1 *Pseudomonas graminis* **strain CPA-7 differentially modulates the**
2 **oxidative response of fresh-cut 'Golden delicious' apple**
3 **depending on the storage conditions.**

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Abstract

14 Oxidative response of fresh-cut antioxidant-treated 'Golden delicious' apples during

15 chilling storage was differentially modified by the biopreservative bacterium CPA-7

16 depending on the conditions; passive modified atmosphere packaging (MAP) or

17 aerobic, the latter being more favorable for the antagonist growth. Inoculation with

18 CPA-7 had no influence on fruit quality parameters in any of the conditions tested.

19 During the first 24 h both in air or in MAP, ascorbate peroxidase (APX) activity

20 triplicated the initial level in response to CPA-7 inoculation, reaching up to 4-fold the

21 activity of non-inoculated fruit (control). From 24 h of storage in MAP, polyphenol

22 oxidase (PPO) activity was sharply enhanced in response to CPA-7 attaining values up

23 to 6-fold higher than that of the control at the end of storage. Contrastingly, from 1 d to

24 3 d of storage in MAP CPA-7 suppressed catalase (CAT) activity by 1.5-fold.

25 Subsequently, after 3 d in such conditions, superoxide dismutase (SOD) and PPO

26 activities were almost duplicated in the presence of CPA-7 if compared to the control.

27 On the other hand, when stored in air, polyphenol oxidase (POX) showed a biphasic

induction in response to CPA-7 after 3 d and 6 d of incubation. At day 6, the activity of this enzyme reached values 8-fold higher in response to CPA-7 than the observed for the control regardless of storage conditions, yet it was not paired to an increase of browning. Inoculation with CPA-7 led to the slowdown of the decline of antioxidant capacity in air, which contrasted with the response upon MAP conditions. These results suggest that CPA-7 may trigger the activation of the fruit defense-response thereby mitigating its oxidative damage. Such activation may play a role as a putative biocontrol mechanism against foodborne pathogen infections.

1. Introduction

Control of foodborne diseases through bio-friendly strategies circumventing the usage of chemicals that produce potentially harmful residues in fresh-cut fruit is an important area of research. The use of antagonistic epiphytic microorganisms is a method that fulfills this characteristic which has been widely explored over the past (Belak and Maraz, 2015; Leverentz et al., 2006). In addition to the maintenance of microbiological quality, the growing demand of fresh processed fruit and vegetables has challenged food industry to also guarantee the physicochemical acceptance of these products in spite of the metabolic changes inherent to this type of commodities, mainly associated to abiotic stress caused by both refrigerated storage and processing. In this sense, cold storage in regular controlled atmosphere conditions is known to be stressful to harvested fruit inducing the accumulation of ROS (Chiriboga et al., 2013). Thus said, scarce information is available about the oxidative changes of fresh-cut fruit at the enzymatic or metabolic level (Larrigaudiere et al., 2008).

Besides, cutting of fruit flesh compromise compartmentalization in nearby cells allowing phenolic compounds located in vacuoles to get in contact with polyphenol oxidase (PPO), located in plastids, triggering the reaction known as enzymatic browning (Holderbaum et al., 2010). Other antioxidant enzymes such as peroxidase (POX) has also been linked to polyphenol-associated browning through a coordinated mechanism

involving the accumulation of its substrate, hydrogen peroxide (H_2O_2), stimulated by the PPO-mediated generation of quinones (Jiang and Miles, 1993). Concomitantly, H_2O_2 is highly oxidant and may damage membrane and other cellular components through several mechanisms. Thus, in both fresh-cut and intact fruit, its removal is rapidly activated in order to protect plant cells from these damages. H_2O_2- scavenging mechanisms include enzymatic reactions which involve superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) found in the cytosol, chloroplasts and mitochondria of higher plants (Hung et al., 2005) as well as non-enzymatic antioxidants including phenolic compounds (vitamin E, flavonoids, phenolic acids and other phenols); nitrogen compounds (alkaloids, amino acids and amines), carotenoids and chlorophyll derivatives (Kumar, 2012).

The control of enzymatic browning in the fresh- cut produce industry is currently mainly based on the modulation of PPO activity through antioxidant formulations that commonly contain reducing compounds such as ascorbic acid and its derivatives, cysteine and glutathione (Rupasinghe et al., 2005). These agents are hypothesized to control PPO activity either by reducing quinones to the native diphenols or by reacting irreversibly with o- quinones to form stable colourless products (Nicolas et al., 1994; He and Luo, 2007).

In addition, plant oxidative metabolism is also activated in response to biotic stress as a part of induced defense mechanisms (Pieterse et al., 2000, 2014). Under the light of the increasing knowledge about plant-microorganism interactions, induced resistance has arisen as a method for controlling postharvest diseases. The investigation of these processes has revealed that biocontrol agents (BCA) activate defense-related enzymes such as phenylalanine ammonia lyase (PAL), POX, PPO and chalcone synthase among others, therefore contributing to better antioxidant defenses and tolerance to pathogens attack (Chen et al., 2000; Jain et al., 2012; Alkan and Fortes, 2015).

81 *Pseudomonas graminis* strain CPA-7 is an aerobic epiphyte bacillus which has been

82 used as BCA against foodborne pathogens such as *Escherichia coli* O157:H7,

83 *Salmonella enterica* and *Listeria monocytogenes* on fresh cut apples, melons and

84 peaches (Abadias et al., 2014; Alegre et al., 2013a, 2013b; Collazo et al., 2017).

85 Although attempts to elucidate the mechanisms underlying its antagonistic activity have

86 been carried out, they still remain poorly understood (Collazo et al., 2017). Thus, we

87 hypothesized that a putative mode of action for CPA-7 could be the activation of

88 defense mechanisms in fresh-cut fruit leading to a better control of foodborne

89 pathogens populations. Accordingly, the oxidative metabolism of inoculated fresh-cut

90 apples treated with an antibrowning agent both upon aerobic conditions, suitable for

91 CPA-7 growth, and in semi-commercial conditions (modified atmosphere packaging,

92 MAP) were investigated.

2. Materials and methods

2.1 Fruit processing

95 Apples (*Malus domestica* L. cv. Golden Delicious) were obtained from local packing-

96 houses (Lleida, Catalonia, Spain). Prior to experimental assays, the fruit were washed

97 with running tap water, surface disinfected with 700 mL L^{-1} ethanol, peeled with an

98 electric fruit peeler and cut into eight wedges with a handheld corer/slicer. Wedges

99 were kept in chilled (5°C) chlorinated tap water (pH 6) until they were subsequently

100 treated or stored.

