Running title: Genotype and organic farming effect on tomato phenolics and L-ascorbic

Polyphenol and L-ascorbic acid content in tomato as influenced by high lycopene genotypes and organic farming at different environments

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Abstract

The accumulation of polyphenols and L-ascorbic acid was evaluated under conventional (integrated pest management, IPM) and organic farming, as means to increase the accumulation of chemoprotective compounds. The effect of genotype was considerably higher than the growing system, in fact it is determining. 'Kalvert', a high-lycopene cultivar, outstood for the accumulation of most polyphenols, though low-carotenoid cultivars with high accumulation were also detected. Organic farming significantly increased the levels of caffeic acid by 20%, but reduced those of ferulic acid and naringenin by 13% and 15% respectively. A strong interaction with the environment was detected: in Navarra the differences were limited, while in Extremadura lower contents of ferulic acid and higher contents of chlorogenic acid and rutin were found in organic farming for certain cultivars. The effect of organic farming on L-ascorbic acid was dependent on cultivar and environment and it only led to an increase in Extremadura by 58%.

Keywords: *Solanum lycopersicum* L.; Organic farming; L-ascorbic acid; Functional quality.

Chemical compounds studied in this article Caffeic acid (PubChem CID: 689043); *p*- coumaric acid (PubChem CID: 637542); *trans*-ferulic acid (PubChem CID: 445858); Chlorogenic acid (PubChem CID: 1794427); Kaempferol (PubChem CID: 5280863); Quercetin (PubChem CID: 5280343); Myricetin (PubChem CID: 5281672); Naringenin (PubChem CID: 932); Rutin (PubChem CID: 5280805); L-Ascorbic acid (PubChem CID: 54670067)

Highlights

- The cultivar ‘Kalvert’ outstood for the accumulation of most of polyphenols
- Genotype has a major effect on the accumulation of studied chemoprotective compounds
- Strong environmental interactions were detected for phenolic content in certain cases
- Effect of organic farming on L-ascorbic was dependent on cultivar and environment

1. Introduction

Consumer awareness on the role of food in the improvement of health and in the prevention of many age-related diseases is becoming increasingly important. In this context, the main goal of aging research in not centered only in increasing lifespan but to improve health during life. Consequently, the development of better foods has become a major goal for the food industry. The fruit and vegetable market is well aware of these demands and tries to supply foods with increased levels of chemoprotective compounds and reduced levels of pesticides and, at the same time, assuring a production with a minimal impact on the environment.

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetable worldwide, both fresh and processed. Although tomato does not outstand for its nutritional value nor for the content of chemoprotective compounds, it has become one of the main contributors of healthy components to diet considering the high consumption levels of this product (Chun et al., 2005). Among tomato chemoprotective compounds, carotenoids, polyphenols and vitamin C play an important role in this species.

Carotenoids are responsible for the ripe color of tomatoes. Lycopene is the most abundant carotenoid and its concentration ranges from 18.6 to 65.0 mg kg^{-1} fresh weight (fw) in different tomato varieties (Martínez-Valverde, Periago, Provan, & Chesson, 2002). The second carotenoid of importance is beta-carotene, its concentration is much lower than lycopene, reaching concentrations up to 12 mg kg^{-1} fw (Galpaz, Ronen, Khalfa, Zamir, & Hirschberg, 2006). The intake of tomato carotenoids has been linked with the prevention of certain types of cancer, and especially with prostate cancer (Giovannucci, 1999). In 2007 the US Food and Drug Administration concluded that there was limited evidence supporting an association between tomato consumption and reduced risk of prostate cancer (Kavanaugh, Trumbo, & Ellwood, 2007). But later studies continue pointing out the chemoprotective role of tomato (reviewed by Martí, Roselló, & Cebolla-Cornejo, 2016).

79 The main phenolic compounds found in tomato are the flavonoids rutin, naringenin, naringenin
80 chalcone and quercetin and the hydroxycinnamic acids chlorogenic and caffeic acids (García-
81 Valverde, Navarro-González, García-Alonso, & Periago, 2013; Martínez-Valverde et al., 2002).
82 Kaempferol can be found in tomato, but only in certain materials and low concentrations (Martí
83 et al., 2016). Among them, rutin, a glycoside of quercetin, is the main tomato phenolic
84 compound, with concentrations up to 31.1 mg kg^{-1} fw at the mature red stage (García-Valverde
85 et al., 2013). Polyphenols are gaining importance during the last years as they seem to interfere
86 with the initiation, promotion and progression of cancer. In fact, several studies link the intake
87 of polyphenols and the protection against different types of cancer (reviewed by Martí et al.,
88 2016).
89 Among vitamins, vitamin C is one of the most important in tomato. In this species, vitamin C can
90 be found at concentrations ranging from 85.5 to 560.0 mg kg^{-1} fw in different cultivars (George,
91 Kaur, Khurdiya, & Kapoor, 2004). These contents are low compared to other crops such as
92 *Brassicas*, berries, pepper, kiwi, *Citrus* or strawberry. Nevertheless, tomatoes represent one of
93 the main sources of dietary intake of vitamin C in Mediterranean diets (Garcia-Closas et al.,
94 2004). Apart from being recognized as an important antioxidant, vitamin C has been linked with
95 the protection against cardiovascular diseases (reviewed by Raiola, Rigano, Calafiore, Frusciante,
96 & Barone, 2014).
97 One of the strategies followed to satisfy consumer demands of functional quality in tomato has
98 been led by breeders, who have developed tomato varieties with increased levels of these
99 compounds. Cultivars such as 'Doublerich' with increased vitamin C were commercialized from
100 the mid-20th century. But the success of such cultivars has been limited due to the high
101 dependency on growing conditions and some deleterious effects. Nevertheless, new variants
102 are developed and new sources of variation are continuously described (Leiva-Brondo et al.,
103 2012). The development of cultivars with high lycopene contents has been more successful,
104 especially because it was linked to another important objective of the processing industry:

