

Accumulation of capsaicin in two chili pepper varieties (*Capsicum annuum* L.) at different stages of fruit maturity

Emese EGYED¹, Roxana L. STOIAN-DOD¹,
Alexandru-Ioan APAHIDEAN¹, Emese GÁL^{2*}, Sándor RÓZSA^{1*},
Tincuța M. GOCAN¹, Dănuț-Nicolae MĂNIUȚIU¹

¹University of Agricultural Sciences and Veterinary Medicine, Faculty of Horticulture and Business in Rural Development, 3-5 Mănăstur St., Cluj-Napoca, Romania; mse73tomtim@gmail.com; roxana.stoian@usamvcluj.ro; alexandru.apahidean@usamvcluj.ro; rozsa.sandor@usamvcluj.ro (*corresponding author); tincuta.gocan@usamvcluj.ro; dan_maniuțiu@yahoo.com

²University of Babeș-Bolyai, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos St., Cluj-Napoca, Romania; gal.emese.81@gmail.com (*corresponding author)

Abstract

Chili peppers are appreciated and consumed especially for their sensorial attributes, but also because of their rich content in bioactive compounds. Capsaicin is one of the most important compounds found in peppers that gives them their spicy heat profile. Therefore, the main aim of this study was to determine the optimal harvesting time of hot peppers with the highest content in capsaicin. The capsaicin content of chili peppers depends on several factors, including the variety of pepper, growing conditions, and cultivation method. Capsaicin is mainly concentrated in the internal membranes of the pepper, where the seeds are also found. These biochemicals are used mostly in food industry, but anti-cancer effects have also been reported. To determine the capsaicin content, gas chromatographic analysis of volatile compounds was performed in both young and adult plants. 'Hyffae F1' and 'Paprika giallo' hot pepper hybrids grown in greenhouse conditions were subjected to both chemical and organic cultivation treatments and two planting densities. The results obtained, showed that the capsaicin content varied between 12.11 and 39.01%. The highest quantity was recorded in 'Paprika giallo' under the influence of chemical treatments with the fruits were harvested at physiological maturity. The results also revealed that variations in capsaicin contents were rather influenced by harvesting time than the cultivar.

Keywords: fertilisation; GC-MS analysis; peppers; ripening stage

Introduction

Chili pepper is a horticultural plant that can be used as an ingredient or spice. It belongs to the *Capsicum* genus, within the Solanaceae family, which includes over 35 recognized species, of which five *C. annuum*, *C. chinense*, *C. baccatum*, *C. frutescens* and *C. pubescens* have been domesticated (Pérez-Grajales *et al.*, 2019). It is native to Central and South America but is now cultivated in different climatic regions around the world

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(Barboza *et al.*, 2019; Pickersgill, 2018). Since consumers' demand increased, new varieties have been obtained in terms of shape and color and sensory properties (Guo *et al.*, 2021). Sensory attributes and chemical composition of peppers are influenced by several factors, such as genotype, growing conditions, ripening and climate (Perucka and Oleszek, 2000; Popelka *et al.*, 2017, Nazneen *et al.*, 2023; Ursu *et al.*, 2012).

Chili pepper is one of the most important vegetables, cultivated for its multiple uses such as culinary and medicinal uses. Pepper fruits can be consumed fresh at technical or physiological maturity, dried as paprika, or processed in the canning industry (Carolina *et al.*, 2021). The red pigment extracted from the mature fruits is used as a natural dye in the food and cosmetic industry (Baenas *et al.*, 2021). Some species and varieties of peppers also have decorative value, being cultivated as ornamental plants (Sahid *et al.*, 2023). Chili pepper has a high adaptability, so it can be grown in different environmental conditions from tropical to temperate ones, being widespread and widely used in gastronomy in many regions of the world. Spicy varieties of pepper are consumed in small quantities, being considered spices for taste, which stimulate the appetite. The paprika obtained by grinding fruits is a frequently used spice in culinary products from different countries, while in Romania it is produced and used mainly in Banat and Transylvania.