2.2 Antagonist culture conditions

102 For the antagonist inoculum preparation, a single colony of *Pseudomonas graminis*

103 strain CPA-7 grown in tryptone soy agar plates (TSA, Biokar, Beauveais, France)

104 during 48 h at 30 ºC, was inoculated into 50 mL of tryptone soy broth (TSB, Biokar,

105 Beauveais, France) and incubated in aerobic conditions for 20-24 h in agitation (15.71

106 rad s^{-1}) at 25 ºC. Bacterial cells were harvested by centrifugation at 9800 × g for 10 min

107 at 10 °C and re-suspended in sterile distilled water. The concentration of the

108   suspension was checked by plate count of appropriate 10-fold dilutions in saline

109   peptone (8.5 g L^{-1} NaCl, 1 g L^{-1} peptone) onto TSA plates after incubation at 30 °C for

110   48 h.

111   **2.3 Inoculation, sampling and microbiological analysis**

112   Suspensions of CPA-7 at 10^7 CFU mL^{-1} were prepared in a 60 g L^{-1} NatureSeal® AS1

113   AgriCoat Ltd., Great Shefford, UK) antioxidant aqueous cold solution (4 ºC), as

114   recommended by the manufacturer. Non-inoculated antioxidant solution was included

115   as control. Fruit wedges were dipped-inoculated at a ratio 1:2 (weight of product:

116   volume of suspension) for 2 m in agitation (15.71 rad s^{-1}) in tabletop orbital shaker

117   Unimax 1010 (Heidolph, Germany). After drainage of the excess of water, 10 g of apple

118   wedges were used to determine initial microbiological quality in triplicate and three

119   samples of about 70 g were frozen with liquid nitrogen, grinded in a commercial grinder

120   (Minimoka 6R-020, Coffeemotion, Lleida, Spain) and stored at -80 °C until biochemical

121   analysis. The rest of the treated fresh-cut fruit was packaged (120 g per replicate) in

122   400 mL polyethylene terephthalate ShelfMaster™ Pronto™ trays (PlusPack, Denmark).

123   Each tray was considered as a replicate and three replicates per treatment and

124   sampling time were used. Trays were sealed with a peelable plastic film (polyester anti-

125   fog film (OALF) of 14 μm of thickness + oriented polypropylene (OPP) of 20 μm of

126   thickness) with a line of holes of 60 - 80 μm each and 75 mm separation spacing

127   between them, to achieve passive modified atmosphere. In a parallel set of trays the

128   film was macro perforated (nine extra holes per tray) in order to attain aerobic

129   conditions. Trays were stored at 5 °C in darkness and biochemical and microbiological

130   analyses were performed at 0, 1, 3 and 6 d post-inoculation. Population dynamics of

131   CPA-7 as well as total mesofils of inoculated and non-inoculated samples were

132   analyzed at each sampling time. For this, 10 g of apple from each tray was

133   homogenized in 90 mL of buffered peptone water (APT, Biokar, Beauvais, France) and

134   tested by viable cells count onto TSA and plate count agar plates (PCA, Biokar,

135   Beauveais, France) incubated at 30 °C (for CPA-7) and at 25 °C (for mesofils) for 48 h

136   and 72 h, respectively.

2.4 Fruit physicochemical quality parameters

138   Firmness, color and pH of fresh-cut wedges were determined initially and at each

139   sampling time as described elsewhere (Alegre et al., 2013a). Low values of CIE

140   coordinate L* and high values of a* were considered as indicators of surface browning

141   intensity (Sapers and Douglas, 1987). Soluble solids and titratable acidity were

142   measured in two samples per tray initially and at the end of storage. Soluble solids

143   concentration at 20 °C was expressed as mass fraction of sugars relative to the fruit

144   (%). Acidity was measured in 10 mL of pulp and was expressed as malic acid content

145   (g L^{-1} juice).

2.5 Biochemical analyses

2.5.1   Total antioxidant capacity (TAC) and total phenolic content (TPC)

148   Extracts for total phenolic content (TPC) and total antioxidant capacity (TAC)

149   determination were prepared by mixing 3 g of frozen fresh pulp with 10 mL of a solution

150   containing 19.7 mol L^{-1} methanol, 0.05 mol L^{-1} HCl. The mixture was held in agitation

151   (20.94 rad s^{-1}) for 2 h and centrifuged at 24 000 x g at 4 ºC. TPC was quantified by

152   measuring the optical density (OD) at 765 nm in a spectrophotometer (EONC, Biotek

153   Instruments, Highland Park, VT, USA) after the reaction of 0.05 mL of each extract with

154   0.25 mL of Folin-Ciocalteu reagent and 0.5 mL of 1.9 mol L^{-1} Na_2CO_4.

155   TAC was determined by measuring OD at 593 nm of the above mentioned extracts

156   following the Ferric Reducing Antioxidant Power (FRAP) protocol (Giné-Bordonaba and

157   Terry, 2016).

2.5.2   Hydrogen peroxide production and malondialdehyde (MDA) content

159   Malondialdehyde (MDA) was quantified in 0.5 g of frozen fresh pulp as a marker of lipid

160   peroxidation using the thiobarbituric acid reactive substrates (TBARS) assay described

by Martínez-Solano et al. (2005). The OD of the supernatants after reaction was measured at 532 nm and substracted to the inespecific absorption read at 600 nm. Quantification of TBARS was calculated by its extinction coefficient of 155 mmol L^{-1} cm $^{-1}$.

To determine hydrogen peroxide levels, 5 g of frozen fresh pulp was homogenized in 7.5 mL of 0.5 mol L^{-1} trichloroacetic acid, filtered through two layers of Miracloth (Textil Planas Oliverassa, Manresa, Spain) and centrifuged at 20 000 x g for 15 min at 4 ºC. H_2O_2 content was determined using the aqueous peroxide colorimetric assay, PeroxiDetect™ Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions. This procedure is based on the measurement of the color change at 560 nm of adduct formed by Fe^{3+} ion and xylenol orange (XO, PubChem CID 73041), during the oxidation by peroxide of Fe^{2+} to Fe^{3+} ions at acidic pH. The molar extinction coefficient of the XO-Fe^{3+} colored adduct in aqueous solution at 560 nm is 15 000.

