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Original Article

Evolution of Polyphenols, Flavonoids, and Tannins Content in Walnut Leaves and Green Walnut Husk during Growing Season

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Abstract

The walnut leaves and the green walnut husk are been widely used in folk medicine. These vegetal materials have been reported as a source of bioactive molecules such as the phenolic compounds. In this research, there were analysed different quality parameters afferent to several walnut varieties, harvested from the germplasm collection located at S.C.D.P. Vâlcea, Romania. Romanian cultivars 'Jupâneşti', 'Valrex' and 'Valmit' and French one 'Franquette' were analyzed. The variation in bioactive compounds content from the walnut leaves and walnut husk at different developmental stages (from June to September) was investigated. The results showed that the content in polyphenols, flavonoids and tannins has significantly increased until the ripening stage, after that a decrease of the values of these parameters follows. Greater values of the three parameters were also observed at walnut leaves and green walnut husk of cultivars attacked by walnut blight, caused by the bacterium *Xanthomonas arboricola* pv. *juglandis (Xaj)*. The findings of this study reveal the mode of variation in the content of polyphenols, flavonoids and tannins during the growing season of the walnut leaves and green walnut husk, emphasizing also the influence of *Xaj*. The results obtained demonstrated the potential of the walnut leaves and the green walnut husk as an economical source of antioxidant and antimicrobial agents.

Keywords: flavonoids; green walnut husk; *Juglans regia* L.; polyphenols; tannins; walnut leaves; *Xanthomonas arboricola* pv. *juglandis*

Introduction

The common walnut (*Juglans regia* L.), known as English or Persian walnut, considered one of the oldest tree species, is important both from both economically and nutritionally, due to the quality of its fruits, the superior quality of the wood and its other organs. As a technical plant, the walnut provides the raw material for many branches of industry. It is appreciated for its wood with high quality, having high strength, plasticity and finesse, being used in the manufacture of the luxury furniture, the sculpture, in the aircraft and car industry. The walnut is also used as a medicinal plant. The walnut leaves juice is used as a vermifuge, as a tonic, as an ointment for varicose veins, as urine disinfectant. The homeopathy followers recommend the mesocarp for the treatment of acne, irritation, vomiting. As an ecological plant, walnut is irreplaceable because it is one of the few trees that absorb the heavy metals from the atmosphere. Although it grows slowly due to its highly developed roots, the walnut is used against the soil erosion and the landslides. Thus, being at the same time a technical, forestry, medicinal, dendrological and ameliorative plant, the walnut is unquestionably considered to be one of the most important tree species in our country.

Green and dry walnut leaves are been widely used in folk medicine for the infusions, treatment of skin inflammations, ulcers and for its antiseptic and astringent properties (Pereira *et al*., 2007; Almeida *et al*., 2008). Green walnut husk is an agro-forest waste generated in the walnut harvest that could be valued as a source of natural compounds with antioxidant and antimicrobial properties (Fernández-Agulló *et al*., 2013). It is the basic material for the traditional walnut liqueur (Stampar *et al*., 2006).

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Walnut and by-products derived from the walnut tree contains flavonoids, phospholipids, sterols, triterpenes, quinones, oils, tannins, essential fatty acids (Fukuda *et al.*, 2003; Amaral *et al.*, 2004; Li *et al.*, 2006; Stampar *et al.*, 2006; Li *et al.*, 2007; Pereira *et al.*, 2007; Pereira *et al.*, 2008; Cosmulescu and Trandafir, 2011; Fernández-Agulló *et al.*, 2013; Tapia *et al.*, 2013; Cosmulescu *et al.*, 2014; Kafkas *et al.*, 2017). Phenolic compounds such as pyrogallol, p-hydroxybenzoic acid, vanillic acid, ethyl gallate, protocatechuic acid, tannic acid, syringic acid, gallic acid, ferulic acid, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, chlorogenic acid, and caffeic acid were also isolated from walnuts. Walnut has about 16 polyphenols, with antioxidant activity so protective that it is described as "remarkable". Free fatty acids, diglycerides, sterols, sterol esters, phosphatides and vitamins are present in minor quantities in walnut.

