*

Ethylene production ($\mu L\ kg^{-1}\ h^{-1}$) was measured on fruit kept in an acclimatised chamber at 20 °C as described by Giné-Bordonaba et al. (2014) with some modifications. After harvest, 2 apples for each replicate, and 3 replicates per location or harvest maturity were placed, in 1.5 L flasks continuously ventilated with humidified air at a flow rate of 1.5 L $h^{-1}$. Each flask containing 2 fruit was considered as an experimental replicate. Gas samples (1 mL) were taken periodically from the effluent air and injected into a gas chromatograph fitted with a FID detector (Agilent Technologies 6890, Wilmington, Germany) and an alumina column F1 80/100 (2 m x 1/8 x 2.1, Tecknokroma, Barcelona, Spain).

1-Aminocyclopropane-1-carboxylic acid (ACC) and malonyl-1-aminocyclopropane-1-carboxylic acid (MACC) levels, expressed as nmol $g^{-1}$ FW, were determined according to Bulens et al. (2011) using 2 g frozen pulp tissue. 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) activities were determined as described by Lindo-García et al. (2019) using frozen pulp tissue and the results expressed as nmol $C_2H_4$ $g^{-1}$ FW $h^{-1}$.

*2.4. Determination of specific sugar and acid content*

The protocols described by Giné-Bordonaba et al. (2017) were used for extracting sugars (sucrose, glucose and fructose) and malic acid from 2 g of frozen pulp tissue. The supernatants of each extraction were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase) and sucrose (β-fructosidase) using commercial kits (BioSystems S.A., Barcelona, Spain). Determination of malic acid was also done using commercial kits (L-malate dehydrogenase; BioSystems S.A., Barcelona, Spain). The measured levels of sugars and malic acid were expressed as mg 100 $g^{-1}$ FW.

The extraction of ascorbic acid was carried out using the protocol of Rassam and Laing (2005) with slight modifications. Briefly, 3 g of frozen pulp tissue were mixed with 5 mL of metaphosphoric acid suspension (3% MPA, 8% acetic acid) and centrifuged at 24000 *g* for 22 min at 4ºC. The supernatant was filtered through a 0.22 µm filter. Levels of ascorbic acid were determined by injection of 10 µL of supernatant on an Agilent 1260 Infinity II liquid chromatograph UHPLC and measuring the absorbance at 254 nm. Separation was carried out on a Poroshell 120 EC-C18 (3 x 100 mm, particle size 2.7 µm, Agilent) at a flow-rate of 0.125 ml $min^{-1}$ using 10% of methanol (v/v) as mobile phase. Total ascorbic acid was measured by UHPLC using 10 µL of sample after 3 hours

158 reduction of 950 µL of extract with 50 µL of 40mM Tris [2-carboxyethyl] phosphine
159 hydrochloride (TCEP·HCl).

160 *2.5. Total phenolic compounds, antioxidant capacity and peroxidation markers*

161 Total phenolic compounds and antioxidant capacity of the apple flesh were determined
162 using frozen tissue as previously described Giné-Bordonaba and Terry (2008) by mixing
163 3 g of apple pulp tissue with 10 mL of 79.5% (v/v) methanol and 0.5% (v/v) HCl in Mili-
164 Q water. Sample extraction was held at 20 ºC with constant shaking for 2 h and mixing
165 the samples every 30 min. The extract was centrifuged at 24,000 *g* for 30 min at 20 ºC.
166 From the same extract, total phenolic compounds (mg gallic acid equivalents (GAE) 100
167 $g^{-1}$ FW) were measured by means of the Folin-Ciocalteu method and total antioxidant
168 capacity (mg $Fe^{2+}$ 100 $g^{-1}$ FW) measured by the Ferric Reducing Antioxidant Power
169 (FRAP) assay as described in recent works (Giné-Bordonaba et al., 2016).
170 Malondialdehyde (MDA) was analysed as an index of lipid peroxidation using the
171 thiobarbituric acid reactive substrates (TBARS) based on the protocol previously
172 described (Martínez-Solano et al., 2005) using 0.5 g of frozen tissue mixed with
173 4 mL of 0.1% trichloroacetic acid (TCA) solution. MDA levels were expressed as
174 µmol 100 $g^{-1}$ FW.

175 *2.6. Susceptibility to major postharvest pathogens*

176 *P. expansum* (CMP-1) and *R. stolonifer* (RSF) are the most aggressive isolates from the
177 Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain) collection capable of
178 infecting pome fruit. Conidial suspensions were prepared by scraping the surface of 7-
179 day-old cultures grown on potato dextrose agar (PDA) with sterile water containing
180 0.01% (w/v) Tween-80 using a sterile glass rod as previously described by Vilanova et
181 al. (2012). Concentration of each fungus was determined using a haemocytometer and
182 adjusted to obtain $10^4$ conidia $mL^{-1}$ of *P. expansum* and *R. stolonifer*. 'Golden Reinders'

apples were wounded with a nail (1 mm wide and 2 mm deep) to produce an injury on the equatorial part. The wounds were inoculated with 15 µl of an aqueous suspension of *R. stolonifer* or *P. expansum* and the fruit were allowed to dry at room temperature. For each harvest date and location, four replicates of 10 fruit per pathogen were used (Baró-Montel et al., 2020). Then, inoculated apples were incubated at 20 ºC and 85% relative humidity and the rot lesion diameter (severity) and the percentage of infected wounds (incidence) were determined at 3 and 7 days for *R. stolonifer* and *P. expansum*, respectively.

### *2.7. Statistical data analysis*

Data was subjected to analysis of variance (ANOVA) tests using JMP 13.1.0 SAS Institute. Prior to analysis, the data regarding the incidence in decay were transformed by the arcsine of the square root. No transformation was performed for severity, quality and biochemical data. Statistically, differences with *p*-values under 0.05 were considered significant and means were compared by 95% Tukey's HSD test. Least significant differences values (LSD; $p = 0.05$) were calculated for mean separation using critical values of t for two-tailed tests. Spearman's rank correlation matrix ($p < 0.05$) was done using the R corplot package. Partial least square (PLS) regression models were used to correlate quality parameters, biochemical composition and postharvest ethylene emissions (as X variables or explanatory variables) with *P. expansum* and *R. stolonifer* severity as response variables (Y). The Non-Linear Iterative Partial Least Squares (NIPALS) algorithm was used for computing the first few factors. KFold validation was used to select the number of factors that minimize the Root Mean PRESS statistic. Data for PLS models were centred and weighed by the inverse of the standard deviation of each variable in order to avoid dependence on measured units. PLS regression model analyses were performed using JMP 13.1.0 SAS Institute.