105 increasing red color intensity. Several mutants have been used for this purpose, some of them
106 altering specific steps of the biosynthesis pathway, and other altering the regulation of the
107 pathway. Among them, the most efficient rely on the use of *high pigment (hp)* mutations
108 (reviewed by Cebolla-Cornejo, Roselló, & Nuez, 2013).
109 Breeding efforts for enhanced polyphenol content lag behind. Nevertheless, the use of *hp*
110 mutations was initially targeted to increase carotenoid contents but it showed as side effects
111 increased polyphenol and vitamin C contents (Sestari et al., 2014). Consequently, the use of this
112 type of cultivars has gained importance to satisfy the demands of quality markets.
113 Another strategy to improve functional value focuses on the control of the growing
114 environment. In this context, organic farming can both offer tomato fruits with no traces of
115 pesticides nor fertilizers and assure a minimum impact on the environment. Still, consumers of
116 organic food seem to be more interested in the perception of good health, nutrients and taste
117 than in environmental concerns (Hughner, McDonagh, Prothero, Shultsz II, & Stanton, 2007).
118 But, can organic farming really increase the contents of chemoprotective compounds? In the
119 case of carotenoids, contradictory results have been obtained. Caris-Veyrat et al. (2004)
120 obtained higher carotenoid levels under organic farming, Riahi et al. (2009) found no effect of
121 the growing system and Rossi et al. (2008) observed only lower lycopene contents under organic
122 farming. Regarding polyphenols and vitamin C, Hallmann (2012) observed higher contents of
123 these compounds under organic farming. Although in a later study total phenolic acids content
124 was not affected by growing system (only chlorogenic acid content was higher under organic
125 farming), total flavonoid content and quercetin and rutin contents were higher under organic
126 farming (Hallmann, Lipowski, Marszalek, & Rembialkowska, 2013). Vinha, Barreira, Costa, Alves,
127 & Oliveira (2014) also found higher lycopene, vitamin C, total phenolics and flavonoids under
128 organic farming.
129 The existence of uncontrolled factors limits the possibility to extract clear conclusions on the
130 effect of growing system on the accumulation of bioactive compounds. Thus, new experiences

with new environments and farmers are required. Of special interest is the performance of high-lycopene cultivars, which may offer increased accumulation of chemoprotective compounds. In a recent study, the role of high-lycopene cultivars and organic farming on the yield and quality of processing tomato was evaluated (Lahoz et al., 2016a). We found that the levels of lycopene were not affected by the growing system, while beta-carotene contents were higher under organic farming. The objective of this work is to deepen our knowledge on the accumulation of main tomato polyphenols and L-ascorbic acid, comparing the performance of standard and high-lycopene cultivars under organic farming and conventional Integrated Pest Management (IPM) at different environments.

2. Materials and methods

2.1. Chemicals and reagents

The polyphenols caffeic acid, *p*-coumaric acid, *trans*-ferulic acid, chlorogenic acid, kaempferol, quercetin, myricetin, naringenin and rutin, L-ascorbic acid, methaphosporic acid (MPA), hexadimetrine bromide (HDM), butylated hydroxytoluene (BHT), formic acid, HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Sigma-Aldrich (Steinheim, Germany). Boric acid and sodium hydroxide (NaOH) were provided by Panreac (Castellar del Vallés, Spain). Water was purified employing a Milli-Q water system (Millipore, Molsheim, France). Stock solutions of polyphenols were prepared at 500 mg L^{-1} in a MeOH/water (80:20 v/v) mixture and kept stored at -20 °C. The working solutions were prepared by direct dilution in MeOH/water (48:52 v/v). Stock solution of L-ascorbic acid was prepared at 1000 mg L^{-1} in water, stored at 4 °C.

2.2. Plant material and cultivation

A total of 6 commercial industrial tomato cvs. were grown in three different environments representing two Spanish growing areas: Extremadura (south-west of Spain), and Navarra

157 (north-east of Spain). In a first step the evaluation was conducted in both locations during 2012
158 and a second evaluation with different climatic conditions was performed during 2013 in
159 Navarra. Cvs. studied were: 'CXD-277' (Campbell's seeds), 'Heinz(H)-9661', 'H-9997', 'H-9036'
160 (Heinz Seed), 'ISI-24424' (Diamond seeds S.L.; Isi Sementi S.P.A.) and 'Kalvert' (Esasem S.P.A.).
161 'H-9036' and 'H-9661' were considered as standard controls because they are extensively used
162 by local producers for their good agronomical performance. The materials were selected
163 considering their accumulation of carotenoids in previous studies. 'H-9661' and 'H-9036' were
164 included as low lycopene cvs. , H-9997' and 'CXD-277' as cvs. with intermediate accumulation
165 and 'ISI-24424' and 'Kalvert' as high-lycopene cvs (Lahoz et al., 2016a).
166 For each growing system, a randomized complete block design with 3 blocks per condition was
167 used, with 25 plants per block and condition. For both growing systems, plants were drip-
168 irrigated. The fertilization doses applied, as well as phytosanitary treatments were those
169 typically employed in each cultivation site and system.
170 In the case of conventional management with IPM, the plantation in Navarra was carried out in
171 the research fields of INTIA in Cadreita (Navarra) on May 10th in 2012 and on 23rd May in 2013.
172 In Extremadura, the plantation was carried out in the fields of the research center Finca "La
173 Orden-Valdesquera" (Badajoz, Extremadura) on April 24th in 2012. For Navarra, a spacing of 1.60
174 m x 0.35 m and two plants per plug (3.57 plants m^{-2}) was applied under a 15 μm polyethylene
175 plastic. In Extremadura, the same growing procedures were applied, however the spacing was
176 1.50 m x 0.2 m (3.33 plants m^{-2}).
177 In the case of organic management, the plantation in Navarra was carried out in the fields of the
178 local organic farming business GUMENDI in Lodosa (Navarra) on May 4th in 2012 and on May
179 17th in 2013. The edaphoclimatic conditions of both fields in Navarra (conventional IPM and
180 organic) were similar and close geographically. The spacing employed was the same as in
181 conventional IPM but a 15 μm biodegradable plastic Mater-Bi® was employed instead

polyethylene plastic. In the case of Extremadura, the plantation was carried out on April 24th in 2012 in the research center "Finca La Orden-Valdesquera".

