The role of the epidermis defense system in black spot development during postharvest storage of avocado cv. Hass

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25 ***ABSTRACT***

26 The main destination of Chilean Hass avocados is the European market. Starting in season
27 2014-15 some fruits displayed at destination markets small black spots while the
28 epidermis was still green. This disorder appeared after prolonged cold storage combined
29 with controlled atmosphere. Peak incidence occurred in season 2016-17 and then
30 appearing erratically in the following seasons. We postulate that black spot development
31 is the consequence of oxidative stress caused by pre-harvest abiotic factors that and
32 symptoms are only evident postharvest. The objective of this work was to evaluate the
33 enzymatic and non-enzymatic defense system of Hass avocado fruit epidermis from three
34 different geographical areas defined by the distance from the orchard to the Pacific
35 Ocean, and to study its correlation with the incidence of black spot during postharvest
36 storage.

37 The incidence of the disorder did not show a pattern in relation to the geographical areas,
38 rather it did so by orchard and by the days in storage. As for the biochemical variables
39 studied, the total phenolic compounds and the total antioxidant capacity showed a
40 negative correlation with the incidence of black spot during post-harvest storage, showing
41 a considerable decrease from harvest to the first 10 days of storage in fruits from orchards
42 with incidence. Moreover, principal component analysis (PCA) showed that the climatic
43 variables (mean temperature, minimum temperature, maximum temperature and relative
44 humidity) and variables of the non-enzymatic defense system of the epidermis such as
45 epicatechin and its derivatives as the most important variables in the data matrix.
46 Likewise, partial least squared regression (PLS-R) showed as negatively correlated

variables with black spot development: maximum temperature, dry matter and phenolic compounds and positive correlation: caliber, minimum temperature, epicatechin, peroxidase enzyme activity and maximum relative humidity. The results of this work open a door to explore the use of different products or process that early trigger the defense system of the fruit epidermis in order to control the disorder during post-harvest storage.

Keywords: Avocado, post-harvest, black spot, phenolics compounds, oxidative stress

1. INTRODUCTION

Main destinations for Chilean avocados are Europe and the United States, with 41.2% and 13.3% respectively during the season 2018/2019 (Hass Avocado Committee 2019). One of the main causes of rejection of exported Chilean Hass avocados is a disorder originated in the epidermis or exocarp of the fruit known as "black spot". Black spot is evidenced only after prolonged postharvest storage (25 days or more), with the appearance of dark spots on the fruit epidermis and is detected only at the exit of cold storage and / or controlled atmosphere at destination markets thus causing fruit rejection (Zamora Magdaleno et al., 2001). In Chile this problem has manifested erratically, reaching in extreme cases rejections of 20% of the exported fruit as the 2016-17 season.

Previous studies have associated such oxidations in the epidermis with friction blows (Zamora-Magdaleno et al. 1999; Everett et al. 2008). In Chile, so far there are few attempts related to understanding the role of endogenous fruit factors, mainly those related to the defense mechanism of the epidermis. The defense mechanisms of plants

68 can be structural, such as thickening of cell walls or lignification of tissues, or biochemical,
69 such as the production of phenolic compounds, phytoalexins or enzymes (Thatcher et al.,
70 2005; Fang and Bhandari, 2011). The defense mechanism of plants is regulated by
71 secondary metabolism, which includes routes related to the synthesis of terpenes,
72 phenolic compounds and nitrogen compounds (Fang and Bhandari, 2011). The oxidative
73 stress that could give rise to "black spot" development in Hass avocado is the result of an
74 imbalance between the production of reactive oxygen species (ROS) and the defense
75 mechanisms (enzymatic and non-enzymatic) of the epidermis, which is exposed during the
76 growth, development and post-harvest of the fruit to abiotic stresses (Ahmad et al., 2010).

77 There are no previous studies that have correlated the incidence of black spot with the
78 avocado epidermis defense system. However, previous studies have correlated the
79 development of anthracnose (pathological disorder caused by *Colletotrichum*
80 *gloeosporioides*) and its control with the activation of the epidermis defense system.
81 Melgar et al. (2018) reported epicatechin as the most abundant phenolic compound found
82 in Hass avocado epidermis in a pool of 29 different phenolic compounds. Other studies
83 reported epicatechin content as a natural antifungal by inhibiting the action of the
84 lipoxygenase enzyme during the activation stage of inactive *Colletotrichum*
85 *gloeosporioides* infection (Karni et al., 1989). Recently, Bowen et al. (2018) reported that
86 the maturity stage, storage and ripening influence the antifungal compounds content
87 (persins, catechins and epicatechin) in Hass avocado epidermis. Another mechanism to
88 maintain homeostasis of the cellular redox system is the activation of the enzymatic
89 defense system being the first redox reaction catalyzed by the enzyme superoxide

90 dismutase (SOD) that converts superoxide (O_2-) to H_2O_2. Excess H_2O_2 is toxic to cells
91 therefore it must be metabolized. Peroxidase (POX), ascorbate peroxidase (APX) and
92 catalase (CAT) convert H_2O_2 to water. POX detoxifies H_2O_2 using flavonoids as substrates.
93 CAT metabolizes H_2O_2 to water and oxygen through the iron-heme groups attached to the
94 enzyme and APX detoxifies H_2O_2 using ascorbic acid as a reducing agent giving
95 dehydroascorbate (MDHA) (radical form) and dehydroascorbate (DHA).

96 Pre harvest factors (temperature, relative humidity, water deficit, etc) as well as harvest
97 (maturity stage) and post-harvest (storage time and atmosphere conditions, temperature,
98 etc) represent abiotic factors that might promote the production of ROS and thus must be
99 balanced by the defense system of the fruit epidermis (enzymatic and non-enzymatic) in
100 order to avoid oxidative stress and physiological disorders such as "black spot". A previous
101 study of our group correlated different pre-harvest abiotic factors (climatic variables) and
102 the defense system of the epidermis at harvest (enzymatic and non-enzymatic) with black
103 spot incidence after prolonged storage. Therefore, this research work aims to provide
104 information regarding the behavior of the defense system of the epidermis of the fruit
105 during postharvest storage. Thus, the objective is to evaluate and correlate the evolution
106 of the enzymatic and non-enzymatic defense system of the Hass avocado epidermis
107 during prolonged postharvest storage (25 and 40 days in regular air conditions at 5°C) on
108 the incidence of black spot in orchards displaying contrasting behavior of this disorder
109 (affected vs. unaffected).

110

111 ***2. MATERIALS AND METHODS***

112 **2.1. Collection sites and plant material sampling**

113 Fruit epidermis of Hass avocados from nine orchards from the central area of Chile were
114 sampled. The orchards were selected in three different geographical areas: interior,
115 intermediate and coastal zones. The orchards selected by geographical area have the
116 following characteristics: a) interior area: distance from the orchard to the sea ≥ 45 km.
117 The orchards that have these characteristics correspond to: orchards A, B and C, located in
118 Panquehue, Bartolillo and La Viña respectively; b) intermediate zone: distance from the
119 orchard to the sea between 20 and 45 km. The orchards that presented these
120 characteristics corresponded to the orchards D, E and F, located in Cabildo, Nogales and
121 María Pinto respectively, and c) coastal zone: distance from the field to the sea ≤ 10 km.
122 The orchards that presented these characteristics corresponded to G, H and I, all located
123 in Santo Domingo. The key differences between the three selected zones were
124 temperature regimen (absolute maximum and minimum, and average), relative humidity
125 and harvest precocity, with the interior zone being the earliest and the coastal zone being
126 the latest (Supplementary Table 1). The climatic data were obtained from orchards that
127 had a weather station, otherwise, the information was collected from the nearest
128 available weather station. The variables used were temperature (absolute minimum,
129 absolute maximum and average), relative humidity and rainfall (Supplementary Table 1).
130 One hundred and fifty fruits were collected per orchard in two harvest seasons defined as:
131 early harvest (dry matter between 23 and 26%) and late harvest (dry matter between 27
132 and 30%). The fruits were transported to the laboratory immediately after being

133 harvested, where they were weighed, cooled and stored at 5 ° C in regular air (RA). Fifty
134 fruits were stored for 25 days, 50 fruits for 40 days and 50 fruits were used to sample
135 epidermis every 10 days during storage (up to 40 days). At harvest, 10 fruits were taken in
136 order to perform a dry matter analysis. From day 0 (corresponding to the day of harvest)
137 and every 10 days during storage, 10 fruits (50 in total) were taken where epidermis
138 samples were used for biochemical analysis. In total 2700 fruits were used in this
139 corresponding to 2017/2018 season. The epidermis pieces were finely ground in liquid
140 nitrogen and stored at -20 ° C for further analysis. The follow-up of the defense system of
141 the epidermis during the post-harvest (10, 20, 30 and 40 days in cold storage) was limited
142 to orchards that presented incidence of black spot at the exit of cold storage and also to
143 orchards that did not present incidence of the disorder or that its incidence was mild
144 within the same geographical area of complex orchards in order to compare.