## 3. Results and discussion

### 3.1. *Fruit quality and physiology*

'Golden Reinders' apples from different locations were harvested at comparable maturity stages based on fruit firmness (around 70N for all orchards) and relatively similar starch index (6±1) (Figure 1A and 1E) and in line with the local harvesting criteria for this cultivar (Alegre et al., 2006). This said, a tendency was observed towards higher starch index in apples from the mountains (Figure 1E). It is well documented that starch accumulation and degradation is affected by temperature (Smith et al., 1979). Low temperatures prior to harvest, as those experienced in mountain orchards (Supplementary Figure 1), can promote the starch degradation into sugars, while temperatures above 30 ºC tend to inhibit starch breakdown (Yamada et al., 1994). Both firmness and starch index are considered as ethylene-dependent processes (Johnston et al., 2001; Thammawong and Arakawa, 2007). This said, the ethylene production rate, determined as the slope of the ethylene production between the onset of ethylene production and the climacteric peak, observed in mountain apples (13.60 µl $Kg^{-1}$ $day^{-1}$) in comparison to those from the valley (7.65 µl $Kg^{-1}$ $h^{-1}$) were not correlated with differences in apple flesh firmness but rather with higher starch index in the mountain location (Supplementary Figure 2A). In contrast, the significant decrease of firmness (-0.35 N $day^{-1}$, Figure 1B) and the increase in the starch index (+0.15 SI $day^{-1}$, Figure 1F) during on-tree ripening were strongly correlated with differences in the ethylene production pattern (Supplementary Figure 2B). In detail, three different ethylene production patterns were observed after harvest in fruit collected at different maturity stages (Figure 2K). In apples harvested 7 days before the optimal harvest ($OHD_{-7}$), the autocatalytic ethylene production began 16 days after the harvest and reached maximum values (35 µL $Kg^{-1}$ $h^{-1}$) nine days thereafter (25 days after harvest). Apples harvested between OHD and $OHD_{+21}$ showed a similar ethylene production

pattern, starting the autocatalytic ethylene production a week after harvest and reaching a plateau 15 days thereafter with a maximum ethylene production of *ca.* 100 µL $Kg^{-1}$ $h^{-1}$. Finally, in apples harvested at $OHD_{+28}$ ethylene production started 5 days after harvest and reached its maximum three days later (at 8 days; 180 µL $Kg^{-1}$ $h^{-1}$). Likewise, firmness loss was also more pronounced when comparing $OHD_{-7}$ to OHD and $OHD_{+21}$ to $OHD_{+28}$ harvests with a decrease of 0.85 N $day^{-1}$ and 0.55 N $day^{-1}$, respectively. However, the on-tree firmness loss observed in our trial was less severe than that reported in previous studies on different apple and pear cultivars (Varanasi et al., 2011; Lindo-García et al., 2019).

Differences on the onset of the ethylene production between locations were associated to the levels of ACC (Figure 2A). Accordingly, apples with similar ACC levels at harvest shared the same starting point for the onset of autocatalytic ethylene production after harvest whereas higher levels of ACC, such as those found in Gotarta apples, led to an earlier rise of the autocatalytic ethylene production after harvest. These results are in agreement with previous studies that shown that ACC availability act as the limiting factor for the ethylene production in apples (Lara and Vendrell, 2000). Indeed, similar results were also observed when comparing fruit from different harvest (Figure 2B), for which ACC accumulation during on-tree ripening was positively correlated ($r=0.849$; $P<0.05$) with the anticipation of the climacteric rise.

In the case of the enzymes involved in the final steps of ethylene biosynthesis, our results showed that nor the growing location or the fruit maturity influenced ACS activity (Figure 2E and 2F respectively). Regarding ACO (Figure 2G and 2H), a significant higher activity was found in apples grown in Vilanova yet the greater ACO activity was not translated into higher ethylene production.

Overall, our results suggest that differences found in the postharvest ethylene production pattern between locations were not limited by the different enzymatic activities involved in the last steps of ethylene biosynthesis but rather triggered by environmental factors (Druege, 2006), likely affecting upstream the ethylene biosynthetic pathway. As an example, an enhancement of ethylene production has been previously reported in 'Golden Delicious' apples when temperatures prior to harvest drop below 15 ºC (Knee, 1989), a situation occurring in mountain but not in valley orchards (Supplementary Figure 1).

A different behaviour was found when fruit were picked in the same orchard at different harvest dates. In this condition, ACC levels and ACO activity showed a rising trend throughout the different harvest dates while ACS activity remained unchanged. Similar results were observed in previous studies with 'Golden Delicious' apples (Tan and Bangerth, 2000). The burst in both ACS and ACO enzyme activities, as well as in the ACC levels observed at $OHD_{+28}$, may be considered as an indicator of the transition from a pre-climacteric into a climacteric stage (Hoffman and Fa Yang, 1980). Although more ACC was available for malonylation during on-tree ripening especially at $OHD_{+28}$, MACC levels remained unchanged across the different harvest dates (Figure 2D). Similar results were reported in on-tree ripening studies done in apples (Tan and Bangerth, 2000) and pears (Lindo-García et al., 2020), suggesting that the inhibition of ACC malonylation is likely a characteristic of on-tree ripening. No correlation was found between ACS activity and ACC levels nor the fruit potential to produce ethylene upon harvest during on-tree ripening. In contrast, ACO activity was positively correlated ($r=0.895$; $P<0.05$) with the autocatalytic ethylene production after harvest in apples picked at different maturity stages (Supplementary Figure 2B). The high correlation between ACC, ACO and ethylene production pattern suggest that ACO activity and ACC levels are the main limiting factors for the fruit ethylene production when comparing fruit from different

maturities. Such results would therefore be in line with those obtained in on-tree ripening studies made with 'Blanquilla' pears (Lindo-García at al., 2019) suggesting that such regulation is conserved within pome fruit species.

### *3.2. Taste-related composition*

Environmental conditions prior to harvest strongly influence the SSC/TTA ratio, a common index for apple taste perception (Harker et al., 2002). Apples harvested in valley orchards had, in average, SSC/TTA ratios 30% higher than those from the mountains (Figure 1C). These significant differences in the ratio SSC/TTA may be caused by differences on sugar metabolism (Lemoine et al., 2013), respiration rates (Bepete and Lakso, 1997) or accumulation of malic acid within the vacuoles (Moskowitz and Hrazdina, 1981), all of them substantially influenced by environmental cues. Our results agree with previous findings suggesting that apples grown under warm temperatures usually contain higher sugar and lower acid levels (Tyagi et al., 2017). In turn, harvest date also influenced the SSC/TTA ratio (Figure 1D). Although such increases in SSC/TTA ratios improve consumer perception, firmness loss during on-tree ripening may negatively affect final quality and consumer's acceptance (Harker et al., 2008).