2.5.3 Activities of enzymes involved in antioxidant metabolism

2.5.2.1 Peroxidase (POX) y Polyphenol oxidase (PPO)

Total peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.14.18.1) were extracted from 10 g of frozen fresh pulp as described by Giné-Bordonaba et al. (2017). POX activity was measured as the optical density at 470 nm following the reaction of the extract with 10 mmol L^{-1} guaiacol and 10 mmol L^{-1} H_2O_2, according to the method described by Lurie et al. (1997). PPO activity was determined at 400 nm in a reaction mixture containing 0.1 mol L^{-1} potassium phosphate buffer (pH 6) and 65 mmol L^{-1} pyrocatechol together with the correspondent enzyme extract as described by Vilaplana et al. (2006).

2.5.2.2 Superoxide dismutase (SOD) and catalase (CAT)

For the extraction of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), 5 g of frozen fresh pulp were homogenized in 15 mL 0.1 mol L^{-1} potassium phosphate buffer (pH 7.8), 2 mmol L^{-1} dithiothreitol (DTT), 50 g L^{-1}

188 polyvinylpolypyrrolidone (PVPP), 0.1 mmol L^{-1} ethylene diamino tetracetic acid (EDTA)

189 and 1.25 mmol L^{-1} polyethylene glycol. The homogenized was filtered, centrifuged and

190 purified as described by Giné-Bordonaba et al. (2017). In turn, SOD activity was

191 determined by measuring its ability to inhibit the photochemical reduction of

192 nitrobluetetrazolium (NBT) at 560 nm following the method of Gianopolitis and Ries

193 (1977).

2.5.2.3 Ascorbate peroxidase (APX)

195 For ascorbate peroxidase (APX; EC 1.11.1.11) extraction, 10 g of frozen fresh pulp

196 was homogenized with 30 mL of 0.1 mol L^{-1} base phosphate buffer (pH 7.5) containing

197 0.8 mmol L^{-1} HCl, 1 mmol L^{-1} EDTA, filtered through two layers of Miracloth and

198 centrifuged at 10 000 x g for 15 min at 4 ºC. APX activity was determined at 290 nm

199 during 10 min by monitoring the H_2O_2-dependent decomposition of ascorbate in a

200 mixture containing twenty microliters of the recovered supernatant and 280 μL of a

201 reaction solution (0.22 mmol L^{-1} ascorbic acid, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} H_2O_2)

202 (Nakano and Asada, 1981).

2.5.2.4 Protein content

204 Protein content of all extracts was determined by the Bradford method at 595 nm using

205 a protein assay kit (Bio-Rad, München, Germany). Protein reagent was mixed with the

206 correspondent phosphate buffer (pH 6, 7 or 7.8) used for each enzyme extraction in a

207 ratio 1:3.6. Bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) was used

208 as standard.

2.6 Expression of results and statistical analysis

210 Enzyme activity was expressed in units of enzyme activity per milligram of protein (U

211 mg^{-1} protein). For SOD, 1 U represents the amount of enzyme required to inhibit NBT

212 reduction by 50 % of initial amount. For the rest of the analyzed enzymes, 1 U

213 represents the quantity of enzyme responsible for a change in 1 absorbance unit per

214 minute. Microbiological data was estimated as microbial counts per gram of fruit (CFU

215   g^{-1} fruit). Non-enzymatic antioxidant activities were expressed as g of the measured

216   analyte (i.e. Gallic acid (GAE) or Fe^{3+}) per kilogram of fruit. Oxidative stress markers

217   (H_2O_2 and MDA) were expressed as μmol kg^{-1} fruit. The data were analyzed using the

218   general linear model procedure to determine differences among treatments and

219   interaction effects using the statistical software JMP (version 11 SAS Institute Inc., NC,

220   USA). All data were verified for normal distribution and homoscedasticity of residues

221   and accordingly, enzymatic activities and microbiological data were transformed to

222   log_{10} of U mg^{-1} protein or CFU g^{-1}, respectively, Transformed results were schematically

223   represented as back-transformed means ± standard error of the mean. Transformed

224   and non-transformed means were compared by analysis of variance (ANOVA) and

225   separated by Tukey's test (P < 0.05).

3   Results

3.1 Microbial dynamics

228   Initial populations of CPA-7 on the fruit were 5.53 ± 0.03 log_{10} (Fig. 1A). Low oxygen

229   concentration associated to modified atmosphere packaging (MAP) slowed down CPA-

230   7 growth if compared to the samples stored in air, delaying the start of the exponential

231   phase from 1 d to 3 d post-inoculation. On day 6, samples stored in modified

232   atmosphere packaging showed a 0.5 log_{10} reduction of CPA-7 growth in respect of the

233   samples stored in aerobic conditions. Mesofils populations were initially the same (1.7

234   ± 0.2 log_{10}) in all samples (Fig. 1B). However differences in growth (1 log_{10} CFU g^{-1}

235   fruit) among aerobic and modified atmosphere-stored samples became evident on day

236   6.

3.2 Fruit physicochemical quality parameters

238   No significant differences were observed among values of L* (lightness) and a*

239   (redness) in the presence of CPA-7 (Table 1). Likewise, no differences were observed

240   in the content of soluble solids or pH in response to the antagonist under any of the

241   tested conditions throughout storage. Firmness was better maintained throughout the

242 studied period in the presence of CPA-7 if compared to non-inoculated fruit, in which it

243 was reduced at the end of storage.

3.3 Biochemical analyses

3.1.1 Total antioxidant capacity (TAC) and total phenolic content (TPC)

246 At the beginning of the experiment total antioxidant capacity (TAC), measured by the

247 content of Fe^{3+}, was 13.6 ± 0.2 g kg^{-1} fruit in all samples and gradually decreased

248 during storage (Fig. 2A). However, the drop of TAC was significantly slower in

249 presence of CPA-7 in aerobic conditions where it remained at 12.3 ± 0.4 g kg^{-1} fruit

250 until day 6. No differences were observed among MAP-stored samples in the first three

251 days of the experiment. However, on day 6 in MAP conditions, it was recorded a

252 reduction by 0.8-fold in response to CPA-7 if compared to the control.