145 **2.2 Incidence of "black spot" and evaluation of fruit quality attributes**

146 After 25 and 40 days of cold storage in regular air atmosphere (AR) at 5 ° C, the incidence
147 of black spot was quantified as percentage of affected fruits and severity (Figure 1). Once
148 the evaluation was done at the exit of cold storage, the fruits were kept at 20 ° C and 65%
149 RH to evaluate the days it takes to reach their consumption maturity (ready to eat),
150 quantify disorders such as internal browning (pulp and vascular bundles), rot (presence or
151 absence of stem end rot and anthracnose) and color according to Rivera et al. (2017).
152 These quality evaluations are not shown in this manuscript.

153

154 **Figure 1**: Relative scale of black spot severity on the skin of avocado fruits var. Hass: own
155 elaboration. The numbers correspond to: (0): fruit without damage; (1) fruit with less than
156 25% of the damaged skin; (2) fruit with less than 50% of its skin with damage and (3) fruits
157 with more than 50% of its skin with damage by black spot.

158

159 **2.3 Determinations of the non-enzymatic defense system of the epidermis**

160 **2.3.1 Extraction and quantification of total phenolics and total antioxidant capacity**

161 These tests were carried out following the methodology proposed by Saavedra et al.
162 (2017), with slight modifications. Briefly, 50 mg of lyophilized epidermis was taken and 2
163 mL of acetone: water mixture (70:30) was added. Subsequently, the tubes were incubated
164 at room temperature with shaking for 1 h in a vortex. Then, the samples were centrifuged
165 at 17000 g for 20 min at 4 ° C. Two hundred µL of the supernatant was taken which was
166 evaporated with nitrogen gas. Finally, the pellet was re-suspended in methanol and the
167 samples were stored at -20 ° C. All steps were carried out in the absence of light. To
168 quantify total phenolics, 240 µL of water was added to a microplate and then mixed with
169 20 µL of the extract diluted 1:10. Then, 20 µL of the 1 N Folin Ciocalteu reagent and 20 µL
170 of 5% w / v sodium carbonate were added. The reaction was incubated for 2 h at room
171 temperature and protected from light. The absorbance at 725 nm was measured. Total
172 phenolics were calculated based on a standard curve of gallic acid (Y = 0.0054X-0.0005 /

173 R^2: 0.985), and the results were expressed in mg of equivalent gallic acid per 100 grams of
174 dry matter.

175 The antioxidant capacity of the extracts was evaluated by the DPPH radical inhibition
176 assay. Briefly, 20 µL of the diluted 1: 100 extract was mixed with 125 µL of 60 mM DPPH.
177 The reaction was incubated for 30 min at room temperature and protected from light. The
178 absorbance at 517 nm was measured. A standard Trolox curve was performed (Y =
179 0.2353X + 7.768 / R^2: 0.9934). The results were expressed in millimoles of Trolox
180 equivalents per 100 grams of dry matter.

181 **2.3.2 UPLC-DAD analysis of epicatechin and derivatives, chlorogenic acid and other**
182 **phenolics**

183 The procedure of Adamson et al. (1999) was used for sample preparation with small
184 modifications. Briefly, from 250 mg of avocado peel previously lyophilized was added to 3
185 ml petroleum ether, and the mixture was centrifuged at 15000 g for 5 min. The
186 supernatant was eliminated by aspiration. The residual petroleum ether was evaporated
187 using a vacuum concentrator during 10 min. After that, 10 ml of cold extraction solution
188 (acetone:water:acetic acid, 70:29.5:0.5, % v v^{-1}) was added to each sample, vortexed in a
189 horizontal form using a shaker during 2 h protected from light. Finally, samples were
190 centrifuged at 15000 g for 5 min and then an aliquot of the supernatant was filtered using
191 a filter syringe (0.22 µm). Samples were collected in vials of 2 ml and stored at 4 ° C until
192 subsequent analysis by UPLC-PAD.

193 Phenolic compounds were analyzed using an UPLC system composed of an Aquity HClass
194 separation module (Waters, Milford, USA) equipped with an auto-injector, an Aquity
195 photodiode array detector (PDA eλ detector) and the Empower software. The column
196 used for UPLC separation was an Acquity BEH C 18 (1.7 µm, 100 x 2.1 mm) (Waters,
197 Milford, USA) with an Acquity VandGuard BEH C 18 pre-column (1.7 µm, 5 x 2.1 mm),
198 operated at 30 °C. The mobile phase consisted in 0.1% formic acid in water (solvent A) and
199 0.1% formic acid in acetonitrile (solvent B). The gradient program was as follows: 2% B for
200 2 min, 2 to 7% B in 2 min, 7 to 12% B in 11 min, 12 to 26% B in 5 min, 26 to 55% B in 5 min,
201 55 to 95% B in 1 min and 95% B for 3 min. The flow rate and sample injection used were
202 0.2 ml min^{-1} and 2.0 µl, respectively. Spectral data were recorded from 200 to 700 nm
203 during the whole run. Phenolic compounds were identified and quantified by comparing
204 their retention time and UV-visible spectral data to known previously injected standards
205 (280, 320 and 360 nm). The results were expressed in mg g^{-1} of sample db.

206 **2.4. Determinations of the epidermis enzyme defense system**

207 **2.4.1. Enzyme Peroxidase (POD)**

208 The peroxidase test was carried out based on the methodology proposed by Sellamuthu et
209 al. (2013). Briefly, to 180 mg of finely ground avocado peel with liquid nitrogen, 1.5 mL of
210 extraction buffer (90 mM sodium phosphate buffer at pH 7.0, 0.1 mM EDTA and 0.1%
211 TRITON X-100), 15 mg of PVPP (Polyvinylpolypyrrolidone) and 15 µL of 100 mM PMSF
212 were added. The samples were homogenized and then sonicated for 10 min in an ice bath.
213 The samples were centrifuged for 20 min at 15000 g at 4 ° C. One hundred and sixty µL of

214 20 mM Guaiacol and 40 µL of the protein extract reacted, then incubated for 5 min at 30 °
215 C. Finally, 80 µL of 50 mM hydrogen peroxide was added and the absorbance increase at
216 460 nm was measured every 30 s for 5 min. Enzymatic activity was expressed as enzymatic
217 unit (Δ A460 min^{-1} mg $protein^{-1}$).

218 **2.4.2. Enzyme Catalase (CAT)**

219 The catalase test was developed according to methodology reported by Uarrota et al.
220 (2019). Briefly, 500 µL of the extraction buffer (90 mM sodium phosphate buffer at pH 7.0,
221 0.1 mM EDTA and 0.1% of TRITON X-100) was added to a 180 mg sample of finely ground
222 avocado peel in liquid nitrogen). Then 20 µL of 125 mM PMSF were added. The mixture
223 was homogenized and centrifuged for 10 min at 15000 g and 4 ° C. The supernatant was
224 transferred to a new eppendorf tube and stored at -20 ° C until use. Two hundred and fifty
225 µL of 50 mM potassium phosphate buffer at pH 7.0 and 22.5 µL of hydrogen peroxide
226 stock was added. Absorbance at 240 nm was measured to obtain a range between 0,38 to
227 0,40. The reaction kinetics for 5 min was monitored by measuring absorbance every 10 s
228 at 240 nm. The enzymatic activity of CAT was expressed as enzymatic unit (Δ A240 min^{-1}
229 mg $protein^{-1}$).

230 **2.4.3. Enzyme Polyphenol Oxidase (PPO)**

231 The test was carried out following the methodology of Bi et al. (2015) with slight
232 modifications. To 100 mg of finely ground avocado epidermis sample, 500 µL of extraction
233 buffer (0.1 M phosphate buffer at pH 6.5 and 2% PVP) was added. A vortex was used to
234 release the pellet and centrifuged for 10 min at 10000 g and 4 ° C. Subsequently, only the

235 supernatant was used. This test was performed with the samples always in indirect
236 contact with ice. Two hundred and forty µL of 0.1 M phosphate buffer pH 6.5 and 20 µL of
237 the extracts were added. Finally, 40 µL of 0.1 M catechol was added. Progress curves were
238 plotted by measuring the reaction every 2 min for 30 min at 410 nm. Enzymatic activity
239 was expressed as enzymatic Unit (Δ A410 min^{-1} mg $protein^{-1}$).

