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Variation of flavonoids in a collection of peppers (*Capsicum* sp.) under organic and conventional cultivation: effect of the genotype, ripening stage and growing system

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ABSTRACT

BACKGROUND. In the last years, the acreage for organic agriculture and the demand for organic fruits and vegetables have increased considerably. Regarding this scenario, landraces can provide valuable germplasm, such as *Capsicum* landraces. *Capsicum* peppers are very interesting for their high content in phenolics, particularly flavonoids, which provide a high added value. Moreover, the broad genetic diversity in local varieties expands the opportunities for adaptation to organic and to exploit genotype×environment interaction to select materials with the highest content in phenolics. **RESULTS.** In this work, the main flavonoids of peppers were exhaustively evaluated over two years in a wide collection of heirlooms, both unripe and fully ripe, under organic and conventional cultivation. Genotype and ripening stage highly contributed to the variation of flavonoids. The growing system influenced to the variation to a lesser extent. Luteolin and quercetin showed the highest contributions to total phenolics (70% and >20%, respectively) at both ripening stages, while myricetin, apigenin and kaempferol showed lower levels. Average content of flavonoids was higher in ripe fruits, and organic management significantly increased the accumulation of total flavonoids and luteolin. Positive correlations between flavonoids were found at both ripening stages, especially between main flavonoids luteolin and quercetin and between kaempferol and quercetin ($\rho>0.7$). **CONCLUSION.** A remarkable genotype×environment interaction enabled the identification of accessions with high flavonoid content grown under organic conditions at both ripening stages, particularly total flavonoids and luteolin at fully ripe stage. Our results reinforce the importance of a wide genetic variation and considering different ripening stages and growing conditions for breeding peppers quality.

***Key words*:** HPLC, antioxidants, sustainable agriculture, quercetin, luteolin, kaempferol

INTRODUCTION

Human consumption habits are constantly changing depending on social, historical and/or environmental factors. Nowadays, the demand for products coming from sustainable production systems like organic agriculture is increasing remarkably. The organic cultivated area in Europe has increased from 6.8 million hectares in 2005 to 13.5 million hectares in 2016.[1] The main reasons for such trend in consumers are: i) to prevent excessive phytosanitary treatments and fertilizers in order to decrease the impact on agroecology equilibriums and contributing to a more sustainable use of resources; ii) the assumption that organically-produced fruits and vegetables are healthier and richer in nutraceutical compounds.[2–5]

By contrast, plant breeding, particularly in vegetables, has been traditionally focused on conventional and highly intensive agricultural practices. Therefore, there is a need of adaptation and improvement of plant materials to organic agriculture, as in fact most cultivars are modern F_1 hybrids whose breeding process has been mainly carried out under high-inputs conditions. In this regard, traditional varieties (i.e. ecotypes, landraces and heirlooms) can be considered very interesting plant resources as starting point in organic breeding. Thus, they have been cultivated during generations of farmers and evolved and adapted to the low-input conditions, typical of traditional growing systems, particularly cultivars grown before the green revolution.[6] Moreover, in contrast to modern F_1 materials, they may show several traits of interest like resistances to local diseases and higher nutritional and organoleptic qualities highly appreciated by consumers (i.e. taste-of-the-past).[7] Furthermore, these plant resources hold a huge genetic diversity, which is essential to attempt any breeding program, and also to promote their use enables the conservation of agrodiversity and mitigates the genetic erosion of plant resources.[8]

In this frame, *Capsicum* peppers represent a good case of study. Native peppers from America (or chillies), are grown worldwide since they were introduced to Europe since the fifteenth century and later to Africa and Asia in the sixteenth century, with a current harvested area of about 4 million ha of both dry chillies and fresh peppers.[9] They also encompass a wide diversity of varietal types and genotypes. Thus, cultivated peppers belong to five species of genus *Capsicum*: three from the *annuum* complex, which includes the most economically important species *C. annuum* (sweet and chilli peppers, e.g. bell types, jalapenos, serranos, anchos, pasillas and many cayenne peppers), *C. chinense* (e.g. Bhut jolokia and Habanero) and *C. frutescens* (e.g. Tabasco), and other two species, mainly grown in the Andean region, *C. baccatum* (South American *ají*) and *C. pubescens* (rocoto in Andean countries or *Manzano* in Mexico).[10,11] As a gateway to Europe, Spain became a hot spot of diversity for *C. annuum* due to its great heterogeneity in agroclimatic conditions and culinary uses, arising a plethora of ecotypes in all the regions of the peninsula and islands (Canary and Balearic). In fact, nowadays Spain has an extensive list of protected designations of origin (PDOs) and protected geographical indications (PGIs), much larger than other vegetables.[7,12]

Moreover, the fast popularity and rapid spreading of peppers from America were due to their double use as vegetable and/or spice as well as unripe and fully ripe stages.[13–15] Later, *Capsicum* fruits were found to have a high nutritional value due to their content in bioactive compounds such as vitamins (A, B complex, C and E), carotenoids and, particularly, phenolic compounds.[16–18]