Due to composition of the fruit, the walnut is classified as a strategic nut crop for human nutrition and is included in the FAO list of priority plants (Gandev, 2007). The seed part of the fruit (kernel) is consumed fresh, toasted, or mixed with other confectionaries. Not only dry fruits (nuts) are used but also green walnuts, kernels, bark, green walnut husk and leaves have been used in both cosmetic and pharmaceutical industry (Stampar *et al.*, 2006).

Our paper aimed to determine the variation in time of some composition parameters from the walnut leaves and the green walnut husk for different *Juglans regia* L. cultivars, harvested in 2018 from UCv-SCDP Vâlcea, Romania. For all analysed cultivars, the content of polyphenols, flavonoids and tannins from the walnut leaves and the green walnut husk was evaluated knowing that the identification and the quantification of the active compounds from these vegetal materials may explain their therapeutic effects, already observed clinically. Finally, the study reveals the mode of variation in the content of polyphenols, flavonoids and tannins during the developmental stages of the walnut leaves and the green walnut husk, emphasizing also the influence of *Xaj* bacterium.

Materials and Methods

Plant material

Three Romanian cultivars 'Jupâneşti', 'Valrex' and 'Valmit' and French one 'Franquette' were analyzed. All four cultivars are vigorous, have terminal bearing and good productivity. The Romanian varieties have good resistance to low temperatures (up to -30 °C) while the French cultivar is less resistant to low temperatures. The Romanian varieties have the ripeness period of the fruit in September, the first decade of the month for 'Valrex' cv. and the second decade of the month for 'Jupâneşti' and 'Valmit' cvs. The 'Franquette' cv. presents a later maturity of the fruit as compared to the other cultivar, namely: the second decade of October. 'Franquette' and 'Jupâneşti' cultivars have elliptical nuts and the average weight of 12.1 g of dry in-shell nuts. 'Valrex' cv. has ovate shaped fruit and an average weight of 14.3 g. 'Valmit' cv. has round fruit and a mean weight of 12.6 g. More agronomical and genetic information about these cultivars were presented in our previous works (Giura *et al.*, 2016a, b).

The walnut leaves and the green walnuts were sampled in early morning hours, and stored at 0-4 °C until their use. Before being analysed, the walnut leaves and the green walnuts were washed and wiped with a paper towel. Then, the walnut leaves and the green walnut husk cut into pieces and transformed into a homogeneous mixture with a vertical mixer.

Chemical substances

Gallic acid, catechin, tannic acid and Folin-Ciocalteu reagent were purchased from Redox Bucharest - Sigma Aldrich, Dako, Epp. Romania. Methanol, sodium hydroxide, sodium carbonate, sodium nitrite and aluminium chloride were purchased from Merck Romania SRL.

Chemical analysis and equipment

Quantitative determination of polyphenols was performed by spectrophotometric method using a UV-Vis spectrophotometer PerkinElmer Lambda25. The methodology proposed by Singleton and Rossi (1965) was respected. The method principle is based on forming a blue coloured compound between phosphotungstic acid and polyphenols, in an alkaline medium. For analysis, a methanolic extract of homogenised vegetal material with concentration 100 mg/mL was used. The concentration of polyphenols was calculated using the calibration curve, performed under the same conditions as the sample, using the absorbance values at the maximum absorption, located at 765 nm. In order to obtain the calibration curve, a stock solution of gallic acid was used. 0.5 mL of the methanol extract of vegetal material was added to a 10 mL flask containing 7 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent. After 5 minutes of rest, 2 mL solution of sodium carbonate 10% was added. After 60 minutes of rest, absorbance of the samples was measured and the concentration of polyphenols was estimated. Finally, the content of polyphenols was expressed as mg gallic acid equivalent/ 100 g of vegetal material.