240 **2.4.4. Enzyme Phenylalanine Ammonium Lyase (PAL)**

241 The test was carried out following the methodology of Popović et al. (2016) with slight
242 modifications. One hundred mg of finely ground avocado peel were homogenized with 1
243 mL of 0.1 M sodium borate buffer (pH 8.8), which contained 10 µl mL^{-1} of 5 mM β-
244 mercaptoetanol, 2 mM EDTA and 2% (w / v) of PVP. The mixture was centrifuged at 20000
245 g for 15 min at 4 ° C. The supernatant was used to carry out the PAL test. Ten µL of the
246 extract was added, with 230 µL of the sodium borate buffer pH 8.8, 50 µL of L-
247 phenylalanine (substrate) and the mixture was incubated at 37°C for 1 h. Once the
248 incubation was finished, the reaction was stopped by adding 10 µL of 1M TCA and the
249 absorbance at 290 nm was measured. Enzymatic activity was expressed as equivalent
250 µmoles of cinnamate $min^{-1}.mg^{-1}$ of protein.

251 **2.4.5. Enzyme Superoxide Dismutase (SOD)**

252 The test was carried out following the methodology of Bahin et al. (2011) with slight
253 modifications. To 100 mg of finely ground avocado peel with liquid nitrogen 0.5 mL of 1 M
254 extraction buffer which contained 0.1 nM EDTA (pH: 7.8) was added. The mixture was
255 centrifuged at 13000 g for 10 min at 5°C. The supernatant was used to carry out the test.

256 1.3 μM of riboflavin, 13 mM of methionine and 63 μM of NBT (nitro blue tetrazolium)
257 were used. A 50 mM phosphate buffer (pH: 7.8) was prepared. Three solutions were
258 prepared, solution A control (194 mg of methionine + 6 mg NTB + 3.73 ml Riboflavin);
259 solution B (solution A + extract) and solution C (solution A + extract) by covering the test
260 tube with aluminum to avoid contact with light. Solutions A and B (in microplates) were
261 illuminated with fluorescent white light for 15 min. The unlit samples (solution C) served
262 as blank. After incubation in fluorescent light, absorbance at 560 nm was measured.
263 Enzymatic activity was expressed as enzymatic unit (Δ A560 min^{-1} mg $protein^{-1}$).

264 **2.5 Statistical analysis**

265 All data were initially collected and submitted to a univariate statistics analysis ("two-way
266 ANOVA" considering the "orchards" and "storage time" as factors) independently for each
267 harvest season, using five fruits (n = 5) for each storage time. Where statistical differences
268 were observed, a Tukey test was used for separation of means ($p < 0.05$). Then, all the data
269 were subjected to an unsupervised multivariate analysis (principal component analysis -
270 PCA and hierarchical group analysis - HCA) with the objective of determining the most
271 important variables and to explore the structure of the data. Finally, the data were
272 subjected to a supervised multivariate statistics analysis (partial least squared analysis
273 associated with regression – PLS-R) with the aim of looking at variables correlated with the
274 incidence of black spot and making prediction models of the incidence of black spot. All
275 analyzes were performed in R software (R core Team, 2019 version 3.6.1) using scripts
276 developed by our research group.

277 ***3. RESULTS AND DISCUSSION***

278 **3.1 Black spot incidence**

279 Black spot incidence was higher in fruits from early harvest for all geographical areas
280 studied, being orchard H (coastal) the most affected with 54% black spot incidence after
281 40 d of storage in regular air conditions (Figure 2). The coastal area is characterized by
282 displaying several days of fog, especially in the morning. The incidence reported above
283 may be related to what was reported by Everett et al., (2008), who established that the
284 black spot from lenticel damage increased in fruits harvested after rains (trees and wet
285 fruits). For the late harvest, orchard B (interior) displayed 24% of black spot incidence and
286 was the orchard that presented the highest severity of black spot on the epidermis. Both
287 orchards presented incidence after 25 days of storage. Therefore, both orchards were
288 selected for the evaluation of the defense system during the postharvest storage period
289 for season 2017/2018. The lowest incidence orchards selected to be contrasted with the
290 complex ones were I (coastal with 10% of the fruits affected in the early harvest) and A
291 (interior with 12% of its fruits affected with black spot incidence) (Figure 2). Attention was
292 drawn to the incidence pattern for the two most complex orchards, with a relatively stable
293 incidence for orchard B (interior) in both seasons, while orchard H (coastal) changed from
294 an early harvest with a high incidence to a late harvest with almost nonexistent black spot
295 incidence. Zamora-Magdaleno et al. (1999) suggested that friction problems (black spot
296 from bumps) can be accentuated in immature fruits due to the higher phenolic content in
297 the skin. Figure 2 shows the incidence of black spot for the nine orchards in both seasons
298 of 2017/2018.

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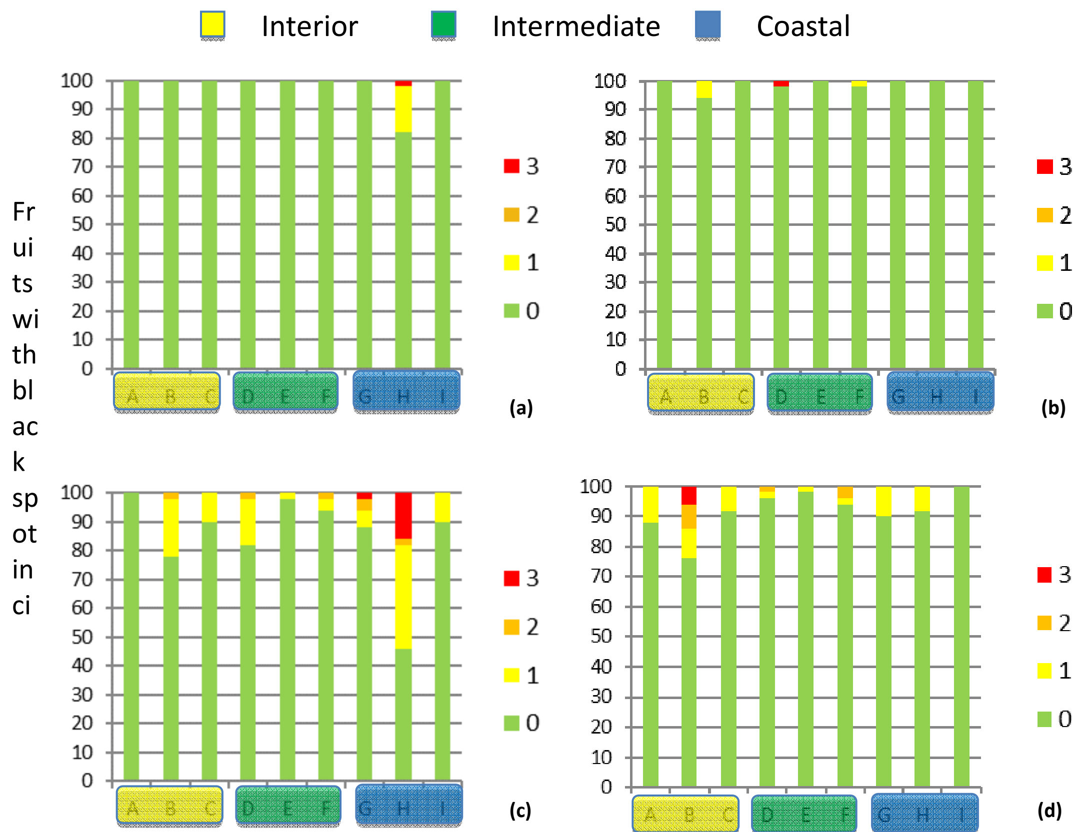

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310 **Figure 2**: Percentage of fruits affected with black spot in the early (a) and late season (b)
311 after 25 d, and after 40 days of cold storage in early (c) and late season (d) for season
312 2017/2018. The severity scale is based on Figure 1.