As regard the specific sugar accumulation, significant differences in glucose levels were also found between locations (Table 1) but not between harvest dates (Table 2). Fruit grown in the valley had 30% higher concentrations of glucose compared to that from the mountains. An interesting behaviour in sucrose accumulation was also found for Gotarta's apples that presented 1.4-fold higher concentrations of sucrose in comparison to the other orchards. In these lines, enhanced sucrose accumulation level has been observed in plants grown under low temperatures (Horacio and Martinez-Noel, 2013) and in 'Fuji' apples grown at high altitude probably caused by an up-regulation of UDP-

sulfoquinovose synthase (Karagiannis et al., 2020). Interestingly, sucrose levels, despite not showing significant changes during on-tree fruit ripening were negatively or positively correlated with maturity-related markers like firmness and malic acid content or starch index and ACO activity, respectively (Supplementary figure 2B). This result may highlight the potential involvement of sucrose as a key signalling molecule able to modulate ethylene biosynthesis and regulate ripening events in conjunction with other phytohormones as recently pointed out in multiple studies conducted with pears (Lindo-García et al., 2019) and tomato (Jia et al., 2016). Further and more targeted studies are encouraged to elucidate the regulatory role of sucrose in apple ripening.

Not only sugars but organic acids are also important contributors to the fruit taste (Kader, 2008). As found in this work, orchard location seemed to have a significant influence in the content of malic acid. Indeed, 25 % higher concentrations were found in apples grown in the mountains (Table 1). This result may be due to the fact that malic acid accumulation within the vacuoles is favoured by low temperatures during fruit growth as proposed by Lakso and Kliewer (1975) and Moskowitz and Hrazdina (1981). In contrast, high temperatures may increase the tonoplast permeability producing the leak of malic acid towards the cytoplasm (Lobit et al., 2006) and its degradation to maintain the cytoplasmic pH (Etienne et al., 2013). In the case of apple from the same orchard (Table 2), malic acid content tended to decrease as the fruit ripened on-tree.

*3.3. Oxidative stress and antioxidant capacity*

Among the studied acids, we also analysed ascorbic acid, an antioxidant molecule well known by its role in photosynthesis and photoprotection mechanisms (Lee and Kader, 2000) as well as a cofactor in enzymatic reactions (Smirnoff and Wheeler, 2000). Albeit apples are an important source of ascorbic acid, the contribution of ascorbic acid to the antioxidant capacity in apples is relatively low (Drogoudi et al., 2008). In this work, both

331 the location and the fruit maturity stage significantly influenced the total ascorbic acid
332 levels (Table 1 and 2). Apples grown in Gotarta exhibited 25% higher levels of total
333 ascorbic acid than apples grown in other locations (Table 1). Such difference may be
334 related to low night temperatures (Supplementary figure 1) favouring the accumulation
335 of ascorbic acid as reported in mandarin and grapefruits (Lee and Kader, 2000) and
336 'Cortland' apples (Barden and Bramlage, 1994). In contrast, an advanced harvest maturity
337 reduced the levels of total ascorbic acid and ascorbicate. Twenty five percent lower
338 amounts of total ascorbic acid were found in apples harvested at advanced maturity
339 ($OHD_{+21}$ and $OHD_{+28}$) if compared to earlier maturity stages ($OHD_{-7}$ and $OHD_{+14}$), hence
340 in agreement with the available literature (Lee and Kader, 2000).

341 Phenolic compounds are the main components accounting for the antioxidant activity in
342 the apple flesh (Lee et al., 2003; Kalinowska et al., 2014). Accordingly, a positive
343 correlation between the fruit antioxidant capacity and the content of total phenolic
344 compounds was observed in our study (Supplementary Figure 2). Significant differences
345 in the total phenolic content were found between valley and mountain apples (Table 1).
346 Apples grown in the mountains had, in general, 25% higher levels of total phenolic
347 compounds than fruit from the valley, in agreement with previous studies made on 'Fuji'
348 apples (Karagiannis et al., 2020) as well as other fruit (Crespo et al., 2010). In contrast,
349 phenolic compounds were not significantly affected by the fruit maturity stage (Table 2),
350 yet apples harvested in advanced maturity stages ($OHD_{+21}$ and $OHD_{+28}$) had 15% lower
351 levels of phenolic compounds than apples harvested earlier which is in agreement with
352 available literature (Alberti et al., 2017).

353 When the level of antioxidants are not sufficient to scavenge reactive oxygen species
354 (ROS), these compounds may accumulate and compromise the integrity of molecules
355 such as proteins and lipid membranes (Hodges et al., 2004; Suzuki and Mittler, 2006).

Malondialdehyde (MDA), a product of lipid peroxidation (Hodges et al., 1999), was used in our study as a marker of oxidative stress within the apple flesh. Accordingly, mountain apples were likely exposed to greater stress, since mountain apples had slightly higher, yet not significant, levels of MDA (8%) if compared to valley apples (Figure 3C). The positive correlation between MDA levels, antioxidant capacity and ethylene production (Supplementary Figure 2A) suggested that the higher ethylene production found in mountain apples may be a fruit response to cope with specific environmental stresses (Wang et al., 2002) aiming to activate different antioxidant mechanisms to counteract the action of ROS (Thao et al., 2015; Lv et al., 2020). Future studies are encouraged to further decipher the relationship between environmental cues, ethylene production and antioxidants in apple fruit.

### *3.4. Disease resistance*

Despite the notable differences in the flesh composition and physiology detailed above, no significant differences between locations were found in *P. expansum* incidence 7 days after inoculation (Figure 4A), yet the lesion diameter was significantly lower in apples grown in mountain orchards. In the case of *R. stolonifer*, no clear behaviour was found for the orchard location (30% and 52.5% incidence in Llesp and Alcanó respectively, 75% in Gotarta and 90 % in Vilanova). Differences in flesh composition and physiology from fruits grown in different locations were likely not paralleled by substantial changes in the flesh structure and hence leading to no differences in postharvest pathogens incidence. The structure of apple peel/flesh from different locations should be further studied in relationship to the fruit postharvest behaviour. Our results also showed that an advanced fruit maturity at harvest led to higher incidence and lesion diameter in apples inoculated with *R. stolonifer*.