Quantitation of flavonoids was performed by spectrophotometric method using a UV-Vis spectrophotometer PerkinElmer Lambda25. The methodology proposed by Zhishen *et al.* (1999) was respected. The method principle is based on the formation of a yellow-orange-coloured compound by the reaction of flavonoids and aluminium chloride. For analysis, a methanolic extract of vegetal material with concentration 100 mg/mL was used. The concentration of the flavonoids has been calculated using the calibration curve, performed under the same conditions as the sample solutions, using the absorbance values of the maximum absorption, located at 510 nm. To obtain the calibration curve, a stock solution of catechin was made. 1 mL of methanolic extract of vegetal material was added to a 10 mL volumetric flask containing 6 mL of distilled water and 0.5 mL of sodium nitrite 5%. After 5 minutes of rest, in the volumetric flask was added 0.5 mL of aluminium chloride 10%. After 5 minutes, 2 mL solution of sodium hydroxide 1M was added. The absorbance of the solution at 510 nm was measured. Flavonoids concentration was estimated using a calibration curve of catechin and, finally, the content of flavonoids was expressed as mg catechin equivalent/100 g of vegetal material.

Quantitative determination of tannins was performed by spectrophotometric method using a UV-Vis spectrophotometer PerkinElmer Lambda25. The methodology proposed by Makkar *et al.* (1993) was used. For analysis, an aqueous extract of homogenised vegetal material with concentration 100 mg/mL was used. The concentration of tannins was calculated using the calibration curve, performed under the same conditions as the sample, using the absorbance values at the maximum absorption, located at 765 nm. In order to obtain the calibration curve, a stock solution of tannic acid was used. 1 mL of the aqueous extract of vegetal material was added to a 10 mL flask containing 2 mL of distilled water and 2 mL of Folin-Ciocalteu reagent. After 5 minutes of rest, 5 mL solution of sodium carbonate 10% was added. After 60 minutes of rest, absorbance of the samples was measured and the concentration of tannins was estimated. Finally, the content of tannins was expressed as mg tannic acid equivalent/100 g of vegetal material.

Results and Discussion

Walnuts have antioxidant features due to the presence of some bioactive compounds, such as vitamin C, polyphenols, flavonoids, anthocyanins and carotenoids (Cernîşev and Şleagun, 2007). The level of these compounds in the body is an indicator of health; they operated as biomarkers food quality (Costin and Segal, 1999).

Composition of polyphenolic compounds within the by-products derivates from the walnut (Fukuda *et al.*, 2003; Li *et al.*, 2006) is quite well known. Polyphenolic compounds are a class of antioxidants that prevent oxidative damage to lipids and thus increase the nutritional value of the food (Tudor-Radu *et al.*, 2016). They possess a broad spectrum of biochemical activities, such as antioxidant, antimutagenic and anticarcinogenic (Nakamura *et al.*, 2003).

The evolution in time of the polyphenolic compounds content at the walnut leaves and the green walnut husk, both on healthy vegetal materials and attacked by *Xaj* is shown in Figs. 1 to 4. At the walnut leaves, in 15 June 2018, the content of polyphenolic compounds was found between 2510 mg gallic acid equivalent/100 g of vegetal material 'Franquette' cv. and 7632 mg gallic acid equivalent/100 g of vegetal material 'Jupâneşti' cv. The highest content of polyphenolic compounds in the walnut leaves was found at 27 July 2018 for all Romanian cultivars and at 17 August 2018 for French one. Pereira *et al.* (2007) and Cosmulescu *et al.* (2014) obtained similar results.

In the green walnut husk, the amount of polyphenolic compounds was lower compared to the values obtained from the walnut leaves. Thus, in 15 June 2018, the amounts of polyphenolic compounds between 1788 mg gallic acid equivalent/100 g vegetal material 'Jupâneşti' and 2518 mg gallic acid equivalent/100 g vegetal material 'Valmit' were found. The highest content of polyphenolic compounds from the green walnut husks was found in 6 July 2018 for 'Valmit' and 'Valrex' cultivars and in 27 July 2018 for 'Jupăneşti' and 'Franquette' cvs. Oliveira *et al.* (2008) and Fernández-Agulló *et al.* (2013) obtained similar results.