253 Total phenolic content (TPC) was initially 5.1 ± 0.1 g kg^{-1} fruit in all samples regardless

254 of the presence of the antagonist (Fig. 2B). On day 3 and for both storage conditions,

255 the inoculation with CPA-7 was associated to a significantly higher amount of TPC (4.5

256 ± 0.1 g kg^{-1} fruit), than in the control samples (4.2 ± 0.04 g kg^{-1} fruit in air and 3.7 ± 0.04

257 g kg^{-1} fruit in MAP). In agreement to TAC, on day 6, TPC was reduced in MAP-stored

258 samples in response to CPA-7 (4.1 ± 0.1 g kg^{-1} fruit) if compared to the control (4.9 ±

259 0.1 g kg^{-1} fruit).

3.1.2 Hydrogen peroxide production and malondialdehyde content (MDA)

261 No sign of peroxidation of the membranes could be estimated from the measurement

262 of the content of the marker malondialdehyde (0.019 ± 0.04 µmol kg^{-1} fruit) under any

263 of the conditions tested during the analyzed period (data not shown). When combined

264 to the antioxidant, CPA-7 was able to erase the oxidative burst or at least that referred

265 to H_2O_2 accumulation in the fruit stored in air (Fig. 3). Contrastingly, in the samples

266 stored in MAP H_2O_2 drastically increased in the first 24 h reaching up to 1.4-fold higher

267 in response to CPA-7 than in the non-inoculated control. In general, metabolization of

268 this molecule was significantly slower in the MAP-stored samples in the presence of

269 CPA-7 than in the rest of the samples. In this sense, values remained 13-fold higher if

270 compared to the control at the end of storage. Contrastingly, in inoculated samples

271 stored in air, the levels of H_2O_2 remained close to zero throughout the whole

272 experiment.

273 *3.1.3 Activities of enzymes involved in antioxidant metabolism*

274 Changes in the enzymatic antioxidant potential of 'Golden delicious' fresh-cut apple

275 differed throughout storage according to the inoculation with the antagonist and/or to

276 the storage conditions. SOD activity decreased from 5554 ± 42 U mg^{-1} protein at initial

277 time to 4863 ± 111 U mg^{-1} protein after 24 h in all samples, except for the CPA-7-

278 inoculated MAP-stored sample, in which it remained stable up to day 3. This contrasted

279 with the drop (0.7-fold lower than the CPA-7-inoculated sample) observed for the

280 correspondent control (Fig 4A). In contrast, on day 6, SOD activity was oppositely

281 modulated by CPA-7 depending on the storage conditions; it was 0.7-fold lower than

282 the control when stored in MAP and 0.6-fold higher than the control when stored in air.

283 Catalase activity immediately after processing was similar in all samples (237 ± 17 U

284 mg^{-1} protein) regardless of any of the factors assayed (Fig. 4B). However, it

285 differentially changed in response to the antagonist in different storage conditions. It

286 increased by 0.7-fold compared to the initial value when stored in air, reaching the

287 same level than the control after 24 h. In contrast, CAT activity in inoculated samples

288 was inhibited in response to the antagonist when stored in MAP (by more than 0.6-fold

289 if compared to the control) during the first 3 d of storage.

290 Initially, PPO activity was 243 ± 7 U mg^{-1} protein which was reduced by 0.5-fold in the

291 first 24 h (Fig. 4C). Thereafter, it drastically increased in response to CPA-7 in MAP

292 conditions reaching almost 8-fold the activity showed by the correspondent non-

293 inoculated fruit. However, when stored in air, CPA-7 had no significant effect in PPO

294 activity throughout the analyzed period.

295 Activity of APX was initially around 348 ± 43 U mg^{-1} protein in all samples (Fig 4D).

296 During the first 24 h post-inoculation, APX activity was induced up to 3-fold, if

297 compared to the initial values, in response to the antagonist when stored in air. At this

298 sampling time, peaks of APX activity in response to CPA-7 triplicated the activities

299 recorded for the controls, regardless of the storage conditions. An enhanced APX

300 activity associated to the antagonist was maintained until day 3 (1.8 fold higher than

301 the control) in air-stored samples.

302 Immediately after inoculation, POX activity was inhibited in response to CPA-7 (by 0.6-

303 fold if compared with the control) (Fig. 4E). Thereafter, this enzyme showed a biphasic

304 activation in response to CPA-7 in aerobic conditions reaching values 1.5-fold and 1.7-

305 fold higher than those observed for the control after 1 and 6 d post-inoculation,

306 respectively. In contrast, POX activity was reduced in all MAP-stored samples during

307 the first 24 h post-inoculation, but it attained significantly lower values (98 ± 10 U mg^{-1}

308 protein) in the presence of the antagonist than in the control (84 ± 5 U mg^{-1} protein). As

309 for air-stored samples on day six, POX activity was enhanced in the CPA-7-inoculated

310 MAP-stored samples, reaching values eight fold higher than the control.

311 ## 4 Discussion

312 Processing of fresh produce implies the removal or damage of most of the constitutive

313 barriers of plants and generally activates inducible defense mechanisms to protect

314 themselves against pathogens (Reviewed by Hodges and Toivonen, 2008).

315 Pseudomonads that have been used as biocontrol agents (BCA) have shown to

316 enhance plant response including cell-wall straightening, oxidative burst and

317 expression of defense related genes that are usually activated by pathogens (Van der

318 Ent et al., 2009). This phenomenon 'primes' plant metabolism allowing it to react faster

319 and stronger upon a subsequent pathogen attack (Pieterse et al., 2001). One of the

320 enzymes associated to resistance responses is peroxidase (POX) which mediates the

321 last steps of the biosynthesis of lignin and hydrogen peroxide (H_2O_2) (Hiraga, 2001).

