South Western Journal of Horticulture, Biology and Environment
P-Issn: 2067- 9874, E-Issn: 2068-7958

Vol.9, No.2, 2018
pp.65-76
Art.no. e18106

CHANGES IN PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF HOT PEPPER FRUITS ON MATURITY STAGES, CULTIVATION AREAS AND GENOTYPE

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ABSTRACT. *This study aims to understand the phytochemical changes influenced by the maturity stages of the hot pepper fruits. Five local pepper populations (Capsicum annuum L.) were selected and cultivated in field in two different areas, Almăj and Filiași, in the Southwestern part of Romania. The fruits were harvested in two different stages: green fruit and red fruit stage. Ascorbic acid, total polyphenols and antioxidant activity (ABTS and DPPH) were determined. The content of phytonutrients increased in fruit maturation stages, especially total polyphenols and antioxidant activity. Positive correlations were recorded between the antioxidant activity and the total polyphenols in both areas of study. The correlation between total polyphenols and the antioxidant activity (DPPH) (r=0.388*) was recorded for fruits harvested in the red fruit stage in Filiaşi area. The correlation between total polyphenols and the antioxidant activity (ABTS) (r=0.983*) was recorded for fruits harvested in the green fruit stage in Almăj area. Results show that each phytonutrient has a unique accumulation way during fruit maturation that is dependent on genotype, maturity stage, and crop area.*

KEY WORDS*: ascorbic acid, Capsicum annuum L , polyphenols.*

INTRODUCTION

The hot pepper is one of the oldest plant species in the world. Currently this genus is considered to comprise 27 species, five are domesticated and

used as fresh vegetables and spices along with about 3000 varieties (Ibiza et al. 2012). The widespread geographical distribution of *Capsicum annuum* L. and *Capsicum frutescens* L. from the New World to other continents took place in the 16th century through the Spanish and Portuguese traders and was integrated in the food habits of several countries. The hot pepper is used as a spice for flavouring many dishes from around the world. In addition to acting as a flavouring and colouring agent, this fruit is also used as an alternative medicine for treating various diseases such as inflammations, diabetes, back pain, acute tonsillitis (Dinu et al. 2013, Pintilie et al. 2012). Chilli was an important plant in Maya's traditional medicine to treat various conditions such as neck pain, ear pain and skin care (Gurnani et al. 2016). It is consumed worldwide both fresh and processed in various forms (pickled, baked, boiled, dry) due to its sensory properties, flavour, and content in bio-components. The pepper is considered a source of phenolic compounds, including phenolic acids, flavonoids, capsaicin (Alvarez-Parrilla et al. 2011). Numerous studies have demonstrated the protective role of phenolic compounds on heart disease and some forms of cancer. These health measures can be attributed to the antioxidant capacity of pepper, which is largely influenced by the stage of fruit growth and maturation, but also by the influence of the cultivation area. Therefore, it is important to understand how changes in different phytochemicals which are beneficial to health can be influenced by the maturation process. Such changes in phytonutrients depend on the maturation and on the cultivation area and they are particularly important for the diet, since the pepper fruits harvested in the green or red fruit phase are frequently eaten as fresh vegetables. Genetic variability is the most important condition in a horticultural plant breeding program. As a result, research into genetic variation between genotypes is always very difficult to initiate an improvement program. In Romania there are a lot of local populations of hot peppers coming from all over the country. In the literature, there is limited information on the biochemical composition of the same. Because of the nutraceutical properties of this species, consumption has increased in recent decades, and researchers therefore pay special attention to creating new varieties and hybrids. Lack of information about the biochemical properties of available local populations makes it difficult for breeders to work. Total polyphenols, anti-tumor, ascorbic acid and antioxidant activity are among the most important quality criteria for hot pepper, when it comes to human health. Therefore, we believe that the

present study will serve as a new source of information for Romanian and foreign breeders looking for variants to create new commercial varieties with a high antioxidant capacity and high phenolic content. Study aimed to observe and (better) understand the changes in different phytonutrients that are dependent on the cultivar, fruit maturity and cultivation area.