At each sampling stage, the polyphenols content was found to be greater in the vegetal materials attacked by *Xanthomonas arboricola* pv. *juglandis (Xaj)* compared to the healthy vegetal materials.

Flavonoids are a large group of hydroxylated phenolic compounds distributed in green plant kingdom, being located in cell vacuoles. They are carrying out important functions in the plants. They are responsible for colour and aroma of flowers and fruits, which helps to attract pollinating insects. Flavonoids protect plants from different biotic and abiotic stresses. They are regulating cell growth and help for UV filtration, nitrogen fixation, cell cycle inhibition, as chemical messengers. Flavonoids have roles in frost hardiness and in drought resistance and may play a functional role in plant heat acclimation and freezing tolerance (Cushnie and Lamb, 2005; Samanta *et al.*, 2011; Mierziak *et al.*, 2014; Mathesius, 2018).

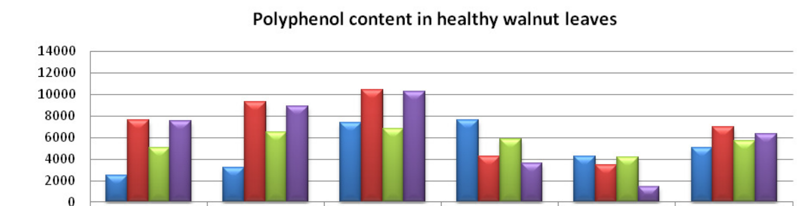
Figs. 5 and 6 indicates the modification in time of flavonoids content in walnut leaves, higher values of flavonoids content being recorded in walnut leaves attacked by *Xaj*. In 15 June 2018, the flavonoids content showed values between 187 mg catechin equivalent/100 g of healthy vegetal material 'Jupâneşti' and 269 mg catechin equivalent/100 g of healthy vegetal material from 'Valmit'. At this sampling stage, for the varieties attacked by *Xaj*, the flavonoids content showed values between 236 mg catechin equivalent/100 g of vegetal material 'Jupâneşti' and 381 mg catechin equivalent/100 g of vegetal material 'Valrex'. The highest content of flavonoids in the walnut leaves was found at 27 July 2018 for all Romanian cvs. and at 17 August 2018 for French one, both at the healthy vegetal material and attacked by *Xaj*.

In the green walnut shell, the flavonoids content was lower compared to the values obtained from the walnut leaves. Thus, in 15 June 2018, the flavonoids content was found between 38 mg catechin equivalent/100 g of healthy vegetal material 'Valrex' and 67 mg catechin equivalent/100 g of healthy vegetal material 'Franquette' while at the vegetal material attacked by *Xaj*, the extreme values were 72 mg catechin equivalent/100 g of vegetal material 'Valmit' and 123 mg catechin equivalent/100 g of vegetal material 'Franquette'. The highest flavonoids content from the green walnut husk was found in 6 July 2018 for 'Valmit' and 'Valrex' cvs., in 27 July 2018 for 'Jupâneşti' cultivar and in 17 August 2018 for 'Franquette' cv.

Tannins are found in very small quantities in most plants and in large quantities they are found in the bark, the wood and the leaves of oak, alder, spruce, poplar, walnut. From a chemical point of view, tannins are polyphenolic compounds, water-soluble, astringent. They are secondary metabolites of vegetal materials, being either galloyl esters and their derivatives or oligomeric and polymeric proanthocyanidins (Khanbabaee and van Ree, 2001). In the vegetal organism, tannins play an important biochemical role because they increase the resistance of plants to viruses and microorganisms. Tannins have wide uses in medicine and leather industry. They have the antidiarrheal, antimycotic and antiseptic action as a result of the precipitation of bacterial and fungal proteins. They are antioxidants because they are highly reducing compounds (Chezem and Clay, 2016).

