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Comparison of different analytical methods to evaluate the heat shock protein (HSP) response in fruits. Application to tomatoes subjected to stress treatments

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1 **Comparison of different analytical methods to evaluate the heat shock protein (HSP)**
2 **response in fruits. Application to tomatoes subjected to stress treatments.**

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12

13 **Highlights**

- 14
- 15 • HSP evaluation can be used for practical purposes.
 - 16 • To assess the HSP response in fruits, different complementary methods should be
17 used.
 - 18 • A simple method (dot blot) can quantify HSP induced in fruits by heat exposure.
 - 19 • HSP level induced by stress treatments correlates with acquired physiological
20 tolerance.

21

21 **Abstract**

22 Heat shock proteins (HSP) are synthesized in living tissues exposed to transient increase
23 in temperature and play a central role in the protective response against heat and other
24 stresses. In fruits, this response to heat treatment provides resistance to a physiological
25 alteration known as chilling injury. Despite the physiological importance of this group of
26 proteins, publications comparing different methodological alternatives for their analysis are

27 rather scarce. In the present paper, we conducted a comparative study using different
28 electrophoretic and immunological techniques to evaluate the HSP response in fruits.
29 Proteins were extracted from tomato fruit exposed to an HSP-inducing temperature (38° C)
30 for different times (0, 3, 20, and 27 hours). Different alternatives of analysis (SDS-PAGE,
31 SDS-PAGE followed by IEF, western blot, and dot blot) were performed, and their potential
32 application discussed. The study was complemented with a practical application, in which
33 tomatoes were subjected to heat and anaerobic treatments and then stored in a chill-
34 inducing temperature. This application evidences the relevance of knowing the level of
35 proteins attained by stress treatments which correlates with the acquired tolerance.

36

37 **Keywords:** HSP kinetics; chilling injury prevention; heat treatments; stress monitoring; dot
38 blot; immunological methods

39

40 **1 Introduction**

41 It is well known that the exposure of living tissues to a transient temperature rise of
42 5 to 10°C above their normal temperature, induces the synthesis of a specific group of
43 proteins referred to as heat shock proteins (HSPs), which are usually present at low levels
44 in non-exposed cells (Luengwilai et al., 2012). These proteins play a central role in the
45 protective response against heat and other stresses, and in the case of fruits, they are
46 linked to the acquired resistant of heat-treated commodities against chilling injury (Aghdam
47 et al., 2013). From the biochemical point of view, HSP are classified into five different
48 families, according to their molecular masses, each of them having a particular function.
49 The two most relevant families in plants are the 70 kDa family (HSP70) and the small heat
50 shock protein family (sHSP) (Zeng et al., 2016). HSP70 is the most studied group,
51 because of the important function of their members as chaperones. Proteins belonging to
52 this group are involved in relevant processes such as the prevention of protein

53 aggregation, the refolding of denatured proteins, and the translocation of proteins across
54 membranes (Waters, 2013). In turn, the sHSP group constitutes the most diverse group of
55 plant HSP, considering sequence identity, cellular localization, and function. The
56 diversification of this family reflects the evolutionary adaptation to stress conditions unique
57 to plants, such as heat, cold, salinity, oxidative stress, drought, and mechanical injury (Sun
58 et al., 2013). This group shares a common C-terminal sequence of approximately 90
59 aminoacids known as α -crystallin domain (ACD), which is responsible for the reported
60 immunological cross-reactivity among different members (Basha et al., 2012).

61 The assessment of the presence and over-expression of HSP has also been used
62 with technological purposes. For instance, these proteins can be used to monitor the
63 exposure of living organisms to environmental pollution, since their induction constitutes
64 one of the first detectable biochemical responses against external disturbances, and the
65 increased levels usually persist for periods much longer than other biochemical markers
66 (Basile et al., 2013). In this regard, high concentrations of HSP70 were detected in animals
67 and plants subjected to physical stress or exposed to chemicals such as PCB, DDT, or
68 lindane (Dunlap and Matsumura, 1997). In the field of postharvest technology of fruits and
69 vegetables, HSP constitutes the principal marker to evaluate the level of protection exerted
70 by heat treatments, applied to prevent the development of chilling injury and other
71 physiological and pathological distresses in sensitive commodities. In this regards,
72 different studies were carried out in fruit species such as avocado (Florissen et al., 1996),
73 tomatoes (Ré et al., 2017; Polenta et al., 2015; Aghdam et al., 2015; Polenta et al., 2007),
74 peaches, plums, bananas, and grapefruits (Aghdam et al., 2013).

75 Despite the growing interest that HSP has raised in plant and postharvest
76 scientists, because of their role in biotic or abiotic stresses, there is a lack of studies
77 comparing diverse alternatives of analysis. In the present paper, we conducted a
78 comparative study of different electrophoretic and immunological analytical alternatives

79 (some of them developed by our group), to detect and evaluate the HSP response in fruits.
80 These techniques were used to assess the biochemical response in tomatoes subjected to
81 different stress treatments, and correlated with the chilling injury protection. The
82 advantages and limitations of each technique are specifically focused and described. This
83 work hypothesizes that for a complete picture of the HSP response, different
84 complementary analyses should be conducted, which can be used as biochemical
85 markers to assess and predict the stress treatment performance in fruits.

86

87 **2. Material and methods**

88 2.1 Plant material and treatment application

89 2.1.1 Model experiment to induce the synthesis of increasing amounts of HSPs, according 90 to the treatment intensity

91 Mature-green tomatoes (*Lycopersicon esculentum* cv. Cardinal) according to USDA
92 standard (USDA, 1991) of uniform size were obtained from an experimental greenhouse
93 (harvested in October 2015). Fruit were visually selected (60 fruit from an entire lot of 150
94 fruit, with an average weight of 180 g), and their surfaces were sterilized for 3 min with a
95 chlorine solution (150 mg/kg Cl₂) at room temperature in a recipient of 100 L, then
96 thoroughly rinsed with tap water in a similar recipient at room temperature for another 3
97 min, and then left on filter paper to drain.

98 Thermal treatments were applied by incubation of the fruit in an experimental chamber at
99 38 °C ± 1 °C and 95 percent relative humidity. Sixty fruit were divided into four lots, and
100 fruit were placed into clean vented plastic trays. Three of these lots were heat-treated for 3
101 (3 h), 20 (20 h), and 27 hours (27 h) respectively, whereas the remaining group received
102 no treatment and was used as a control (C). The experiment was run twice with similar
103 results.

104 2.1.2 Experiment to assess the HSP response and its correlation with chilling injury (CI)
105 prevention

106 Nine hundred and sixty mature-green tomatoes (*Lycopersicon esculentum* cv. Colt 45)
107 (USDA, 1991) of uniform size were picked directly from the greenhouse (harvest date:
108 November 2015). Fruits were treated similarly as described in 2.1.1. For the evaluation of
109 the effect of stress on CI prevention, tomatoes were placed into clean vented plastic trays
110 and divided into six lots, each of them submitted to one of the following treatments:

111 I: No treatment, used as control (C).

112 II: Short heat shock treatment (immersion for 30 min in a water bath at 42 ± 1 °C) (HS30').

113 III: Short heat shock treatment (immersion for 60 min in a water bath at 42 ± 1 °C) (HS60').

114 IV: Long heat shock treatment (incubation in a traditional chamber at 38 ± 1 °C and 95
115 percent relative humidity for 72 h) (HS72h).

116 V: Anaerobic treatment (incubation in a 20 L plastic chamber at 20 ± 1 °C, with first a rapid
117 atmosphere exchange by ventilation with humidified nitrogen at a flow rate of 100 ml/min
118 for 2 h, and then a continuous influx of humidified nitrogen at 50 ml/min-flow rate for 3
119 days) (ANA3d).

120 VI: Anaerobic treatment (incubation in a 20 L plastic chamber at 20 ± 1 °C, with first a rapid
121 atmosphere exchange by ventilation with humidified nitrogen at a flow rate of 100 ml/min
122 for 2 h, and then a continuous influx of humidified nitrogen at 50 cm³/min-flow rate for 6
123 days) (ANA6d).

124 To evaluate the effect of treatment on the development of CI, fruit were stored for 21 days
125 at 2 °C, and samples were taken under 2 conditions: immediately after treatment and after
126 the storage for 4 additional days in a chamber at 20 °C.

127

128 2.2 Protein Extraction

129 Proteins were extracted from tomato pericarp following the method of Hurkman and
130 Tanaka (1986) with some modifications. Briefly, fruit were divided into lots of 5 units
131 (individual fruit). Five grams of pericarp were taken from each fruit. The pericarps from
132 these fruit were homogenized in a Waring Blender in liquid nitrogen. The operation was
133 completed by grounding in a mortar, with the addition of liquid nitrogen. One gram from
134 this homogenate was thoroughly mixed in the presence of 1 mL extraction [100 mmol L⁻¹
135 Tris/HCl pH 8.0, containing 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ PMSF, and 2 % (v/v) β-
136 mercaptoethanol] and 4 mL of phenol saturated with 100 mmol L⁻¹ Tris buffer (pH 8.0), and
137 then centrifuged at 21000 x g for 10 min at 4°C. The phenolic phase was recovered, mixed
138 with four volumes of 0.1 mol L⁻¹ ammonium acetate (AMA), and incubated overnight at -20
139 °C. Protein pellets were obtained by centrifugation at 21000 x g for 20 min at 0 °C. Pellets
140 were then washed twice with AMA, once with cold acetone (80 % v/v), and dried at room
141 temperature. The dried residue was redissolved directly in electrophoretic sample buffer
142 [25 mmol L⁻¹ Tris pH 6.8, 1 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol,
143 and 0.002 % (w/v) bromophenol blue], and boiled for 2 min before being loaded onto a gel
144 and submitted to electrophoresis. Protein concentrations were determined by the Lowry
145 method (Lowry et al., 1951).