MATERIAL AND METHODS

Five local hot pepper populations (*Capsicum annuum* L.) originating from Dolj county (south-west of Romania) and cultivated in the Jiu meadow in Filiaşi (44°24'0"N 23°31'E) on alluvial soil and in Almăj (44°27'N 23°42'E) on a reddish brown soil were studied. The local populations received the name of the village from which they originate: P1-from Filiaşi, P2-from Răcari, P3-from Slăvuţa, P4-from Tibărcuţa, P5-Balta. The culture was established by seedling in the field. Biochemical determinations were performed at the technological maturity of fruits (green fruit stage) and at the physiological maturity (red fruit stage). The fruits of the two areas were harvested at the same time but in two stages of successive maturity, based on their colour, green and red. The fruits were harvested from 10-15 plants (≈1.0 kg of fruits) for the best homogeneity of the samples, they were cleaned from dust with a paper towel, then the peduncles and the seeds were removed and they were cut in small pieces and the extracts for samples were obtained.

Ascorbic acid (AA) was extracted and analyzed by reversedphase HPLC. Fresh chili pepper homogenate, 5g, was mixed and diluted to 100 ml with 0.1 N HCl. After 30 minutes the extraction solution was centrifuge dat 4200 rpm for 10 minutes. The supernatant was filtered through 0.2 µm pore size filter. The separation was performed using a Hypersil Gold a Q column (25cm x 4.6 mm) with a particle size of 5 µm while a 50 mM water solution of KH_2PO_4 buffer adjusted to pH 2.8 with orto-phosphoric acid was used as the mobile phase. The column temperature was kept at 10 ^{0}C and the flow rate at 0.7 ml min^{-1}. All the results werw expressed in mg kg^{-1} fw. Acetonitrile was HPLC grade while potassium dihydrogen ortophosphate and phosphoric acid were of analytical purity. Ultrapure water was obtained from a Milli-Q water purification system.

Total phenolic content (TP). The amount of total phenolic compounds in the chili pepper fruits extract was determined colorimetrically with Folin-Ciocalteu reagent using the method described by Singleton & Rossi (1965) with some modifications (Cosmulescu et al. 2017). Briefly, each probe was prepared like this: to 0.1 mL extract (1g FW + 10 mL 80% methanol) add 0,9 mL ultrapure water and 5 m L reactive Folin-Ciocalteu (diluted 1:10 with ultrapure water). After two minutes, 4 mL of a 7.5% sodium carbonate solution was added, and the samples

were kept in the incubator at the room temperature for two hours. After that, the absorbance of probes was measured at 765 nm by using a Thermo Scientific Evolution 600 UV-Visible spectrophotometer with VISION PRO software. A standard curve was prepared by using 50, 100, 150, 200 and 250 mg/L solutions of gallic acid in methanol and water (80:20, v/v). Gallic acid was used as the reference standard and the results (total phenolic content, TPC) were expressed as gallic acid equivalents (GAE) and as mg/ kg $^{-1}$ FW.

Antioxidant activity - **DPPH** was measured in the methanolic extract using the DPPH (2.2-diphenyl-1-picrylhydrazyl) assay. Methanol (Merck, Germany), DPPH (2.2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich, Germany) and Trolox (6-hydroxi-2, 3, 7, 8-tetramethylchroman-2-carboxylic acid) (Merck, Germany) were employed. The sample was extracted according to the same protocol described for total phenolic content. The free radical scavenging ability of the extracts against the DPPH free radical was evaluated as described by Dinu et al. (2018) with some modifications (Cosmulescu et al. 2017). Each ethanol of extract of chili pepper fruits (50 mL) was mixed with 3 mL of a 0.004% (v/v) DPPH methanolic solution. The mixture was incubated for 30 min at room temperature in the dark and the absorbance was measured at 517 nm using Thermo Scientific Evolution 600 UV-Visible spectrophotometer with VISION PRO software. Trolox was used as standard. A blank reagent was used to study stability of DPPH over the test time. The scavenging activity of extracts was evaluated according to the formula: % scavenging = $[A_0-(A_1–A_S)]/A_0]100$, where A_0 is the absorbance of DPPH alone, A_1 is the absorbance of DPPH + extract and A_S is the absorbance of the extract only. The Trolox calibration curve was plotted as a function of the percentage of DPPH radical scavenging activity. The results were expressed as μM Trolox equivalents (TE)/1g FW.

Antioxidant activity-ABTS (2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging activity was measured colorimetric at 734 nm. Trolox was used as standard. The standard calibration curve was plotted as a function of the calculated percentage of ABTS radical cation scavenging activity. The results were expressed as micromole Trolox equivalents per 1gram fresh weight (μM Trolox/1g FW). All determinations were performed in triplicate, and all results were calculated as mean.