Given the diverse *R. stolonifer* incidence but especially severity found in apples grown in different locations and harvest dates, a partial least square regression (PLS) model was performed in order to identify the biochemical or physiological variables associated to the fruit susceptibility to this pathogen (Figure 5). Likewise, a PLS model was also made to study *P. expansum* infection but the model prediction capacity was low mainly due to the scarce data (Y) variability (data not shown). Our results showed that lesion severity caused by *R. stolonifer* was positively correlated with SCC, starch index, and several enzymes or intermediates involved on ethylene metabolism (ACC, ACS, ACO), as well as the postharvest ethylene production pattern itself (Figure 5). On the other hand, lesion diameter was negatively correlated with the fruit firmness. These results are consistent with previous studies describing that fruit ripening favours colonization of multiple postharvest pathogens (Cantu et al., 2008; Vilanova et al., 2012; Nybom et al., 2020). On the other hand, the earlier onset of ethylene production in Gotarta apples, if compared with other locations, seemed to favour *R. stolonifer* growth further reinforcing the dual role that ethylene may have on enhancing or weakening the fruit resistance to postharvest pathogen infection (Baró-Montel et al., 2019).

## 4. Conclusion

Orchard location was the main source of variability for most of the investigated quality or biochemical traits, while the fruit maturity at harvest mainly influenced ethylene related metabolites and enzymes but also ethylene-dependent quality traits such as the firmness. Ethylene biosynthesis, and especially the accumulation of ACC is differentially regulated by the environment while differences in the fruit ethylene biosynthetic pathway in fruit from different maturities are mainly due to differences in ACO activity. Compared with valley orchards, apples grown in the mountain showed higher levels of sucrose, malic acid, and antioxidants and lower SSC/TTA and glucose. Our data also showed that differences in the fruit biochemical composition between locations did not influence the fruit susceptibility to *P. expansum* and *R. stolonifer* infection yet the lesion severity caused by *R. stolonifer* was favoured by an enhanced fruit ethylene production.

The results from this study may assist growers on deciding the optimal harvest date to obtain fruit with an optimum taste- and health-related composition as well as lower susceptibility to specific postharvest pathogens.

## Author's contribution

JGB, NT and PFC conceived and designed the experiment. PFC and JGB performed all field and storage samplings including quality measurements and sample preparation for biochemical analysis. PFC, GE and CL performed the analysis of ethylene and ethylene-related enzymes or precursors. RT, NT and PFC were responsible for the fruit inoculation and evaluation of rots. PFC and JGB wrote the manuscript and all remaining authors contributed in improving and revising the final version.

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## Tables

***Table 1:*** Levels (mg 100 $g^{-1}$ FW) of the main sugars, organic acids and total phenolic compounds in 'Golden Reinders' apple flesh from different locations. Data shown are means of three replicates ± standard deviation. Letters indicate significant differences according to Tukey test ($P < 0.05$).

| Compound | Alcanó | Vilanova | Llesp | Gotarta | CV (%) |
|---|---|---|---|---|---|
| Glucose | 13.9 ± 1.59 ab | 14.7 ± 0.17 a | 11.7 ± 0.88 b | 8.7 ± 0.82 c | 22.0 |
| Fructose | 50.3 ± 8.10 | 38.8 ± 1.00 | 40.6 ± 6.58 | 46.3 ± 2.71 | 11.7 |
| Sucrose | 19.5 ± 1.31 ab | 19.5 ± 2.10 ab | 16.1 ± 4.16 b | 28.3 ± 3.95 a | 20.6 |
| Total sugars | 80.6 ± 4.84 | 73.0 ± 2.46 | 68.3 ± 11.37 | 83.8 ± 7.42 | 7.7 |
| Malic acid | 3.6 ± 0.26 | 3.5 ± 0.38 | 4.9 ± 0.53 | 5.5 ± 0.12 | 22.2 |
| Ascorbic acid | 2.2 ± 0.19 | 2.6 ± 0.47 | 2.5 ± 0.21 | 2.8 ± 0.39 | 9.5 |
| Total ascorbic acid | 3.2 ± 0.46 | 3.7 ± 0.90 | 3.8 ± 0.18 | 4.5 ± 0.85 | 13.6 |
| Total Phenolic Compounds | 79.0 ± 9.59 b | 88.3 ± 10.48 ab | 104.6 ± 7.18 a | 108.6 ± 5.71 a | 14.6 |

690 ***Table 2:*** Levels (mg 100 $g^{-1}$ FW) of the main sugars, organic acids and total phenol compounds in 'Golden Reinders' apple flesh for different
691 harvest dates. Data shown are means of three replicates ± standard deviation. No significant differences were found between harvest dates ($P <$
692 0.05).

693

| Compound | OHD-7 | OHD | OHD+7 | OHD+14 | OHD+21 | OHD+28 | CV (%) |
|---|---|---|---|---|---|---|---|
| Glucose | 16.6 ± 0.80 | 15.2 ± 2.42 | 15.7 ± 2.42 | 16.0 ± 1.08 | 16.2 ± 1.17 | 14.9 ± 2.56 | 4.1 |
| Fructose | 46.9 ± 7.21 | 40.7 ± 7.26 | 45.2 ± 7.26 | 43.0 ± 3.36 | 51.6 ± 2.95 | 34.8 ±1.84 | 13.1 |
| Sucrose | 15.4 ± 2.46 | 15.3 ± 0.82 | 17.8 ± 0.82 | 16.4 ± 2.85 | 18.9 ± 2.86 | 17.9 ± 4.35 | 8.6 |
| Total sugars | 77.3 ±7.04 | 71.1 ± 9.91 | 78.6 ± 9.91 | 67.3 ± 7.31 | 86.7 ± 1.95 | 72.8 ± 11.23 | 9.0 |
| Malic acid | 4.2 ± 0.39 | 3.6 ± 0.28 | 3.6 ± 0.28 | 3.6 ±0.23 | 3.1 ± 0.23 | 3.4 ± 0.78 | 10.1 |
| Ascorbic acid | 2.0 ± 1.53 | 1.7 ± 0.44 | 1.8 ± 0.34 | 1.5 ± 0.78 | 1.6 ± 0.07 | 1.4 ± 0.55 | 12.6 |
| Total ascorbic acid | 3.4 ± 0.94 | 3.6 ± 0.85 | 2.8 ± 0.79 | 3.1 ± 0.39 | 2.2 ± 0.29 | 2.3 ± 0.47 | 16.1 |
| Total Phenolic Compounds | 73.9 ± 11.07 | 67.9 ± 7.57 | 80.0 ± 13.90 | 71.5 ± 4.65 | 62.8 ± 6.45 | 63.0±6.60 | 9.1 |

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**List of figures**

**Figure 1:** Quality characteristics at the time of harvest including Firmness (A, B), SCC/TTA (C, D) and Starch index (E, F) for the different locations (A, C, E) and for different harvest dates for the same location (B, D, F). The lower and top boundary of the box indicates the 25th and 75th percentile, respectively while the middle line within the box marks the median. Error bars above and below the box indicate the 90th and 10th percentiles respectively. Letters indicate significant differences according to Tukey test ($p < 0.05$). Number of fruit was different depending on the quality trait being measured (n=60 for firmness, n=6 for SSC/TTA and n=15 for starch index).