In this regard, phenolic compounds are secondary metabolites naturally produced by plants which have a range of functions in plants related to plant development, structural integrity and colour and sensory characteristics as well as protection against predators, infections and plagues.[19–21] Furthermore, it has been described that the content in

phenolics in several crops may be influenced by genetic and agroclimatic factors.[22–24] The contribution of these bioactive compounds in human diet is highly appreciated thanks to their scavenging-derived metabolites and antioxidant functions, such as anti-inflammatory, antiatherosclerotic and antitumoral properties or against diseases.[25–27]

According to previous reports, the main group of phenolics in *Capsicum* pods with antioxidant properties are flavonoids which have a structure of two phenyl rings and one heterocyclic ring (C6-C3-C6),[28] of which quercetin, luteolin, myricetin, kaempferol and apigenin are the most common compounds.[29–31] The objective of the present study was to assess the content of the main phenolic compounds in a comprehensive collection of varietal types of pepper and chilli in two growing conditions, organic and conventional management, as well as the changes in the profile of phenolics due to the fruit ripening in different years. To our knowledge, this is the first exhaustive study on the effect of organic conditions, ripening stage, genotype and their interactions on the accumulation of the main antioxidant phenolics in a broad collection of *Capsicum* varietal types and species.

MATERIAL AND METHODS

Plant material

This work has been developed in a collection of 14 *Capsicum* accessions including *C. annuum* (12), *C. chinense* (1) and *C. baccatum* (1). The collection encompassed a wide diversity of morphotypes and geographical origins, including ecotypes of PDOs and PGIs (Table 1. Appendix 1a. Appendix 1b).

Pepper cultivation

The trials were carried out during two consecutive growing seasons (2015 and 2016). Plants were grown under open field conditions in a spring-summer growing cycle, in both organic and conventional systems. Both plots were located in Sagunto (Valencia, Spain). The organic plot was within the protected area "Marxal dels Moros" (UTM coordinates, X: 734494.88 and Y: 4390434.86) while the plot managed under conventional conditions was located close to this protected area (X: 732900.40 and Y: 4391754.37). In this way, agroclimatic and environmental characteristics (i.e. climate, soil texture) were similar in both plots. Organic and conventional plots were distributed in ridges and plants were transplanted on the top of the ridges at a distance of 0.5 m within ridge and 1 m between ridges. Plants were irrigated by furrow irrigation three times per month during spring months and four times per month in summer months.

In the soil of the organic plot and prior to our trials, a crop rotation every four years was applied, and for this work before each trial sheep manure was applied as fertilizer (4 kg/m^2). Treatments for pest control were not necessary in organic plot since micro-fauna balance was established after years of organic management in the "Marxal dels Moros" area. Adventitious plants were controlled by hand clearing (once per month).

By contrast, the conventional management included fertilization based on several inorganic products. Thus, a mix of nitrogen, phosphorus and potassium (15-15-15; 50 g/m^2) was applied before transplanting, and after transplanting iron chelate (one application of 3 kg/1000 m^2) and calcium nitrate (one application at 20 g/L and two applications at 10 g/L). Pests were controlled using abamectin (1.8%, EC) and chlorpyrifos (48%, EC) as pesticides in combination with copper oxychloride (58.8% WP) as fungicide. Thus, chlorpyrifos (50 cc) was combined with copper oxychloride (100 cc) in 20 L of water (six times per season) and abamectin (30 cc) with copper

oxychloride (100 cc) in 20 L of water (3 times per season). Adventitious plants were cleared manually once per month.

Design and preparation of samples

A random distribution model was chosen for the design of both plots. 10 plants per accession were distributed randomly in 2 blocks of 5 plants per plot. This experimental design was framed by a bordure of excess plants from transplanting. Fruits were harvested at both ripening stages to prepare separately unripe and fully ripe samples. Each sample was prepared with fruits of 2 plants (one per block) and, therefore, 5 samples (n=5) per combination of accession (14) × ripening stage (2) × growing conditions (2) were analysed in both 2015 and 2016 trials. 30 g of fruit mesocarp of each sample was lyophilized in a freeze dryer *Wizard 2.0*, (VirTis, Warminster, PA, USA) and milled. The powder samples were then placed in sealed falcon tubes and stored in dry and dark conditions until the extractions and analyses of phenolics.

Analysis of phenolics

The extraction of phenolics was carried out according to Plazas et al.[32] with some modifications. A subsample of 100 mg of each lyophilized sample were placed in a 2 mL microcentrifuge tube with 1.5 mL of extraction solution (methanol/water 80:20 v/v; 0.1% BHT), mixed with vortex and incubated in ultrasonic bath (Elmasonic s30, Elma Schmidbauer GmbH, Germany) at 30 ºC for 1 h. The mixture was then centrifuged at 9500 × g for 5 min and 750 μL were collected in a 2 mL threaded microcentrifuge tube. The extract solution was hydrolysed with 3 M HCl at 95 ºC in a thermoblock for 1h (Thermoblock TD, Falc Instruments, Italy). The hydrolysed sample was cooled to room temperature and stored at -80 ºC until the analysis, then they were filtered through a 0.22 μm PTFE membrane.