146

147 2.3 Electrophoretic analysis

148 SDS/PAGE was carried out according to the procedure of Laemmli (1970). For analytical
149 purposes, 15 µg of protein were loaded onto each well of a 0.75 mm-thick gel, whereas for
150 preparative use, 800 µg of protein were loaded onto a 1.5 mm-thick-gel.

151 Proteins were separated by using 12.5 % homogeneous polyacrylamide slab gels. Gels
152 were stained with 0.1 % (w/v) CBB solution.

153 Isoelectric focusing (IEF) was carried out in a vertical system, in a gel composed of 5 %
154 polyacrylamide, 0.4 % pH 3–10 ampholyte (Pharmalyte, Amersham), 2 % pH 4-6.5
155 ampholyte (Pharmalyte, Amersham), and 8 M urea.

156 The bands of interest from previous SDS-PAGE analysis were excised, soaked in 20 mmol
157 L⁻¹ NaOH for 20 min, and loaded onto the IEF gel. The electrophoresis was run in a
158 Protean II electrophoresis system (BIORAD) at the following voltage steps: 150 V for 30
159 min, 200 V for 60 min, and 250 V for 90 min. Calibration proteins (Isoelectric point (pI) 4.5-
160 11) were used to estimate the pI of the different protein bands. Gels were stained with 0.1
161 % (w/v) CBB solution. Samples were run in triplicate with similar results.

162

163 2.4 Antigen preparation and immunization protocol

164 Protein bands of interest were excised from IEF gels, rinsed several times with Phosphate-
165 buffered saline (PBS) and homogenized in the same buffer. Rabbit immunization for the
166 production of polyclonal antibodies was carried out as described by Polenta et al. (2007).
167 Briefly, rabbits of around 2 kg (3) were injected subcutaneously with 400 mg of HSPC1
168 excised from IEF gels, and suspended cleaning and sonication directly in 1 mL of PBS
169 buffer emulsified in complete Freund's adjuvant (day 1). Booster injections were
170 administered at days 4 and 14, with the same dose in incomplete Freund's adjuvant. Two
171 or four additional injections were performed and blood samples were withdrawn 1 week
172 after each injection. Animals were maintained under conditions that fulfilled all ethical and
173 scientific requirements for animal use included in EU Directive 2010/63/EU. Pre-immune
174 serum (day 0) was considered as negative control. Antiserum containing the polyclonal
175 antibodies against HSPC1, one of the sHSP, was aliquoted and stored at -80 °C until use.

176

177 2.5 Immunoblotting

178 Separated polypeptides were transferred (50 min at 100 V) onto a nitrocellulose
179 membrane (0.45 μm) by using a Mini Protean II Electrophoresis System (BIORAD). In the
180 case of the s HSP, the polyclonal antiserum was raised against HSPC1 (diluted 1:750),
181 which was used as the primary antibody. Anti-rabbit IgG raised in goat and conjugated to
182 alkaline phosphatase (BiORAD, dilution 1:1500) was used as the secondary antibody. In
183 the case of HSP70, a commercial monoclonal antibody (SIGMA, cat H5147, diluted
184 1:1500) was used as the primary antibody, while anti-mouse IgG raised in goat and
185 conjugated to alkaline phosphatase (BIORAD, dilution 1:1500) was used as the secondary
186 antibody. Membranes were revealed with nitroblue tetrazolium chloride and 5-bromo-4-
187 chloro-3-indolyl phosphate. In each experiment, samples were run by triplicate with similar
188 results.

189

190 2.6 Dot blot

191 For the dot blot analysis, 40 μg of total protein was directly deposited with an automatic
192 pipet onto the nitrocellulose membrane (Hybond, Amersham, 0.45 μm pore size).
193 Quantification was carried out by setting up a standard in which known amounts of
194 calibrants were deposited. For sHSP evaluation, HSPC1 (from a previous experiment)
195 electrophoretically purified from tomato (cv. Colt 45) and electroeluted from the gel was
196 used as the calibrant, while in the case of HSP70, it was used a commercial protein
197 purified from bovine brain (SIGMA, cat H9776). The absolute amount of protein was
198 expressed in ng of protein, while the relative amount was referenced to the initial amount
199 present in untreated fruit (considered as 100 %). Calibrants were deposited in triplicate,
200 with the values shown in Table 1 representing the average value for each concentration.

201

202 2.7 Image analysis

203 Gels were analyzed with a Bio-Rad GS-800 Imaging Calibrated Densitometer and digitally
204 processed by Quantity One 1-D Analysis software. Lane- and band-based functions were
205 used to determine apparent molecular weights (MWs), pls, and relative and absolute
206 amounts of proteins. A known amount of Bovine Serum Albumin (BSA) was used as
207 protein standard for lane-based protein quantitation. Samples were quantified by triplicate,
208 with the values shown in Table 1 representing the average value.

209

210 2.8 Chilling injury evaluation

211 The establishment of a CI-inducing condition was determined by a storage temperature
212 considerably lower than the reported threshold for the damage (2°C, threshold
213 temperature: 12.5°C) and by storage time longer enough to induce the development of
214 symptoms (21 days). Considering that in tomatoes, the main symptoms of CI are the
215 increased rate of fungal infection and the presence of pitting, decay was evaluated
216 visually, as the presence of macroscopic fungal growth, and pitting as the presence of
217 more than one spot. The corresponding percentages of diseased fruit, and fruit with visual
218 pitting were recorded (Efiuvwevwere & Thorne, 1988; El Assi 2004; Biswas et al., 2016).

219

220 **3 Results**

221 3.1 SDS-PAGE analysis

222 Figure 1A shows the SDS-PAGE analysis of protein extracts from tomatoes exposed to 38
223 °C for different periods (0, 3, 20, and 27 h). As evidenced, this technique made possible
224 the detection of a prominent group of proteins induced by heat exposure, with molecular
225 masses ranging from 15 to 35 kDa, which is compatible with the sHSP characteristics.
226 Since these proteins are located in a region of the gel with a low density of proteins, the
227 electrophoresis was complemented with densitometric analysis. Therefore, the relative

228 amount of protein induced by each treatment (Fig 1B) could be estimated, showing that the
229 most significant increase in intensity corresponds to the 21 kDa and 25 kDa protein bands.

230

231 3.2 SDS-PAGE followed by IEF for the analysis of specific bands of interest

232 Since 1D electrophoresis cannot resolve individual proteins with similar MW, the 21 kDa
233 protein band was excised from the SDS/PAGE and subsequently separated by IEF (Fig
234 1C). This technique resolved the band of fruit heated for 27 h into a set of up to 9 different
235 proteins. It is important to highlight that the increment of sHSP was already detected after
236 3 h of treatment, which shows that this combined technique constitutes an early and
237 specific monitoring tool. Additionally, this method allows the estimation of the main
238 physicochemical parameters of the individual proteins (isoelectric point and molecular
239 mass). The IEF gel was subjected to densitometric analysis (Fig 1D), which permitted to
240 estimate the intensity of the bands, each of them representing an individual protein.
241 Therefore, the relative amount of proteins induced by the different treatments could be
242 compared, which shows that the two main proteins, termed HSPC1 and HSPC2,
243 represent, altogether, approximately more than 75 % of the small heat shock proteins
244 induced by the treatment. These two proteins, together with most of the proteins present in
245 the original SDS/PAGE band, reacted with the anti-HSPC1 rabbit antiserum (Fig 1E),
246 which evidences that they belong to the sHSP family.

247

248 3.3 Western blot analysis

249 Figure 2 shows western blot analysis of tomatoes submitted to different intensities of heat
250 treatments (0, 3, 20, and 27 h). Membranes were revealed with two types of antibodies:
251 anti-HSPC1 rabbit antiserum obtained by our group (Fig 2A), and commercial anti-HSP70
252 monoclonal antibodies (Fig 2B). Remarkably, this last antibody, which was raised against
253 a protein from cow brain, recognized the stress proteins induced in tomato. The use of

254 highly specific antibodies provides unambiguous evidence that the over-expressed
255 proteins belong to the two most important HSP families and permitted the analysis of each
256 family.

257 As shown in Fig 2B, an important basal level of HSP70 was already present in control fruit,
258 and increased thereafter, proportionally to the treatment intensity. In the case of sHSP, a
259 low basal level was also detected, which increased after heat exposure, according to the
260 treatment intensity (Fig 2A), and in a pattern similar to the HSP70 family.