Globally, due to their economic importance the Asian Research and Development Center for Vegetable Crops (AVRDC), considers this species one of the main objectives, so in order to create new cultivars with superior properties, it made a collection of 5177 genotypes from 81 countries. In the catalogue of the European Union, 2444 genotypes are currently registered (Soameş and Icoana, 2020; Ursu *et al.*, 2013).

The amounts of capsaicinoids found in pepper fruits can vary depending on light intensity, temperature and minerals with which the plant is fertilized; the age of the fruit and the position of the fruit on the plant can also influence their content of capsaicinoids (Marincaş *et al.*, 2018; Mali *et al.*, 2019). In commercial chili pepper fruits, capsaicin content usually varies from 33 to 59%, while dihydrocapsaicin content ranges from 30 to 51% and nondihydrocapsaicin from 7 to 15%, with the rest 5% capsaicinoids (Baytak and Aslanoglu, 2017; Olguin-Rojas *et al.*, 2019; Muñoz-Concha *et al.*, 2020). The heat we feel when we eat hot peppers is due to bioactive compounds such as capsaicinoids, which are volatile alkaloids (Fayos *et al.*, 2019). The main compounds are capsaicin and dihydrocapsaicin comprising together 97% of the total capsaicinoid content, the remaining 3% corresponding to minor capsaicinoids. Some studies have shown that these compounds can act as chemopreventive agents, preventing the development of cancer by inhibiting the proliferation of malignant cells and increasing apoptosis (Xie *et al.*, 2022). Another role is that it promotes vascular health, preventing cardiovascular disorders and reducing inflammatory responses (Thongin *et al.*, 2022; Cortes-Ferre *et al.*, 2022), has been shown to protect the liver by reducing oxidative stress (Das *et al.*, 2022) and demonstrated analgesic properties (Nava-Ochoa *et al.*, 2021). Biosynthesis of capsaicinoids have been identified in the placenta of peppers; they accumulate in the vacuoles of the epiderma of the epicarp's internal teguments until the cells are metabolized, and then their extracellular secretion occurs (Sun *et al.*, 2022). Capsaicinoids have also been found in other parts of chili peppers, such as the pericarp, seeds, or even in the leaves or stems, but in considerably smaller amounts. Several studies have shown that the capsaicinoid synthesis occurs during fruit ripening. As peppers mature, they undergo biochemical reactions, physiological and structural changes that have a significant impact on their quality (Chiaiese *et al.*, 2019; Popelka *et al.*, 2017). Due to the fact that peppers are harvested and consumed at different ripening stages, from immature to overripe, studying the accumulation of these bioactive compounds during fruit development is crucial. The capsaicin level in pepper is around 0.025%, and in chili peppers around 0.25% (Holzer, 1994).

Therefore, for a more comprehensive understanding of the behavior of these health-promoting bioactive compounds, more in-depth studies with a larger number of varieties and with more complex technologies will need to be pursued and studied. Among the first researchers were Iwai *et al.* (1979), which suggested a behavior in the accumulation of capsaicin during ripening until reaching a maximum value. Then, a rapid reversal occurred as these compounds were degraded, approximately 60% (Iwai *et al.* 1979). Other research, has

demonstrated that each pepper variety has a different ripening period (Lima *et al.*, 2017). Consequently, the aim of this study was to investigate chili pepper fruits obtained from a hybrid and a variety under the same growing conditions, harvested at technical and physiological maturity to determine the most suitable harvest time to obtain the highest content of capsaicin.

Materials and Methods

Biological material

In order to carry out this research, 'Hyffae F1' hybrid and 'Paprika Giallo' have been used as biological materials. 'Hyffae F1' hybrid is vigorous hybrid with rich foliage that covers the fruit very well until maturity. It has a high ability to adapt to heat and drought stress conditions. The fruits are dark green and turn bright red at physiological maturity. The average fruit is 10-15 × 170-180 mm (Figure 1 a).

'Paprika giallo' is a variety used for the preparation of famous hot dishes. The plant is vigorous, it can be grown both in solar tunnels and greenhouses but also in the field. Fruits are light green and turn yellow at biological maturity and are very spicy (Figure 1 b).