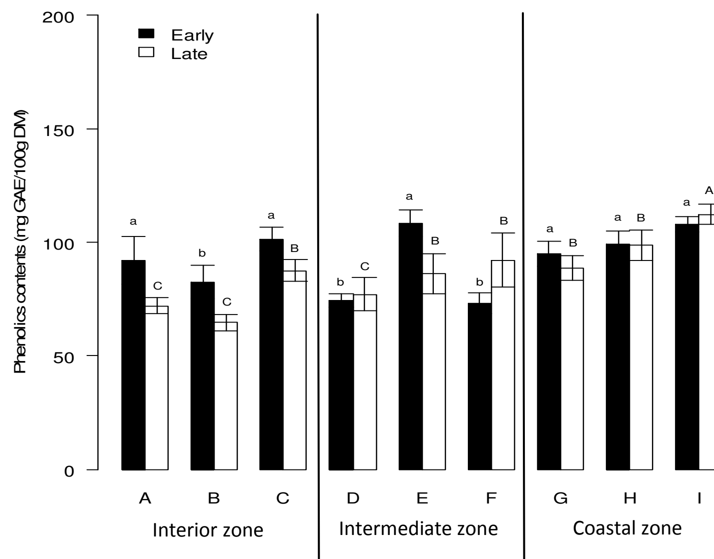
313

314 **3.2 Non-enzymatic defense system at harvest and during storage**

315 **3.2.1 Phenolic compounds**

316 The two-way ANOVA with the variables of the non-enzymatic system at harvest revealed
317 differences in the phenolic compounds between the orchards and an interaction between

318 the orchards and harvest time. A greater amount of phenolics was observed in early
319 harvest compared to late harvest (Tukey test, p <0.05). In general terms, the total phenolic
320 content was higher in the coastal zone than in the interior zone in both harvest seasons,
321 which is consistent with what was published by Medina-Carrillo et al. (2017), where higher
322 levels of phenolic compounds were identified in temperate and semi-warm areas
323 compared to warm areas. Orchards I, H, E, C and G displayed higher amounts of phenolics
324 in their extracts compared to F, A, D, B. Melgar et al. (2018) reported that the different
325 structures of avocado fruit present different phenolic profile, with the highest
326 concentration in the epidermis, being mainly derived from catechins and chlorogenic acid.
327 Figure 3 shows the total phenolic content at harvest in all orchards at both harvest times.



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329 **Figure 3**: Content of phenolic compounds at harvest for the nine evaluated orchards and
330 two harvest seasons of 2017/2018. Two-way ANOVA indicates differences between
331 orchards and an interaction between orchards and harvest time. Lowercase letters
332 indicate comparison for early season and capital letters for late season using a Scott-Knott
333 test (p <0.05).

334

335 Figure 4 shows a clear downward trend of the average total phenolic content for all
336 orchards during 40 d regular air storage. A more pronounced declined was observed for
337 late season fruit.

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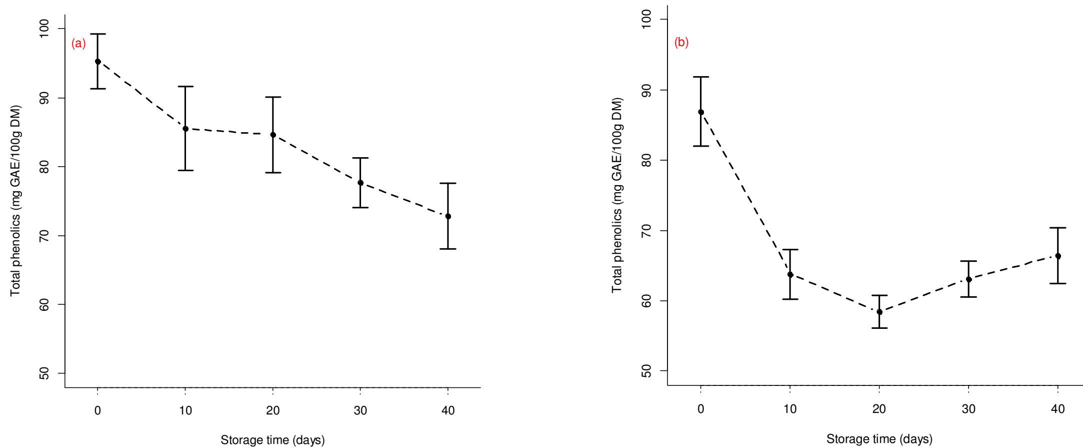

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340 **Figure 4:** Evaluation of the average content of phenolic compounds during 40 d storage in
341 regular air (5°C) for the four orchards evaluated displaying contrasting incidence of black
342 spot. (a) Early harvest and (b) Late harvest.

343

344 These results complement the work carried out in Mexico and New Zealand where it was
345 reported that the content of phenolic compounds in the epidermis of Hass avocado fruit
346 decreases during development until harvest (Medina-Carrillo et al., 2017; Bowen et al.,
347 2018). For early harvest of coastal orchards, it was observed that the greatest difference
348 in phenolic content between both orchards (high and low incidence) occurred after 10 d

349 of storage in RA (Figure 5). From 20 d in cold storage, the phenolic contents tended to be

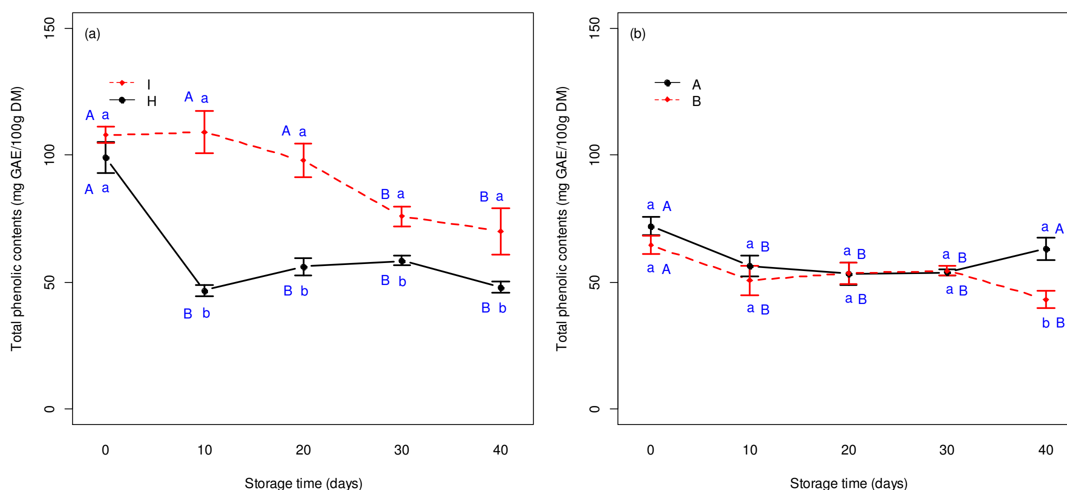
350 equated. Although in average the fall of phenolics for the four orchards was more abrupt

351 in the late harvest (possibly due to the state of maturity and climatic factors), if the cases

352 are analyzed separately, the strongest fall between harvest and the first 10 d of storage

353 occurred in orchards from the coastal zone in the early harvest and also a higher incidence

354 of black spot was observed (Figure 5).



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356 **Figure 5**: Evaluation of the total phenolic content from harvest day to 40 d storage in

357 regular air (AR) at 5 ° C. (a) Coastal orchards in early harvest and (b) Interior orchards in

358 late harvest.

359 Two-way ANOVA showed differences between orchards, storage time and an interaction

360 between orchards and storage time. Lowercase letters compare both orchards (H with I

361 and A with B). Capital letters compare the sampling times within each orchard (0, 10, 20,

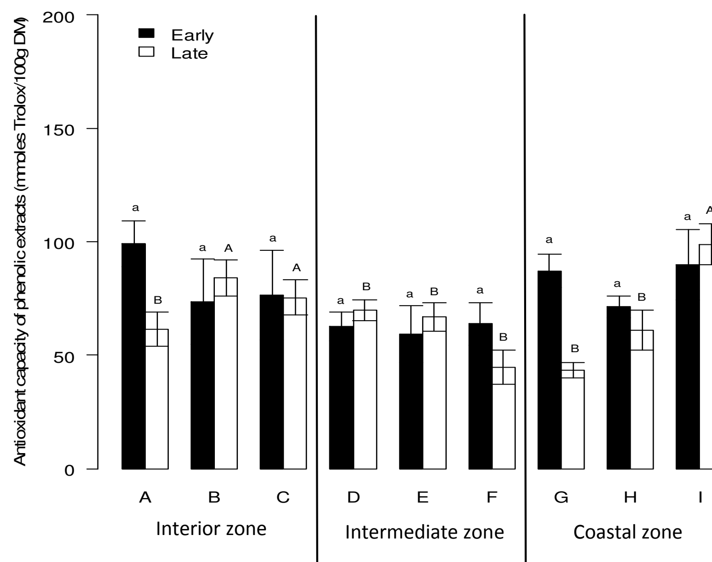
362 30 and 40 d in RA at 5°C). H and B: high incidence black spot orchards and I and A: low
363 incidence black spot orchards.