261

262 3.4 Dot blot analysis

263 Results show that dot blot offers a simple and accurate way to specifically quantify the
264 amount of HSP induced in fruits by heat exposure. To estimate the absolute amount of
265 proteins, we set up first a calibration curve, by loading different amounts of the target
266 proteins onto a nitrocellulose membrane and revealing them with the immunologic system
267 described in Methods (primary and secondary antibodies, and chromogenic substrate). Fig
268 3A shows the standard curve for the sHSP group, which was obtained by using an
269 electrophoretically-purified protein (termed HSPC1), while for the HSP70 family, the
270 calibration curve was obtained with a purified commercially available HSP70 from
271 SIGMA® (Fig 3B).

272 The estimated limits of detection for the method were 100 ng and 300 ng for HSPC1 and
273 HSP70 respectively. Calibrations curve were adjusted to a second order polynomial, with
274 an R square of 0,95, and CV among 15 and 21 % (depending on the calibrant
275 concentration) for sHSP; and an R square of 0.97 and CV among 12 and 23 %, for
276 HSP70.

277

278 For the analysis of the samples, protein extracts from the treated tomatoes were diluted, if
279 necessary, until the measured intensity lied within the range of the calibration curve. Table

280 1 shows the absolute amounts of protein in the treated tomatoes, as calculated in the
281 densitometric analysis of the dots. For quantitation purposes, the images of the
282 membranes were digitalized, and the dots intensities measured with a user-friendly open-
283 source software (ImageJ®). By comparing the intensities of control and treated samples, it
284 was possible to estimate the increase in sHSP concentration, even in tomatoes submitted
285 to the lowest combinations of time-temperature (30 min at 42 °C – data not shown, or 3 h
286 at 38 °C). This table also shows absolute concentrations of sHSP and HSP70, as well as
287 their relative amounts, by reference to the original amount present in control fruit
288 (considered as 100 %). In the case of sHSP, coefficients of variation (CV) showed values
289 among 7.6 and 14.6 %, for repeatability, and among 8.8 and 18.7 % for reproducibility. For
290 the case of HSP70, values were among 4.7 and 9.9 % (reproducibility), and among 8.4
291 and 11.1 (reproducibility). Pearson correlation coefficient between treatment intensity (time
292 in h) and protein amount were 0.91 ($p < 0.01$) for the case of sHSP, and 0.95 ($p < 0.01$) for
293 the case of HSP70.

294 Owing to the universal character of HSP, it is expected that this technique be capable of
295 quantifying the level of HSP attained after the exposure of any plant tissues to heat or
296 other stresses.

297

298 3.5 Practical application of the methodologies

299 Through the design of a practical experience, we evaluated the performances of the
300 proposed methods. The experiment involved the application of different stress treatments,
301 the subsequent evaluation of the HSP content, and the link between HSP synthesis and
302 the performance of the treatments to prevent the development of CI. Tomatoes were either
303 untreated or subjected to different intensities of heat or anaerobic treatments, and the
304 most relevant results are presented in the following items.

305

306 3.5.1 Physiological evidence of chilling injury

307 After treated, fruit were stored in a chilling injury-inducing condition (2°C) for 13 and 21 d,
308 and evaluated immediately after cold withdrawal, and after 4 days at 20°C, to induce the
309 development of the chilling injury symptoms, as described by Biswas et al. (2016). In fruit
310 evaluated after treatments, or after withdrawal from cold storage, no symptoms of chilling
311 injury were evident (data not shown). Symptoms were evident to different extents only
312 after 4 days at 20°C, as shown in Table 2.

313

314 3.5.2 SDS-PAGE Analysis

315 Figure 4A shows the protein pattern of extracts from tomatoes untreated (Control) or
316 subjected to the different treatments (HS30', HS60', HS72h, ANA3d y ANA6d). Samples
317 were analyzed immediately after treatments, and after 21 d of storage at 2 °C. Protein
318 pattern was similar to those described in 3.1, with several new bands in heat-treated fruit,
319 in the region of low MW (as indicated by arrows), the most prominent of them being a band
320 of around 21 kDa. Interestingly, this band became evident immediately after treatment and
321 remained visible during the entire storage at 2 °C (Data not shown). No band with these
322 characteristics was apparent, either in untreated tomatoes or in fruit subjected to
323 anaerobic treatments (ANA3d and ANA6d). Among the different treatments, fruit exposed
324 to heat for 72 h (HSP72h) showed the highest intensity of bands.

325 In the region of molecular weights around 70 kDa, the high density of proteins in the gel
326 made it difficult to detect differences in the protein patterns among treatments.

327

328 3.5.3 Inmunoblots

329 Figure 4B shows western blot analysis revealed with the commercial anti-HSP70
330 monoclonal antibody. As shown in this figure, members of this family were constitutively
331 expressed in untreated tomatoes, while heat treatments induced the synthesis of

332 additional amounts of proteins, in concentrations correlated with the treatment intensities.
333 Particularly, in tomatoes subjected to the HS72h treatment, additional bands of proteins
334 belonging to the same family were also detected.

335 Figure 4C shows western blot analysis revealed with the anti-HSPC1 rabbit antiserum. It is
336 important to mention that the protein used to generate the antibodies in rabbits was
337 induced in this experiment by the exposure of tomatoes for 72 h at 39 °C (HSP72h
338 treatment). This treatment caused the most remarkable overexpression of HSP, in general,
339 and of sHSP, in particular. As shown in this figure, a basal level of sHSP was already
340 present in untreated fruit, although at a very low concentration.

341 Table 2 presents the amounts of HSP induced by each treatment, estimated by using the
342 purified HSPC1 as a quantitative reference (absolute amount), or referred to those present
343 in untreated fruit, considered as 100 % (relative amount). Interestingly, short heat
344 treatments (HS30' y HS60') increased the initial amount of protein by approximately 2.5
345 times, while in the longest heat treatment (HSP72h), the increase was approximately 6.7
346 times. In turn, the anaerobic treatments had no effect on sHSP synthesis, indeed
347 provoking a slight decrease in their concentration. This fact can be also appreciated in
348 SDS-PAGE and western blot analyses (Fig 4).

349

350 **4 Discussion**

351 4.1 Assessment of the techniques

352 The four techniques evaluated in the study were capable of detecting and/or
353 quantifying the increase in HSP in a model experiment, in which different treatment
354 intensities were used. The techniques showed their capability to assess the kinetics of
355 HSP synthesis and give a complete picture of the HSP response, and can be used
356 independently, or as a set of analyses, since there are complementary each other. This
357 capability is qualitatively shown, in the case of the electrophoretic and Western blot

358 analyses, and quantitatively, in the case of dot blot. This information is valuable from the
359 technological point of view, considering that, as shown in previous studies, the amount of
360 the induced proteins properly reflects the level of stress undergone by tissues. Among the
361 different studies on this subject, the level of overexpression of HSP were used to evaluate
362 and monitor the optimal protection induced by stress treatments in chilling sensible
363 commodities such as tomatoes (Polenta et al., 2015), citrus (Polenta et al., 2007), banana
364 (He et al., 2012) and avocado (Kassim et al. 2013). In this last commodity, Florissen et al.
365 (1996), correlated the minimum time required to induce the synthesis of HSP with the
366 performance of the treatment. Interestingly, the ability of living organisms, including plants,
367 bacteria, and animals, to withstand high temperatures can be correlated with their capacity
368 to accumulate HSP (Sung et la., 2014).

369 For individual use, the selection of each technique will depend on aspects such as
370 the levels of detail required, the feasibility of application of each method, the equipment
371 and reagents available (especially immunosera), and the particular objectives of the
372 research.

373 Despite its simplicity, the combination of SDS-PAGE with densitometry provides
374 precise information on MWs of the induced proteins, and also permits the semi-
375 quantitation (estimation) of HSP accumulation. However, in regions with a high protein
376 density such as the 70 kDa region, it is difficult to properly identify the protein/s of interest
377 and, therefore, detect small variations, for which more complex immunological techniques
378 would be necessary.

379 The combination of SDS-PAGE + IEF offers additional information, such as MW
380 and pI of individual proteins, but the type of HSP analyzed is rather limited to specific
381 regions represented, in this particular case, by the lower MW range of the sHSP family.
382 The proposed modification of the 2D-IEF-SDS/PAGE protocol, in which the classical steps
383 were inverted, had two main positive effects: first, the resolution of the protein isoforms

384 was improved, since IEF offers a considerably better-resolving performance than SDS-
385 PAGE. This is because IEF can concentrate, within each gel band, the protein molecules,
386 while in the latter, protein molecules tend to diffuse as the electrophoresis progresses.
387 Therefore, this advantage would have been lost if IEF had been used as the initial step. In
388 addition, the total amount of protein loaded onto the gels could be greatly increased (800
389 μg of total protein), a feature that makes this method also suitable for preparative purposes
390 (i.e. protein purification to generate antisera). In fact, the method was used, in the present
391 research, to purify and use one of the sHSP (HSPC1), which was used as a calibrant for
392 the standard curve in the dot blot technique (Fig 3A and Table 1).