Statistical analysis. The significance of differences between variants was statistically determined u with variance analysis using ANOVA and the Statgraphics Centurion XVI program (Stas Point Technologies, Warrenton, VA, USA), calculating the limit differences, LSD ≤ 0.05%, (LSD = least significant difference). The means were separated by Duncan's multiple range test by the least significant difference (LSD, P ≤ 0.05). In addition, have been done correlations coefficients Pearson (r) between chemical compounds analyzed from chili pepper fruits.

RESULTS AND DISCUSSION

Observations and determinations were made in this study, focusing on phytochemical changes that are dependent on the maturity stages of the hot pepper fruits and the crop areas. The ascorbic acid varied for the 5 populations of hot pepper from 1110.3 mg/kg $^{-1}$ fw to 1273.5 mg/k^{-1} FW in the green fruit phase, from 1187.7 to 1288.4 mg/kg^{-1} FW in the red fruits grown in Filiaşi area (Table 1). This oscillation is also found in the culture established in Almăj area (Table 2). There is a slight increase in each

Table 1. The ascorbic acid, total polyphenols and antioxidant activity of the hot pepper fruits in Filiași area (mean for 2016 and 2017)*.

Population	Harvesting stage	Ascorbic acid (mg/ kg $^{-1}$ FW)	Total polyphenols (mg GAE/kg $^{-1}$ FW)	Antioxidant activity (µM Trolox/1 g FW)	
				ABTS	ABTS
P1	green	1132.2 ^{c}	2138.1 ^{e}	3.84 ^{bc}	4.00 ^{b}
	red	1204.0 ^{b}	4495.3 ^{e}	5.96 ^{ab}	5.60 ^{c}
P2	green	1122.4 ^{d}	4189.4 ^{a}	4.70 ^{ab}	4.20 ^{b}
	red	1187.7 ^{c}	7915.7 ^{a}	6.12 ^{ab}	6.80 ^{a}
P3	green	1110.3 ^{e}	3236.5 ^{c}	5.00 ^{a}	5.32 ^{a}
	red	1189.2 ^{c}	5940.5 ^{c}	6.68 ^{a}	6.92 ^{a}
P4	green	1273.5 ^{a}	2788.6 ^{d}	2.84 ^{d}	3.36 ^{c}
	red	1287.5 ^{a}	5735.9 ^{d}	5.54 ^{ab}	5.96 ^{b}
P5	green	1243.3 ^{b}	3802.9 ^{b}	3.00 ^{cd}	3.40 ^{c}
	red	1288.4 ^{a}	6433.6 ^{b}	5.20 ^{b}	5.81 ^{bc}
P≤ 0.05 at green		4.97	9.71	0.97	0.25
P≤0.05 at red		10.77	11.24	1.25	0.35

*Data presented as mean. Mean values followed by the same letter within columns are not significantly different according to Duncan's multiple range test (p ≤ 0.05).

population as it progresses towards the physiological maturity, but also an increase in the culture from Almăj compared to the one in Filiaşi. This increase on maturity phases was also observed by Niklis et al. (2002). It has been found by Deepa et al. (2007) that the pepper maturation affects the vitamin C content in sweet peppers. Our results are consistent with previous reports and with the results of Niklis et al. (2002) who described

Table 2. The ascorbic acid, total polyphenols and antioxidant activity of hot pepper fruits in Almăj area (mean for 2016 and 2017)*

Population	Harvesting stage	Ascorbic acid (mg/kg $^{-1}$ FW)	Total polyphenols (mg GAE/kg $^{-1}$ FW)	Antioxidant activity (μM Trolox/1 g FW)	
				ABTS	ABTS
P1	green	1170.0 ^{b}	3138.7 ^{d}	9.04 ^{b}	8.73 ^{c}
	red	1254.3 ^{b}	5495.5 ^{c}	10.96 ^{b}	11.60 ^{b}
P2	green	1162.4 ^{b}	4965.2 ^{a}	15.20 ^{a}	15.70 ^{a}
	red	1167.0 ^{d}	8233.7 ^{a}	17.72 ^{a}	17.86 ^{a}
P3	green	1130.7 ^{c}	3552.6 ^{b}	11.58 ^{b}	12.24 ^{b}
	red	1197.2 ^{c}	6543.1 ^{b}	17.68 ^{a}	17.82 ^{a}
P4	green	1172.0 ^{b}	3467.1 ^{c}	11.84 ^{b}	12.36 ^{b}
	red	1282.4 ^{a}	3744.4 ^{e}	17.23 ^{a}	17.86 ^{a}
P5	green	1214.3 ^{a}	3450.1 ^{c}	10.94 ^{b}	11.40 ^{b}
	red	1298.7 ^{a}	5214.6 ^{d}	16.70 ^{a}	16.91 ^{a}
P≤ 0.05 at green		20.20	28.40	3.20	2.26
P≤0.05 at red		21.99	20.49	2.23	1.92