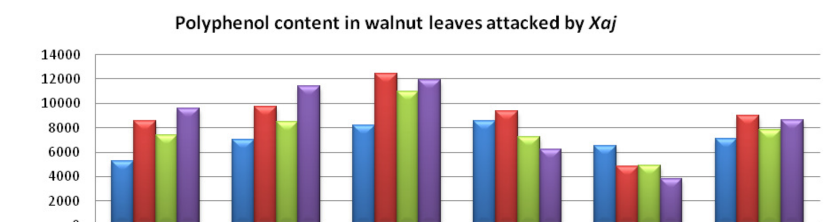
At the walnut leaves, in 15 June 2018, the tannins content showed values ranging from 204 mg tannic acid equivalent/100 g of vegetal material 'Jupâneşti' and 272 mg tannic acid equivalent/100 g of vegetal material 'Valrex'. The same tendency for variation of the tannins content was also observed in walnut leaves of the analyzed varieties attacked by *Xaj*. The highest tannins content in the walnut leaves was found at 27 July 2018 for all Romanian varieties and at 17 August 2018 for French one, both at the healthy and attacked leaves.

In the green walnut husk, the tannins content was lower compared to the values obtained from the walnut leaves. Thus, in 15 June 2018, the tannins content showed values ranging from 122 mg tannic acid equivalent/100 g of vegetal material 'Jupâneşti' and 222 mg tannic acid equivalent/100 g of vegetal material 'Valrex'. In this moment, in the green walnut husk attacked by *Xaj*, the tannins content showed values was ranging from 210 mg tannic acid equivalent/100 g of vegetal material 'Valmit' and 326 mg tannic acid equivalent/100 g of vegetal material 'Valrex', which attests to the fact that at 15 June 2018 the bacteria little attacked the 'Valmit' variety comparatively with the other varieties. The highest tannins content from the green walnut husk was found in 6 July 2018 for 'Valmit' and 'Valrex' varieties, in 27 July 2018 for 'Jupănești' cv. and in 17 August 2018 for the French cultivar, both at the healthy and attacked walnut husk. At each sampling stage, the tannins content was found to be greater in the green walnut husk attacked by *Xaj* compared to the healthy green husk.



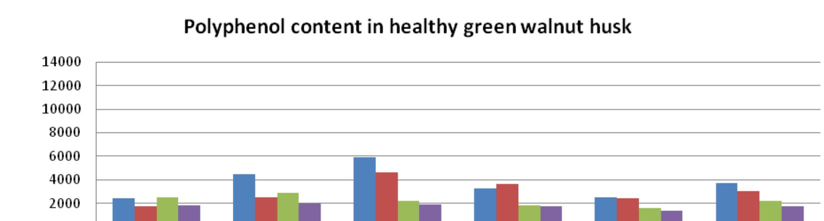
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	2510	3239	7399	7643	4290	5016.20
Jupânești	7632	9329	10425	4247	3416	7009.80
Valmit	5044	6458	6782	5854	4194	5666.40
Valrex	7536	8929	10268	3600	1412	6349.00

Fig. 1. Modification in time of polyphenol content (mg gallic acid equivalent/100 g of vegetal material) in healthy walnut leaves



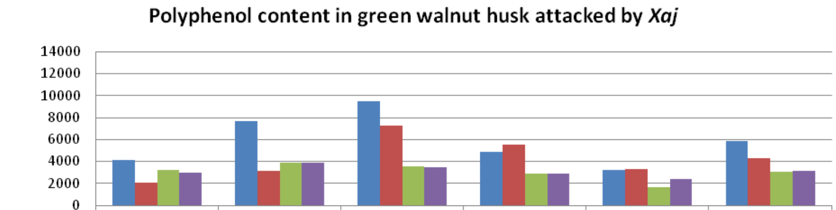
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	5236	7056	8203	8552	6513	7112.00
Jupânești	8585	9746	12405	9401	4800	8987.40
Valmit	7370	8525	10982	7263	4882	7804.40
Valrex	9568	11399	11938	6252	3844	8600.20