393 From the point of view of the method sensitivity, the overexpression of proteins
394 induced by the treatments could be easily detected within 3 h of heat exposure, while
395 longer treatments rendered concentrations increasingly higher. Interestingly, no apparent
396 maximum was attained in the present research, even after 27 h of heat exposure, which is
397 in contrast with some previous studies, where a plateau in HSP concentration was attained
398 after a few hours. Among these investigations, a rapid increase in HSP concentration was
399 verified in rice leaves within the first two hours of exposure to high temperature (Lee et al.,
400 2013). Another study reported that HSP70 increased gradually, although was especially
401 abundant from 2 h to 24 h after heat stress (Miova et al., 2015).

402 The high number of different proteins belonging to the sHSP family evidences the
403 complexity of the heat shock response, and is comparable with previous studies. In this
404 regard, it was reported that *Arabidopsis thaliana* can accumulate up to 19 new proteins,
405 with estimated molecular masses between 15 and 25 kDa (Santhanagopalan et al., 2015).
406 In protein extracts from heated tomato cells, three 20-kDa HSP with pIs ranging from 7.0
407 to 7.3, and five 21-kDa proteins with pIs between 5.1 and 6.0 were isolated (Nover and
408 Scharf, 1984)

409 When more detailed and specific information is required, Western blot analysis has
410 the advantage of combining the specificity and sensitivity of immunological methods, with
411 the advantage of the resolution associated with electrophoretic techniques. In this study,
412 this technique permitted the detection of differences in both HSP70s and sHSP
413 accumulation in fruit submitted to different time exposures. As shown in Figures 2B,
414 control fruit has basal levels of HSP70, which were notably increased after heat exposure,
415 in amounts proportional to the treatment intensity. These basal levels probably correspond
416 to constitutive isoforms of HSP70 (also known as Heat Shock Cognate – Yang and Tohda,
417 2018), while the augmented amounts detected following heat treatments represent
418 inducible proteins.

419 In the case of sHSP, the continuous increment evidenced by western blot was
420 consistent with that observed in the SDS-PAGE analysis. When applying heat treatment
421 with protective purposes, it is important to consider the half-life of the proteins, which was
422 estimated to be approximately 38 h (Puigderrajols et al. 2002).

423 Another method presented in this study, dot blot, proved adequate for the analysis
424 of HSP, considering its simplicity, specificity, and sensitivity, although its main limitation is
425 the lack of specific information on individual proteins, since no separation step is included.
426 This method can be adapted for use even in small laboratories, since no sophisticated
427 equipment is required.

428 Table 1 shows the performance of this technique to determine absolute and relative
429 amounts of HSP in tomatoes submitted to treatments of different intensity. Interestingly,
430 the basal amount of sHSP in the variety assayed in this study was similar to that measured
431 by our group in other tomato varieties (unpublished results). It remains to be determined
432 whether this finding can be extrapolated to other species and varieties, which would be
433 helpful to standardize the application of heat treatments. Quantitative data obtained by this
434 method can be employed with predictive and optimizing purposes, to develop

435 mathematical models of HSP induction, as a function of time and temperature exposure,
436 which would be helpful for the successful application of heat treatments in fruits.

437 Although heat treatment constitutes a promising technology to prevent the
438 development of chilling injury in sensible fruits and vegetables, there are still some
439 technical difficulties preventing its more extensive commercial application (Aghdam et al.,
440 2013). One of them is the narrow range of treatment intensity that separates a successful
441 treatment from a deleterious one (Polenta et al., 2006). Since the level of HSP properly
442 reflects the treatment intensity, we believe that this can be a suitable parameter to
443 implement process control strategies during the treatment application. Other aspects
444 leading to the successful application, such as the treatment uniformity, have been also
445 focused on other studies (Lu et al. 2010). It is expected that, by adjusting these and other
446 parameters, heat treatments could become a widespread technique in the future.

447

448 4.2 Effect of treatments on the development of chilling injury

449 The second part of the study was designed to validate the biochemical findings with
450 a practical experience, by using the developed method to assess the HSP profile in fruit
451 submitted to different stress treatments, applied to prevent chilling injury. To stimulate the
452 development of the latent damage induced during storage, fruits were exposed, after cold
453 withdrawal, for 4 days at 20°C (Aghdam et al., 2014). Results show that, immediately after
454 treatments, only fruit subjected to anaerobiosis for 6 days (ANA6d) had symptoms of
455 physiological damage, even before storage.

456 In turn, the storage of untreated tomatoes (Control) caused the appearance of
457 visible damage after 21 days. However, as also shown in previous studies (Wang et al.,
458 2015a) the application of short heat treatments (HS30' y HS60') prior to storage,
459 decreased the extent of damage, with fruit showing lower percentages of both pitting and
460 decay (Table 2). The beneficial effect of heat treatments and the consequent HSP

461 synthesis was previously shown in different investigations on tomatoes (Ré et al., 2017;
462 Luengwilai et al., 2012; Neta-Sharir et al., 2005). Results show that the effectiveness of
463 treatments was highly dependent on their application at an adequate intensity, since short
464 treatments were much more effective than long treatments, in spite of the higher
465 concentration of HSP attained. These results suggest that mechanisms other than HSP
466 are also involved in stress protection, evidencing an optimal range of intensity that is
467 effective to prevent the development of CI, with treatments beyond this region having a
468 deleterious effect (Aghdam et al., 2015). The development of effective monitoring systems
469 is of utmost importance for the successful application of this technology.

470

471 4.3 Practical implications of HSP analysis

472 The present study shows that SDS-PAGE + image analysis permits a simple
473 **estimation** of the level of sHSP induced by heat treatments, which can accurately reflect
474 the intensity of exposure. Therefore, it constitutes a useful tool for monitoring the induction
475 and continuity of the protecting effect of treatment during storage.

476 In turn, Western blot constitutes a useful and highly specific tool for monitoring
477 purposes. Proteins belonging to the HSP70 family, in particular, could be considered as a
478 universal tool to assess different stress conditions such as heat, drought, cold, chemicals,
479 and oxidants or pathogens, because of their evolutionary conservation (Ferradini et al.,
480 2015).

481 In the present research, HSP70 accumulation in treated tomatoes showed the
482 relationship between treatment intensity and protein concentration. Indeed, after exposing
483 the fruit to 72 h at 39° C, new proteins belonging to this family were detected.
484 Interestingly, anaerobic treatments were not able to induce the synthesis of HSP70,
485 indeed provoking the disappearance of some of the bands present in control samples.
486 Evidently, the biochemical mechanism associated with exposure to anaerobic stress,

487 which proved successful in other studies (Wang et al., 2015b) is different from heat stress,
488 and does not involve the synthesis of HSP70.

489 Regarding the sHSP group, HSPC1 antibodies had a significant cross-reactivity
490 with other members of this family (Figures 2A and 4C). This fact was also observed in
491 other species such as rice (Chen et al., 2014). Similarly to the HSP70 family, the level of
492 sHSP accumulation under heat stress depends on the temperature and the duration of the
493 exposure (Yang et al., 2014).

494 The present research highlights the relevance and practical applicability of the
495 simultaneous detection of HSP70 and sHSP, which are the most relevant HSP families in
496 plants, taking into account their cooperative role in the reestablishment of the cellular
497 homeostasis. In this regard, although studies on HSP have been traditionally carried out
498 separately, more studies focus on the synergistic action of different HSP families
499 (Hasanuzzaman et al., 2013). This universal mechanism of protein protection by HSP is
500 widely distributed among different prokaryotic and eukaryotic species and, therefore, the
501 analysis of these proteins is expected to become increasingly important in any study on
502 stress physiology and stress-based technologies such as chilling injury prevention.

503

504 **5 Conclusions**

505 HSP can be analyzed by different complementary analyses, since these proteins are
506 meaningful markers to optimize the application stress treatments in fruits. Techniques
507 included in the present investigation proved, to different extents, suitable for the
508 identification, estimation, and quantitation of the HSP70 and sHSP groups, which are the
509 most relevant HSP families in plants. The feasibility of the application of each method will
510 strongly depend on the availability of equipment and specific reagents (*ie.* PAGE, Western
511 blot, and IEF equipment and accessories, immunosera, etc.), as well as on the particular
512 objectives of the research. Although each technique has particular advantages and

513 limitations, they are effective to provide relevant information, which can be used for
514 scientific or technical purposes. Although this particular investigation was undertaken in
515 tomato fruit, it can be extended, with minor modifications, to different plant species and
516 tissues, especially for studies dealing with stress physiology. Research in this way is
517 currently underway in our lab

518

519

520 **6 Compliance with Ethics Requirements**

521 The authors declare that they have no conflict of interest. All institutional and national
522 guidelines for the care and use of laboratory animals were followed. Animals were
523 maintained under conditions that fulfilled all ethical and scientific requirements for animal
524 use included in EU Directive 2010/63/EU for animal experiments.

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529 manuscript.