**Figure 2:** Ethylene-related precursors (A, B, C, D) and enzymes (E, F, G, H) and ethylene production (µL $Kg^{-1}$ $h^{-1}$) after harvest (J, K), for the different locations (A, C, E, G, J) at the Optimal Harvest Date (OHD) and for the same location at different harvest dates (B, D, F, H, K). Error bars represent the standard deviations of the means (n=3). Letters indicate significant differences according to Tukey test ($p < 0.05$).

**Figure 3:** Antioxidant capacity (mg $Fe^{2+}$ 100 $g^{-1}$; A, B) and MDA content (µmol 100 $g^{-1}$; C, D) for different locations (A, C) or different harvest dates (B, D). Error bars represent the standard deviations of the means (n=3). Letters indicate significant differences according to Tukey test ($p < 0.05$).

**Figure 4:** Susceptibility of 'Golden Reinders' apples to *P. expansum* after 7 days of incubation (A, B) and *R. stolonifer* after 3 days of incubation (C, D) for different locations (A, C) and different harvest dates (B, D). Disease incidence are represented with bars and severity with dots. Error bars represent the standard deviations of the means (n=4). Letters indicate significant differences according to Tukey test ($p < 0.05$).

**Figure 5:** A) Partial Least Squares (PLS) correlation loading plots of the 2 factors of *R. stolonifer* severity for apples grown in different locations and from different harvests. B) Variable importance plot (VIP) and correlation coefficient (C). D) The measured *vs* the predicted *R. stolonifer* severity through the model and its correlation coefficient. *Legend:* 1, Firmness; 2, SCC; 3, TTA; 4, Starch Index; 5, Ascorbic Acid; 6, Total Ascorbic Acid; 7, Malic Acid; 8, Glucose; 9, Fructose; 10, Sucrose; 11, Total Sugars; 12, Total Phenolic Compounds; 13, Antioxidant Capacity; 14, MDA; 15, ACC; 16, MACC; 17, ACS; 18, ACO; 19, Days for ethylene production onset; 20, Days for maximum ethylene production; 21, Maximum ethylene production.

**Supplementary Figure 1:** Temperature 90 days before harvest in mountain (average between Gotarta and Llesp) and valley orchards (average between Alcanó and Vilanova).

**Supplementary Figure 2:** Bivariate correlations among the different quality and biochemical traits of 'Golden Reinders' apples between orchard locations (A) and harvest dates on the same valley orchard (B). The size of the circle for each correlation and the colour depict the significance and the correlation coefficient, respectively. Positive correlations coefficients are displayed in blue and negative correlations coefficients in red.

**Figure 1:**

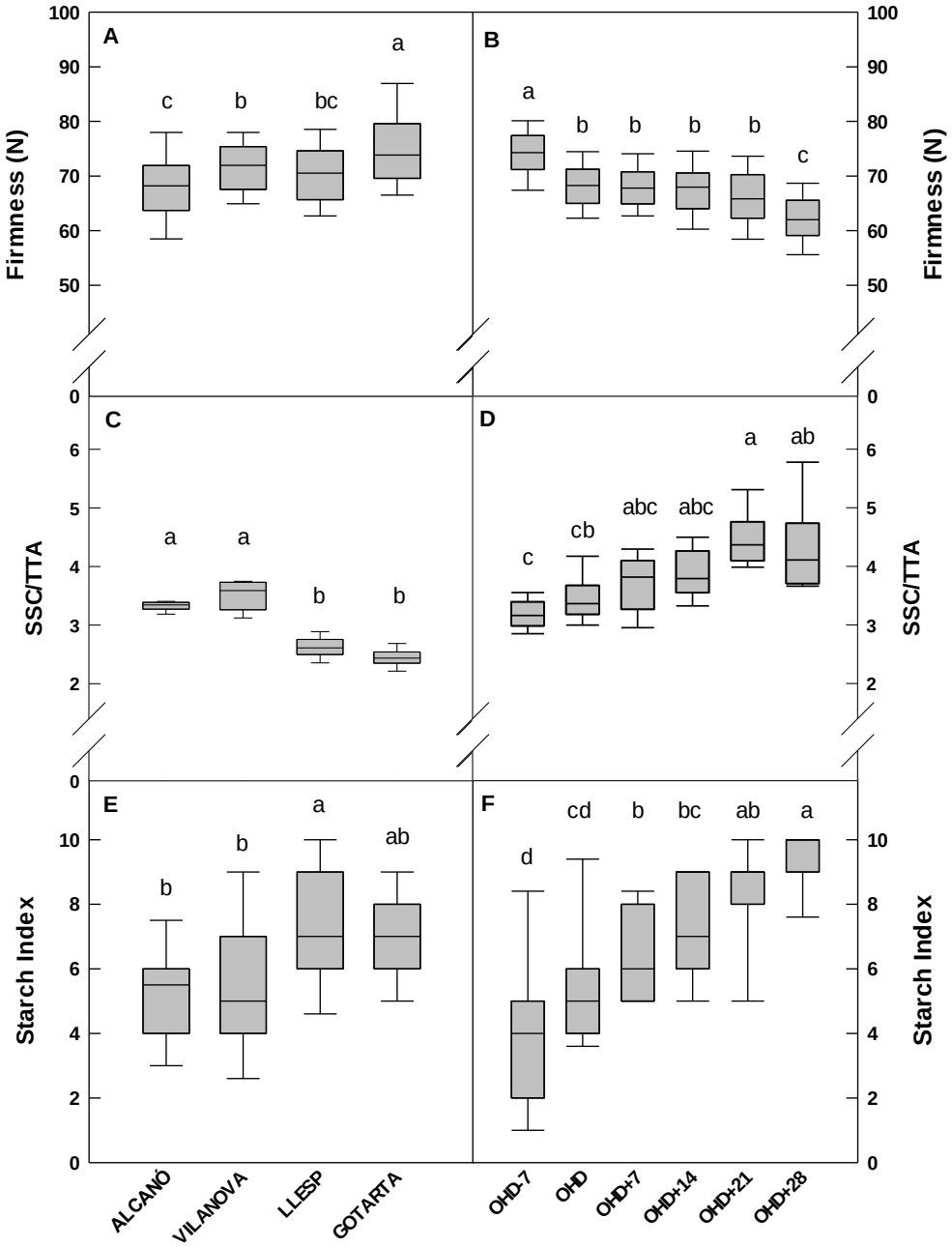

A
B
C
D
E
F
Firmness (N)
SSC/TTA
Starch Index
ALCANÓ
VILANOVA
LLESP
GOTARTA
OHD-7
OHD
OHD+7
OHD+14
OHD+21
OHD+28