Conventional production and organic farming were performed following the regulations for Integrated Pest Management and Regulation on Organic Farming of each regional administration (Extremadura and Navarra respectively). Fertilization was calculated considering previous soil analysis (physical and chemical characterization and determination of mineral, nitric and ammoniacal nitrogen, Nmin, in the 0-60 cm soil depth profile) and crop extractions in each growing stage. For this purpose, mean yields of the used varieties in each area of cultivation were considered. In conventional management in Extremadura basal dressing included 50-90-90 kg ha^{-1} using a complex mineral fertilizer 8-15-15. Remaining dressing (until reaching the global 200-140-250 kg ha^{-1} recommendation for the area) was fertirrigated with a complex liquid fertilizer 20-15-15 and a weekly application considering dose/phenology and a mean expected yield in the area of 80 t ha^{-1}. For organic farming, basal dressing included 111-111-37 kg ha^{-1} applied using Agrimartin® FeBiologico (Fertinagro, Teruel, Spain). Remaining dressing was applied at the onset of flowering, maximum crop development and fruit setting. Blood meal, Protesan-15%N (ACP Europe, Ganollers, Spain) 125 kg N ha^{-1} and Patentkali® 30% $K_2$0/10% MgO/42% SO3(K+S Kali Gmbh Kassel, Germany) 700 kg ha^{-1} were used for this purpose. In conventional management in Navarra basal dressing included 54-138-180 kg ha^{-1}, applied using a complex mineral fertilizer 9-23-30. Remaining dressing once considered initial Nmin (146,6 kg N ha^{-1} in 2012 and 152,8 kg N ha^{-1} in 2013) until reaching the recommended 250 kg N ha^{-1} in this area was applied by fertirrigation using complex liquid fertilizer N32 (Herogra, Abolote, Spain). Weekly applications started at the fourth week of cultivation. In the case of organic farming basic dressing included 25 t ha^{-1} of ovine compost with a mean N richness of 15 kg t^{-1}.

Maximum temperature and relative humidity were recorded using a HMP45C probe (Vaisala, Helsinki, Finland) in Navarra and Extremadura, and solar irradiance was recorded using a 110/S

207 pyranometer (Skye, Powys, United Kingdom) in Navarra and a CMP3 pyranometer (Kipp&Zonen,
208 Delft, the Netherlands) in Extremadura.
209
210 **2.3. Sampling**
211 Considering commercial practices, tomato samples were collected (a single harvest of red-ripe
212 fruits for each cv. and growing system) when the 85% of tomato fruits of the plants were in the
213 commercial-red stage. Two representative red-ripe tomato fruits were taken from each of the
214 25 plants of the replicates. Later in the laboratory, samples were washed with tap water, and a
215 biological mean of each replicate was obtained blending longitudinal wedges of equivalent
216 weight from each tomato until a completely homogeneous sample was obtained. Then, it was
217 stored at -80 °C until analysis.
218
219 **2.4. Analysis of polyphenols**
220 Phenolic extraction was performed following the procedure described by Martí, Valcárcel,
221 Herrero-Martínez, Cebolla-Cornejo, & Roselló (2015). Briefly, 1 g of homogenized sample was
222 weighted and 5 mL of MeOH/water (48:52 v/v) containing 1 g kg^{-1} BHT were added. An ultrasonic
223 extraction was done by immersing the samples in an ultrasonic bath Elmasonic S30H (Elma
224 Electronics AG, Wetzikon, Switzerland) at a frequency of 60 Hz during 177 min. All extraction
225 procedure was done in absence of light to avoid the oxidation of target compounds. The
226 resulting extracts were centrifuged at 4000 rpm (2361g) during 5 min in an Eppendorf 5804R
227 refrigerated centrifuge at 4 °C (Hauppauge, NY, USA). Supernatants were filtered through a 0.2
228 µm pore size polytetrafluoroethylene (PTFE) filter before their analysis by High Performance
229 Liquid Chromatography (HPLC).
230 The separation and quantification of polyphenols was performed using an 1100 Series HPLC
231 system (Agilent Technologies, Waldbronn, Germany), equipped with a quaternary pump, a
232 degasser, an auto-sampler, and a diode array detector (DAD). The chromatographic column used

was a fused-core Kinetex-XB C18 column (150 mm x 4.6 mm internal diameter; particle size, 2.6 µm) from Phenomenex (Torrance, CA, USA). The chromatographic analysis was performed following the procedure described by Martí et al. (2015) with some modifications. The flow rate was kept at 0.8 mL min^{-1} and the sample injection volume was set at 10µL, all the separation procedure was performed at room temperature. The mobile phase solvents employed were water, ACN and MeOH, acidified with formic acid 1 mL L^{-1}. A multi-segmented gradient was performed varying MeOH and ACN concentrations from 30% and 0% to 24% and 18%, respectively, until minute 12, followed by a rise of MeOH concentration up to 30% at minute 13 maintaining ACN concentration at 18%, finally MeOH concentration was decreased from 30% to 20% meanwhile ACN concentration was raised from 18% to 30% until minute 20, finally the initial conditions were recovered for the next sample injection. Detection and quantification of polyphenols was performed using the DAD detector at different wavelengths depending on each polyphenol. Thereby, 255 nm was used for rutin, 290 nm for naringenin, 320 nm for caffeic, *p*-coumaric, ferulic and chlorogenic acids, and 365 nm for kaempferol, quercetin and myricetin. Absorption spectra were recorded for further identification of compounds. Peak identification was done by comparing the elution times and the recorded spectra, and when required, samples were spiked to support the identification. Samples were analyzed twice.

2.5. Analysis of L-ascorbic acid

The extraction of L-ascorbic acid was performed following the method described by Galiana-Balaguer, Roselló, Herrero-Martínez, Maquieira, & Nuez (2001) with some modifications. Homogenized samples were centrifuged at 12000 rpm (10483g) during 5 min at 4 °C using a 5415R centrifuge (Eppendorf, Hauppauge, NY, USA). Resulting supernatants were diluted 1/10 with a 20 g L^{-1} MPA solution and filtered through a 0.2 µm pore size cellulose acetate (CA) filter before analysis.

The quantitation of L-ascorbic acid was performed using an Agilent Technologies 7100 capillary electrophoresis system (Waldbronn, Germany) equipped with a diode array detector. Uncoated fused silica capillaries (32cm total length, 24cm effective length, 375 µm outside diameter, 50 µm internal diameter) from Polymicro Technologies (Phoenix, AZ, USA) were rinsed at 50 °C with NaOH 1M during 5 min, followed by 5 min of NaOH 0.1M and 10 min of water prior its first utilization. Before each working session, the capillary was flushed at 25 °C with running buffer during 30 min. Between runs, capillary was flushed with running buffer during 3 min. Running buffer was prepared daily in an 400mM boric acid solution containing 1 g L^{-1} HDM adjusted to pH 8. All solutions and buffers were filtered prior injection through a 0.2 µm pore diameter CA filter. Injection was performed hydrodynamically at 3400Pa for 5s. A voltage of -15kV at 25 °C was applied. Detection and quantification was performed at 254 nm.