530

531 **Legends to Figures**

532 **Figure 1:** (A) SDS / PAGE of protein extracts from tomatoes untreated (Control, C), or
533 treated for 3 (3 h), 20 (20 h), or 27 (27 h) hours at 38°C. (B) Densitometric analysis of the
534 low molecular weight region of the gel (indicated by a dotted line in Fig 1A). Proteins
535 showing an important increase are indicated by arrows. (C) IEF pattern of the 21 kDa band
536 excised from the SDS/PAGE shown in Fig 1A (indicated by arrow b) (D): Densitometric
537 analysis of the IEF gel corresponding to tomatoes untreated (C) or submitted to 38°C for 3
538 (3 h), 20 (20 h), or 27 (27 h) hours. The most prominent proteins (termed HSPC1 and

539 HSPC2), which also showed important increases with the duration of treatments, are
540 indicated by arrows. (E) Western blot analysis of the IEF of the 21 kDa band excised from
541 the SDS/PAGE as shown in (C).

542

543 **Figure 2:** Western blot analysis of protein extracts from tomatoes untreated (Control, C),
544 and treated for 3 (3 h), 20 (20 h), or 27 (27 h) hours at 38°C. Membranes were revealed
545 with antiserum of rabbit immunized with HSPC1 protein (A), or with commercial
546 monoclonal antibody anti-HSP70 (SIGMA, cat H5147).

547

548 **Figure 3:** Calibration standard curve used for the quantification of the dot blot analysis.
549 The calibration proteins used s were HSPC1 from a previous experiment,
550 electrophoretically purified from tomato (cv Colt 45), and electroeluted (A) or commercial
551 HSP70 purified from bovine brain (B - SIGMA, cat H9776).

552

553 **Figure 4:** (A): SDS / PAGE of protein extracts from tomatoes untreated (Control, C),
554 exposed at 38 °C for 30 min (HS30'), 60 min (HS60'), 72 h (HS72h), or at anaerobiosis for
555 3 (ANA3d) or 6 days (ANA6d) 27 (27 h) hours at 38°C. Western blot analysis was revealed
556 with antiserum of rabbit immunized with HSPC1 protein (B), or with commercial
557 monoclonal antibody anti-HSP70 (C) SIGMA (cat H5147).