For the determination of the main flavonoids in *Capsicum*, a methodology based in Bae et al.[29] with slight modifications was used. The flavonoids were determined in their aglycon form after acid hydrolysis by high-performance liquid-chromatography (HPLC, 1220 Infinity LC, Agilent Technologies, USA) coupled to an UV detector. Flavonoids were separated using a Brisa LC^2-C_{18} column (3μm, 150 × 4.6 mm) (Teknokroma, Barcelona, Spain), with 0.1% formic acid in water (A) and HPLC grade methanol (B, Sigma-Aldrich, St. Louis, MO, USA) as mobile phase and 0.8 ml/min of flow rate. Detection was performed at 360 nm and 10 μl of sample infection volume were used.

Total flavonoid content was calculated as the sum of the five flavonoid aglycons analysed and results were presented in mg of each flavonoid per kg of fresh fruit and were plotted using ggplot2 package (2.2.1 version).[33] Fresh weight was estimated according to the initial moisture content which was estimated from the difference in weight of each sample before and after the process of freeze drying. External standard quercetin (Purity ≥ 95%), luteolin (98%), myricetin (96%), kaempferol (97%) and apigenin (95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calibration curves for quercetin (11.4 - 114.0 μg/ml), luteolin (11.8 - 117.6 μg/ml), myricetin (1.0 - 9.6 μg/ml), kaempferol (1.0 - 9.7 μg/ml) and apigenin (1.0 - 9.5 μg/ml) were performed three times.

Statistical analysis

Data were analysed using the *Statgraphics Centurion XVII* software (StatPoint Technologies, Inc; Warrenton, Virginia, USA) and a generalized linear model analysis of variance (GLM ANOVA) with transformed data (log x +1) was used.

For the general ANOVA, the linear model used was $X_{ijklm} = \mu + a_i + b_j + c_k + d_l + (\alpha \times \beta)_{ij} + (\alpha \times \gamma)_{ik} + (\beta \times \gamma)_{jk} + e_{ijkl(m)}$, where X_{ijklm} is the value for fruit sample m of genotype i,

growing system j, ripening stage k and year l; μ is the general mean; a_i is the effect of genotype i; b_j is the effect of growing system j; c_k is the effect of ripening stage k; d_l is the effect of year l; $(\alpha \times \beta)_{ij}$ is the interaction between genotype i and growing system j; $(\alpha \times \gamma)_{ik}$ is the interaction between genotype i and ripening stage k; $(\beta \times \gamma)_{jk}$ is the interaction between growing system j and ripening stage k and $e_{ijkl(m)}$ is the error term, i.e. the effect of fruit sample m from the combination of genotype i, growing system j, ripening stage k and year l. Year interactions were not performed in GLM ANOVA due to its nature as a random factor.

The specific ANOVA was made dividing unripe and fully ripe stages and the linear model used was $X_{ijkl} = \mu + a_i + b_j + c_k\ (\alpha \times \beta)ij + e_{ijk(l)}$, where X_{ijkl} is the value in one specific ripening stage for fruit sample l of genotype i, growing system j and year k; μ is the general mean; a_i is the effect of genotype i; b_j is the effect of growing system j; c_k is the effect of year k; $(\alpha \times \beta)_{ij}$ is the interaction between genotype i and growing system j and $e_{ijk(l)}$ is the effect of fruit sample l from the combination of genotype i, growing system j and year k.

The means were calculated for each set of ten samples and regression analysis was used to study the genotype×growing system interactions.[34] Calculation of the regression coefficients (β) per genotype was obtained from the average contribution of each growing system and the formula $\beta_i=(\mu_{ij}-\mu_{ijk}) / (\textit{Environmental Mean}) = (\mu_{ij}-\mu_{ijk}) / ((\mu_j-\mu_{jk}))$; where β_i is the regression coefficient value for each specific trait and ripening stage for genotype i; μ_{ij} is the mean of genotype i in growing system j; μ_{ijk} is the mean of genotype i for both growing systems j and k, μ_j is the mean for all the genotypes in growing system j, μ_{jk} is the mean for all the genotypes in both growing systems j and k. The cases with β values that did not differ significantly from 0 were considered stable genotypes against the growing system effect.[35]

Correlation between phenolics was studied by means of Spearman's rank coefficient ρ in unripe stage (n=280), fully ripe stage (n=279) as well as the evolution of phenolics in ripening (ratios fully ripe/unripe, n=28). Correlation between genotypes according to their composition in phenolics was studied through principal component analysis (PCA) and heatmap graphical representation of data for both growing systems in unripe and fully ripe stage using online software ClustVis.[36] Original values were transformed by ln (x+1) and SVD with imputation was used in PCA.