364 Orchard I (coastal with low incidence) showed a greater amount of total phenolics during
365 the entire postharvest storage period when compared with orchard H (coastal with high
366 incidence) ($p < 0.05$). When the phenolic content of the orchard H was analyzed, a high
367 content was observed only at harvest while for the remaining storage times there were no
368 significant differences. For orchard I a high content of phenolic compounds was observed
369 at day 0, 10 and 20 with significant differences at 30 d and 40 d ($p < 0.05$).

370 Analysis of interior orchards (A and B) from late harvest by two-way ANOVA also showed
371 differences between the orchards, storage time and an interaction between orchards and
372 storage time. During storage, no significant differences were observed between 0 to 30 d.
373 On day 40, orchard A (low incidence) showed higher phenolic contents than orchard B
374 (high incidence) ($p < 0.05$), similar behavior as previously displayed for coastal orchards
375 from early harvest. The content of phenolic compounds of orchard A during postharvest
376 were similar at day 0 and 40, but significantly different on days 10, 20 and 30,
377 respectively.

378 **3.2.2 Antioxidant capacity**

379 The antioxidant capacity showed differences among late season orchards at harvest. Fruits
380 from orchards I, B and C presented phenolic extracts with greater antioxidant capacity
381 than the rest. Figure 6 displays the antioxidant activity for all orchards at harvest for both
382 seasons of 2017/2018.



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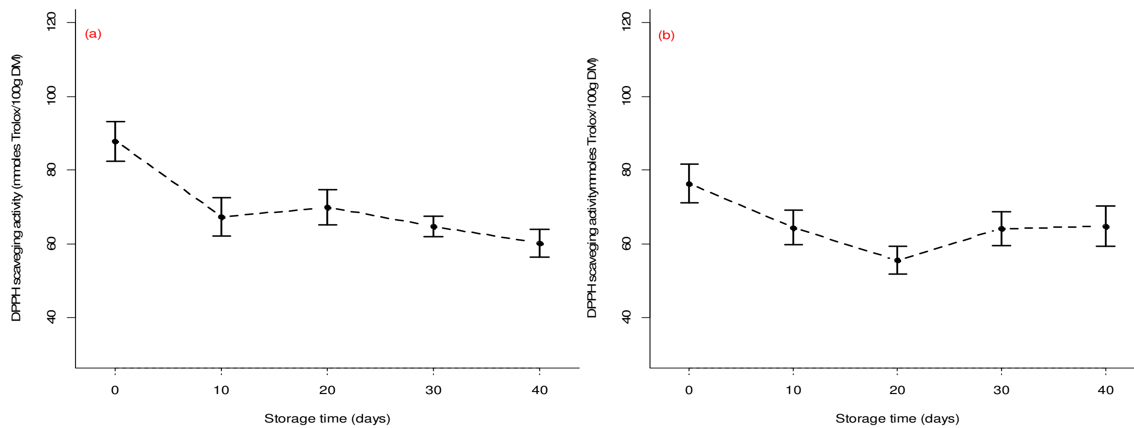
384 **Figure 6:** Antioxidant capacity of extracts from all orchards at harvest for both seasons.
385 Two-way ANOVA showed differences between orchards only. Lowercase letters indicate
386 comparison for early harvest and capital letters for late harvest. Scott-Knott test, $p < 0.05$.
387 No statistical differences were detected between harvest seasons with $p < 0.05$.

388

389 As with phenolic compounds, a marked fall in antioxidant activity was observed from
390 harvest to 40 d of storage, with the most noticeable drop at 10 d of storage (Figure 7).
391 Changes might be related to that reported by Slater et al. (1975), where it states that the
392 antioxidant content in the fruit varies depending on the state of maturity. Figure 7 shows
393 the evolution of the average antioxidant activity of the four orchards evaluated during the
394 storage period and harvest seasons.

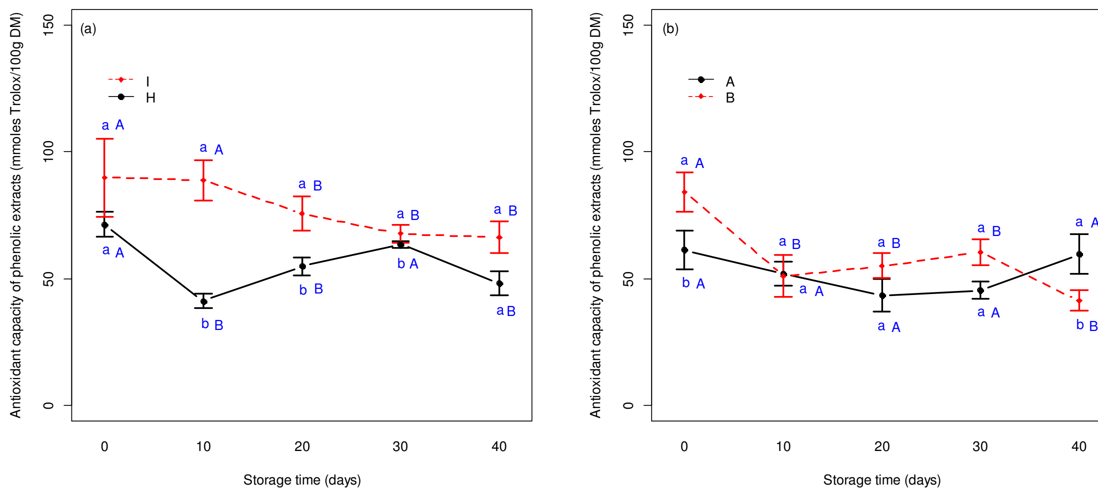
395



396 **Figure 7:** Evaluation of the average change of the total antioxidant capacity of the
397 orchards evaluated during the 40 d storage in regular air at 5 °C. (a) Early harvest and (b)
398 Late harvest.

399

400 Figure 8 displays the behavior of the antioxidant capacity of the phenolic extracts
401 evaluated individually by orchard, where the most noticeable difference is observed at 10
402 d for coastal orchards as previously reported above for phenolic compounds.



403 **Figure 8:** Evaluation of the antioxidant activity during regular air storage at 5 ° C for
404 contrasting black spot incidence orchards within the same geographical area. (a) Coastal
405 orchards from early harvest. (b) Interior orchards from late harvest. Lowercase letters
406 indicate comparison between orchards and uppercase letters between sampling moments
407 within the same orchard (0, 10, 20, 30 and 40 d in RA at 5°C). H and B: high incidence
408 orchards and I and A: low incidence orchards.

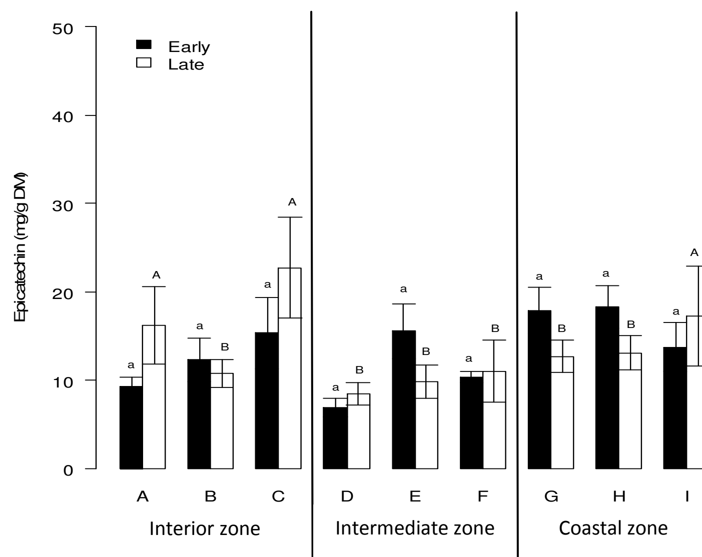
409

410 For the early harvest, the two-way ANOVA showed differences between orchards, storage
411 times and interaction between both factors. Orchard I (coastal with low incidence)
412 showed greater antioxidant activity than orchard H (coastal with high incidence)
413 throughout storage, including at harvest. For the orchard H, the antioxidant capacity on
414 day 0 and 30 were similar, but different on days 10, 20 and 40 in RA, respectively. In
415 relation to orchard I (coastal with low incidence), the antioxidant activity was similar to
416 day 0 and 10, and these were different from the rest. In the late harvest, significant
417 differences were observed between storage time and the interaction between storage

418 time and orchard. Orchard B (interior with high incidence) showed greater antioxidant
419 activity than A (interior with low incidence) at harvest, while A displayed greater
420 antioxidant activity at day 40 of storage. The antioxidant activity of orchard A did not
421 show significant differences between all sampling points, while for B, the highest
422 antioxidant activity was observed at harvest day, with significant differences during the 40
423 d of storage.