558

Fig 1A

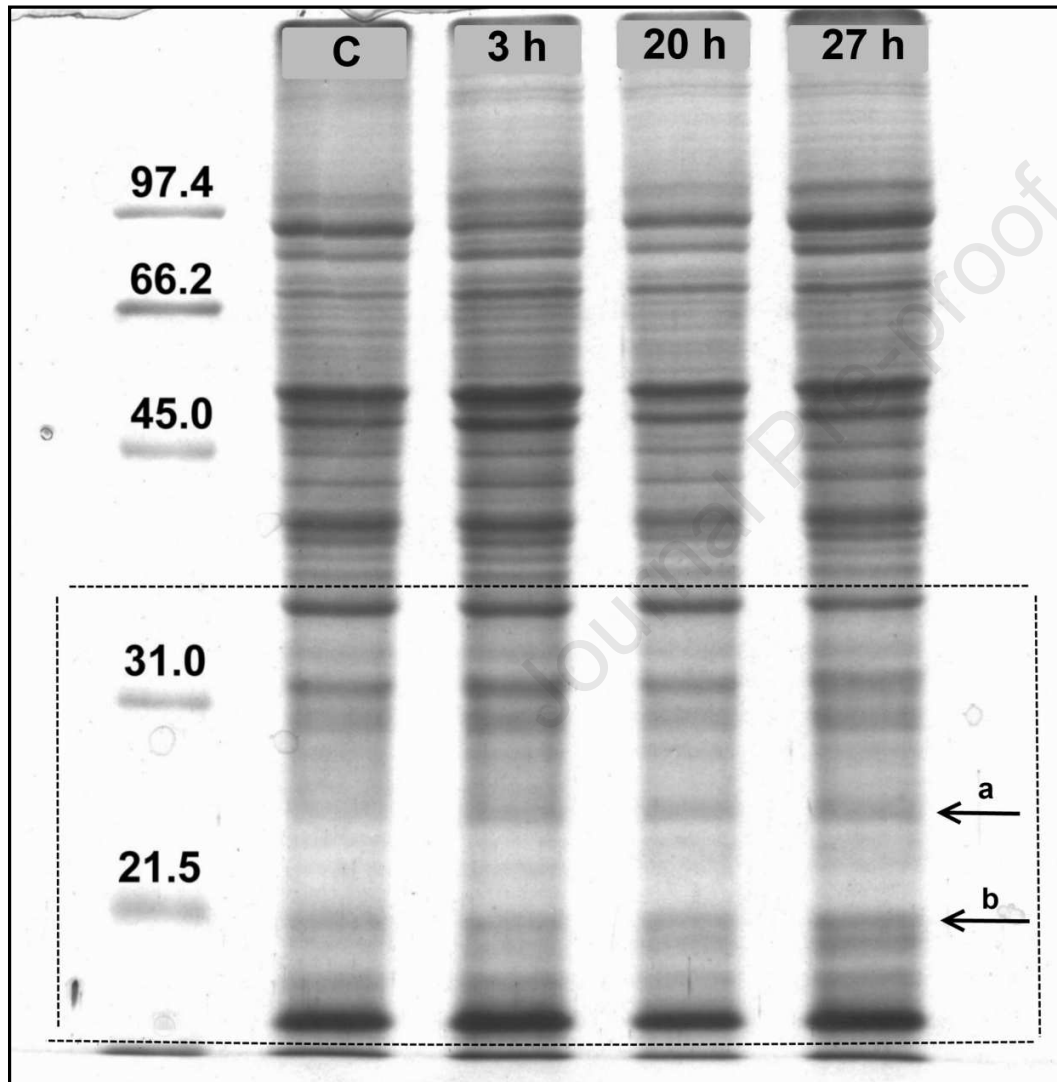


Fig 1B

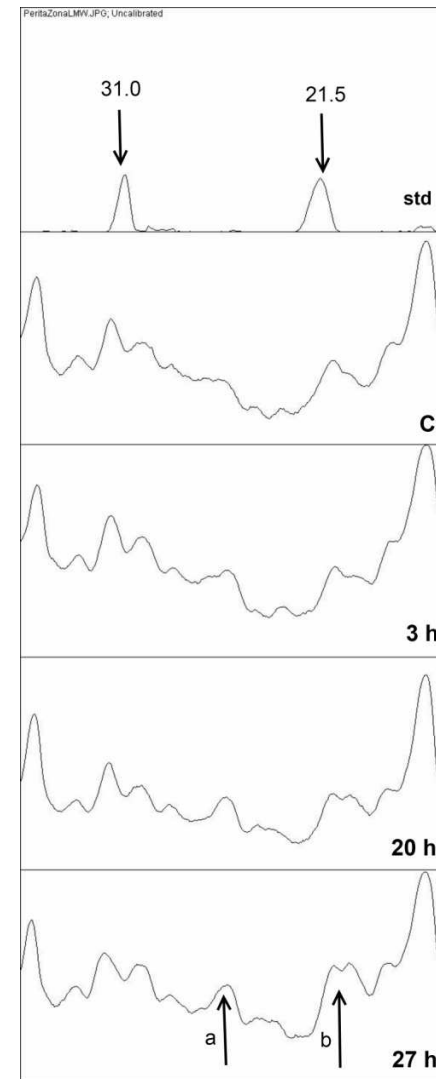


Fig 1C

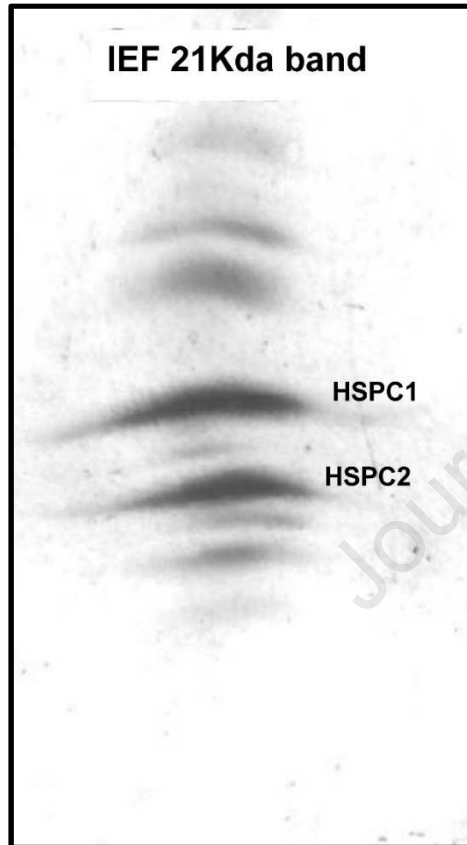


Fig 1D

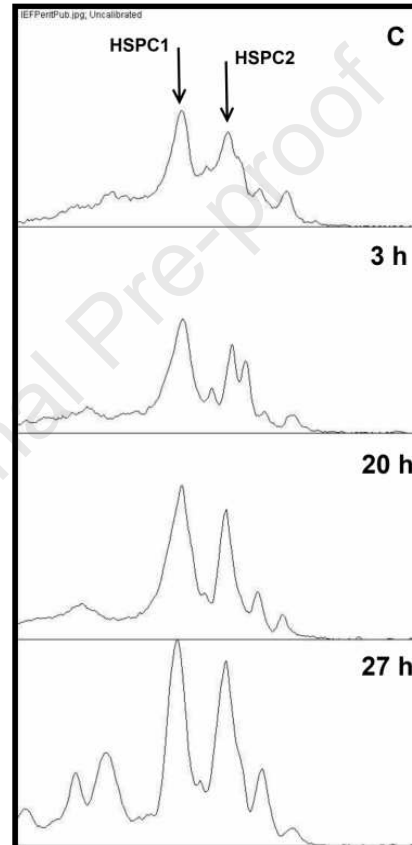


FIG 1E

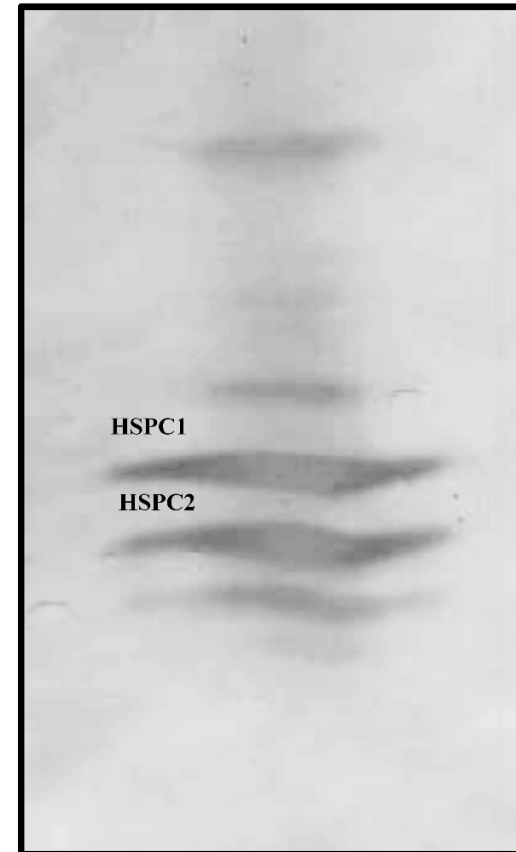


Fig 2

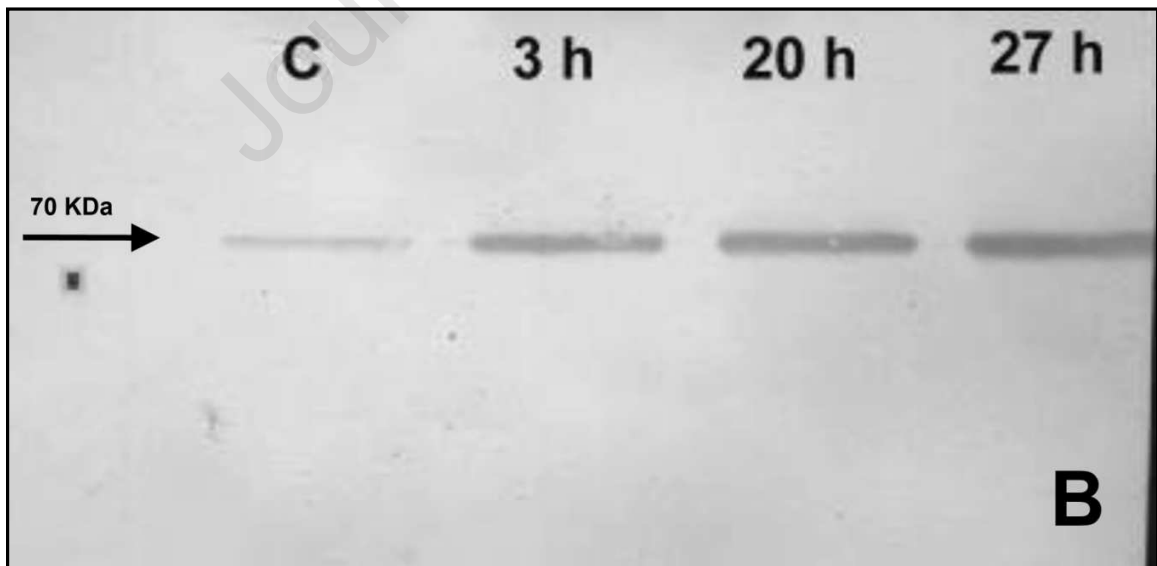
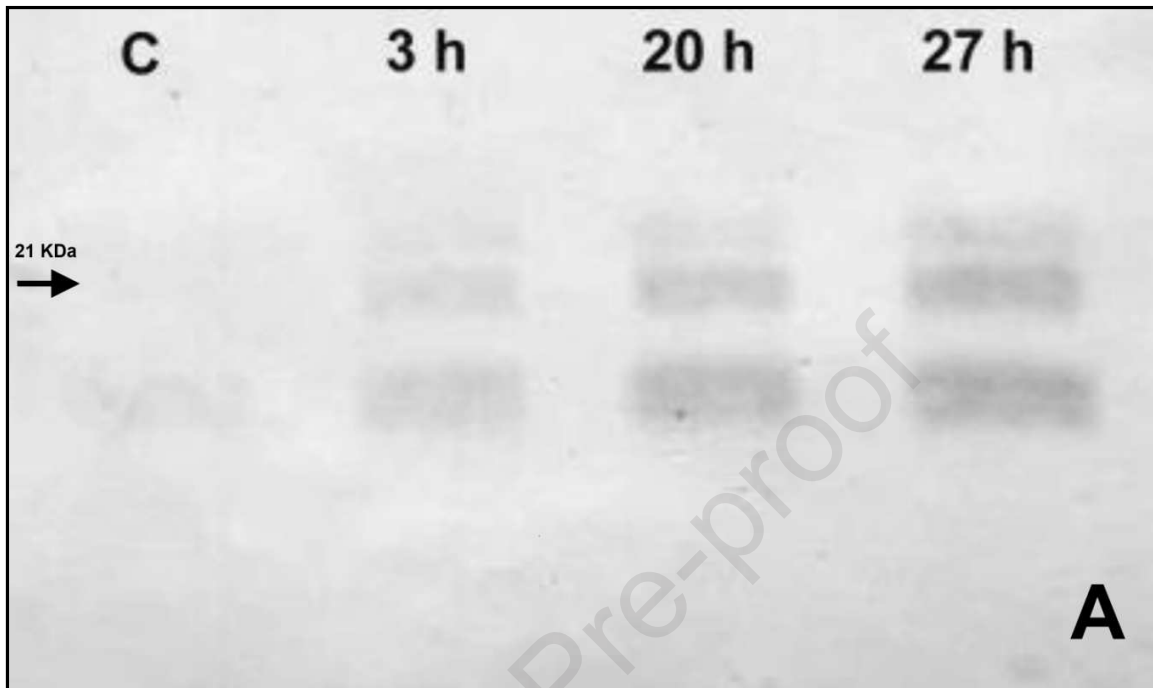