322   POX also catalyzes single-electron oxidation of phenolic compounds in the presence of

323   hydrogen peroxide. This latter molecule is one of the first molecules generated in plant

324   reaction to wounding and pathogen infection and has been postulated to play a dual

325   role as signaling factor to induce defense genes related to wound-stress and

326   hypersensitive response and it is also involved in fine tune regulation of oxidative

327   stress caused by reactive oxygen species (ROS) both locally and systemically (Hung et

328   al., 2005; Sewelam et al., 2014; Baxter et al., 2014). However, little is known about the

329   oxidative response in fresh-cut fruit associated to BCA.

330   The results obtained in the present study showed that oxidative response of 'Golden

331   delicious' fresh-cut apples was differentially modified in the presence of CPA-7

332   depending on the storage conditions. For instance, immediately after processing and

333   treatment with the antioxidant, H_2O_2 quickly began to accumulate in all samples to up to

334   the first 24 h in the MAP-stored samples and up to 3 d in the air-stored control. Similar

335   H_2O_2 accumulation pattern has been previously observed in non-inoculated fresh-cut

336   'Fuji' apples treated with ascorbic acid and stored in aerobic conditions (Larrigaudiere

337   et al., 2008). However, in the presence of CPA-7, the oxidative burst was practically

338   erased in the air-stored samples, and H_2O_2 levels were kept close to zero during the

339   whole experiment. Activation of ROS-scavenging enzymes correlated with the initial

340   oxidative burst associated with processing, thus the levels of SOD activity were initially

341   elevated in all samples. Subsequently, SOD activity decreased in all samples and APX

342   was sharply and transiently induced by CPA-7 as a first line of defense against

343   elevated H_2O_2 production in both MAP and air-stored samples after the first 24 h. On

344   the other hand, when stored in air CPA-7 was also associated to an early increase of

345   CAT and POX activities which could contribute to quickly eliminate H_2O_2 as soon as it

346   was produced. The decline of SOD activity 24 h after processing was also observed by

347   Larrigaudiere et al. (2008) in antioxidant-treated Fuji apples.

In the conditions assayed herein, as time passed, stress associated to mechanical damage was added to that caused by low-oxygen atmosphere as in MAP-stored samples, hence leading to changes in the modulation of some scavenging enzymes aiming to maintain redox homeostasis. In this way, from day 1 to 3, SOD activity peaked in the CPA-7-inoculated MAP-stored samples and subsequently, PPO and POX were sharply induced until the end of storage counteracting the inhibition of CAT. In contrast, in the air-stored samples the activation of SOD due to CPA-7 lasted until day 6 and was paired to the induction of POX. Thus, stressful conditions were associated not only to increased ROS-scavenging enzymes activities but to changes in their balance. It has been previously observed that reduced CAT activity is sometimes compensated by induction of APX and glutathione peroxidase (GPX) (Apel and Hirt, 2004). Similarly, Wang et al. (2014) observed that the treatment of loquat fruit with the antagonist *Bacillus cereus* AR-156 induced SOD activity while reduced the activities of CAT and APX enhancing the plant response to infection by *Colletotricum acutatum*.

The modulation of oxidative metabolism in fruit and vegetable in response to *Pseudomonas* spp. as observed in the present study, has been previously reported and correlated to the induction of plant resistance to several plant pathogens (Sangeetha et al., 2010). For instance, combinations of a non-fluorescent *Pseudomonas* spp. strain NFP6, *P. fluorescens* and *B. subtilis* induced up to 4-fold PAL, POX and PPO activities and increased by 3.6-fold the phenolic content in banana fruits challenged with crown rot pathogens, 5 d after treatment with the antagonists (Sangeetha et al., 2010). Likewise, *Pseudomonas corrugate* strain 13 and *Pseudomonas aerofasciens* strain 63-28 significantly induced PAL, POX and PPO in cucumber 2-5 d following treatment and elicited the defense response to subsequent infection with *Phytium aphanidermatum* (Chen et al., 2000). Similarly, mixtures of fluorescent rhizosphere and endophytic Pseudomonads induced these enzymes when sprayed on banana plants which subsequently showed enhanced systemic resistance

375   against bunchy top virus (Harish et al., 2009). Similar response regarding POX, PPO

376   and SOD activities was previously observed for other biocontrol agents such as

377   *Bacillus amyloliquefaciens* LJ02 after being sprayed on three leave-stage seedlings (Li

378   et al., 2015). In general accordance with our study, they observed a 2-fold increase of

379   POX activity on day 6 as well as an enhanced SOD activity in aerobic conditions until

380   day 5 priming plants for resistance to powdery mildew caused by the fungus

381   *Spaheroteca fugilinea.*

382   Changes in PPO activity in response to CPA-7 has been previously investigated in

383   fresh-cut 'Piel de sapo' melon stored in air and in MAP conditions during 8 d of storage

384   at 5 ºC (Plaza et al., 2016). In contrast to our results, no variation was recorded in

385   response to CPA-7 for MAP-stored samples. Furthermore, although an induction of this

386   enzyme associated to the antagonist was observed by Plaza et al. (2016) after 8 d of

387   storage in aerobic conditions compared to the initial activity, this increase did not differ

388   from the control. Differences in sampling days between that study and ours could

389   suggest that PPO could peak after 6 d of MAP-storage and drop before day 8 and in

390   the same way, the increase of PPO activity in aerobic samples could be produced after

391   6 d of storage. Alternatively, discrepancies between both studies could be associated

392   to the differential behavior of the bacterium in relation to different fruit matrices.

393   The question still remains on whether the increase of ROS-scavenging enzymes

394   activities such as superoxide dismutase, catalase and peroxidase is only a plant

395   response or is also a strategy deployed by the antagonist. This would confer this

396   bacterium with the ability to tolerate highly oxidant conditions generated after

397   processing of fresh produce and could act as a pivotal mechanism of action to

398   outcompete foodborne pathogens or spoilage microorganisms. Differences in the

399   modulation pattern of the oxidative metabolism throughout storage observed in the

400   present study did not correspond to great changes in CPA-7 populations as a function

401   of storage conditions. The tracking of population dynamics showed that CPA-7 was

able to tolerate the changes produced in the fruit oxidative metabolism during the assayed period. In this sense, CPA-7 showed similar growth upon refrigerated conditions both in air and in MAP with a slight inhibition corresponding to lower oxygen availability after 6 d of incubation. Previous semi-commercial trials performed with fresh-cut apples in conditions resembling those assayed herein showed similar CPA-7 population dynamics and revealed an inhibitory or bacteriostatic effect of this antagonist on *L. monocytogenes* depending on the temperature of storage rather than on MAP or air storage conditions (Alegre et al., 2013a). From this perspective, high resistance to ROS of the antagonistics yeasts (*Cryptococcus laurentii* LS-28 and *Rhodotorula glutinis* LS-11) inoculated in wounded apple and peach fruits has been associated with enhanced antagonistic activity against *Botrytis cinerea* and *Penicilium expansum* (Castoria et al., 2003; Zhang et al., 2017).