**Figure 2:**

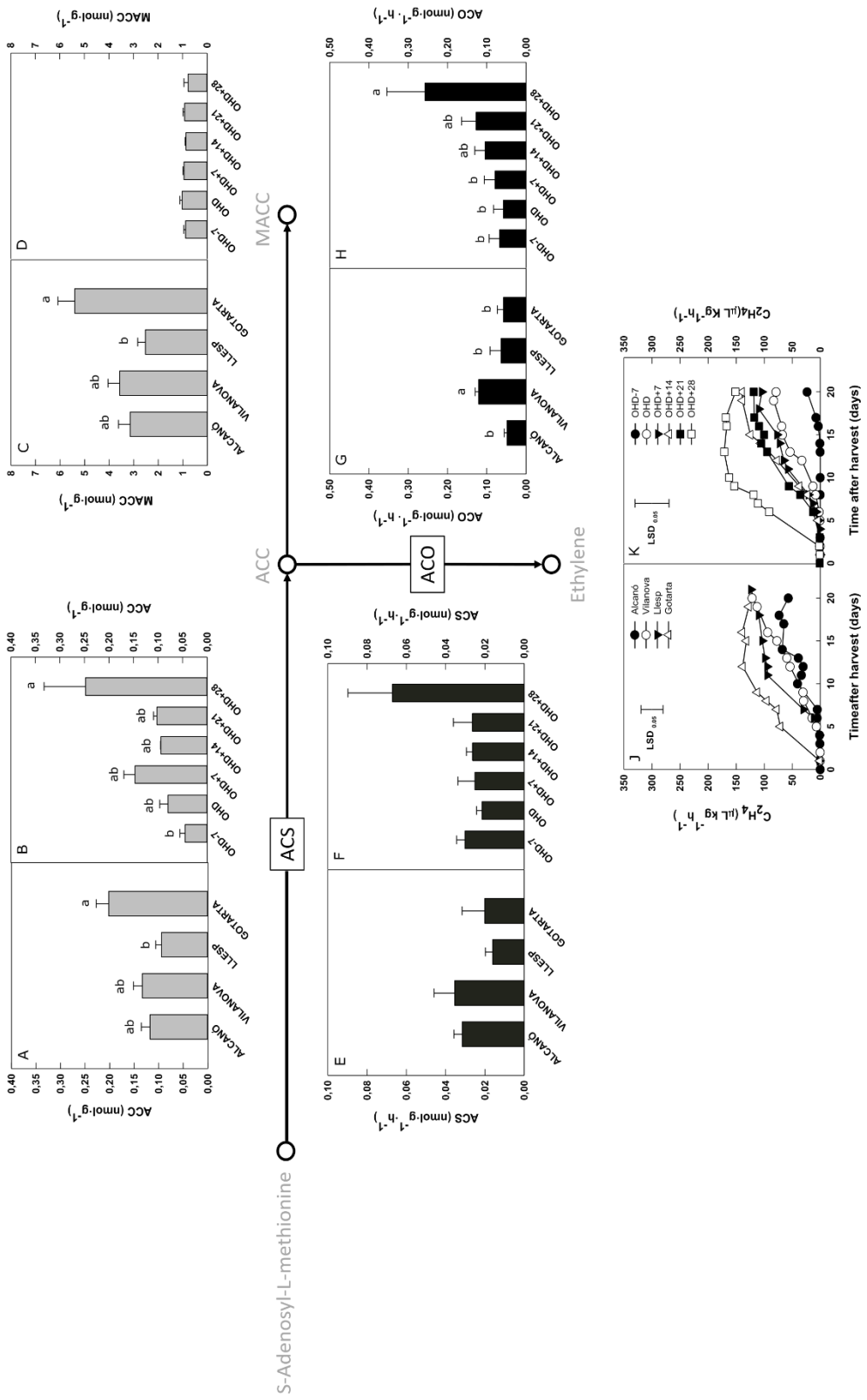

S-Adenosyl-L-methionine
ACS
ACC
MACC
ACO
Ethylene
A
B
C
D
E
F
G
H
J
K
ALCANO
VILANOVA
LLESP
GOTARTA
OHD-7
OHD
OHD+7
OHD+14
OHD+21
OHD+28
Alcanó
Vilanova
Llesp
Golarta
Time after harvest (days)
Timeafter harvest (days)