Figure 1. The two pepper cultivars studied, fruits at consumption maturity and physiological maturity respectively. a) 'Hyffae F1' – chemical fertilization with training system, 30,000 plants/ ha – 14 plants/ parcel. b) 'Paprika Giallo' – chemical fertilization with training system, 30,000 plants/ ha – 14 plants/ parcel

Experimental site and technology

The experiment has been carried out in the solar tunnels located in Păniceni, Căpușu Mare commune situated in the northeastern part of the Gilău Mountains, on the Căpuș river, in the western part of Cluj County. The size of one experimental plot was 4.5 m², while the whole experimental design covered in total 216 m².

The 'Hyffae F1' hybrid and the 'Paprika Giallo' varieties were used to establish the culture. To reach a plot of 30,000 plants/ha, the planting distance between plants per row was 41.5 cm, and at a plot of 45,000 plants/ha, the distance between two plants/row was 30 cm. Soil fertilization was carried out starting from 09.06.2020 once every 15 days, with 40 g/m² of Cropcare 11-11-21, and 80 g/m² (organic fertilizer) of Siforga 5-3-8 (chemical fertilizer), during the growing period applying 8 fertilizations, in total a grammage of 320 g/m² Cropcare and 640 g/m² Siforga.

The chili peppers seed have been sown on the 24th of March, 2020, in heated solar tunnels in seedling trays of 45 cells. In each cell, one seed was distributed at a depth of 0.8-1 cm. Soil preparation for planting involved minimum tillage followed by the installation of the drip irrigation system. The chili pepper seedlings were planted on the 15th of May, 2020, and fertilizations were applied starting from the 9th of June. During the vegetation period, weeding practices were carried out as often as needed and two treatments were applied

against aphids, on the 18th of June with Mospilan 20 SG and another on the 3rd of July with Movento 100 SC. The plants were watered every 2 days, for 30 minutes each, with a flow rate of 2 m³/h. Pepper ripening was tracked by attaching ribbons of different color and started immediately after the flowering period. Harvesting was carried in a chronological order according to the color of the ribbon. The fruits were harvested manually starting on the 24th of July, and repeated at every 2 weeks until October the 10th. After this date the fruits no longer showed any specific characters.

Sample preparation and extraction

Ultrasound-assisted extraction (UAE) has been used to extract the capsaicin from chili pepper samples. UAE is known as one of the easiest extraction techniques because it uses common laboratory equipment, such as an ultrasonic bath. Barbero *et al.* (2008) proposed using ultrasound in various industries because of its positive effects on extracting capsaicinoids from chili peppers. Analytical-grade ethyl alcohol absolute anhydrous (99.9%) and dichloromethane (99.8%) were purchased from VWR suppliers, and used without further purification.

Ethanol (99.9%) was used for the extraction of capsaicinoids. Solid-liquid extraction combined with ultrasound extraction is one of the most commonly used procedures. Five grams of fresh chili pepper fruits (crushed) were extracted with 10 ml of EtOH and maintained for 30 min at 25 °C, in an ultrasonic bath (at 37 kHz). After separation (filtration), the ethanolic part was evaporated to dryness by a rotary evaporator, at 40 °C. The extracts were reconstituted with 200 µL of dichloromethane (DCM, and from this solution 1 µL was injected into the GC-MS system. All extractions were carried out in duplicates. GC MS Analysis was performed as described by Araceli *et al.* (2012).

Gas chromatographic analysis of volatile compounds was performed using a GC Shimadzu QP 2010 PLUS coupled with a mass spectrometer (QP 2010 Shimadzu) equipped with an AOC-20i+s injector, and a ZB-5 MS plus capillary column (30 m × 0.25 mm, 0.25 µm film thickness, Phenomenex). The temperature of the ion source and the interface temperature were 220 °C. The oven temperature was held at 40 °C, for 1 min, then programmed to rise from 40 °C to 300 °C, at 10 °C/min, and held at this temperature for 5 min. The carrier gas was helium (99.99990% from Linde Hungary), at a constant flow rate of 0.83 mL/min. The injection volume was 1 µL, made in split mode (20:1) at 260 °C. The detector was set to electron impact mode (EI, 70 eV) with an acquisition range (m/z) from 35 to 600 in scan mode. To identify capsaicinoids in peppers, retention times and chromatographic peaks were compared to NIST library data.