RESULTS AND DISCUSSION

Effect of genotype, growing system, ripening stage and interactions

The ANOVA analysis showed that the genotype (G) had the highest contribution to the observed variation with a significant effect for both total and individual flavonoids, except for myricetin and kaempferol (Table 2) and, to a lesser extent, the year effect also contributed significantly to the variation in total flavonoids and most individual flavonoids (except for myricetin), which confirms the importance of multi-year design to decrease the environmental effect related to agroclimatic conditions from year to year, especially in open field.[37] The ripening stage (R) also contributed significantly in all flavonoids, particularly in myricetin (Table 2), indicating changes in the content of phenolics due to the ripening process.[38] By contrast, the general effect of the growing system (E) was not significant, although significant G×E interaction was found for variation in total flavonoids ($p<0.05$) and luteolin ($p<0.01$), suggesting a different behaviour of the accessions depending on the growing system. Also, G×R interaction was significant for total and individual flavonoids. This fact and the high contribution of the ripening stage could be hiding the magnitude of the other factors to the observed

variation and, therefore, specific ANOVA for each ripening stage were then performed separately (Table 2).

As found in the general ANOVA, the genotype effect was also significant and predominant in total flavonoids and individual flavonoids at both unripe and fully ripe stages, followed by the year effect (Table 2). By contrast to the general ANOVA the ANOVAs considering each ripening stage separately showed a significant contribution of the growing system and its interactions with the genotype in some flavonoids, especially at the fully ripe stage (Table 2). For that reason, the discussion of results in the next sections will be performed separately for each ripening stage.

Flavonoids in unripe fruits

The analysis of the main phenolic compounds at the unripe stage revealed remarkable differences among flavonoids and among varieties (Table 3). Luteolin and quercetin had on average the highest contribution to total flavonoids (around 70% and 25%, respectively) while myricetin, apigenin and kaempferol showed considerably lower levels (≤ 5% total, each) (Table 3). Moreover, a considerable variation within each compound was observed depending on genotype, and to a lesser extent depending on the growing system and G×E interaction, as detected in the ANOVA (Table 2). Thus, luteolin ranged between 29 and 220 mg kg^{-1} (for Jalapeño organic and Gernika conventional, respectively), quercetin between 2 and 72 mg kg^{-1} (for Jalapeno and Guindilla Ibarra both in organic, respectively), myricetin between 1.3 and 9 mg kg^{-1} (for ECU-994 organic and BOL-58 conventional, respectively), apigenin between 0.2 and 4 mg kg^{-1} (for Serrano organic and conventional and Guindilla Ibarra conventional, respectively) and kaempferol between 0.04 and 2.5 mg kg^{-1} (for ECU-994 organic and Guindilla Ibarra conventional, respectively. Table 3). In a previous work of Howard et al.[39] these differences among phenolics in unripe *Capsicum* fruits were also detected

with quercetin and luteolin ranging from 2 to 68 and from 6 to 44 mg kg^{-1} respectively. However, the average content of quercetin in that report was higher than luteolin, while the contrary was found in our study with a clear predominance of the mean luteolin content. This fact reveals that the use of genetic diversity is highly recommended to assess the behaviour of any crop.

Differences among genotypes were remarkable as found in the ANOVA (Table 2). Thus, genotypes with the highest total phenolic compounds in unripe stage were Bierzo, Gernika, Guindilla Ibarra and Padron in both growing systems, Piquillo in organic and Espelette in conventional growing system (>120 mg kg^{-1}, Table 3). Mean values for luteolin ranged from 29 to 205 mg kg^{-1} in organic and from 29 to 221 in conventional, and several genotypes showed levels >85 mg kg^{-1} (Espelette, Gernika, Guindilla Ibarra and Padron in both systems, Piquillo in organic and Mojo Palmero in conventional) (Table 3). Also, a remarkable range of variation was found for quercetin, with mean values comprised between 2 and 72 mg kg^{-1} in organic and between 2 and 64 mg kg^{-1} in conventional. Thus, Bierzo, Espelette, Gernika, Guindilla Ibarra, Padron and Valenciano in both systems and Piquillo in organic, showed values >25 mg kg^{-1} (Table 3).

Finally, regarding minor flavonoids, myricetin levels ranged between 1 and 7 mg kg^{-1} and 2 and 9 mg kg^{-1} in organic and conventional, respectively, with *C. baccatum* BOL-58, Gernika, Jalapeño, Padron and Serrano showing levels >5 mg kg^{-1} (Table 3). Apigenin ranged from 0.2 to 3.4 mg kg^{-1} and from 0.2 to 3.7 mg kg^{-1} in organic and conventional, respectively, with BOL-58, *C. chinense* ECU-994 and Guindilla Ibarra showing ≥ 3 mg kg^{-1} in both systems (Table 3). Kaempferol mean values ranged between 0.04 and 2.18 mg kg^{-1} in organic and between 0.24 and 2.45 mg kg^{-1} in conventional, with Bierzo, Gernika, Guindilla Ibarra and Padron ≥1.5 mg kg^{-1} (Table 3).

These results indicate the wide variability available in terms of phenolics content depending on the genotype and suggest the needing of using comprehensive genetic pools to breed for nutritional quality. In fact, these finding based on a considerable collection of varietal types gave comparatively wider ranges of variation in unripe peppers than those from Howard et al.[39] based in four *C. annuum* varieties and one *C. frutescens*, with quercetin + luteolin values comprised from 17 to 85 mg kg^{-1} FW. Other study, based on seven *C. annuum* cultivars and one *C. chinense* reported a range of variation between genotypes at the unripe stage for total flavonoids (luteolin, quercetin, myricetin, apigenin and kaempferol) from 4 to 50 mg kg^{-1} FW.[40] Nevertheless, our genetic pool of 14 varieties gave ranges for the sum of both flavonoids much higher than the mentioned reports (30 to 275 mg kg^{-1}), which remarks the importance of working with a wide genetic pool for breeding and selection purposes.