**Figure 3:**

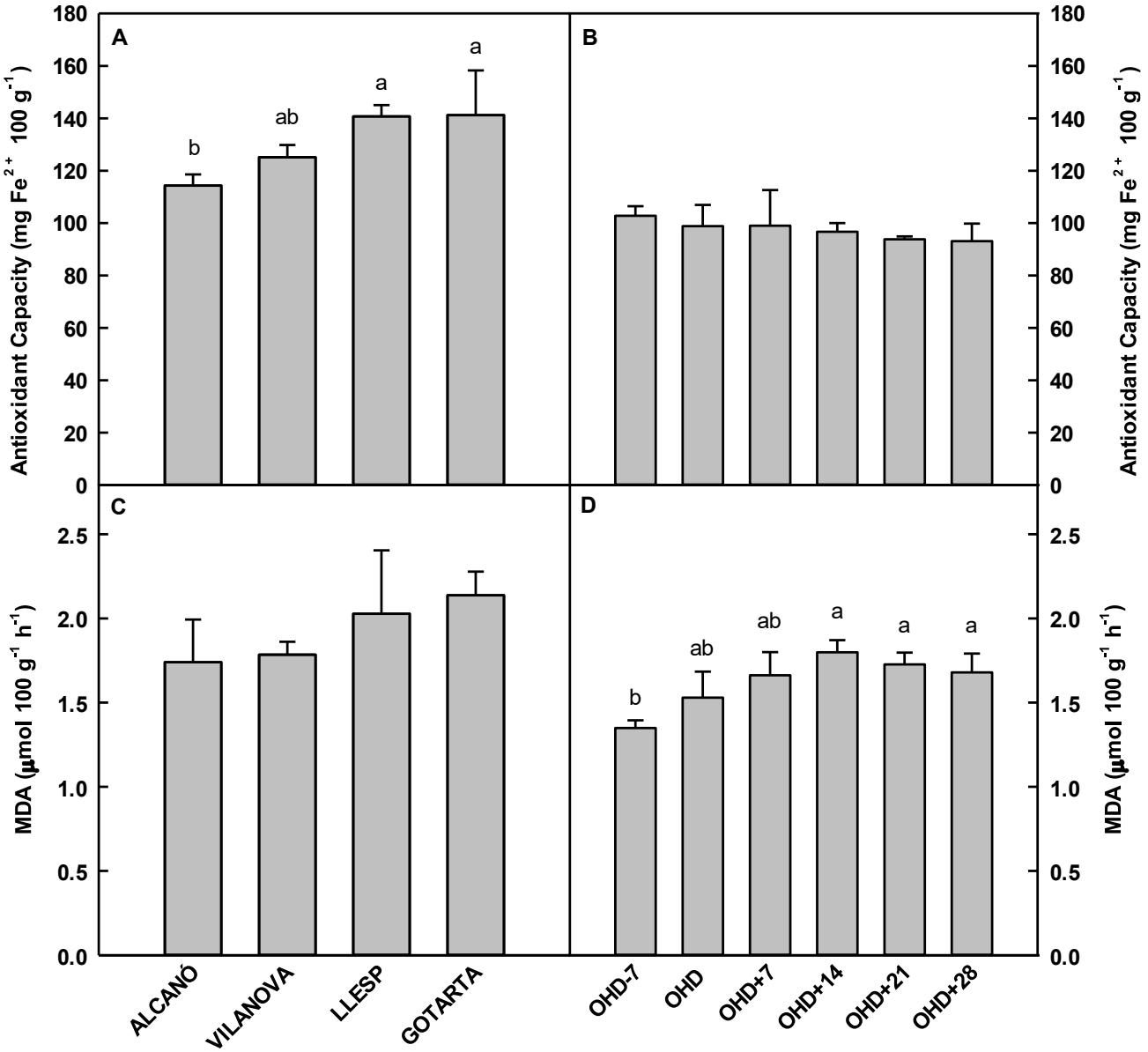

A
B
C
D
Antioxidant Capacity (mg Fe2+ 100 g-1)
MDA (µmol 100 g-1 h-1)
b
ab
a
a
b
ab
ab
a
a
a
ALCANÓ
VILANOVA
LLESP
GOTARTA
OHD-7
OHD
OHD+7
OHD+14
OHD+21
OHD+28