424 **3.2.3 Epicatechin and derivatives**

425 At early harvest, there were no statistical differences between the orchards. On the other
426 hand, for late harvest, the orchards A, C and I had higher epicatechin contents ($p < 0.05$).
427 Figure 9 shows the contents of epicatechin at harvest for all the orchards evaluated.



428

429 **Figure 9:** Epicatechin content in the epidermis of fruits from the nine orchards at of
430 harvest for both seasons of 2017/2018. Two-way ANOVA indicates significant differences
431 only between orchards. Lowercase indicate differences for early harvest and capital letters
432 for late harvest. Scott-Knott test, $p < 0.05$. The comparison is independent of the season.

433

434 During the postharvest evaluation, orchard H (coastal displaying the highest incidence)
435 presented the highest epicatechin levels in both harvest seasons, showing statistical
436 differences with orchard A in the early harvest and with the orchard B in the late harvest.
437 It has been reported that epicatechin is present in the epidermis of avocado fruit mostly
438 as different types of dimer B, trimers, tetramers and glycosides of flavonol (Melgar et al.,
439 2018).

440 In relation to epicatechin derivatives, differences were observed only for the sum of total
441 catechins (the main one was epicatechin), where the C orchard had a higher content in
442 the late harvest over the early one, and it was statistically different from orchards F and D.
443 The other orchards had no significant statistical differences. Registered epicatechin
444 derivatives showed a response behavior, increasing the contents from harvest to
445 consumption maturity. In the post-harvest evaluation, there were significant differences
446 between orchard A (interior with low incidence) and B (interior with high incidence), being
447 B the one with the lowest content of derivatives at the time of late harvest. Unlike this
448 research, in the work carried out by our group in the 2018-2019 season with the same
449 orchards, the derivative 1 of epicatechin showed a considerable decrease between the
450 harvest and the first 10 days of storage (Supplementary Figure 2). For all epicatechin
451 derivatives (2, 3, 4, and 5) separately no differences were observed at harvest between
452 orchards and between harvest seasons for season 2017/2018 (data not shown).

453 During storage, orchard A had a lower content of chlorogenic acid than the rest of the
454 orchards in the early harvest, showing a significant difference ($p < 0.05$). On the other

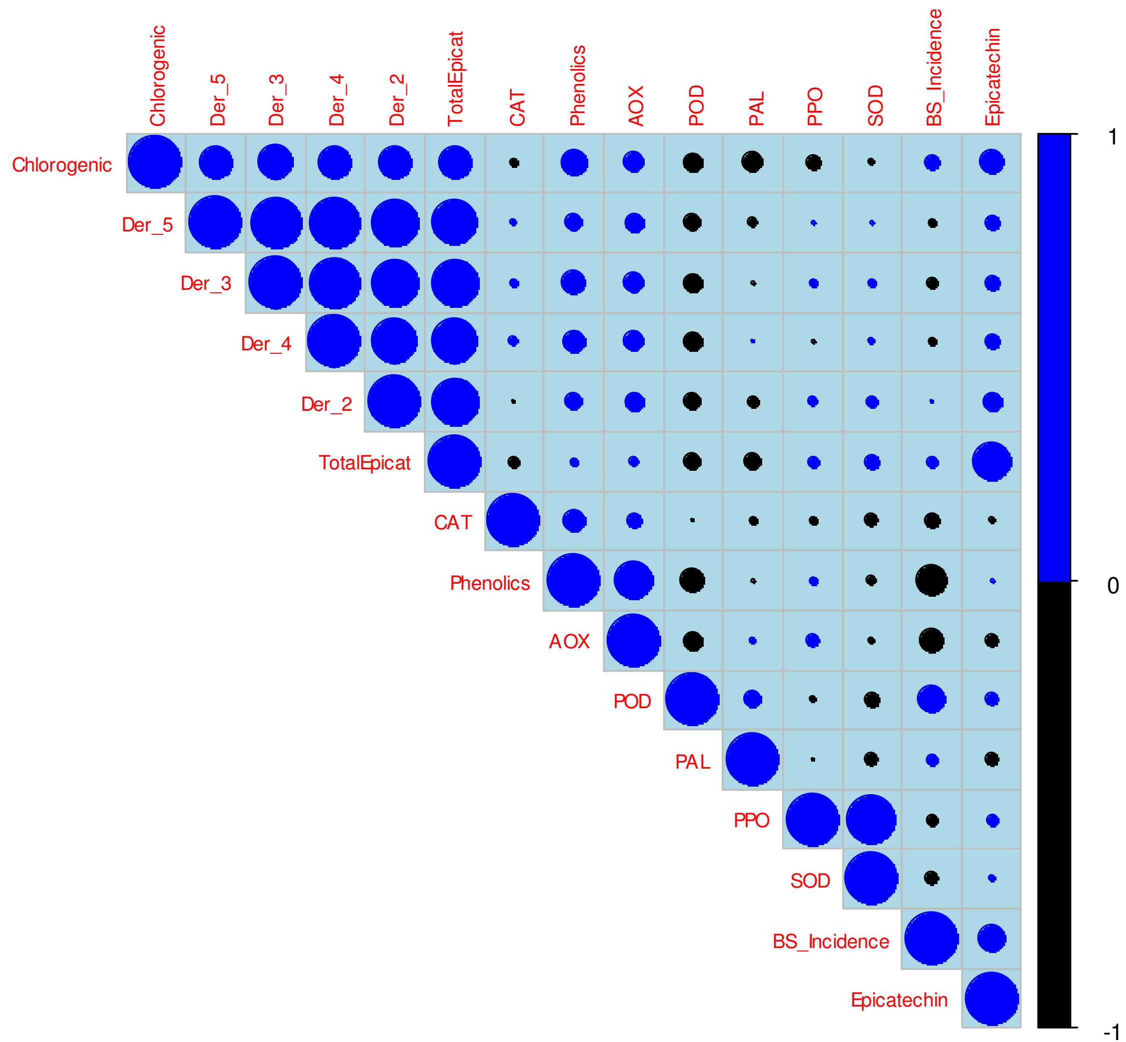
455 hand, coastal orchards showed a higher level of chlorogenic acid in relation to the
456 interiors, being significantly different (data not shown).

457 ***3.3 Enzymatic defense system***

458 Two-way ANOVA showed that at harvest, the enzymes studied only showed differences
459 between the orchards (data not shown). Within the five enzymes quantified in this study,
460 only the activity of the enzyme peroxidase (POD) had a positive correlation (27%) with the
461 appearance of black spot. The behavior of POD was clearly in response to the disorder,
462 since only statistical differences were generated in orchard H (coastal with high incidence)
463 in the early period at 40 days of storage, when the highest incidence of black spot was
464 recorded in this essay. POD, together with PPO, are the two best known enzymes involved
465 in browning processes (e.g., oxidative degradation of phenolic compounds) of fruits and
466 vegetables (Nokthai et al., 2010). Unlike what was found in this study, in another study
467 conducted by our group (Supplementary Figure 3) in the season 2018/2019, the activity of
468 POD at harvest was negatively associated with the appearance of black spot in storage. In
469 relation to PAL, in the late harvest the interior orchards showed greater activity of this
470 enzyme in relation to the coastal orchards, being statistically different. PAL catalyzes the
471 first step in the biosynthesis of a wide range of secondary metabolites derived from
472 phenylpropanoids in plants, such as flavonoids and isoflavonoids, coumarins, lignins,
473 hydroxycinnamic acid esters, wound protectors and other phenolic compounds. The
474 activity of CAT and PPO did not show differences between orchards or between harvest
475 seasons (data not shown). Enzyme activity may be regulated by environmental conditions.
476 Mittler and Zilinskas (1994) reported in a study on peas that, in the phase of water stress,

477 the SOD and POD enzymes increased their activity and these remained high once the plant
478 returned to its normal state, but not the CAT enzyme that returned to its normal level
479 once the stress was over.

480 Pearson's correlation ($p < 0.05$) of all enzymatic and non-enzymatic variables with the
481 incidence of black spot is presented in Figure 11. As can be seen, the incidence of black
482 spot presented a strong negative association with phenolic compounds, catalase activity,
483 antioxidant capacity of phenolic extracts and derivatives of epicatechin 3, 4 and 5. On the
484 other hand, the positive association of black spot occurred with the activity of the enzyme
485 peroxidase, phenyl alanine ammonium-lyase, the content of chlorogenic acid, epicatechin
486 derivative 2 and the sum of epicatechin derivatives.