Regarding genotype × environment interaction, the main effect of the growing system was low as revealed in the ANOVA (Table 2). Total means in organic *vs* conventional were very similar in total flavonoids (121 *vs* 123 mg kg^{-1}) and most individual flavonoids and only kaempferol showed significant differences (slightly higher in conventional) (Table 3). In addition, considering the regression coefficient β, most accessions showed a stable behaviour, with similar mean values and non-significant β values comparing organic and conventional conditions (Table 3). Nonetheless, some genotypes showed significantly different responses depending on the growing system at unripe stage. Thus, Piquillo and Serrano for total flavonoids, Guindilla Ibarra and Piquillo for quercetin and Numex Big Jim, Piquillo and Serrano for luteolin showed significant higher levels in organic, while Espelette, Gernika and Mojo Palmero for total flavonoids and luteolin and BOL-58 for myricetin had higher values in the conventional system (Table 3).

Flavonoids in fully ripe fruits

Measurements of flavonoids in fully ripe fruits revealed in general higher averages than those in unripe fruits (Table 4). As observed in unripe fruits luteolin, quercetin and myricetin had a higher contribution to total flavonoids in fully ripe fruits (70%, 21% and 6% mg kg^{-1} for both growing systems, respectively) (Table 4). The genotype effect detected in the ANOVA suggested wide ranges of variation within each compound. Thus, luteolin ranged between 33 and 270 mg kg^{-1} (for ECU-994 in organic and Gernika in conventional, respectively), quercetin ranged from 2 to 79 mg kg^{-1} (for Jalapeno and Gernika in conventional, respectively) and myricetin ranged between 6 and 17 mg kg^{-1} (for Bola in organic and BOL-58 in conventional, respectively) (Table 4). Finally, apigenin ranged between 0.1 and 5.8 mg kg^{-1} (for Serrano and BOL-58 in organic, respectively) and kaempferol was comprised between 0.2 and 3.1 mg kg^{-1} (for ECU-994 and Bierzo in organic, respectively) (Table 4).

In terms of total flavonoids, accessions BOL-58, Espelette, Gernika, Guindilla Ibarra and Padron for both organic and conventional growing systems and Bierzo for organic showed the highest values (i.e. ≥150 mg kg^{-1}). For luteolin the highest levels corresponded to Gernika, Guindilla Ibarra (>200 mg kg^{-1}), BOL-58 and Espelette (>100 mg.kg^{-1}), which showed these remarkable levels in both growing systems, as well as Bierzo and Piquillo among fleshy genotypes (70-80 mg kg^{-1}) (Table 4). Regarding quercetin, the highest values in both growing systems were found in Bierzo, Gernika, and Guindilla Ibarra (≥60 mg kg^{-1}). Variation in myricetin was relatively low as most accessions showed contents close to the system means (8.7 mg kg^{-1}) and only BOL-58, and Serrano had higher levels (10-17 mg kg^{-1}) (Table 4). Finally, BOL-58, ECU-994, Gernika and Guindilla Ibarra in both growing systems and Bierzo in organic had the highest levels in apigenin (≥2.5 mg kg^{-1}), while the highest levels in kaempferol

corresponded to Bierzo, Gernika and Guindilla Ibarra in both growing systems (≥2 mg kg^{-1}) (Table 4). In comparison to other works, some genotypic variability in phenolics has been observed in fully ripe fruits of *Capsicum*. Thus, Bae et al.[40] reported ranges from 2 to 90 mg kg^{-1} for total flavonoids in habanero (*C. chinense*) and some *C. annuum* cultivars, while Ghasemnezhad et al.[41] reported values for quercetin comprised from 37 to 118 mg kg^{-1} in *C. annuum* fruits. Furthermore, the maximum levels for fully ripe fruits reported by Bae et al.[40] for luteolin, quercetin, myricetin, apigenin and kaempferol were 21, 31, 13, 3 and 6 mg kg^{-1}, respectively, while other authors based only in three bell peppers,[42] reported 9, 33, 26 and 4 mg kg^{-1} of luteolin, quercetin, myricetin and kaempferol, respectively. In comparison, and as found with unripe peppers, our study detected considerably higher values, reinforcing the utility of using as much genetic diversity as possible for quality breeding, regardless the ripening stage.