Statistical analyses

The data obtained were processed with the Analysis of Variance (ANOVA), followed by Duncan's multiple range test to compare the means of the experimental variants and determine statistically significant differences between the means.

Results

During this research the behavior of two chili pepper varieties were investigated, using the same environmental conditions and agricultural practices.

In order to determine the differences between the two chili pepper varieties under study, some parameters such as the mass of fresh and dried peppers (g) calculated as an average of 5 fruits and the loss of weight after drying (%) for the variety 'Paprika Giallo' were also determined and calculated (Table 1). The results showed that the highest weight of fresh peppers, under chemical fertilization, was recorded in the variant with 30,000 plants trained (15.9 g) while the lowest weight was obtained in fruits obtained from the untrained system and a planting density of 45,000 without chemical fertilization (12.3 g). After drying, the weight of chili peppers decreased by 84.11 % and 88.97%.

Table 1. Weight of fresh and dried chilli peppers and weight loss after drying ‘Paprika giallo’

Fertilization	Training system	Density (plants/ha)	Weight of fresh peppers		Weight of dried peppers		Weight loss [%]
			5 pcs (g)	Mean	5 pcs (g)	Mean	
Organic	Untrained	45000	69.5	13.9	8.57	1.71	87.66
Organic	Untrained	30000	71.5	14.3	9.43	1.88	86.80
Organic	Trained	45000	71.5	14.3	6.06	1.21	85.85
Organic	Trained	30000	69.5	13.9	5.77	1.15	84.11
Chemical	Untrained	45000	66	13.2	7.27	1.45	88.97
Chemical	Untrained	30000	61.5	12.3	6.88	1.37	88.81
Chemical	Trained	45000	73.5	14.7	10.37	2.07	85.88
Chemical	Trained	30000	79.5	15.9	12.52	2.5	84.25

The mass of fresh and dried peppers (g) calculated as an average of 5 fruits and the weight loss after drying (%) for the ‘Hyffae F1’ hybrid is presented in Table 2. The results show that the highest weight of fresh chili pepper under organic fertilization was recorded in the variant with 30 000 untrained plants (37.9 g) while the lowest mass was found in the plot of 45 000 pl/ha, untrained (25.8 g) under the same organic fertilization. After drying, the weight of chili pepper decreased substantially 81.90 % to 88.54 %. These results are in accordance with those reported by Ursu *et al.* (2013) regarding the weight loss of chili peppers due drying.

Table 2. Weight of fresh and dried chilli peppers and weight loss after drying ‘Hyffae F1’

Fertilization	Training system	Density (plants/ha)	Weight of fresh peppers		Weight of dried peppers		Weight loss [%]
			5 pcs (g)	Mean	5 pcs (g)	Mean	
Organic	Untrained	45000	129	25.8	17.89	3.57	86.13
Organic	Untrained	30000	189.5	37.9	34.29	6.85	81.90
Organic	Trained	45000	155.5	31.1	25.79	5.15	83.41
Organic	Trained	30000	162	32.4	27.08	5.41	83.28
Chemical	Untrained	45000	150	30.0	17.19	3.43	88.54
Chemical	Untrained	30000	168.5	33.7	29.63	5.92	82.41
Chemical	Trained	45000	173	34.6	30.58	6.11	82.32
Chemical	Trained	30000	121.5	24.3	16.83	3.36	86.14

The GC-MS chromatogram was used to identify the phytoconstituents from the chilli-extracted sample, and the obtained mass spectrum of compounds was compared with the spectra stored in NIST and Wiley library to establish the compound structure. The three major constituents of the samples, in almost all cases, were capsaicin with retention time of 25.7 min, nonivamide 25.9 min and acexamic acid with a retention time of 22.3 min (Figure 2). Nonivamide, also known as pseudocapsaicin, is a constituent of chili peppers, it is insoluble in water and more stable than capsaicin. Acexamic acid is a medium-chain fatty acid with biological activity.

An example of the chemical profile is presented in Figure 2 for ‘Paprika Giallo’ peppers obtained during chemical fertilization harvested at technological maturity.