2.6. Statistical analysis

The effects of environment (site of cultivation and year), genotype, growing system, and their interactions on polyphenol content were evaluated with a MANOVA test using SPSS 22.0 software (NYSE: IBM, Armonk, USA). Pillai trace test was used to calculate p-value. Individual ANOVAs and Tukey B multiple range tests were performed to complement the analysis. Additionally, to ease a comprehensive study of the effect of genotype and growing system on polyphenols, a graphical MANOVA Biplot representation was obtained for the three environments. Bonferroni circles were used to represent the confidence intervals ($\alpha = 0.05$), and their projection on each variable enable the identification of significant differences between groups. For the compounds in which the MANOVA biplot did not detect significant effects of the type of cultivation the vectors were marked in dashed lines. Multibiplot, a freeware licensed software by Vicente-Villardón was used to perform the Biplot analysis. Effects on L-ascorbic acid contents were analyzed separately with an ANOVA and Tukey B test, as the biosynthesis pathway is different.

3. Results and discussion

3.1. Effects on polyphenol profile: main effects

The MANOVA test showed a significant effect ($p<0.01$) on the phenolic profile for all the studied factors (environment, genotype and growing system), as well as their double and triple interactions involving the environment. The specific effects on each compound were then independently analyzed with ANOVAs (Table 1). In the case of kaempferol, contents were below the limit of quantitation in the samples analyzed, and it was not included in the tables.

The environment affected polyphenol content, with higher contents of chlorogenic and ferulic acids and lower levels of rutin in Extremadura than in Navarra in 2012 (Table 1). For the rest of polyphenols similar concentrations were found in both sites. In Navarra in the conditions of 2013 higher contents of caffeic acid and myricetin and lower contents of quercetin were observed.

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The contents of rutin in Navarra 2013 were lower compared to Navarra 2012.

The effect of environment within a year involves changes in the site of cultivation. This effect is extremely complex, as it implies differences in climate, soils, plant densities and even farmers. Regarding climate, Extremadura is usually characterized by higher solar irradiance and higher temperatures than Navarra, which is located in a higher latitude. Accordingly, plantation and harvest in Extremadura are traditionally earlier. In this particular year, the differences were not dramatic, though a higher number of days with maximum temperatures over 35 °C, as well as higher accumulated solar radiation and maximum temperatures, were recorded in this site of cultivation (Fig. 1).

309 It has been described that a dramatic increase of soluble phenolic compounds can be found as
310 a response of plants to the stress produced by high temperatures (Rivero et al., 2001), although
311 this study was performed under continuous stressing temperatures. Later, in a more detailed
312 study Gautier et al. (2008) found higher levels of rutin in tomatoes exposed to high temperatures
313 (32 °C) but only in higher irradiance conditions, while chlorogenic acid contents were not
314 affected by temperature nor irradiance. Our results seem to indicate that for the accumulation
315 rutin (a major polyphenol in tomato) intermediate conditions would be optimum, as it happens
316 for lycopene accumulation (reviewed by Cebolla-Cornejo et al., 2013). Higher temperatures and
317 irradiance levels such as those of Extremadura would limit the accumulation of rutin while the
318 accumulation of hydroxycinnamic acids would not be affected. Intermediate conditions would
319 favor the accumulation of rutin, at the expense of hydroxycinnamic acids, as in Navarra 2012.
320 On the other hand, a further reduction in both temperature an radiation (Navarra 2013) would
321 limit again the accumulation of major flavonols due to a limitation of photosynthesis, as
322 carbohydrates are the substrates for flavonoid biosynthesis via the shikimic acid and
323 phenylpropanoid pathways (Dorais, Ehret, & Papadopoulos, 2008).
324 Nevertheless, other explanations cannot be ruled out. Raffo, La Malfa, Fogliano, Maiani, &
325 Quaglia (2006) could not find a correlation between different polyphenols and climatic
326 parameters in cherry tomato, and in fact the accumulation of rutin and chlorogenic acid was not
327 correlated. It cannot be discarded that in our case, as Raffo et al. (2006) also suggested, other
328 uncontrolled factors (fertirrigation, plant density…) may have exerted a higher influence than
329 the temperature and irradiance.
330 As expected, genotype had an important effect on polyphenol accumulation, with the
331 exceptions of ferulic and *p*-coumaric acids (Table 1). 'Kalvert' clearly outstood for the
332 accumulation of most polyphenols, including chlorogenic acid (13.80 mg kg^{-1} fw), caffeic acid
333 (2.19 mg kg^{-1} fw), rutin (36.33 mg kg^{-1} fw), myricetin (2.86 mg kg^{-1} fw), quercetin (1.41 mg kg^{-1} fw)
334 and naringenin (11.89 mg kg^{-1} fw). Although other genotypes with lower polyphenol

335 accumulation, in some cases attained similar contents in certain compounds. For example, 'H-
336 9997' also presented high concentrations of quercetin and naringenin and intermediate of rutin,
337 and 'H-9661' also outstood for rutin.
338 In previous works, high accumulation of carotenoids in 'Kalvert' and intermediate levels in 'H-
339 9997' were reported (Lahoz et al., 2016a). The presence in these cvs. of a *hp* gene could explain
340 the concomitant high levels of polyphenols, as higher amounts of these metabolites have been
341 found in materials carrying *hp-1* or *hp-2* mutations (reviewed by Martí et al., 2016).
342 Nevertheless, in that study 'ISI-24424' also presented intermediate carotenoid accumulation
343 and, accordingly, higher contents of polyphenols would also have been expected. Still, it is
344 difficult to establish whether a cv. is *hp* or not, since the genes used in the development of each
345 cv. are not revealed by breeding companies. On the other hand, the identification of high
346 contents of rutin in 'H-9661', a cv. with low carotenoid content (Lahoz et al., 2016a), opens the
347 door to find alternative genes, other than *hp*, targeted to improve polyphenol content. Even
348 more, both strategies could be joined to further increase these contents.
349 The effect of growing system was more limited and it was significant only for caffeic and ferulic
350 acids and naringenin (Table 1). The accumulation of caffeic acid was higher (by 20%) under
351 organic farming, while the accumulation of ferulic acid and naringenin was higher (by 13% and
352 by 15%) in conventional IPM farming. Hallmann (2012) found higher amounts of phenolic
353 compounds in organic tomatoes. In their work, organic fruits accumulated higher amounts of
354 rutin, myricetin and quercetin in comparison with conventional IPM ones. Mitchell et al. (2007)
355 reported higher levels of quercetin and kaempferol under organic farming, suggesting that
356 plants with limited N accumulate more flavonoids than those that are well-supplied. Caris-
357 Veyrat et al. (2004) also found higher concentrations of rutin and naringenin in organic
358 tomatoes, but chlorogenic acid contents were higher in conventionally grown tomatoes. As an
359 explanation, Oliveira et al. (2013) suggested that the higher concentrations of sugars, vitamin C
360 and polyphenols that they found under organic production would be related to stressing