*Data presented as mean. Mean values followed by the same letter within columns are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

higher levels of vitamin C in different varieties of fresh hot pepper harvested in the red fruit stage. However, the inconsistent findings can be found in several studies showing that vitamin C has increased or remained constant as the fruit reached the physiological maturity. According to Deepa et al. (2007) and Gnayfeed et al. (2001), the vitamin C decreased with the over maturation of red pepper fruits (depending on the variety of the hot pepper). These inconsistencies may be the result of the differences in the experimental conditions, such as the used varieties, harvesting stages and sampling protocols.

The total polyphenols represent the largest class of secondary metabolites in plants. The phenolic content and composition of horticultural species depend on species, environmental conditions, harvest maturity, storage conditions after harvesting but also rootstocks (Soare et al. 2018). The phenolic compounds are secondary metabolites that can act as antioxidants because of their ability to donate hydrogen, extinguish singlet oxygen and act as metal chelators (Michalak 2006). The total polyphenol content of the hot pepper fruits increased from 2138.1 GAE/kg^{-1} FW (green

fruit) to 7915.7 GAE/kg^{-1} FW (red fruit) (equivalents of gallic acid) in Filiaşi (Table 1) and from 3138.7 GAE/kg^{-1} FW (green fruit) to 8233.7 GAE/kg^{-1} FW (red fruit) in Almăj (Table 2). There are numerous studies in the literature confirming the presence of phenolic compounds in pepper fruits and more in the red fruits compared to the green fruits (Soare et al. 2017). In the case of these constituents, it is noted that as the fruit advances towards physiological maturity, the total polyphenols content increases. The results are consistent with the study by Marinova et al. (2005) on several horticultural species but also with Materska & Peruka, (2005) study on four varieties of hot pepper. Menichini et al. (2009) claim that total polyphenols have decreased with increasing the fruit maturity. In this study, obvious variations between cultivars were recorded within the same area. These differences are also observed between the areas, highlighting from this point of view Almăj area. It should be noted that the total polyphenols were quantified only from the fruit pericarp and not from the seeds. Numerous studies have highlighted an important content of polyphenols also in the hot pepper seeds (Gurnani et al. 2016, Chan et al. 2017). The content of phenolic compounds in fruits may be influenced by the geographical region, environmental factors, genetic characteristics of cultivars and the stage of harvesting and preservation (Leguizamon et al. 2016).

The hot pepper fruits have antioxidant activity (ABTS and DPPH methods expressed in µM Trolox/1g FW) with insignificant differences between the methods of determination but significant among the cultivation areas. The antioxidant activity is a parameter that evaluates the nutritional quality of fruits and vegetables. DPPH radicals are generally used as a screening method for evaluating a wide variety of compounds. In our study, through the DPPH method, the antioxidant activity in the populations from Filiaşi area recorded values ranging between 3.36 and 6.92 µM Trolox/g FW. These values varied according to the data in Table 1, being lower at green maturity (green fruits) and higher at physiological maturity (red fruits). For the culture set up in Almăj, the recorded values are much higher than those from the previous area. The lowest value was 8.73 µM Trolox/1 g FW in the population 1 at green maturity (green fruits), and the highest of 17.86 µM Trolox/1 g FW in two populations (P2 and P4) at physiological maturity (red fruits). As it can be seen from the tables, the red pepper fruits extract showed a higher antioxidant potential than that of the green fruits, confirmed by Materska & Peruka (2005) data, which claim that the antioxidant activity is higher in red fruits than in the green ones. The