**Figure 4:**

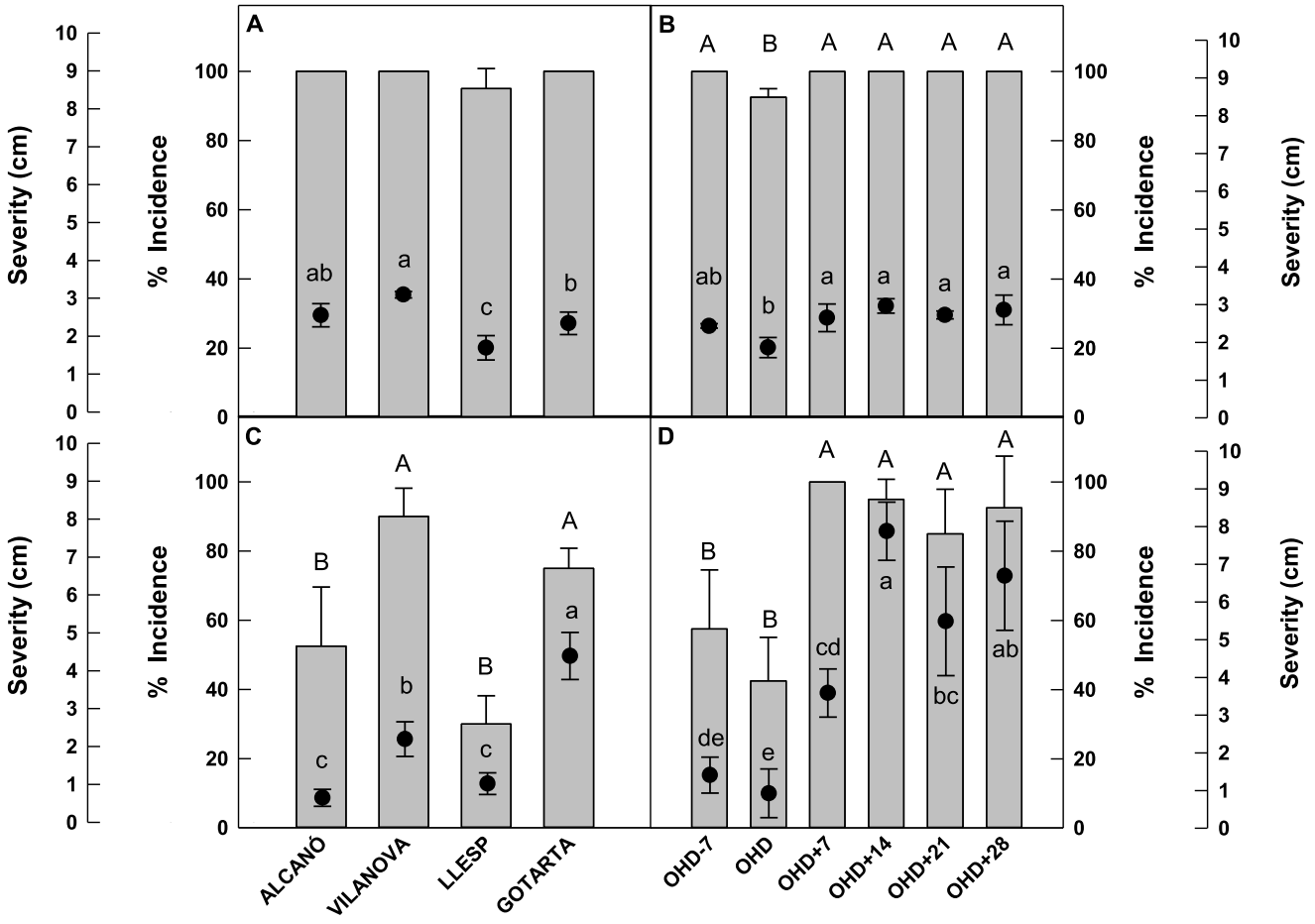
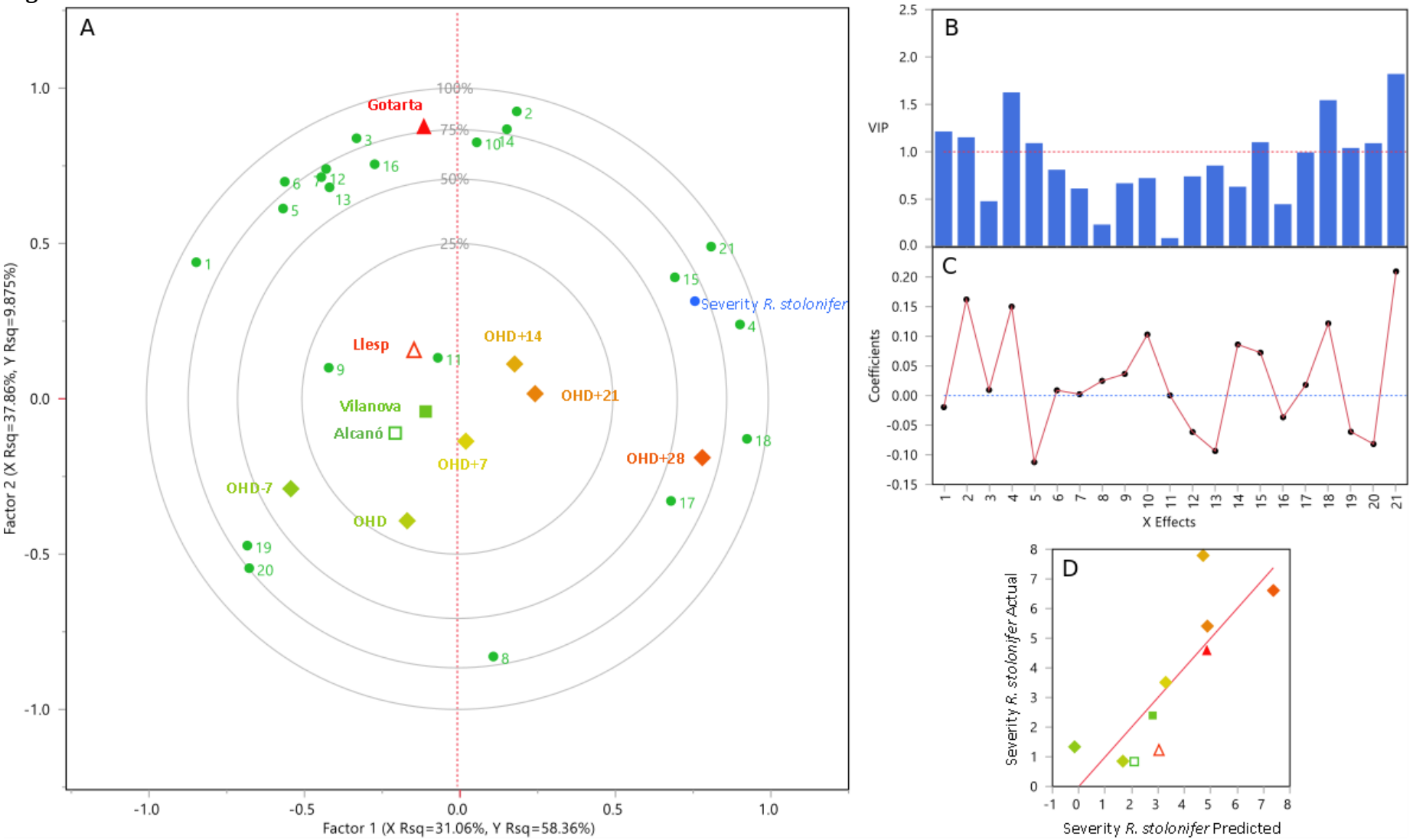
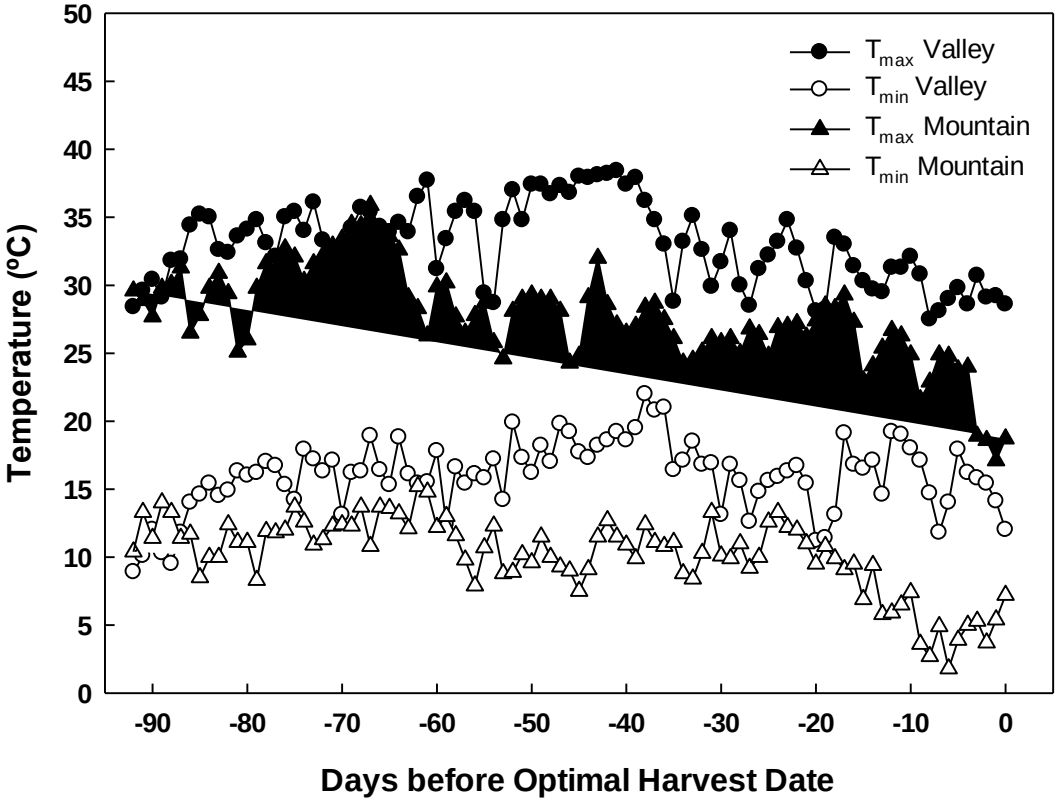

A
B
C
D
Severity (cm)
% Incidence
ALCANÓ
VILANOVA
LLESP
GOTARTA
OHD-7
OHD
OHD+7
OHD+14
OHD+21
OHD+28

**Figure 5:**


A
100%
75%
50%
25%
Gotarta
Llesp
Vilanova
Alcanó
OHD
OHD+7
OHD+14
OHD+21
OHD+28
OHD-7
Severity R. stolonifer
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Factor 1 (X Rsq=31.06%, Y Rsq=58.36%)
Factor 2 (X Rsq=37.86%, Y Rsq=9.875%)
B
VIP
C
Coefficients
X Effects
D
Severity R. stolonifer Actual
Severity R. stolonifer Predicted

**Supplementary Figure 1:**



**Supplementary Figure 2:**