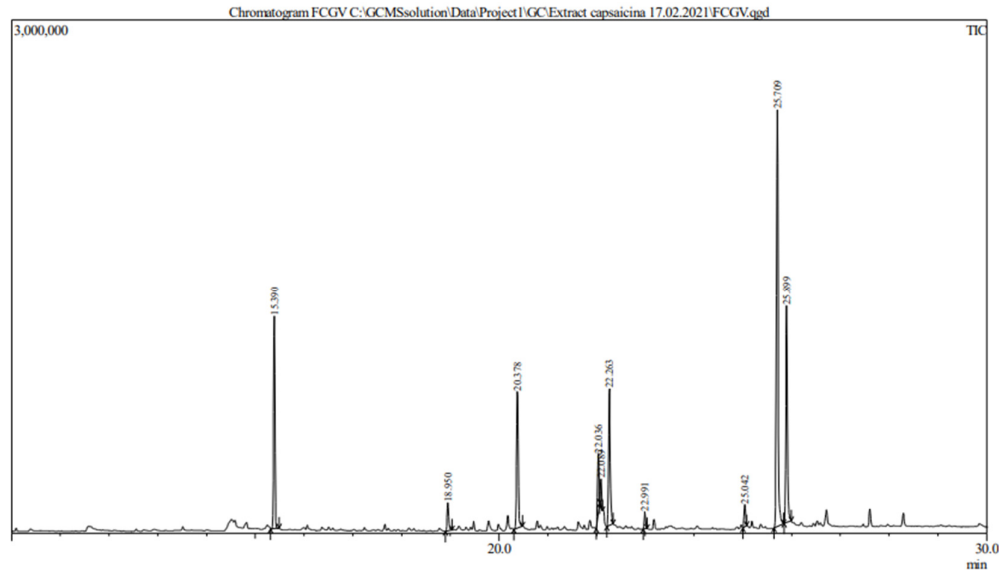


Figure 2. GC-MS chromatogram from Paprika giallo extract.

The aim of the GCMS analysis was to detect capsaicin in fruits of different maturity stages. The GC-MS analysis revealed the presence of different classes of compounds, including acids, esters, aldehydes, amides etc. All samples contain capsaicin and nonivamide, which are typical compounds for chili pepper extracts, the difference lies only in the amount of capsaicin. Fruits harvested at various stages of maturity show clear changes in the peak areas of capsaicin. The peak period of the harvested fruits being August 24.

Beside capsaicin, other compounds were also detected in the chili pepper samples as follow: nonivamide, DDMP, HMF, 9,12-Octadecadienoic acid (Z,Z), 9,12,15-Octadecatrienoic acid, methyl ester and acexamamic acid (Table 3). The results also revealed that the most beneficial effect of chemical fertilization was observed in ‘Paprika Giallo’ exhibiting a content of 39.01% of capsaicin. However, it is worth to mention that regardless the experimental variant, the contents of capsaicin and nonivamides in the fruits were higher than other compounds (Table 3).

Table 3. Variation of the amount of compounds according to the fertilization and maturation grade

Variety	Fertilization	Maturation grade	Compounds (%)						
			Capsaicin	Nonivamide	DDMP	HMF	9,12-Octadecadienoic acid (Z,Z)	9,12,15-Octadecatrienoic acid, methyl ester	Acexamamic acid
Paprika Giallo	Organic	Physiological	35.94	20.75	2.13	1.24	6.86	4.09	10.72
	Organic	Technical	30.52	18.44	0.31	0.44	13.29	2.98	8.36
	Chemical	Physiological	39.01	19.49	0.72	0.96	6.82	3.75	11.56
	Chemical	Technical	34.86	16.83	0.25	1.09	4.05	1.42	11.51
Hyffae F1	Organic	Physiological	15.56	7.33	4.88	12.12	3.29	17.63	10.00
	Organic	Technical	12.11	5.98	1.27	3.10	6.81	9.37	6.41
	Chemical	Physiological	34.48	14.76	5.26	10.11	1.00	4.49	10.46
	Chemical	Technical	25.56	9.92	0.70	2.83	7.98	3.83	7.34

Quantities are expressed in A%, calculated as the average of two analyses.