Fig. 2. Modification in time of polyphenol content (mg gallic acid equivalent/100 g of vegetal material) in walnut leaves attacked by *Xaj*



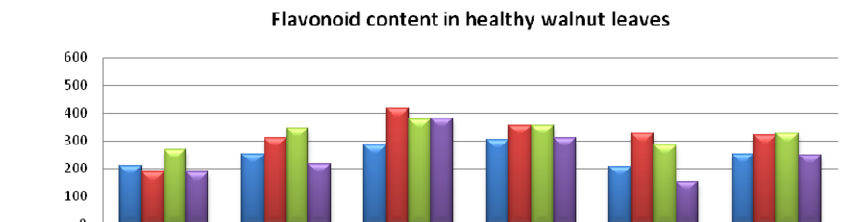
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	2407	4456	5919	3262	2555	3719.80
Jupânești	1788	2540	4627	3666	2469	3018.00
Valmit	2518	2867	2217	1835	1583	2204.00
Valrex	1835	1970	1877	1763	1377	1764.40

Fig. 3. Modification in time of polyphenol content (mg gallic acid equivalent/100 g of vegetal material) in healthy green walnut husk



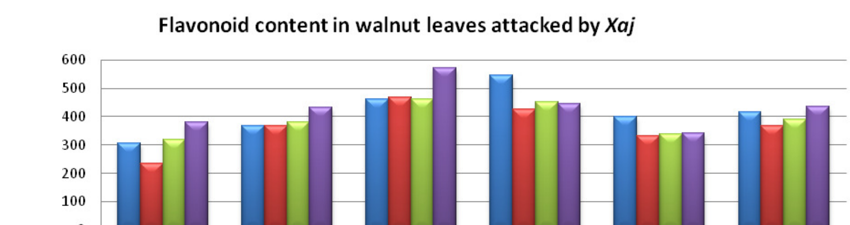
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	4160	7675	9523	4834	3256	5889.60
Jupânești	2103	3177	7249	5519	3287	4267.00
Valmit	3216	3852	3546	2858	1679	3030.20
Valrex	2951	3899	3428	2865	2394	3107.40

Fig. 4. Modification in time of polyphenol content (mg gallic acid equivalent/100 g of vegetal material) in green walnut husks attacked by *Xaj*



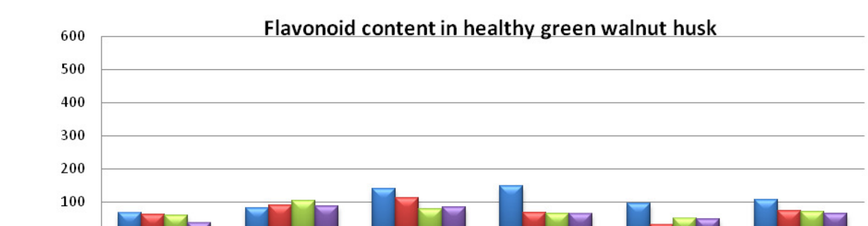
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	211	251	284	305	206	251.40
Jupânești	187	312	416	356	327	319.60
Valmit	269	345	378	357	284	326.60
Valrex	188	218	381	312	154	250.60

Fig. 5. Modification in time of flavonoid content (mg catechin equivalent/100 g of vegetal material) in healthy walnut leaves



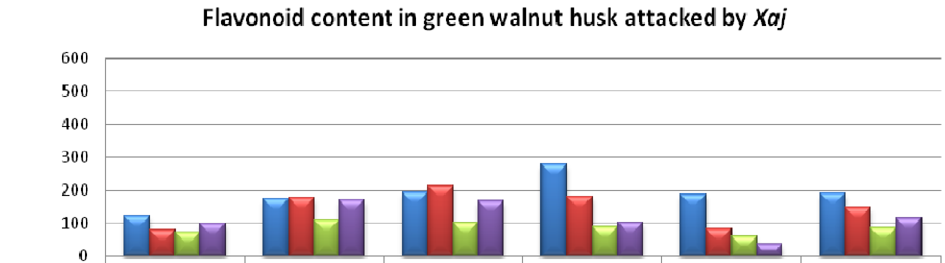
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	307	369	462	546	399	416.60
Jupânești	236	369	467	426	331	365.80
Valmit	318	379	462	452	337	389.60
Valrex	381	431	573	444	341	434.00