The effect of the growing system was higher and significant in the fully ripe stage compared to the unripe stage, with the organic system mean reaching 144 mg kg^{-1} and 139 mg kg^{-1} in the conventional growing system (Table 4). Other previous works detected that accumulation of secondary metabolites as phenolics may be affected by differences between cultural practices like limited nitrogen supply, characteristic in organic cultivation, which may favour the synthesis of flavonoids and could explain our results in fully ripe fruits.[43,44] Thus, the higher contribution of the growing system effect at the fully ripe stage may be due to a longer exposure of the plants to the stressful conditions of organic system during the development of fully ripe fruits, compared to the unripe stage. As found at the unripe stage, although to a greater extent, β parameter for genotype × environment interaction revealed both organic and conventional adapted genotypes. Six genotypes, Bierzo, BOL-58, Guindilla Ibarra, Jalapeno, Numex Big Jim and Serrano, showed a better behaviour for organic growing system considering total

flavonoids and some individual compounds as luteolin. Also, the organic growing system improved individual phenolic content as quercetin (Bierzo and BOL-58) and apigenin (BOL-58). Espelette, Gernika and Mojo Palmero had better response in the conventional growing system regarding total flavonoids and individual compounds like luteolin and quercetin (only for Gernika) (Table 4).

Evolution of flavonoids with the ripening process

As detected in the ANOVA, the ripening stage contributed significantly and remarkably to the content in flavonoids. Some authors have reported that unripe fruits may show higher levels than fully ripe fruits,[39,41,45,46] while the contrary has been reported by others.[47–49] Probably, the scarce genetic diversity used in these works could be the reason for such discrepancy, which reveals the relevance of using highly variable germplasm collections for more robust studies to assess the effect of other factors on the accumulation of bioactive compounds.

Our findings, based in a comprehensive collection of *Capsicum* peppers, indicate that in general the ripening process increases the level of flavonoids, regardless the growing system. Thus, on average and in both growing systems, total flavonoids and individual flavonoids were higher or similar in fully ripe fruits, with the only exception of the minor flavonoid kaempferol under conventional conditions (Figures 1 - 6). This was particularly remarkable in total flavonoids and the major flavonoid luteolin, whose total means increased with ripening by 20% and 13% in organic and conventional growing systems, respectively (Figures 1 and 2) and the minor flavonoid myricetin with a mean increase of 80% in both growing conditions (Figure 4). These findings are in agreement with a recent work, which detected considerably higher levels of total phenolics through spectrophotometric methods in fully ripe fruits in a collection of 37 accessions.[34]

Additionally, this average trend was also found considering accessions separately. Thus, most accessions showed higher or similar flavonoid levels at the fully ripe stage compared to their corresponding unripe levels, although the increase in flavonoids with the ripening process differed among accessions (Figures 1 - 6). Thus, even some genotypes showed higher levels at the unripe stage, as a result of the significant genotype × ripening stage interaction (Table 2). These lesser usual cases of decrease during ripening in peppers were also reported in other works.[39,41,45]

The genotypes with the highest increases in flavonoid averages were BOL-58, Gernika, Jalapeno and Mojo Palmero, which showed in general higher fully ripe/unripe ratios (Figures 1 - 6). Furthermore, myricetin levels in ECU-994 (both growing systems) and Bola (conventional) increased 4-5-fold with ripening (Figure 4) and the same was found for kaempferol in ECU-994 (organic) (Figure 6). By contrast, a few exceptions like Serrano and Valenciano showed a decrease in most flavonoids (except myricetin) in both organic and conventional growing systems (Figures 1 - 6). Additionally, Numex Big Jim for both growing systems and ECU-994 and Piquillo for organic growing system decreased slightly in total flavonoids (Figure 1).

The differences in the accumulation of flavonoids with ripening also depended on the flavonoid compound and this was particularly obvious in minor flavonoids. Thus, for luteolin as the main flavonoid, genotypes showed very similar increases to those observed in total flavonoids, with BOL-58 showing increases of 80% (conventional) or 140% (organic) or at a lesser extent Jalapeno with increases of 20% (conventional) or 75% (organic) and Espelette, Gernika or Mojo Palmero with increases comprised between 20% and 40%, while the rest of accessions showed increases ≤20% or no increases (Figure 2). In the second main flavonoid quercetin, the increases were more variable, from 70-140% (BOL-58) to 70% decrease (Valenciano) (Figure 3). Myricetin

was the flavonoid that increased with ripening in all the genotypes regardless the growing system, with increases that ranged from 30% in Bierzo to genotypes like Bola (conventional) or ECU-994 (organic) with 4 and 6-fold increases, respectively (Figure 4). Minor flavonoids apigenin and kaempferol had a similar behaviour to luteolin and quercetin, with general average contents in fully ripe fruits similar or higher to unripe fruits (Figures 5 and 6).

The effect of the growing system could be detected according to general highest fully ripe/unripe ratios. Thus, the organic growing system seemed to favour slightly the accumulation of total flavonoids and luteolin during the ripening process since the highest ratios were detected in the organic system on average and in most accessions (Table 4, Figures 1 and 2). This was in agreement with previous works based on total phenolics and a range of *Capsicum* materials.[34,50] Finally, according to the ANOVA, the significant system × ripening interaction was detected in kaempferol content (Table 2) which was due to significant differences between organic and conventional ripening ratios (Figure 6). Thus, for kaempferol most genotypes grown under conventional conditions showed fully ripe/unripe ratios ≤1, while most genotypes grown in organic management showed ratios ≥1 (Figure 6).