4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-DDMP

2-Furancarboxaldehyde 5-(hydroxymethyl)-HMF

Previous research results suggest that the capsaicin content usually range from 100 µg.g in chili peppers to 2500 µg.g in red pepper (Parrish, 1996). Pepper species of *Capsicum annum*, such as *C. frutescens* and *C. chinense* contain 220-20 000 µg total capsaicinoids/g dry matter (Thomas *et al.*, 1996). In another study,

cayenne pepper samples had average capsaicin and dihydrocapsaicin contents of 1320 and 830 $\mu\text{g/g}$ dry matter (Lopez-Hernandez *et al.* 1996).

Changes in the total capsaicin content of peppers were also studied, both in fruits from young and mature plants. The fruits were harvested at different stages of ripening during the entire vegetation period. As presented in Figure 3, it can be observed that in the fruits of 'Paprika Giallo' variety harvested at technical maturity, regardless of fertilization type, the amount of capsaicin at the beginning of the harvest period was much higher than at the end of the ripening period, reaching its maximum on the 24th of August, under the influence of organic fertilization (34.86%).

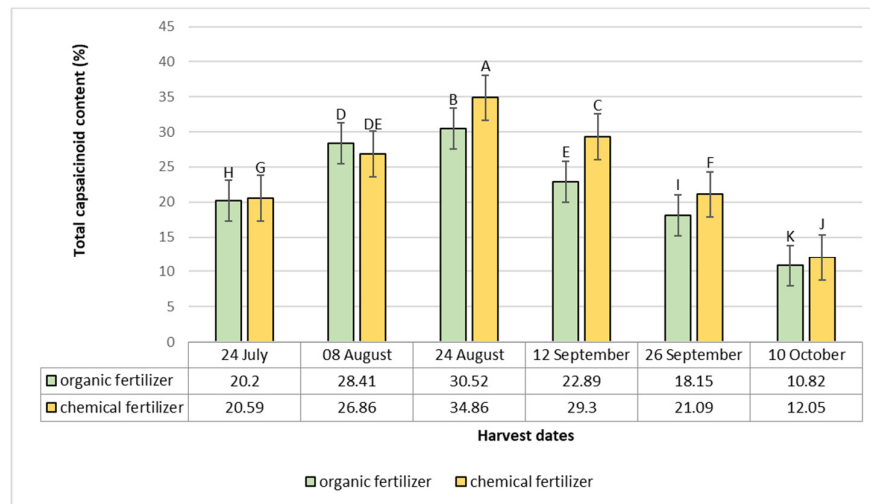


Figure 3. Changes in total capsaisinoid content (%) during the ripening process in Paprika giallo pepper fruits harvested at technical maturity

Different letters above the bars indicate a significant difference between the means according to Duncan's Multiple Range test.

Several authors reported that the accumulation of capsaisinoids in peppers reaches a higher concentration in the first stages of fruit ripening (Mueller-Seitz *et al.*, 2008; Estrada *et al.*, 2000). This hypothesis was also confirmed by the results obtained in this experiment. In the case of fruits that have reached physiological maturity (Figure 4), a slight increase was observed between July 24th and August 24th, regardless of fertilization. The lowest percentage of capsaisin was recorded in fruits harvested on the 10th of October, under both organic and chemical fertilization. This variability regarding the capsaisin content during fruit ripening might be explained by the activity of the capsaisin enzyme that allows the synthesis of new bioactive compounds in competition with the action of peroxisome enzymes that cause their degradation (Lu *et al.*, 2020).