On the other hand, concomitant with enzymatic changes, increased non-enzymatic antioxidant activity provides host tissue with additional defense tools to counteract ROS production induced by both biotic and abiotic stress (Hung et al., 2005). The initial accumulation of phenolic compounds, as observed in our trials regardless of the presence of the antagonist, has been studied for fresh-cut produce (Saxena et al., 2009). It could be related to the high production of H_2O_2 upon mechanical injury through an enhanced respiration or due to the activation of several metabolic pathways such as the hexose –monophosphate shunt pathway, the acetate pathway and/or the release of bound phenolic compounds by hydrolytic enzymes (Harish et al., 2009). However, the subsequent reduction of total phenolic content was slowed down in the presence of CPA-7 when stored in aerobic conditions at least until day 3 which could also account for the maintenance of beneficial nutritional properties of the fruit. Contrastingly, in MAP conditions, CPA-7 was associated to a peak in the production of phenolic compounds on day 3 with a concomitant increase of PPO activity. Interestingly, under such storage conditions, the drastic accumulation of this enzyme at

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429   the end of the storage period was correlated to a significant drop of its substrates, yet it

430   did not correlate with a significant increase of browning.

431   Regarding the effect of the antagonist on fruit quality, CPA-7 had a positive effect on

432   the maintenance of physical parameters such as firmness throughout storage which

433   contrasted with control samples. This result agreed with the maintenance of the cellular

434   membranes integrity in the presence of CPA-7 as estimated chemically from the

435   measure of MDA. No significant effect on pH or soluble solids was observed due to the

436   antagonist during the studied period. The combination of the antagonist with the

437   antioxidant agent resulted in the maintenance of luminosity and redness but similar to

438   the results observed for the control samples at all times of analysis. Similarly, in CPA-

439   7-inoculated fresh-cut 'Golden delicious' apples treated with 60 g L^{-1} Natureseal[®]

440   (AS1), as in the present study, L* and a* Hunter values were maintained during 14 d of

441   storage at 5 ºC (Alegre et al., 2013a). The same antioxidant treatment has been

442   previously shown to efficiently maintain color properties (with a* values from -2.55 to -

443   3.14) and to increase the firmness of fresh-cut of apple wedges from ten cultivars after

444   storage for 5 d at 2 ºC – 4 ºC (Roble et al., 2009). In contrast, when using a different

445   antioxidant treatment (5 g L^{-1} calcium chloride), a decrease of luminosity of CPA-7-

446   treated fresh-cut melon after 8 d of storage at 5 ºC was observed by Plaza et al.

447   (2016).

5 Conclusions

449   The results showed herein indicated that CPA-7 was associated to a differential

450   modulation of the oxidative response of antioxidant-treated fresh-cut apple during

451   chilling storage in MAP or aerobic conditions. CPA-7-inoculated fruit showed a higher

452   antioxidant capacity, a higher accumulation of phenolic compounds and an enhanced

453   activity of key antioxidant enzymes (POX, SOD and APX) which were related to the

454   plant response to either mechanical injury or microbial invasion, at certain moments of

455   storage. The antagonist ability to tolerate or to mediate these changes and to grow

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upon such conditions could imply high resistance to oxidative stress which would, in turn, provide CPA-7 with an adaptive advantage to outcompete putative pathogen infection. The differential temporal enhancement of the analyzed enzymes depending on the storage conditions suggests a synergistic activation of different pathways in response to the several factors associated to production and commercialization of fresh-cut fruit such as mechanical injury, low-temperature, antioxidant-treatment and low-oxygen conditions. Further studies including transductional analysis of the accumulation patterns of different antioxidant isoenzymes, which may be activated by the plant and/or the antagonist upon challenge with foodborne pathogens would improve the understanding of such mechanisms.

Acknowledgements

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620 Table 1. Quality parameters of fresh-cut antioxidant-treated 'Golden delicious' apple

621 wedges inoculated with *P. graminis* CPA-7 (CPA) or non-inoculated (NI) during storage

622 in aerobic conditions (AIR) or in modified atmosphere packaging (MAP).

Parameter	Treatment	Time (days) at 5 ºC			
		0	1	3	6
Lightness (L*)	AIR NI	78.1 ± 0.5 a B	80.9 ± 0.2 a A	79.7 ± 0.4 a AB	78.5±0.6 abc B
	AIR CPA	78.5 ± 0.9 a B	77.5 ± 0.4 bc B	78.8 ± 0.6 ab B	78.5±0.7 abc B
	MAP NI		78.2 ± 0.6 bc A	79.8 ± 0.5 a A	79.1±0.5 ab A
	MAP CPA		80.1 ± 0.3 ab A	79.0 ± 0.5 ab A	79.7±0.4 a A
Redness (a*)	AIR NI	-6.6 ± 0.4 b B	-6.4 ± 0.4 a A	-6.4 ± 0.3 cd A	-5.8±0.5 bc A
	AIR CPA	-6.1 ± 0.6 ab B	-6.0 ± 0.3 bc B	-6.5 ± 0.2 ab B	-6.2±0.4 bc B
	MAP NI		-6.4 ± 0.5 bc AB	-7.7 ± 0.1 d B	-6.4±0.1 bc A
	MAP CPA		-7.2 ± 0.5 ab A	-6.0 ± 0.3 cd A	-6.5±0.3 c A
Firmness (N)	AIR NI	13.1 ± 1.0 a A	12.1 ± 0.8 a A	12.0 ± 0.9 a A	11.5±0.6 a B
	AIR CPA	13.0 ± 0.8 a A	12.8 ± 0.8 a A	12.5 ± 0.6 a A	13.0±0.6 a A
	MAP NI		14.3 ± 0.2 a A	12.2 ± 0.6 a AB	11.7±0.3 a B
	MAP CPA		11.3 ± 1.5 a A	11.7 ± 0.5 a A	13.1±0.9 a A
pH	AIR NI	3.8 ± 0.1 a A	4.0 ± 0.1 a A	3.9 ± 0.02 a A	3.7±0.1 a A
	AIR CPA	4.1 ± 0.1 a A	3.9 ± 0.1 a A	4.1 ± 0.1 a A	3.9±0.2 a A
	MAP NI		3.85 ± 0.03 a A	3.9 ± 0.1 a A	3.9±0.1 a A
	MAP CPA		3.9 ± 0.1 a A	3.9 ± 0.1 a A	4.15±0.04 a A
Titratable acidity as malic acid content (g L^{-1} juice)	AIR NI	3.3 ± 0.2 a A			3.8±0.3 a A
	AIR CPA	3.22 ± 0.02 a A			3.6±0.5 a A
	MAP NI				3.5±0.3 a A
	MAP CPA				3.6±0.2 a A
Soluble solids (%)	AIR NI	12.93 ± 0.04 a A			13.3±0.9 a A
	AIR CPA	12.80 ± 0.06 a A			12.6±0.1 a A
	MAP NI				13.3±0.1 a A
	MAP CPA				12.6±0.3 a A