361 conditions resulting in oxidative stress, as phenylalanine ammonia lyase, cell membrane lipid
362 oxidation and superoxide dismutase activities were higher in fruits grown under organic farming.
363 In our case, the differences are limited, probably suggesting that other factors (i.e. farmers)
364 might be more important. On the other hand, Anton et al. (2014) found, as in our case, a limited
365 effect of growing system, and they reported that polyphenols were more dependent on year
366 and genotype effects. Nevertheless, the existence of strong environmental effects and
367 interactions implied the necessity to evaluate the effect of growing system specifically for each
368 environment.
369 We determined polyphenol content in raw samples, but it should be considered that some
370 authors have found no effect of processing on total phenolics (Dewanto, Wu, Adom, & Liu,
371 2002), while others such as Gahler, Otto, & Böhm (2003) obtained increased levels (they
372 suggested that a possible release of phenolics from the matrix might explain it). In any case, it
373 seems clear that the higher contents observed in raw material would be useful to increase the
374 functional value of processed tomato.
375
376 **3.2. Effects on polyphenol profile: interactions**
377 As the MANOVA revealed strong interactions, it was necessary to perform separate MANOVA
378 biplot analysis for each environment, in order to determine the performance of each genotype
379 and growing system in each one. The analysis of the differences between MANOVA biplots
380 reveals the interaction effects. These biplots revealed the superior performance of 'Kalvert' in
381 the accumulation of most polyphenols, independently of the site of cultivation (Fig. 2), revealing
382 a high stability in the trait. For the rest of the cvs., the performance was strongly dependent on
383 the environmental conditions.
384 Under milder climate conditions, as those in Navarra, minor differences were found for the
385 majority of the cvs. between conventional IPM and organic farming. Even among cvs. only
386 'Kalvert' outstood and in this case, the differences between both growing systems were limited,

387 as the projections of the Bonferroni confidence circles overlapped for most vectors (Fig. 2). It
388 should be mentioned, though, that in Navarra 2013, 'ISI-24424' under organic farming showed
389 a different performance with higher caffeic acid and lower naringenin contents, but this
390 response was not reproduced in the other environments.
391 In conditions with higher irradiance and temperature, as in Extremadura, the specific
392 performance of each cv. would be more clear. In fact, in the biplot for Extremadura a clear
393 differentiation can be observed among cvs. The performance of intermediate content genotypes
394 such as 'ISI-24424' and 'H-9661' is more similar to 'Kalvert', while 'H-9997' outstood for the
395 accumulation of naringenin and caffeic acid. Low content genotypes, 'CXD-277' and 'H-9036'
396 showed a much lower accumulation than the rest. In this site, the difference between organic
397 and convention cultivation is mainly due to the higher accumulation of ferulic acid, as for most
398 cvs. the differentiation depends on the second component, which is parallel to the vector of
399 ferulic acid. For the rest of the compounds with significant effects of the type of cultivation (solid
400 lines in Fig.2) the differences are limited. Only in certain cvs., such as 'H-9661', the projections
401 on chlorogenic or rutin revealed slightly higher accumulation under organic farming. Again, the
402 effect of the growing system seems limited compared to the effect of the genotype.
403
404 **3.3. Effects on L-ascorbic acid accumulation**
405 The environment and genotype had a significant effect on L-ascorbic acid concentration (Table
406 2). Higher accumulation was obtained in the conditions of Navarra 2012. Although other
407 explanations cannot be ruled out, it seems that the environment may justify this difference. As
408 reviewed by Dumas, Dadomo, Di Lucca, & Grolier (2003), light exposure is favorable to vitamin
409 C accumulation, and Liptay, Papadopoulos, Bryan, & Gull (1986) concluded that higher
410 temperatures (24°C vs. 31°C) enhanced ascorbic acid content. Therefore, the conditions of
411 Extremadura would favor accumulation. But, it has also been described that for similar
412 irradiance conditions, ascorbic acid concentration is lower at higher temperatures when the

413 range of temperatures is shorter (27°C vs. 32°C) and maximum temperatures are higher
414 (Gautier et al., 2008), as in our case. It seems that this may be the explanation for the higher
415 contents detected in Navarra, with less stressing temperatures. Regarding the differences
416 between Navarra 2012 and 2013, the lower contents of the latter would be caused by the lower
417 radiation and temperatures registered during this year (Fig. 1).
418 Regarding the genotype effect, L-ascorbic acid accumulation ranged from 107.54 to 136.37 mg
419 kg^{-1} fw. 'CXD-277' again was the cv. with the lowest accumulation. In this case, the low
420 accumulation of polyphenols and L-ascorbic acid of this cv. contrasts with the relatively high
421 accumulation of carotenoids and, especially, of sugar and acids observed in previous studies
422 (Lahoz et al., 2016a). On the other hand, the highest levels corresponded to 'H-9661' and
423 'Kalvert'. The high accumulation observed in 'Kalvert' might be related to the presence of a *hp*
424 mutation, as these mutations have been linked with increased levels of carotenoids, polyphenols
425 and ascorbic acid (reviewed by Martí et al., 2016) This cv. also offers a great accumulation of
426 sugars, acids and even aroma compounds (Lahoz et al., 2016a; Lahoz et al., 2016b), thus proving
427 to be an ideal cv. targeted to high quality markets. Nevertheless, the price premium paid should
428 compensate its lower yield compared to conventional cvs. such as 'H-9036' or 'H-9661') (Lahoz
429 et al., 2016a).
430 Growing system did not affect L-ascorbic acid accumulation (Table 2). Nevertheless, a strong
431 environmental effect and interactions were detected. In fact, in 2013 an outbreak of *Alternaria*
432 was detected, and consequently the results had to be analyzed independently for each
433 environment (Fig. 3). As a result, the effect of growing system in Extremadura (with higher
434 radiation and temperature) was significant, increasing mean cultivar L-ascorbic contents by 58%.
435 In the milder conditions of Navarra 2012 the effect was not significant, while in Navarra 2013 it
436 was negative and contents under organic farming were reduced by 45% (Fig. 3).
437 In Extremadura, the lower contents detected in conventional IPM growing might be related to
438 the fertilization applied. Dumas et al. (2003) described that high rates of nitrogen fertilizers tend