differences are also obvious between the two cultivation areas. The variation of the antioxidant potential is also influenced by the rootstock or the number of stems the plant has (Soare et al. 2018). The increase of the antioxidant activity on fruit maturity phases was also demonstrated by Menichini et al. (2009) in two phases of the development of hot pepper fruits, green phase and red-mature phase. The red fruits have a higher antioxidant capacity than the green fruits, and this is supported by Cervantez-Paz et al. (2012). Alvarez-Parrilla et al. (2011) claim that the values of the antioxidant activity through the ABTS method were between 4 and 18 μM Trolox/1g FW, lower than through the DPPH method in whole hot pepper fruits of the Jalapeno variety. Moreno-Escamilla et al. (2015), Cervantes-Paz et al. (2012) and Materska & Peruka (2005) also reported higher values of the antioxidant activity through DPPH than through ABTS, similar to the present study. Comparing the antioxidant activity determined by the two methods, there is a significant difference between the area of Almăj and that of Filiaşi. The two stages of harvesting revealed that all the populations in Almăj area had superior antioxidant activity than those of Filiaşi. Correlations between phytonutrients and antioxidant activity. As described above, the phytonutrient content of the hot pepper fruits has been influenced by the maturation stage and the cultivation area. The antioxidant activity, which is an indicator of the overall health benefits of the hot pepper fruits, varied during maturation from one cultivar to another and between cultivation areas. In order to better observe the independent maturation relationships between the compounds that determine the antioxidant activity, the correlations between the antioxidant activity and the phytonutrients were evaluated in the two maturation stages for all the tested populations on the crop areas. Positive correlations between antioxidant activity and total polyphenols have been observed throughout the maturation process in both locations (Table 3 and 4).

These phytonutrients are all well-known antioxidant compounds and these results are in line with previous reports describing the antioxidant effects of different fruits and vegetables (Nascimento et al. 2014, Naguib et al. 2012, Alvarez-Parrilla et al. 2011). These results entitle us to affirm that the accumulation of these compounds was the main cause of the increase in antioxidant activity of the hot pepper fruits.

Table 3. The correlations between the analyzed chemical compounds of *Capsicum annuum* L in Filiaş area.

Specification*	Ascorbic acid (mg/kg^{-1} fw)	Total polyphenols (mg GAE/kg^{-1} FW)	Antioxidant activity (µMTrolox/1g FW)ABTS	Antioxidant activity (µM Trolox/1g FW) DPPH
AA	1			
TP	- 0.447	1		
ABTS	- 0.995	0.358*	1	
DPPH	- 0.888	0.037	0.916*	1
Harvesting in green; p 1% = 0,33				
AA	1			
TP	- 0.502	1		
ABTS	- 0.910	0.102	1	
DPPH	- 0.990	0.388*	0.956*	1
Harvesting in green; p 1% = 0,33				

*AA- ascorbic acid; TP- total polyphenols; ABTS and DPPH- antioxidant activity

Table 4. The correlations between the analyzed chemical compounds of *Capsicum annuum* L in Almăj area.

Specification*	Ascorbic acid (mg/kg^{-1} FW)	Total polyphenols (mg GAE/kg^{-1} FW)	Antioxidant activity (µMTrolox/1g FW) ABTS	Antioxidant activity (µM Trolox/1g FW) DPPH
AA	1			
TP	- 0.199	1		
ABTS	- 0.277	0.983*	1	
DPPH	- 0.319	0.979*	0.998*	1
Harvesting in green; p 1% = 0,33				
AA	1			
TP	- 0.894	1		
ABTS	- 0.923	0.652*	1	
DPPH	- 0.639	0.245	0.876*	1
Harvesting in green; p 1% = 0,33				

*AA- ascorbic acid; TP- total polyphenols; ABTS and DPPH- antioxidant activity; * -

CONCLUSIONS

The present study identified the modifications of various phytonutrients and antioxidant activity of hot pepper fruits in the two stages of maturation. The content of ascorbic acid, total polyphenols and antioxidant activity increased with the maturity of the pepper fruits. These results suggest that, although they are affected by the maturity stage, the pepper phytonutrients undergo certain changes depending on the cultivar and the cultivation area. These observations can provide additional information on the content of phytonutrients that are useful to consumers of fresh hot pepper, as green or red fruits. Populations studied are useful to breeders in selection programs of particular importance for the creation of new varieties and hybrids targeting the biochemical characteristics of the fruits.

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