487

488 **Figure 11**: Pearson correlation matrix (p <0.05) of all enzymatic and non-enzymatic
489 variables with the incidence of black spot. In blue positive association and in black
490 negative association.

491 Regressions of the 3 variables most correlated with the incidence of black spot can be
492 seen in Supplementary Figure 1. Phenolic compounds had 36% negative association, and
493 epicatechin, POD and AOX presented 28; 27 and 27% respectively of positive association.
494 In the case of phenolic compounds, the results fit perfectly with that reported by Medina-
495 Carrillo et al., (2017), where they reported that the content of phenolic compounds
496 decreased in the presence of pathogens or any type of stress (e.g., black spot). In the

497 oxidative degradation of these phenolic compounds, two enzymes participate which are
498 very relevant in terms of fruit and vegetable quality, due to the formation of melanins that
499 participate in browning fruit events. These enzymes are polyphenol oxidase (PPO) and
500 peroxidase (POD) (Morante-Carriel et al., 2014). In the case of early harvest, POD activity
501 increased considerably after 40 days of storage, in parallel with the decrease in phenolic
502 compounds and maximum black spot expression. The antioxidant status of different fruits
503 and vegetables can be decreased by the direct oxidation of these phenolics in the
504 presence of PPO and POD (Jiménez et al., 1998; Jiménez and García-Carmona, 1999),
505 which would match the decrease in the antioxidant capacity of phenolic extracts from
506 orchards displaying the highest incidence of black spot. On the other hand, plant-derived
507 phenolic compounds act as antioxidants due to their redox properties, which allows them
508 to act as reducing agents, hydrogen donors, free radical extinguishers and metal chelators
509 (Rodríguez-Carpena et al., 2011). This is why the decrease in phenolic compounds would
510 be directly related to the decrease in the total antioxidant capacity of the extracts.

511 The decrease of the parameters corresponding to the defense system of the fruit
512 epidermis for the orchards with incidence of black spot suggests that the behavior of
513 these variables (with negative correlation) in the first days of storage seem to be relevant
514 in the development of black spot, and after studying them properly they could become a
515 predictive parameter. Moreover, the results of the 2018/2019 season suggest the same
516 behavior with the orchards showing a higher incidence of black spot and presenting a
517 large decrease in the defense system, especially the enzymatic one (post-harvest storage)
518 (Supplementary Figure 3). The first 10 days of regular air storage appeared to be critical

519 for the enzymatic and non-enzymatic defense system, especially for the occurrence of
520 prolonged postharvest disorders.

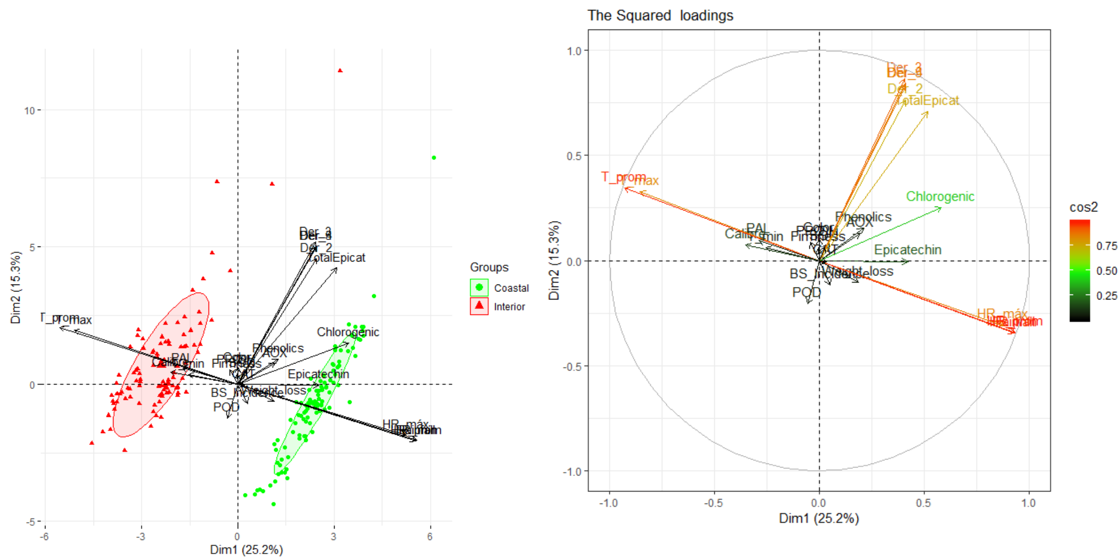
521 In practice, countries (e.g., Chile, Peru) that export to distant countries with travel times of
522 30 to 55 days, use controlled atmosphere. However, the application of the controlled
523 atmosphere occurs in the container and the fruit is previously exposed for 10 days or
524 more to refrigerated regular air conditions. A work of our research group (data not shown)
525 with the same nine orchards and harvest seasons evaluated during the 2018/2019 and
526 2019/2020 seasons, revealed a total control of the incidence of black spot when the
527 controlled atmosphere is applied immediately after harvest (fruit cooled down to 5 ° C)
528 (data not shown). This decrease in the contents of phenolic compounds and the enzyme
529 defense system within the first 10 days, opens the possibility of testing applications of
530 products such as methyl jasmonate or methyl salicylate before cold storage. Both
531 compounds have been previously reported that trigger the synthesis of epicatechin, other
532 defense compounds and enzymes to protect the fruit during prolonged storage (Glowacz
533 et al., 2017).

534 **3.4 Multivariate statistical analysis unsupervised (PCA, HCA) and supervised (PLS-R)**

535 **3.4.1 Principal component analysis (PCA) and Clusters hierarchy (HCA)**

536 A principal component analysis (PCA) was carried out taking into consideration four
537 factors: orchard, days in storage, season harvest and geographical area. The total variance
538 captured by the first two components of the principal component analysis (PCA) was 40%,
539 with 25.18% and 15.30% for component 1 (PC1) and 2 (PC2) respectively (Figure 10).

540



541 **Figure 10:** On the left: Biplot graph of PCA vectors of all the variables with the
542 geographical area as a factor. On the right: Analysis of squared loadings, where the
543 contribution of the variables according to the scale established in the right sector of the
544 table is shown. In red the variables that contribute the most and in black the ones that
545 contribute less.

546

547 The samples classified in component 1 were strongly correlated with average relative
548 humidity, average temperature, rainfall, minimum relative humidity, maximum relative
549 humidity, maximum temperature, chlorogenic acid and sum of epicatechin. On the other
550 hand, the samples grouped in component 2 were more correlated with epicatechin and its
551 derivatives (Supplementary Figure 4).

552 Figure 10 (left) shows a biplot graph of PCA with auto vectors that shows one of the four
553 PCA analyzes that were performed, the one with the geographical areas as a factor (since
554 it generated the best classification of the samples). This figure shows a clear separation of

the samples between the two geographical areas evaluated in the postharvest, coastal and interior.

The analysis of squared loadings showed that the average temperature, minimum relative humidity and average relative humidity, derivatives 3 and 4 of epicatechin and chlorogenic acid were the most important variables (Figure 10-right). Hierarchical grouping analysis (HCA) (Supplementary Figure 5) confirmed the form of grouping shown by the PCA biplot with geographical areas as a factor.

3.4.2 *Analysis of minimum squares allied to regression (PLS-R)*

The PLS-R analysis sought to find variables that are correlated with the incidence of black spot and generate prediction models for new samples. Four models of PLS-R were carried out using different approaches, without variable selection and using 3 different approaches to select the important variables of the model (importance of variable by projection - VIP, selectivity ratio - SR, and the procedure of Jack-Kniffing –JK, which is based on calculating the values of the probabilities (p-value). The VIP criterion generated a good prediction model for the incidence of black spot ($R^2X = 66.29$, $R^2Y = 71.80$, RMSE = .16) and 72% performance. The variables selected by the model using the VIP selection criteria are shown in Supplementary Figure 6.