439 to decrease vitamin C content in tomato. In the same sense, Toor, Savage, & Heeb (2006) found
440 higher levels of ascorbic acid in tomatoes grown with organic fertilization than with mineral
441 nutrient solutions. But again, as stated above, the effect of organic farming may be also a
442 response towards stressing conditions under this management (Oliveira et al., 2013).
443 Several authors have observed a similar trend. Caris-Veyrat et al. (2004), Chassy, Bui, Renaud,
444 Van Horn, & Mitchell (2006) and Vinha et al. (2014) found considerably higher levels of ascorbic
445 acid under organic farming (higher than 30%, 23%, 30%, respectively). Other authors such as
446 Hallmann (2012) have also observed this effect, but much more limited (in this case the
447 difference was limited to a 1% difference). Similarly, Juroszek, Lumpkin, Yang, Ledesma, & Ma
448 (2009) found no significant differences in the content of ascorbic acid between conventional and
449 organic farming management. Maybe these conditions resemble those of Navarra 2012, where
450 the increase in 5.8% of L-ascorbic under organic farming was not significant.
451 On the other hand, Rossi et al. (2008) observed lower levels of vitamin C content under organic
452 farming (almost one half). This behavior is similar to that found in Navarra in 2013. Although the
453 reduction observed in ascorbic acid contents in Navarra 2013 should be carefully handled, as
454 following an *Alternaria* pathogen infection an oxidative burst reaction characterized by the rapid
455 production of reactive oxygen species (ROS) occurs, and defense mechanisms including
456 ascorbate peroxidase activities are triggered to scavenge the excess of H_2O_2 (Meena et al., 2016).
457 Consequently, a reduction in the pool of ascorbate would be expected when oxidative damage
458 occurs (Ding et al., 2009). It is impossible to determine if the reduction observed in this
459 environment was due to the growing system or to the infection. Nevertheless, it seems clear
460 that organic farming with reduced preventive and curative measures will be exposed to
461 reductions in L-ascorbic contents.
462 Apart from the obvious great differences between both cultivations systems and their
463 application by different research groups or farmers, the specific selection of genotypes for each
464 study may play an important role to justify these differences, as Caris-Veyrat et al. (2004) found

that some varieties may increase ascorbic acid levels under organic farming, while others remained with similar levels. This trend was also observed in our study, with a higher impact of organic farming on the contents of 'CXD-277' than in the rest of cultivars (Fig. 3).

The benefits of genotype selection and growing environment on processing tomato may be useful only for certain products. Vitamin C is unstable at high temperatures, therefore processing tends to reduce its contents. Dewanto et al. (2002) and Gahler et al. (2003) confirmed the decline in vitamin C with increasing heating time and processing steps in different tomato products. Nevertheless, Gahler et al. (2003) observed that in some products this decline can be compensated by the loss of water and an increase in dry matter. In any case, the higher contents observed in raw material would, again, be useful to increase the functional value of processed tomato.

4. Conclusions

The use of high lycopene cvs. such as 'Kalvert' can offer increased levels of polyphenols and L-ascorbic acid, that joined to the high levels of carotenoids, sugars and acids and aroma volatiles, positions this type of materials as ideal to satisfy the demands of high-quality markets. Nevertheless, considerably amounts of polyphenols and L-ascorbic acid can be also detected in conventional cvs. with higher yields. The genotype effect has a considerably higher impact on the accumulation of these chemoprotective compounds than the growing system selected. Organic farming has a limited effect on the accumulation of polyphenols, which is highly dependent on the site of cultivation. On the other hand, organic farming increases L-ascorbic acid contents, though this increase again depends on the cultivar and site of cultivation considered.

Conflict of interest

The authors declare no conflict of interest.

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621

622

623 **Table 1.** Effect of the site of cultivation, genotype and growing system on polyphenol content expressed in mg kg^{-1} of fresh weight

		Chlorogenic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Rutin	Myricetin	Quercetin	Naringenin
Environment	*p value*	<0.001	<0.001	0.036	<0.001	0.001	<0.001	<0.001	0.196
(S)	Extremadura 2012	13.90^{b}*	0.98^{a}	0.32^{b}	0.61^{b}	20.36^{a}	0.85^{a}	1.08^{b}	9.86^{a}
	Navarra 2012	6.91^{a}	0.94^{a}	0.30^{ab}	0.46^{a}	25.86^{b}	0.81^{a}	0.94^{b}	8.83^{a}
	Navarra 2013	8.10^{a}	1.82^{b}	0.22^{a}	0.48^{a}	21.21^{a}	1.82^{b}	0.49^{a}	8.86^{a}
Genotype	*p value*	<0.001	<0.001	0.510	0.025	<0.001	<0.001	<0.001	<0.001
(G)	'CXD-277'	7.53^{a}	0.82^{a}	0.26^{a}	0.48^{ab}	14.74^{a}	0.86^{ab}	0.48^{a}	7.53^{a}
	'H-9661'	9.66^{a}	0.97^{ab}	0.29^{a}	0.53^{ab}	29.72^{c}	0.64^{ab}	0.64^{a}	8.72^{a}
	'H-9997'	8.85^{a}	1.15^{b}	0.28^{a}	0.51^{ab}	20.63^{b}	1.12^{b}	1.14^{bc}	11.75^{b}
	'H-9036'	8.81^{a}	0.97^{ab}	0.35^{a}	0.61^{b}	12.39^{a}	0.17^{a}	0.52^{a}	6.49^{a}
	'ISI-24424'	9.15^{a}	1.37^{c}	0.25^{a}	0.42^{a}	21.06^{b}	1.30^{b}	0.81^{ab}	8.71^{a}
	'Kalvert'	13.80^{b}	2.19^{d}	0.26^{a}	0.54^{ab}	36.33^{d}	2.86^{c}	1.41^{c}	11.89^{b}
Growing system	*p value*	0.155	<0.001	0.054	0.031	0.749	0.509	0.656	0.008
(C)	Conventional	9.31	1.13	0.25	0.55	22.29	1.21	0.86	9.90
	Organic	9.96	1.36	0.31	0.48	22.67	1.11	0.81	8.46
SxG	*p value*	0.12	<0.001	<0.001	0.001	<0.001	<0.001	0.068	0.011
SxC	*p value*	<0.001	<0.001	0.124	0.021	<0.001	0.115	0.050	0.284
GxC	*p value*	0.075	<0.001	0.358	0.015	0.012	0.050	0.169	0.436

*Different letters indicate significant differences at p <0.05 (Tukey B test).