Fig 3 A

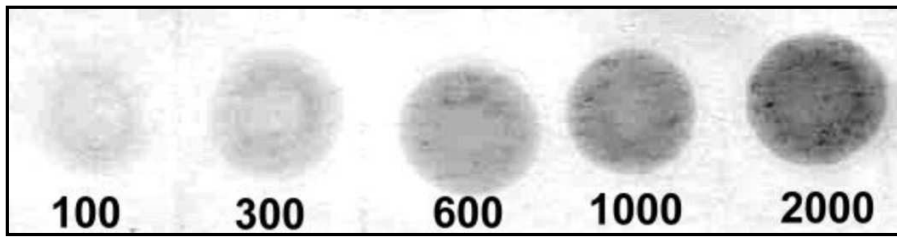


Fig 3 B

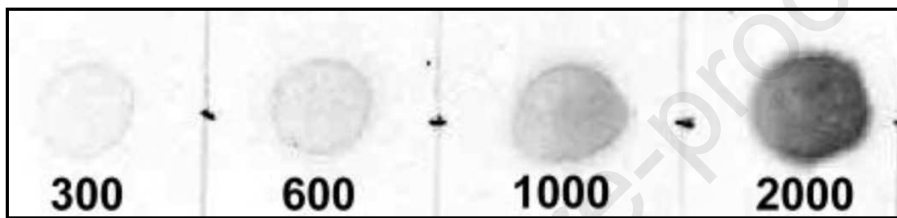


FIG 4A

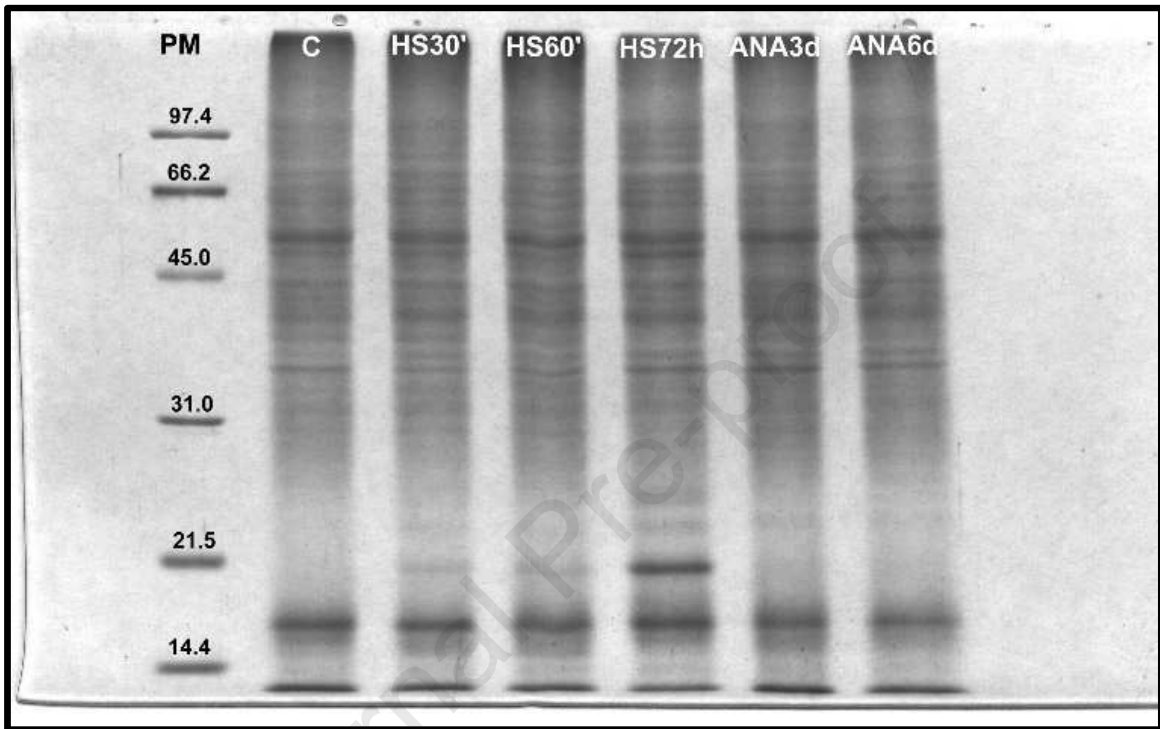


FIG 4B

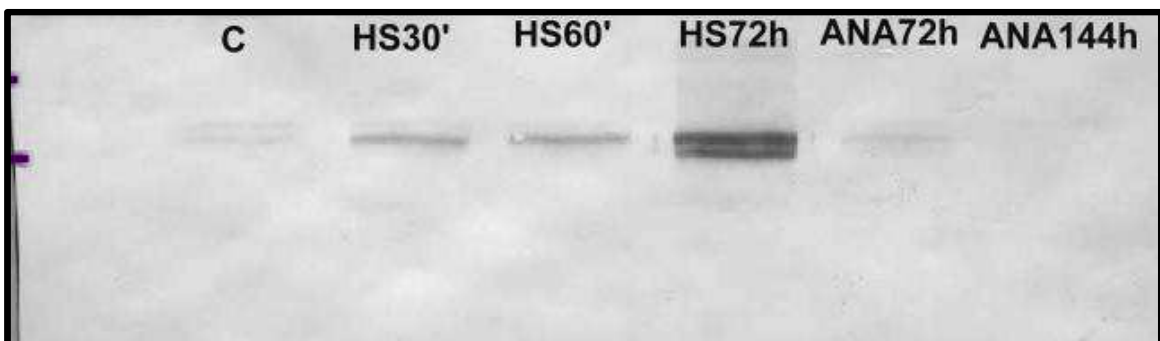


Fig 4C

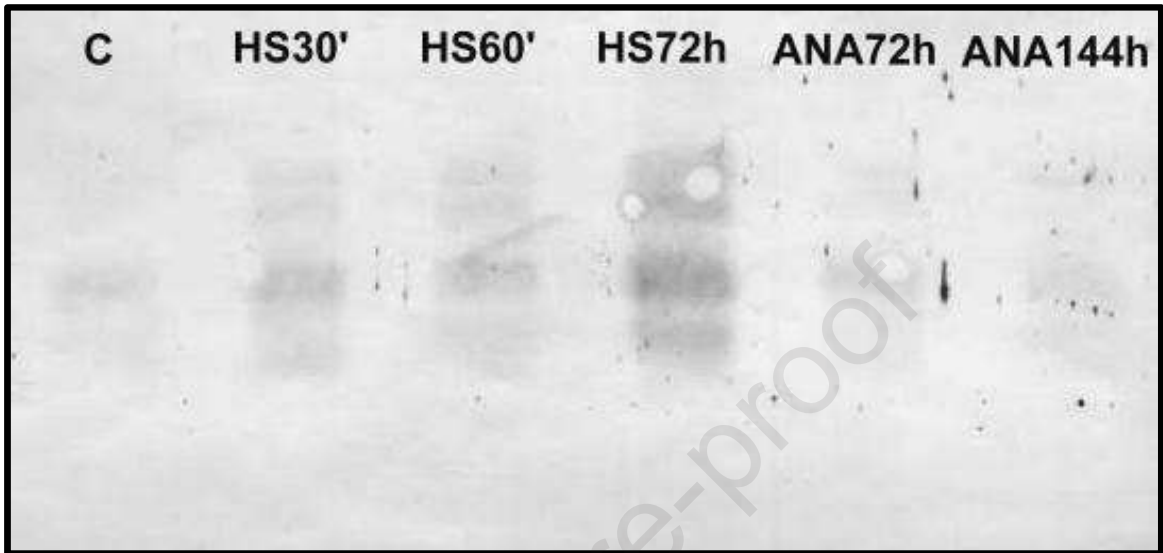


Table 1: Amount of proteins as quantified by dot blot revealed with antiserum obtained from rabbit immunized against HSPC1, or with a commercial monoclonal antibody (anti-HSP70). Analyses were carried out on 40 µg of total protein, and results are expressed as an absolute amount (ng of protein ± Std error) and amount relative to the initial amount present in untreated fruit (considered as 100%). Pearson correlation coefficient between treatment intensity (time in h) and protein amount was 0.91 (p<0.01) for the case of sHSP, and 0.95 (p<0.01) for the case of HSP70. CV: Coefficient of Variation.

		Treatment			
		Control	3 hours	20 hours	27 hours
sHSP	Absolute amount (ng)	645 ± 81	1653 ± 81	2473 ± 303	2768 ± 265
	Amount relative to control (%)	100	256	383	429
	CV (%)	12.8	5.2	19.3	14.7
HSP70	Absolute amount (ng)	899 ± 31	4726 ± 72	7406 ± 232	11023 ± 374
	Amount relative to control (%)	100	526	824	1226
	CV (%)	5.3	2.1	4.2	4.5

Table 2:

sHSP induction and chilling injury symptoms (spoilage and pitting) in tomatoes submitted to the different treatments. The absolute amount of proteins (ng of sHSP included in 40 μ g of total protein of the extract \pm Std error) as quantified with the antibody obtained by immunizing rabbits with HSPC1 protein and amounts relative to those present in untreated fruit (control), considered as 100%. Percentages of fruit with spoilage or with pitting in tomatoes subjected to the different treatments and stored for 20 days at 2° C, after 4 days of exposure to 20° C to induce damage.

Treatments	Absolute amounts of sHSP (ng)	Variation Coefficient (%)	Amounts relative to Control (%)	Immediately After			
				Treatment + 4 days at 20° C		21 days at 2° C + 4 days at 20° C	
				Spoiled Fruit (%)	Fruit w/pitting	Spoiled Fruit (%)	Fruit w/pitting
Control	616 \pm 63	14,8	100	0	0	12,5	6,25
HS30'	1545 \pm 149	13,9	251	0	0	0	0
HS 60'	1663 \pm 110	9,5	270	0	0	6,25	0
HS72hs	4130 \pm 300	10,5	670	0	0	100	NE
ANA3D	970 \pm 76	11,3	157	0	0	12,5	0
ANA6D	480 \pm 60	18,0	78	31,25	12,5	43,75	0

7 Bibliography

- Aghdam, M.S., Sevillano, L., Flores, F.B., & Bodbodak, S. (2015) The contribution of biotechnology to improving post-harvest chilling tolerance in fruits and vegetables using heat-shock proteins *Journal of Agricultural Science*. 153, 7–24.
- Aghdam, M. S., Asghari, M., Khorsandi, O., & Mohayjeji, M. (2014). Alleviation of postharvest chilling injury of tomato fruit by salicylic acid treatment. *Journal of food science and technology*, 51(10), 2815-2820
- Aghdam, M. S., & Bodbodak, S. (2014). Postharvest Heat Treatment for Mitigation of Chilling Injury in Fruits and Vegetables. *Food and Bioprocess Technology*, 7(1), 37-53.
- Aghdam, M. S., Sevillano, L., Flores, F. B., & Bodbodak, S. (2013). Heat shock proteins as biochemical markers for postharvest chilling stress in fruits and vegetables. *Scientia Horticulturae*, 160, 54-64.
- Basha, E., O'Neill, H., & Vierling, E. (2012). Small heat shock proteins and α -crystallins: dynamic proteins with flexible functions. *Trends in biochemical sciences*, 37(3), 106-117.
- Basile, A., Sorbo, S., Conte, B., Cardi, M., & Esposito, S. (2013). Ultrastructural changes and Heat Shock Proteins 70 induced by atmospheric pollution are similar to the effects observed under in vitro heavy metals stress in *Conocephalum conicum* (Marchantiales – Bryophyta). *Environmental Pollution*, 182, 209-216.
- Biswas, P, East, AR, Hewett, EW, and Heyes, JA. (2016) Chilling Injury in Tomato Fruit. In *Horticultural Reviews*, Volume 44, XLIV, First Edition. Book Editor: Janick, J. <https://doi.org/10.1002/9781119281269.ch5>