Values are means ± standard error of the mean. Different lowercase letters represent significant differences among treatments at each sampling time. Different capital letters represent differences for the same treatment throughout time according to analysis of variances (ANOVA) and Tukey's test (P < 0.05).
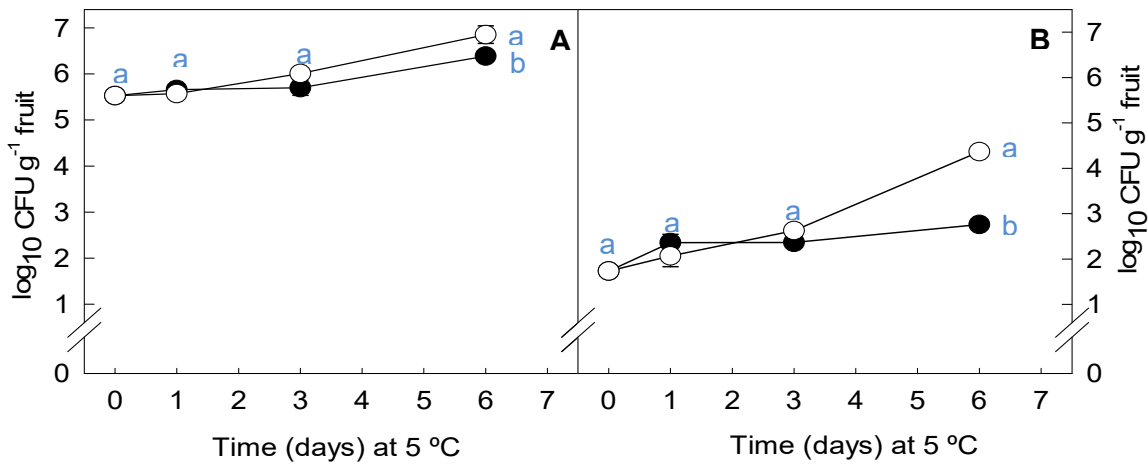
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630 Figure 1. Population dynamics of (A) *P. graminis* CPA-7 and (B) total mesofils on

631 'Golden delicious' fresh-cut apple wedges treated with antioxidant, during six days of

632 storage at 5 ºC in modified atmosphere packaging (MAP) (●) or in air (○). Each point

633 represents the mean and error bars represent the standard error of the mean (n = 6).

634 Different letters represent significant differences among treatments at each sampling

635 point according to analysis of variances (ANOVA) and Tukey's test (P < 0.05).

636 Underlined letters represent equal means that correspond with overlapped symbols in

637 the graph.

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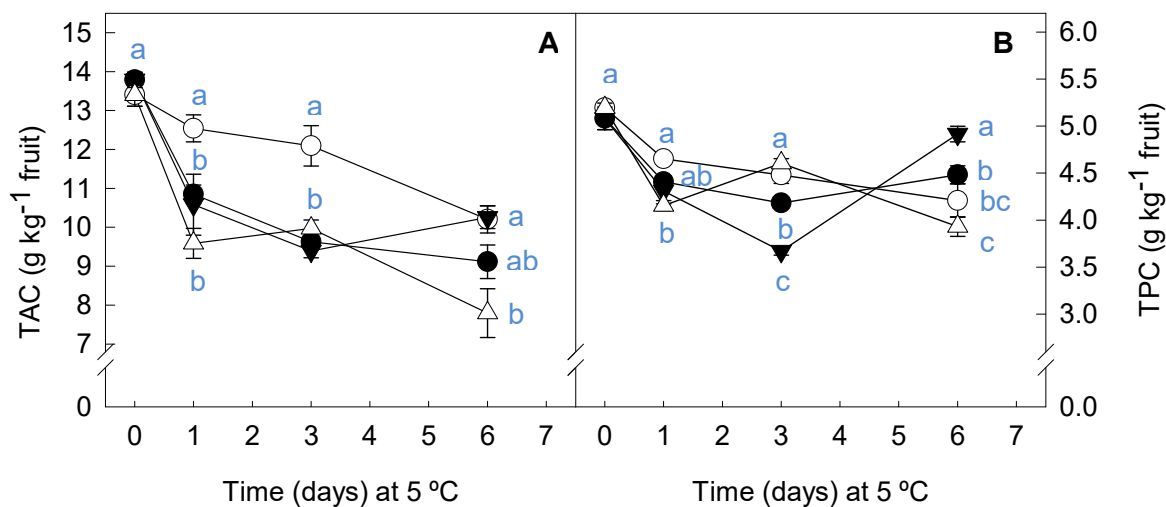
Figure 2. Changes in the levels of (A) Total Antioxidant Capacity, TAC, estimated from the amount of Fe^{3+} and (B) Total Phenolic Content, TPC, estimated from the amount of Gallic acid, in fresh-cut 'Golden delicious' apple wedges treated with antioxidant after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). Each point represents the mean and error bars represent the standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling point according to analysis of variances (ANOVA) and Tukey's test (P < 0.05). Underlined letters represent equal means that correspond with overlapped symbols in the graph.
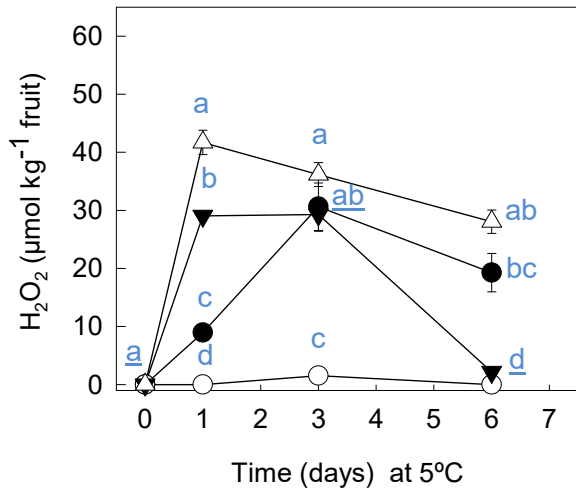
653 Figure 3. Changes in the levels of hydrogen peroxide (H_2O_2) in 'Golden delicious' fresh-