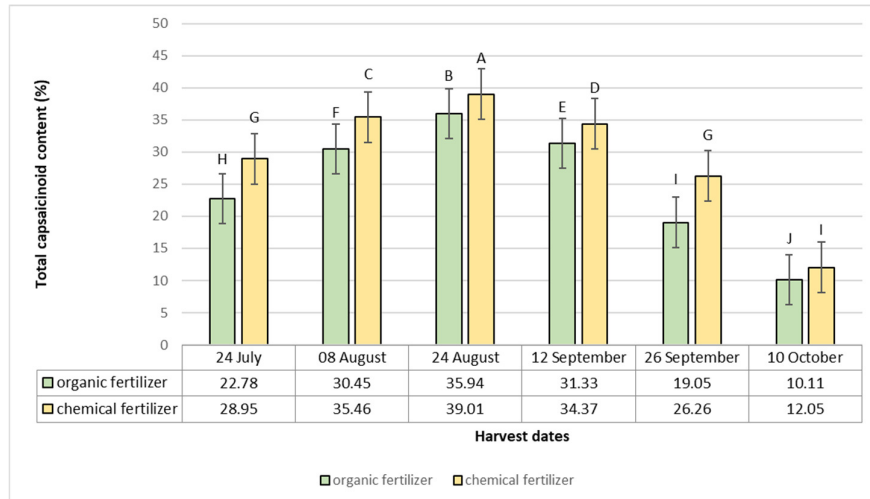


Figure 4. Changes in total capsaicinoid content (%) during the ripening process in ‘Paprika giallo’ fruits harvested at physiological maturity
Different letters above the bars indicate a significant difference between the means according to Duncan’s Multiple Range test.

Considering ‘Hyffae F1’ hybrid, the results highlighted that fertilization had a major influence on the capsaicin content of the fruits harvested at technical maturity. The variants fertilized with chemical fertilizers exhibited higher values in terms of the amount of total capsaicin, during the entire ripening period. It was observed that the amount of capsaicin showed a three-fold decrease under chemical fertilization as compared to the capsaicin content recorded at the beginning of the investigated period. In the case of organic fertilization, during the peak period (August 24), the amount of total capsaicin (12.11%) had a value similar to the amount of capsaicin obtained in ‘Paprika giallo’ variety (10.82%), from the fruits harvested at technical maturity (Figure 5).

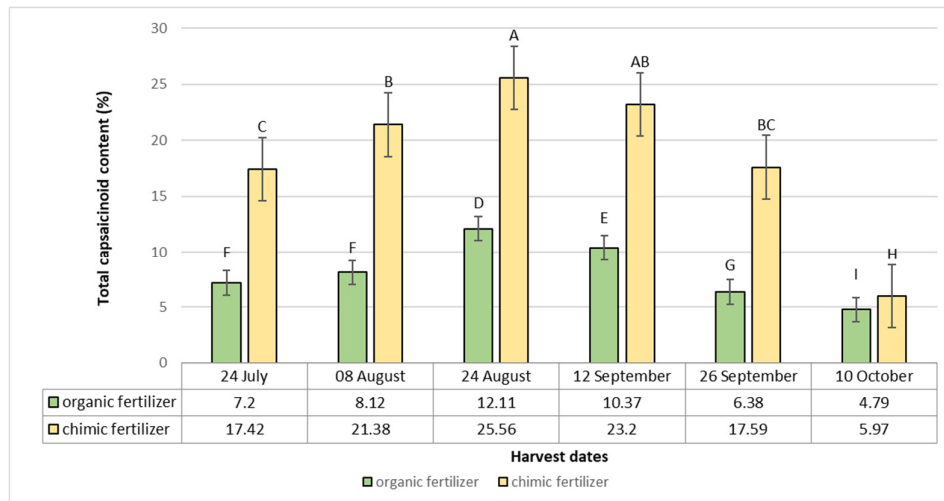


Figure 5. Changes in total capsaicinoid content (%) during the ripening process in ‘Hyffae F1’ hybrid fruits harvested at technical maturity – green fruits
Different letters above the bars indicate a significant difference between the the means according to Duncan’s Multiple Range test.

Furthermore, the results suggest that fertilization had an important role in the accumulation of capsaicin in the red fruits of ‘Hyffae F1’ hybrid (Figure 6). The last determinations regarding the capsaicin content were made on the 10th of October. The data showed a very low capsaicin content in the fruits of ‘Hyffae F1’ hybrid as compared to the ‘Paprika giallo’ variety regardless fertilization type. These results suggests that in addition to climatic factors (water availability, growing conditions, mineral supply, light and temperature and plant maturity, there are certain genetic factors as well which influence the capsaicin content of chili peppers. A similar situation was reported by Vázquez-Espinosa *et al.* (2020) claiming that the concentration of capsaicinoids increases up to 41 dpa (days after flowering) followed by a slight decrease in the following period.