Correlation between phenolic compounds and similarities between accessions

The study of correlation for myricetin, quercetin, luteolin, kaempferol and apigenin in unripe and fully ripe stage showed different degrees of correlation among them (Figure 7). The highest positive correlations were found between luteolin and quercetin, between kaempferol and quercetin and between kaempferol and luteolin in both unripe and fully ripe stage, as well as between apigenin and quercetin and between apigenin and luteolin in fully ripe stage (Spearman's coefficient ρ from 0.6 to 0.8). Furthermore, luteolin and myricetin in fully ripe fruits reached intermediate correlation values (ρ=0.5,

Figure 7). Such positive correlations between flavonoids might be due to a concurrent accumulation because of shared transcriptional regulators of genes related with flavonoid biosynthesis as suggested by Lim et al.[51]

Finally, the correlation of phenolic accumulation with ripening was also analysed by correlating the fully ripe/unripe mean ratios of the genotypes (Figure 7). Highly significant and positive correlations were found between the ripening ratios of luteolin and quercetin, between apigenin and quercetin and between apigenin and luteolin (ρ from 0.6 to 0.9, Figure 7). These findings suggest that such flavonoids changes with ripening in a similar degree could be explained by different genetic expression patterns according to fruit development as reported by Moriguchi et al.[52] In addition, these positive correlations would allow to select indirectly for some phenolic compounds according to the values of other phenolic compounds, of particular interest in the case of the two main flavonoids luteolin and quercetin.

The Principal Component Analysis (PCA) and the heatmaps allowed to visualize the relationship between genotypes according to their flavonoid contents for each growing system and ripening stage (Figure 8). The wide variation and distribution of the genotypes in these graphical representations, for each ripening stage, confirmed the importance of the genotype factor previously detected in the ANOVA (Table 2).

The principal components 1 and 2 (PC1 and PC2) explained 52% and 28% of the total variance at the unripe stage, respectively, according to the flavonoid content (Figure 8). The loadings (eigenvectors) in PC1 for luteolin and quercetin, myricetin and kaempferol contributed negatively, while the contrary was found for apigenin (data not shown). Furthermore, luteolin, quercetin and kaempferol contributed mostly to the variation with loadings around -0.6. In addition, PC2 showed positive loadings for

luteolin, quercetin and apigenin and negative for myricetin and kaempferol with a predominant contribution of myricetin and apigenin (loadings around -0.6 and 0.7, respectively. Figure 8). In this way, genotypes like Guindilla Ibarra and Gernika, with the most remarkable content in luteolin, quercetin and kaempferol, appeared in the negative side of PC1, while Bola, ECU-994 or Jalapeno, with predominant contents of apigenin, appeared in the positive extreme of PC1, which is also showed in the heatmap for unripe stage (Figures 8 and 9). On the other hand, Bola, ECU-994 and Guindilla Ibarra were in the positive side of PC2, while Serrano peppers appeared in the negative side. Both PCA and heatmaps showed the general trend of pairing organic and conventional means within each genotype, which reinforced the fact that the contribution of the growing system factor was considerably lower than that of the genotype and reflected the robustness of our approach.

PCA for the fully ripe stage showed a positive relation between all the flavonoids in PC1 (58% of total variance), with a higher contribution of luteolin, quercetin and kaempferol (around 0.5) and apigenin (around 0.4) (Figure 8) (data not shown). For PC2 (24% of total variance) the positive loadings corresponded to quercetin and kaempferol while myricetin, which contributed the most to the variation (-0.8), luteolin and apigenin showed negative loadings. In this regard, genotypes in positive extreme of PC1, Guindilla Ibarra and Gernika, showed the highest contents in luteolin, quercetin and kaempferol. As in unripe stage, in fully ripe stage PCA and heatmap most genotypes were grouped together regardless the growing system, which confirmed again the impact of the genetic factor (Figures 8 and 9).

Selectable materials

A potential selection of genotypes for organic growing system could be possible according to both adaptation to the growing system and high content in phenolic

compounds. Attending to genotypes with better behaviour at the unripe stage for organic growing system (according to β), *C. annuum* varieties Piquillo and Serrano showed higher differences of total phenolics compared to conventional growing system (Table 3). Additionally, for total phenolics content at the fully ripe stage, *C. annuum* genotypes Bierzo, Guindilla Ibarra, Jalapeno, Numex Big Jim and Serrano and *C. baccatum* BOL-58 showed better behaviour in organic cultivation (Table 4). Moreover, varieties for organic growing system with high content in total phenolic compounds but not a significant difference between systems were *C. anuum* genotypes Bierzo, Espelette, Gernika, Guindilla Ibarra and Padron at the unripe stage (above 119 mg kg^{-1}, Table 3, Figure 1) and Espelette, Gernika, Mojo Palmero and Padron at the fully ripe stage (above 131 mg kg^{-1}, Table 4, Figure 1).