654 cut apple wedges treated with antioxidant after inoculation with CPA-7, during storage

655 in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in

656 MAP (▼). Each point represents the mean and error bars represent the standard error

657 of the mean (n = 9). Different letters represent significant differences among treatments

658 at each sampling point according to analysis of variances (ANOVA) and Tukey's test (P

659 < 0.05). Underlined letters represent equal means that correspond with overlapped

660 symbols in the graph.

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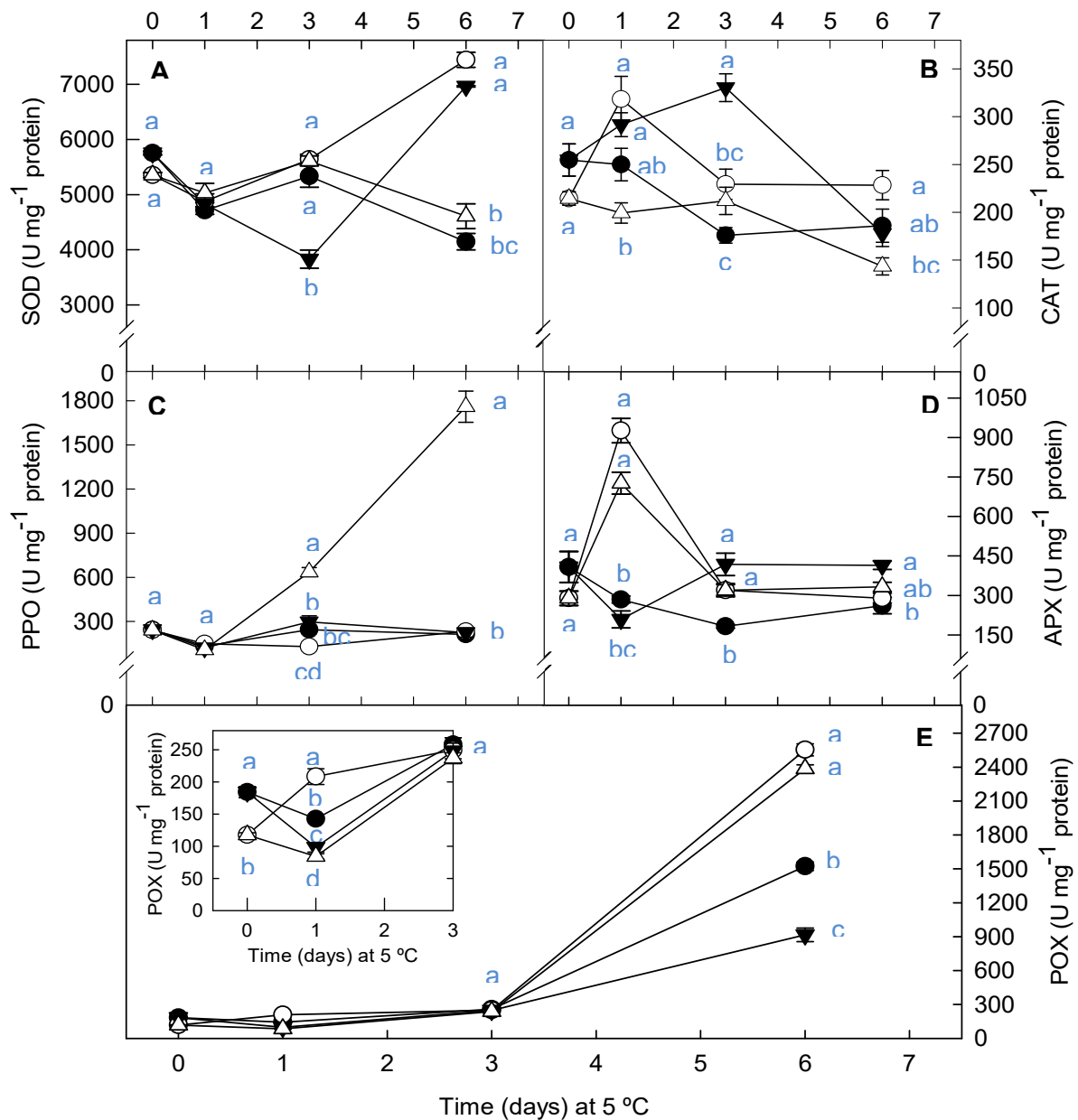
Figure 4. Changes in the activity of the enzymes A) superoxide dismutase (SOD), B) catalase (CAT), C) polyphenol oxidase (PPO), D) ascorbate peroxidase (APX) and E) peroxidase (POX) in 'Golden delicious' fresh-cut antioxidant-treated apple wedges, after inoculation with CPA-7, during storage in air (○) or in MAP (△) and in non-inoculated control fruit during storage in air (●) or in MAP (▼). The internal plot in graph E represents the first three sampling times at a smaller scale. Each point represents the mean and error bars represent the standard error of the mean (n = 9). Different letters represent significant differences among treatments at each sampling

673   point according to analysis of variances (ANOVA) and Tukey's test (P < 0.05).

674   Underlined letters represent equal means that correspond with overlapped symbols in

675   the graph.

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