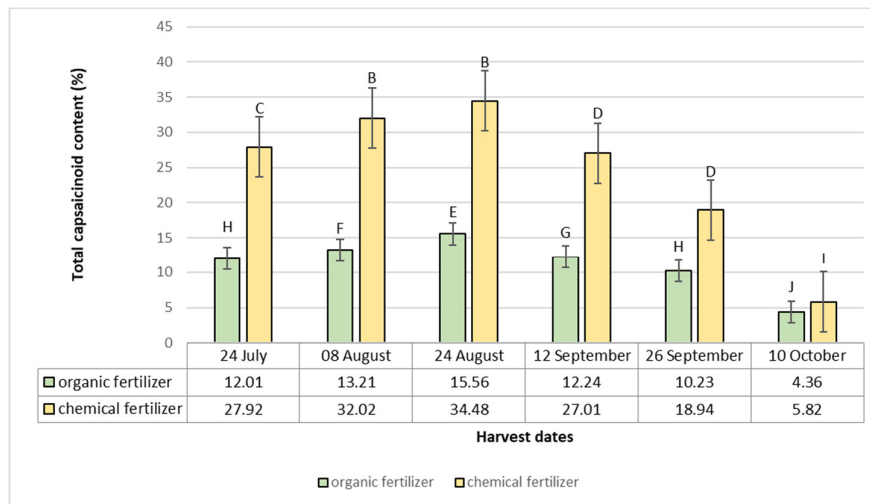


Figure 6. Changes in total capsaicinoid content (%) during the ripening process in Hyffae F1 pepper fruits harvested at physiological maturity. Different letters above the bars indicate a significant difference between the means according to Duncan’s Multiple Range test.

Discussion

Capsaicin binds to TRPV1 receptors (pain vanilloid receptors), which are responsible for the perception of heat and pain. Activation of these receptors induces the burning sensation. It can also stimulate the release of endorphins, which are natural pain-reducing and mood-enhancing chemicals. Although capsaicin has many benefits, excessive consumption of chili peppers can cause gastric irritation and discomfort. People with sensitive digestive conditions should be cautious and consume chili peppers in moderation (Baenas *et al.*, 2019). Changes in the total capsaicin content of pepper were studied, both in fruits from young and mature plants (Pena-Alvarez *et al.* 2012). The fruits were harvested at different stages of ripening during the whole crop period. The development of hot peppers (*Capsicum* spp.) involves several stages, each characterized by specific changes in capsaicin content. These stages are essential for understanding how and when capsaicin concentration varies during plant growth. The capsaicin content of hot peppers varies significantly throughout fruit development, reaching maximum levels at full maturity. During the formation and growth of the fruit, the capsaicin content increases constantly. This growth is influenced by genetic and environmental factors, including temperature, light and soil nutrition. If the fruit remains on the plant too long after full maturity, capsaicin levels may begin to drop slightly (Estrada *et al.*, 2000). This knowledge is essential for growers and producers, who can optimize growing conditions to obtain chili peppers with desired levels of spiciness.

Conclusions

Based on the presented results, it can be stated that the ripening stage is a crucial factor that determines the amount of capsaicin in chili pepper fruits. Changes that occur during the entire ripening period regardless of the cultivar, suggest that the harvest should be done in August and September. The highest amount of capsaicin appeared to be found in 'Paprika Giallo' variety which gives a hot taste. Fertilization influenced the amount of capsaicin in the investigated chili pepper fruits, the highest values being registered under the effects of chemical fertilization. Beside fertilizers, the time of harvest had a significant influence on the capsaicin content of the fruits.

Authors' Contributions

Conceptualization: MDN, RS and GTM; Data curation: EE, AAI and GE; Formal analysis: SR, RS and EE; Funding acquisition: RS; Investigation: EE, GTM, SR and MDN; Methodology: GE and RS; Software: GTM; Supervision: GE, RS and MDN; Validation: MDN; Visualization: AAI, SR, RS and MDN; Writing - original draft: GTM, EE and RS; Writing - review and editing: EE and RS.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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