In conclusion, our findings allowed to explain the contribution of genotype, ripening stage and their interactions in different growing systems, organic and conventional, for the content of main flavonoids in a comprehensive collection of *Capsicum* peppers. A broad variation of flavonoids was found depending mainly on the genotype, the growing system and G×E interaction for each ripening stage. The average contribution of each flavonoid to the total content was as follows: luteolin, quercetin, myricetin, apigenin and kaempferol, in both unripe and fully ripe fruits. The ripening stage contributed remarkably to the content in flavonoids. In general, the ripening process increased the level of flavonoids, and organic cultivation significantly favoured the accumulation of total flavonoids and luteolin during ripening. Correlation between flavonoids was detected at both ripening stages, especially in the main flavonoids luteolin and quercetin and quercetin and kaempferol, which would allow indirect positive selections. Genotype×environment interaction would make possible specific performance selections of accessions according to the high content in phenolic compounds for

organic growing system at the unripe and fully ripe stages. This study and its approach will provide useful information for the research in high value-added vegetables and quality breeding programs.

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Figure Legends

Figure 1: Comparative graphs of the average content (mg kg^{-1} FW, n=10) of total flavonoids at unripe (green) and fully ripe stage fruits (red) for organic and conventional growing systems on each pepper accession. Point graphs signalize proportion of compound evolution with ripening (ratio fully ripe/unripe) for each genotype in organic and conventional system. Thicker grey line separates ratios that reflect an increase of compound average with ripening (ratios to the right of the line) *vs* those that show a decrease (ratios to the left of the line).

Figure 2: Comparative graphs of the average content (mg kg^{-1} FW, n=10) of luteolin at unripe (green) and fully ripe stage fruits (red) for organic and conventional growing systems on each pepper accession. Point graphs signalize proportion of compound evolution with ripening (ratio fully ripe/unripe) for each genotype in organic and conventional system. Thicker grey line separates ratios that reflect an increase of compound average with ripening (ratios to the right of the line) *vs* those that show a decrease (ratios to the left of the line).

Figure 3: Comparative graphs of the average content (mg kg^{-1} FW, n=10) of quercetin at unripe (green) and fully ripe stage fruits (red) for organic and conventional growing systems on each pepper accession. Point graphs signalize proportion of compound evolution with ripening (ratio fully ripe/unripe) for each genotype in organic and conventional system. Thicker grey line separates ratios that reflect an increase of compound average with ripening (ratios to the right of the line) *vs* those that show a decrease (ratios to the left of the line).

Figure 4: Comparative graphs of the average content (mg kg^{-1} FW, n=10) of myricetin at unripe (green) and fully ripe stage fruits (red) for organic and conventional growing systems on each pepper accession. Point graphs signalize proportion of compound evolution with ripening (ratio fully ripe/unripe) for each genotype in organic and conventional system. Thicker grey line separates ratios that reflect an increase of compound average with ripening (ratios to the right of the line) *vs* those that show a decrease (ratios to the left of the line).

Figure 5: Comparative graphs of the average content (mg kg^{-1} FW, n=10) of apigenin at unripe (green) and fully ripe stage fruits (red) for organic and conventional growing systems on each pepper accession. Point graphs signalize proportion of compound evolution with ripening (ratio fully ripe/unripe) for each genotype in organic and conventional system. Thicker grey line separates ratios that reflect an increase of compound average with ripening (ratios to the right of the line) *vs* those that show a decrease (ratios to the left of the line).

Figure 6: Comparative graphs of the average content (mg kg^{-1} FW, n=10) of kaempferol at unripe (green) and fully ripe stage fruits (red) for organic and conventional growing systems on each pepper accession. Point graphs signalize proportion of compound evolution with ripening (ratio fully ripe/unripe) for each genotype in organic and conventional system. Thicker grey line separates ratios that reflect an increase of compound average with ripening (ratios to the right of the line) *vs* those that show a decrease (ratios to the left of the line).

Figure 7: Spearman's rank correlation coefficient for phenolic compounds at unripe stage (n=280), fully ripe stage (n=280) and ripening (ratio fully ripe/unripe, n=28). NS, *, ** and *** indicate not significant for a probability p >0.05 and significant for p <0.05, <0.01 and <0.001, respectively, according to the statistical F ratio.

Figure 8: PCA representation for correlations between phenolics values for each genotype and growing system (organic: O, conventional: C) at unripe stage (above) and fully ripe stage (below). Original values are transformed according to ln(x+1). Unit variance scaling is applied to rows; SVD with imputation was used to calculate principal components (n= 28 data points).

Figure 9: Heatmap representation for correlations between phenolics values for each genotype and growing system (organic: O, conventional: C) at unripe stage (above) and fully ripe stage (below). Original values are transformed according to ln(x+1). Columns were centered; unit variance scaling was applied to columns; both rows and columns were clustered using correlation distance and average linkage (28 rows, 5 columns).

Appendices

Appendix 1a: Images of fruits at unripe and fully ripe stage, 1-Bierzo, 2-Bola, 3-Espelette, 4-Gernika, 5-Guindilla Ibarra, 6-Jalapeno M, 7-Mojo Palmero, 8-Numex Big Jim. Rulers at the bottom are in scale of centimetres (marks between two numbers indicate 1 cm).

Appendix 1b: Images of fruits at unripe and fully ripe stage, 9-Padron, 10-Piquillo, 11-Pimiento Valenciano, 12-Serrano, 13-BOL-58, 14-ECU-994. Rulers at the bottom are in scale of centimetres (marks between two numbers indicate 1 cm).