- Chen, X., Lin, S., Liu, Q., Huang, J., Zhang, W., Lin, J., Wang, Y., Ke, Y., & He, H. (2014). Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1844*(4), 818-828.
- Dunlap, D. Y., & Matsumura, F. (1997). Development of Broad Spectrum Antibodies to Heat Shock Protein 70s as Biomarkers for Detection of Multiple Stress by Pollutants and Environmental Factors. *Ecotoxicology and Environmental Safety*, *37*(3), 238-244.
- Efiuvwevwere, B., & Thorne, S. (1988). Development of chilling injury symptoms in stored tomato fruit (*Lycopersicon esculentum* Mill). *Journal Science Food Agriculture*, *44*, 215–226.
- El Assi, N. (2004). Alleviating chilling injury and maintaining quality of tomato fruit by hot water treatments. *Emir. J. Agric. Sci.*, *16*, 1–7.
- Ferradini, N., Iannaccone, R., Capomaccio, S., Metelli, A., Armentano, N., Semeraro, L., Cellini, F., Veronesi, F., & Rosellini, D. (2015). Assessment of Heat Shock Protein 70 Induction by Heat in Alfalfa Varieties and Constitutive Overexpression in Transgenic Plants. *PLoS ONE*, *10*(5), e0126051.
- Florissen, P., Ekman, J. S., Blumenthal, C., McGlasson, W. B., Conroy, J., & Holford, P. (1996). The effects of short heat-treatments on the induction of chilling injury in avocado fruit (*Persea americana* Mill). *Postharvest Biology and Technology*, *8*(2), 129-141.
- Hasanuzzaman, M., Nahar, K., Alam, M., Roychowdhury, R., & Fujita, M. (2013). Physiological, Biochemical, and Molecular Mechanisms of Heat Stress Tolerance in Plants. *International Journal of Molecular Sciences*, *14*(5), 9643.

- He, L., Chen, J., Kuang, J., & Lu, W. (2012). Expression of three sHSP genes involved in heat pretreatment-induced chilling tolerance in banana fruit. *Journal of the Science of Food and Agriculture*, *92*(9), 1924-1930.
- Hurkman, W. J., & Tanaka, C. K. (1986). Solubilization of Plant Membrane Proteins for Analysis by Two-Dimensional Gel Electrophoresis. *Plant physiology*, *81*(3), 802-806.
- Kassim, A., Workneh, T., & Bezuidenhout, C. (2013). A review on postharvest handling of avocado fruit. *African Journal of Agricultural Research*, *8*(21), 2385-2402.
- Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, *227*(5259), 680-685.
- Lee, D.-G., Ahsan, N., Kim, Y.-G., Kim, K.-H., Lee, S.-H., Lee, K.-W., Rahman, M., & Lee, B.-H. (2013). Expression of Heat Shock Protein and Antioxidant Genes in Rice Leaf Under Heat Stress. *Journal of The Korean Society of Grassland and Forage Science*, *33*(3), 159-166.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem*, *193*(1), 265-275.
- Lu, J., Charles, M. T., Vigneault, C., Goyette, B., & Raghavan, G. S. V. (2010). Effect of heat treatment uniformity on tomato ripening and chilling injury. *Postharvest Biology and Technology*, *56*(2), 155-162.
- Luengwilai, K., Beckles, D. M., & Saltveit, M. E. (2012). Chilling-injury of harvested tomato (*Solanum lycopersicum L.*) cv. Micro-Tom fruit is reduced by temperature pre-treatments. *Postharvest Biology and Technology*, *63*(1), 123-128.
- Miova, B., Dinevska-Kjovkarovska, S., Esplugues, J. V., & Apostolova, N. (2015). Heat Stress Induces Extended Plateau of Hsp70 Accumulation – A Possible

- Cytoprotection Mechanism in Hepatic Cells. *Journal of Cellular Biochemistry*, 116(10), 2365-2374.
- Neta-Sharir, I., Isaacson, T., Lurie, S., & Weiss, D. (2005). Dual Role for Tomato Heat Shock Protein 21: Protecting Photosystem II from Oxidative Stress and Promoting Color Changes during Fruit Maturation. *The Plant Cell*, 17(6), 1829-1838.
- Polenta, G., Budde, C., Sivakumar, D., Nanni, M., & Guidi, S. (2015). Evaluation of Biochemical and Quality Attributes to Monitor the Application of Heat and Cold Treatments in Tomato Fruit (*Lycopersicon Esculentum Mill.*). *Journal of Food Quality*, 38(3), 153-163.
- Polenta, G., Lucangeli, C., Budde, C., González, C. B., & Murray, R. (2006). Heat and anaerobic treatments affected physiological and biochemical parameters in tomato fruits. *LWT - Food Science and Technology*, 39(1), 27-34.
- Polenta, G. A., Calvete, J. J., & González, C. B. (2007). Isolation and characterization of the main small heat shock proteins induced in tomato pericarp by thermal treatment. *The FEBS Journal*, 274(24), 6447-6455.
- Puigderrajols, P., Jofré, A., Mir, G., Pla, M., Verdaguer, D., Huguet, G., & Molinas, M. (2002). Developmentally and stress-induced small heat shock proteins in cork oak somatic embryos. *Journal of experimental botany*, 53(373), 1445-1452.
- Ré, M., Gonzalez, C., Escobar, M., Sossi, M., Valle, E. and Boggio, S. (2017). Small heat shock proteins and the postharvest chilling tolerance of tomato fruit. *Physiol Plant*, 159(2), 148-160. doi: 10.1111/ppl.12491
- Santhanagopalan, I., Basha, E., Ballard, K. N., Bopp, N. E., & Vierling, E. (2015). Model Chaperones: Small Heat Shock Proteins from Plants. In M. R. Tanguay & E. L.

- Hightower (Eds.), *The Big Book on Small Heat Shock Proteins*, (pp. 119-153). Cham: Springer International Publishing.
- Sun, J.-h., Chen, J.-y., Kuang, J.-f., Chen, W.-x., & Lu, W.-j. (2010). Expression of sHSP genes as affected by heat shock and cold acclimation in relation to chilling tolerance in plum fruit. *Postharvest Biology and Technology*, *55*(2), 91-96.
- Sung, Y. Y., Liew, H. J., Ambok Bolong, A. M., Abdul Wahid, M. E., & MacRae, T. H. (2014). The induction of Hsp70 synthesis by non-lethal heat shock confers thermotolerance and resistance to lethal ammonia stress in the common carp, *Cyprinus carpio* (Linn). *Aquaculture Research*, *45*(10), 1706-1712.
- USDA. (1991). United States standards for grades of fresh tomatoes. US Department of Agriculture–Agricultural Marketing Service, Washington, DC.
- Wang, L., Baldwin, E. A., Zhao, W., Plotto, A., Sun, X., Wang, Z., Brecht, J. K., Bai, J., & Yu, Z. (2015a). Suppression of volatile production in tomato fruit exposed to chilling temperature and alleviation of chilling injury by a pre-chilling heat treatment. *LWT - Food Science and Technology*, *62*, 115-121.
- Wang, J. You, Y., Chen, W., Xu, Q . Wang, J. Liu, Y., Song, L., and Wu, J. (2015b). Optimal hypobaric treatment delays ripening of honey peach fruit via increasing endogenous energy status and enhancing antioxidant defence systems during storage. *Postharvest Biology and Technology*. 101, 1-9
- Waters, E. R. (2013). The evolution, function, structure, and expression of the plant sHSPs. *Journal of experimental botany*, *64*(2), 391-403.
- Yang, G., Wang, Y., Zhang, K., & Gao, C. (2014). Expression analysis of nine small heat shock protein genes from *Tamarix hispida* in response to different abiotic stresses and abscisic acid treatment. *Molecular Biology Reports*, *41*(3), 1279-1289.

- Yang, X. & Tohda, C. (2018). Heat Shock Cognate 70 Inhibitor, VER-155008, Reduces Memory Deficits and Axonal Degeneration in a Mouse Model of Alzheimer's Disease. *Front. Pharmacol.* 9:48. doi: 10.3389/fphar.2018.00048
- Zeng, J.-k., Li, X., Zhang, J., Ge, H., Yin, X.-r., & Chen, K.-s. (2016). Regulation of loquat fruit low temperature response and lignification involves interaction of heat shock factors and genes associated with lignin biosynthesis. *Plant, Cell & Environment*, 39(8), 1780-1789.

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Silvina Guidi: Investigation; Methodology; Data curation; Writing - review.

Vanina Ambrosi: Investigation; Methodology; Writing - review & editing.

Gabriela Denoya: Investigation; Methodology; Visualization; Writing - original draft and final editing.

Journal Pre-proof

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare they have no financial interests/personal relationships which may be considered as potential competing interests:



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