Fig. 6. Modification in time of flavonoid content (mg catechin equivalent/100 g of vegetal material) in walnut leaves attacked by *Xaj*



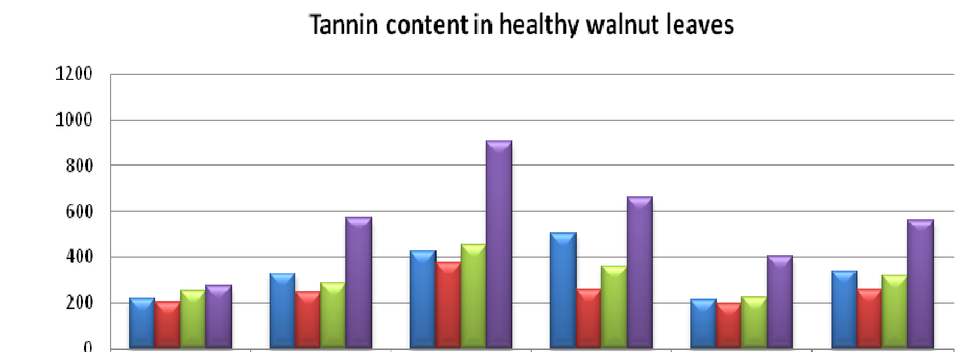
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	67	81	139	149	97	106.60
Jupânești	63	90	112	69	32	73.20
Valmit	60	104	78	66	51	71.80
Valrex	38	88	86	64	49	65.00

Fig. 7. Modification in time of flavonoid content (mg catechin equivalent/100 g of vegetal material) in healthy green walnut husks



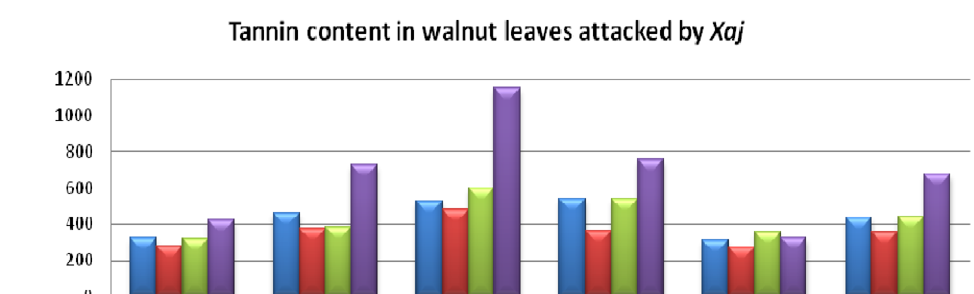
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	123	175	195	277	188	191.60
Jupânești	79	178	212	182	82	146.60
Valmit	72	110	101	89	61	86.60
Valrex	99	170	168	101	36	114.80

Fig. 8. Modification in time of flavonoid content (mg catechin equivalent/100 g of vegetal material) in green walnut husks attacked by *Xaj*