4. CONCLUSIONS

The present work showed that the incidence of black spot did not manifest itself with a certain pattern in relation to a geographical area or harvest season. The coastal orchard H was the one that presented the highest incidence of the disorder in the early season. On

576 the other hand, orchard B was the most complex in the late harvest. This study revealed
577 that the climatic variables were the ones that mostly influenced the behavior of the
578 samples. On the other hand, the non-enzymatic defense system would apparently play a
579 more important role in the development of this oxidative disorder in comparison to the
580 enzymatic defense system. The critical point regarding the total phenolic content and the
581 total antioxidant capacity was concentrated between harvest and the first ten days of
582 storage in a regular air atmosphere (RA at 5 ° C), where the orchards with the highest
583 incidence had lower contents in both variables, noticing an abrupt fall from harvest to the
584 first ten days of storage in relation to the orchards that did not present black spot
585 incidence.

586 The variables that were positively correlated with the incidence of black spot were the
587 caliber, minimum temperature, epicatechin, POD activity and maximum relative humidity.
588 On the other hand, phenolic compounds, dry matter and maximum temperature were
589 negatively correlated.

590 Future studies on the subject should focus, first, on monitoring the content of these
591 biochemical compounds in the skin during the first days of storage (between days 0 and
592 10 of storage), which appear to be critical in terms of development of the disorder and
593 thus could lead to practical solutions from a commercial point of view. Secondly, it is
594 interesting to test early post-harvest applications that trigger the defense system of the
595 epidermis and allow the levels of these compounds to remain high during the delay in
596 applying controlled atmosphere conditions, thus controlling black spot development in
597 Hass avocado.

598

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600

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1. REFERENCES

608

609 Adamson, G., Lzarus, S., Mitchell, A., Prior, R., Cao, G., Jacobs, P., Kremers, B.,
610 Hammerstone, J., Rucker, R., Ritter, K., Schmitz, H., 1999. HPLC method for the
611 quantification of procyanidins in cocoa and chocolate samples and correlation to total
612 antioxidant capacity. *Journal of Agricultural and Food Chemistry*, *47*(10), 4184-4188.
613
614 Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., Sharma, S., 2010. Roles of enzymatic and
615 nonenzymatic antioxidants in plants during abiotic stress. *Critical reviews in*
616 *biotechnology,* 30, 161-175.
617
618 Bahin, E., Bailly, C., Sotta, B., Kranner, I., Corbineau, F., Leymarie, J., 2011. Crosstalk
619 between reactive oxygen species and hormonal signalling pathways regulates grain
620 dormancy in barley. *Plant, Cell & Environment*, *34*(6), 980-993.
621
622 Bi, X., Hemar, Y., Balaban, M. O., Liao, X., 2015. The effect of ultrasound on particle size,
623 color, viscosity and polyphenol oxidase activity of diluted avocado puree. *Ultrasonics*
624 *sonochemistry*, *27*, 567-575.
625
626 Bowen, J., Billing, D., Connolly, P., Smith, W., Cooney, J., Burdon, J., 2018. Maturity,
627 storage and ripening effects on anti-fungal compounds in the skin of Hass avocado fruit.
628 Postharvest Biology and Technology 146, 43-50.
629
630 Comite de Palta Hass de Chile, 2019. Estadísticas de producción de palta Hass.
631 www.paltahass.cl/estadísticas. (accessed 10 November 2019).
632

633 Everett, K. R., Hallett, I. C., Rees-George, J., Chynoweth, R. W., Pak, H. A., 2008. Avocado
634 lenticel damage: The cause and the effect on fruit quality. *Postharvest biology and*
635 *technology,* 48**,** 383-390.
636
637 Fang, Z., Bhandari, B., 2011. Effect of spray drying and storage on the stability of bayberry
638 polyphenols. *Food Chemistry*, vol. 129, no 3, p. 1139-1147.
639
640 Glowacz, M., Roets, N., Sivakumar, D., 2017. Control of anthracnose disease via increased
641 activity of defence related enzymes in 'Hass' avocado fruit treated with methyl jasmonate
642 and methyl salicylate. *Food chemistry,* 234**,** 163-167.
643
644 Jiménez, M., Escribano-Cebrián, J., García-Carona, F., 1998. Oxidation of the flavonol
645 fisetin by polyphenol oxidase. *Biochimica et Biophysica Acta (BBA)-General*
646 *Subjects*, *1425*(3), 534-542.
647
648 Jiménez, M., García-Carmona, F., 1999. Oxidation of the flavonol quercetin by polyphenol
649 oxidase. *Journal of agricultural and food chemistry*, *47*(1), 56-60.
650
651 Karni, L., Prusky, D., Kobiler, I., Bar-Shira, E., Kobiler, D., 1989. Involvement of epicatechin
652 in the regulation of lipoxygenase activity during activation of quiescent Colletotrichum
653 gloeosporioides infections of ripening avocado fruits. *Physiological and molecular plant*
654 *pathology,* 35**,** 367-374.
655
656 Medina-Carrillo, R. E., Salazar-García, S., Bonilla-Cárdenas, J. A., Herrera-Gonzalez, J. A.,
657 Ibarra-Estrada, M. E., Álvarez-Bravo, A., 2017. Secondary Metabolites and Lignin in 'Hass'
658 Avocado Fruit Skin during Fruit Development in Three Producing
659 Regions. *HortScience*, *52*(6), 852-858.
660
661 Melgar, B., Dias, M. I., Ciric, A., Sokovic, M., Garcia-Castello, E. M., Rodriguez-Lopez, A. D.,
662 Barros, L., Ferreira, I. C., 2018. Bioactive characterization of Persea americana Mill. by-
663 products: A rich source of inherent antioxidants. *Industrial crops and products*, *111*, 212-
664 218.
665
666 Mittler, R., Zilinskas, B. A., 1994. Regulation of pea cytosolic ascorbate peroxidase and
667 other antioxidant enzymes during the progression of drought stress and following
668 recovery from drought. *The Plant Journal*, *5*(3), 397-405.
669
670 Morante Carriel, J., Agnieszka-Obrebska, A., Bru-Martínez, R., Carranza Patiño, M., Pico-
671 Saltos, R., Nieto Rodriguez, E., 2014. Distribución, localización e inhibidores de las
672 polifenol oxidasas en frutos y vegetales usados como alimento. *Ciencia y Tecnología* 7(1),
673 23-31.
674

675 Nokthai, P., Lee, V. S., Shank, L., 2010. Molecular modeling of peroxidase and polyphenol
676 oxidase: substrate specificity and active site comparison. *International journal of*
677 *molecular sciences*, *11*(9), 3266-3276.
678
679 R Core Team (2019). R: A language and environment for statistical computing. R
680 Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.
681
682 Rivera, S. A., Ferreyra, R., Robledo, P., Selles, G., Arpaia, M. L., Saavedra, J., Defilippi, B. G.,
683 2017. Identification of preharvest factors determining postharvest ripening behaviors in
684 'Hass' avocado under long term storage. *Scientia Horticulturae,* 216**,** 29-37.
685
686 Saavedra, J., Córdova, A., Navarro, R., Díaz-Calderon, P., Fuentealba, C., Astudillo-Castro,
687 C., Toledo, L., Enrione, J., Galvez, L., 2017. Industrial avocado waste: Functional
688 compounds preservation by convective drying process. *Journal of Food Engineering*, *198*,
689 81-90.
690
691 Slater, G. G., Shanhman, S., Shepherd, J. S., Alfin-Slater, R. B., 1975. Seasonal variation in
692 the composition of California avocados. *Journal of agricultural and food chemistry*, *23*(3),
693 468-474.
694
695 Thatcher, L. F., Anderson, J. P., Singh, K. B., 2005. Plant defence responses: what have we
696 learnt from Arabidopsis?. *Functional Plant Biology*, *32*(1), 1-19.
697
698 Uarrota, V. G., Segatto, C., Voytena, A. P. L., Maraschin, M., Avila, L. V., Kazama, D. C.,
699 Cohelo, C.M., Souza, C. A., 2019. Metabolic fingerprinting of water-stressed soybean
700 cultivars by gas chromatography, near-infrared and UV-visible spectroscopy combined
701 with chemometrics. *Journal of Agronomy and Crop Science*, *205*(2), 141-156.
702
703 Zamora-Magdaleno, M. T., Cajuste-Bontemps, J., Colina-León, M. T., Santacruz, U. H.,
704 1999. Efecto de los daños mecánicos sobre el comportamiento postcosecha de fruto de
705 aguacate. . *Rev. Chapingo S. Hort,* 5**,** 319-328.
706
707 Zamora-Magdaleno, T., Cárdenas, E., Cajuste, J. F., Colinas, M. T., 2001. Anatomía del daño
708 por rozamiento y por *Colletotrichum gloeosporioides* Penz. En fruto de aguacate HASS.
709 *Agrociencia,* 35.

710

711