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625

626 **Table 2.** Effect of the site of cultivation, genotype and growing system on L-ascorbic acid
627 content expressed in mg kg^{-1} of fresh weight

	p value		L-ascorbic acid
Environment (E)	<0.001	Extremadura 2012	113.94[a]*
		Navarra 2012	155.82[b]
		Navarra 2013	100.56[a]
Genotype (G)	0.007	'CXD-277'	107.54[a]
		'H-9661'	136.37[b]
		'H-9997'	117.19[ab]
		'H-9036'	132.11[ab]
		'ISI-24424'	112.40[ab]
		'Kalvert'	135.02[b]
Growing system (C)	0.933	Conventional	123.21
		Organic	123.67
ExC	<0.001		
ExG	0.002		
CxG	0.106		
ExGxC	0.318		

*Different letters indicate significant differences at p <0.05 (Tukey B test).

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629

630 **Figure 1.** Climate conditions in Navarra (Nav 2012 and Nav 2013) and Extremadura (Ext 2012) during the
631 cultivation periods. Vertical lines indicate plantation (P) and harvest (H) dates in Navarra (N) or
632 Extremadura (E) during the year 2012 (12) or 2013 (13) and under conventional (C) or organic farming (O)
633 management. When starting (s) end ending dates (e) were different, it is indicated with lower case letters.
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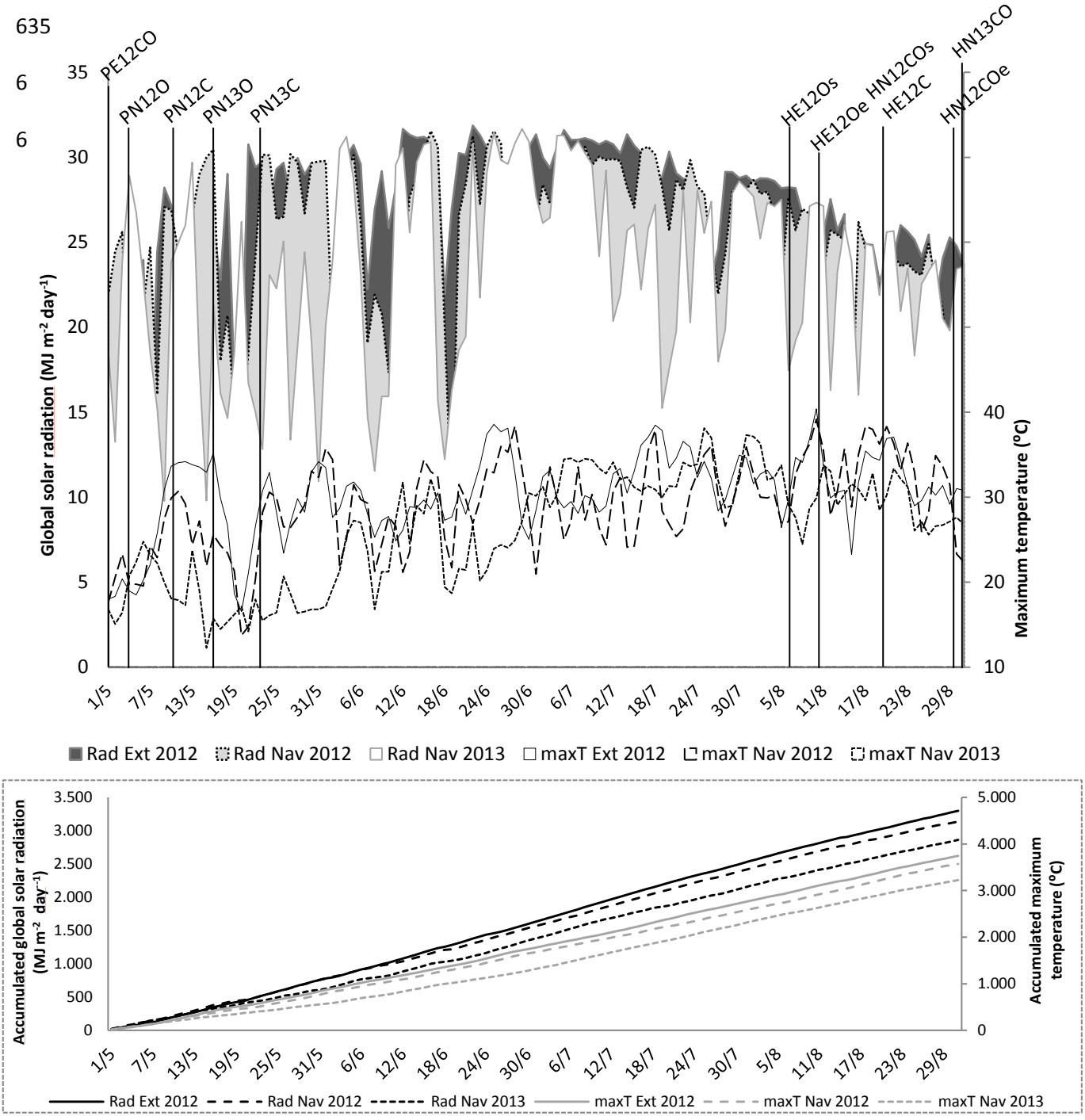
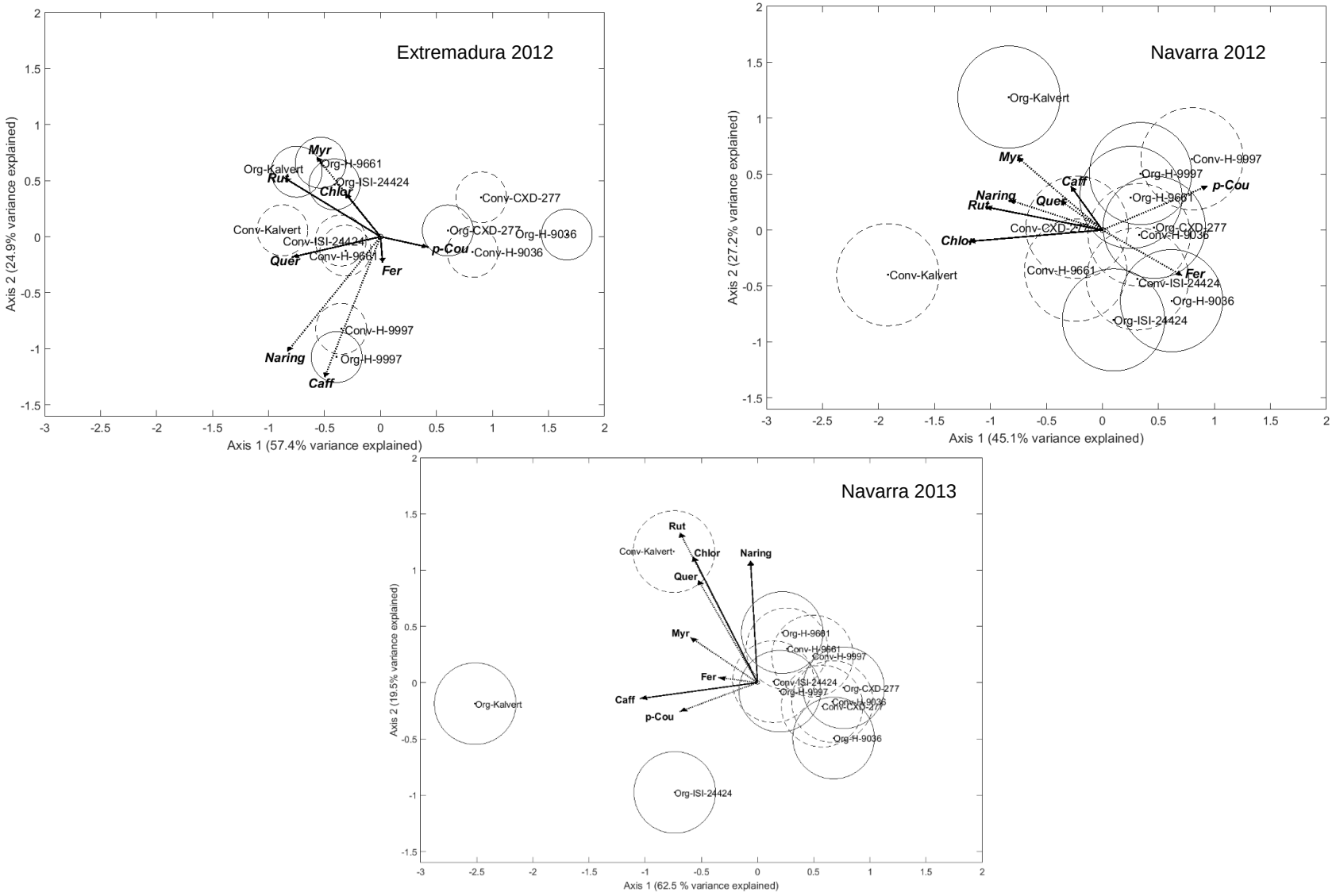