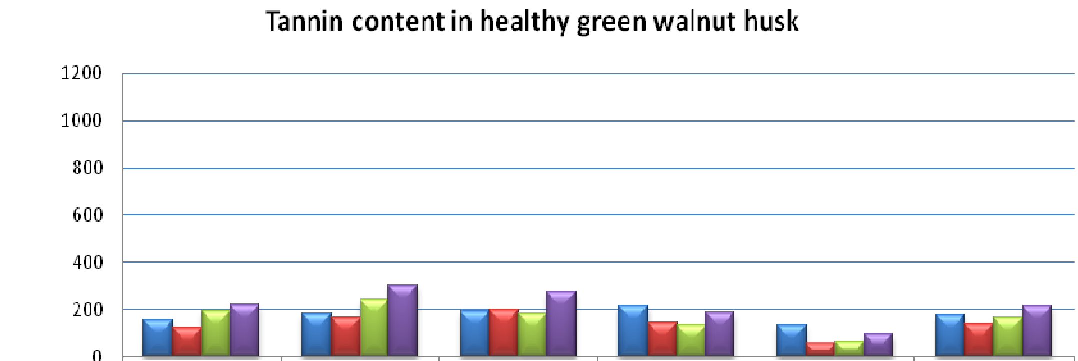
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	219	324	422	499	214	335.60
Jupânești	204	247	376	258	198	256.60
Valmit	251	282	454	361	224	314.40
Valrex	272	569	903	658	402	560.80

Fig. 9. Modification in time of tannin content (mg tannic acid equivalent/100 g of vegetal material) in healthy walnut leaves



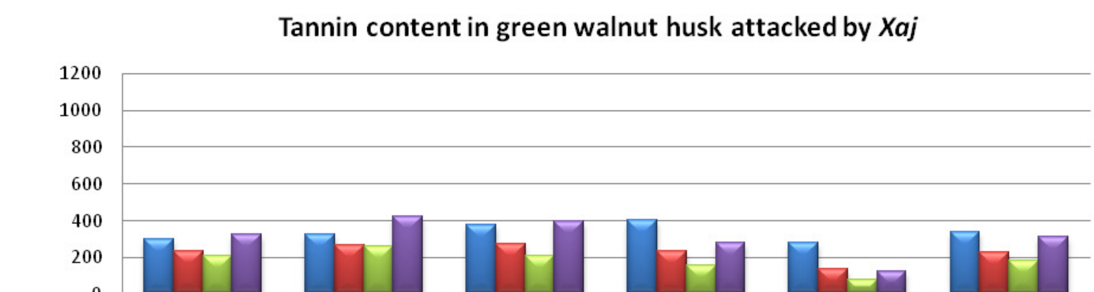
	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	322	463	526	540	314	433.00
Jupânești	281	375	481	365	275	355.40
Valmit	321	385	600	542	358	441.20
Valrex	423	725	1147	752	328	675.00

Fig. 10. Modification in time of tannin content (mg tannic acid equivalent/100 g of vegetal material) in walnut leaves attacked by *Xaj*



	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	154	183	195	215	132	175.80
Jupânești	122	168	200	144	57	138.20
Valmit	192	245	184	136	65	164.40
Valrex	222	302	277	186	98	217.00

Fig. 11. Modification in time of tannin content (mg tannic acid equivalent/100 g of vegetal material) in healthy green walnut husk



	15 June 2018	6 July 2018	27 July 2018	17 August 2018	7 September 2018	Medie
Franquette	298	328	376	403	284	337.80
Jupânești	236	270	278	235	141	232.00
Valmit	210	259	213	160	82	184.80
Valrex	326	427	398	279	125	311.00

Fig. 12. Modification in time of tannin content (mg tannic acid equivalent/100 g of vegetal material) in green walnut husk attacked by *Xaj*

Conclusions

The four walnut cultivars evaluated in this paper highlighted of variation in the content of polyphenols, flavonoids, and tannins, both in walnut leaves and in the green walnut husk, during the annual developmental stages of trees from June to September. An increase in the values of these composition parameters up to the maturity stage of each studied vegetal material was observed, followed by a decrease in the values for each analyzed parameter. At the same time, a higher content of biochemical compounds in the varieties attacked by *Xaj* compared to healthy vegetal materials was observed.

It can be emphasized that there is a correlation between the fruit maturity period of each walnut cultivar with the maturity period of both the walnut leaves and the green walnut husk, the 'Franquette' cv. having a later maturity compared to the Romanian cultivars.

In conclusion, the relatively similar behaviour of the four varieties of walnut taken in the analysis is determined by the cultivars characteristics because the walnuts were grown under the same climatic conditions and the same culture technology was applied.

Conflict of Interest

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

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