Figure 2. MANOVA biplot for polyphenol accumulation in Extremadura 2012, Navarra 2012 and Navarra 2013 under conventional (Conv-cv. name) or organic farming (Org-cv. name). Polyphenol abbreviations: chlorogenic acid (Chlor), caffeic acid (Caff), *p*-coumaric acid (*p*-Cou), ferulic acid (Fer), rutin (Rut), myricetin (Myr), quercetin (Quer), naringenin (Naring). Circles represent Bonferroni confidence intervals. Circles with solid lines correspond to organic farming and circles with dashed lines correspond to conventional farming. Significance of differences is inferred when the projections of confidence circles on each vector do not overlap. Solid vector lines represent significant effects of the type of cultivation and dashed lines represent no significant effects of this factor.



644 **Figure 3.** Mean increase (%) of L-ascorbic acid content when the plants were cultivated under organic
645 farming compared to conventional management. Left: Mean effect of the cultivar in each environments.
646 Right: Mean effect of the environment (Extremadura 2012, Navarra 2012 and Navarra 2013) considering
647 all the cultivars.**ANOVA *p*-value<0.001; ns not significant.

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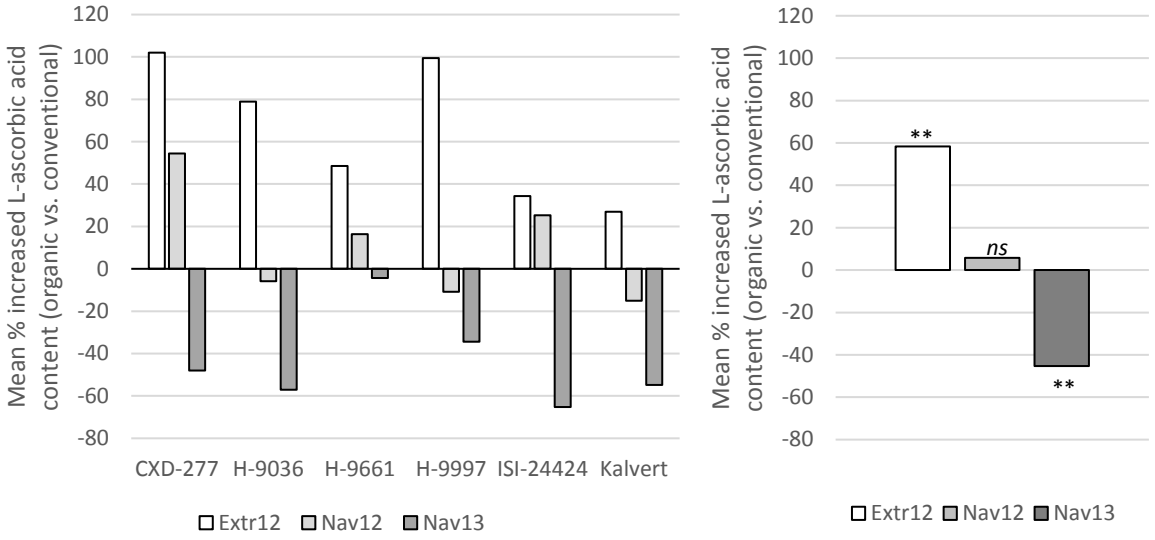

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