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Polyphenol composition of lettuce cultivars affected by mineral and bio-organic fertilisation

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Abstract: Three types of *Lactuca sativa* L. plants (green lettuces Batavia cv. Maritima and cv. Winter Butterhead, red lettuce Lolo rosa cv. Tuska) were investigated for their polyphenol composition. The lettuce plants were grown in polyethylene greenhouses and treated with different fertilisers. The qualitative and quantitative polyphenol composition was evaluated according to the use of mineral, organic (Italpollina and Arkobaleno) and bio (Lombricompost and EKOprop NX) fertilisers. The individual polyphenol components (caffeoyl derivatives and quercetin glycosides) were determined by high-performance liquid chromatography and the sample differences were estimated. The differences in the polyphenol content in the green lettuce cultivars in dependence on fertilisation were much higher than those in the red cultivar. In general, the red lettuce Lolo rosa cv. Tuska was characterised by the highest content of polyphenols. The highest content of all components was determined in the samples of red lettuce with the use of organic fertiliser Arkobaleno. In. the red lettuce and the green lettuce cv. Winter Butterhead organic fertilisation resulted in the higher content of polyphenols in comparison with mineral fertilisation and unfertilised samples. An exception was observed in cv. Maritima, where the unfertilised samples showed higher content of polyphenols compared to the fertilised samples.

Keywords: *Lactuca sativa;* organic and bio fertilisers; HPLC-PDA

Lettuce (*Lactuca sativa* L.) is a domestic annual species in the family Asteraceae (Compositae) cultivated mainly for its fresh leaves, widely consumed worldwide. Diet rich in lettuce could be relevant as dietary sources of polyphenolic compounds, known as powerful natural antioxidants (Dai & Mumper 2010). The polyphenol composition of lettuce has been extensively investigated in relation to different agronomic and fertilisation systems, genotypes, anti-diabetic and antioxidant properties (Gan & Azrina 2016; Sofo et al. 2016). The consumption of lettuce-derived phenolic compounds could be associated with a decreased risk of developing many chronic degenerative diseases, including cancer (Szeto et al. 2004; Reiss et al. 2012). Polyphenols are plant compounds that have both antioxidant and anti-inflammatory capabilities being of help against development of cancers, cardiovascular diseases and diabetes, and neurodegenerative diseases (Lau et al. 2006; Degl'innoocenti et al. 2008; Park et al. 2009; Fraga et al. 2011; Essa et al. 2012; Carter et al. 2013). The effect of $CaCl_2$ treatment of lettuce before harvest on phenolic profiles in leaves during cold storage has revealed the positive effect of $CaCl_2$ on nutritional characteristics of lettuce after a 7-day cold storage at 4 °C (Materska et al. 2019). The main phenolic compounds detected in lettuce leaves (5-*O*-caffeoylquinic acid and

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3,5-dicaffeoylquinic acid, caffeoyltartaric acid, caffeoylmalic acid, 2,3-dicaffeoyltartaric acid and the flavonoids quercetin 3-*O*-6-*O*-malonylglucoside and quercetin-3-*O*-glucuronide) were studied by using HPLC-PDA, LC-MS, ^{1}H-NMR and ^{13}C-NMR techniques.

Recently polyphenol-rich Rutgers Scarlet lettuce (*Lactuca sativa* L.) was developed, containing particularly high levels of 5-*O*-caffeoylquinic acid, cyanidin malonylglucoside, and quercetin 3-*O*-6-*O*-malonylglucoside (Cheng et al. 2014). Most of the studies revealed that 2,3-dicaffeoyltartaric acid was found at much higher levels than other phenolic compounds in the leaves of lettuce (Degl'innoocenti et al. 2008; Assefa et al. 2019). It has been evident that caffeic acid derivatives were the main polyphenols in green varieties, while flavonols were observed in higher quantities in red varieties of lettuce (Llorach et al. 2008). It was demonstrated that among the different varieties of lettuce, red coral lettuce showed the highest total polyphenols and antioxidant activity (Gan & Azrina 2016).

The knowledge of the polyphenol distribution in lettuce cultivars at mature leaves under different fertilisation practices could be of interest to agriculture and food industry, especially in selecting a suitable cultivar and type of fertiliser and making lettuce related nutrient dense dishes. Thus, information on the polyphenol composition can be useful for understanding their potential bioavailability and biological activities. Hence, the objective of this study was to determine the polyphenolic compounds in order to verify how different fertilisation practices can affect the qualitative and quantitative phenolic patterns of lettuce cultivars.

MATERIAL AND METHODS

Material. Lettuce *(Lactuca sativa* L.) plants (green leaf lettuce Batavia cv. Maritima; red leaf lettuce Lolo rosa cv. Tuska and green head lettuce cv. Winter Butterhead) developed in polyethylene greenhouses of the Agricultural University Plovdiv were analysed for their polyphenolic compounds. The lettuce plants were harvested in commercial maturity. A number of 10 lettuce heads per each fertilisation treatment were randomly harvested in the central part of each fertilisation block for avoiding boundary effects among the fertilisation treatments and placed in white plastic bags to minimise water loss. The leaves were weighed, spread on the plane surface and air dried at 20–25 °C by applying ventilation until full dehydration (7 days) as described in the standard EN 13804:2013. The dry material was ground and stored in plastic tins and the dry matter was determined.

Experimental design. The initial phase of the experimental activity was carried out in 2018/2019 by planting it in a greenhouse in the first ten days of October 2018 and harvested in February and March 2019 in economic maturity (200–300 g).

Seedling production. For the purpose of the experiment, bio-seeds of the above cultivars were purchased. The seeds were sown on October 10, 2018 to produce seedlings in a nursery. 1 000 plants were grown, and container technology was applied using 150-ounce stereo boards (Polyforma s.a., Greece). An organic seedling mixture was used: 80% Perlite and 20% Lombricompost developed by Kostadinov & Filipov (2013) for bioproduction of seedlings.

Planting. The plants were planted in the phase of 4–5 leaves on November 8 in the polyethylene greenhouses. After the plough, the test surface was profiled on a high flat bed. The experiments were designed in a 4-row scheme with 70 cm distance between the beds and 30 cm between the rows in the bed. The plants in one row were planted at 30 cm distance between them.

The experiment was based on a block method of four replicates with 28 plants per replicate, with a plot size of 3.36 m^2. On every plot 8 plants in the front (4 plants) and in the rear (4 plants) were used as guards. Plants (20) from every plot were taken for analysis. The experiment in the greenhouse was laid out with 6 variants with a total area of 450 m^2, of which 375 m^2 with organic fertilisation. Irrigation was carried out with a drip system.

The following mineral, organic and bio-fertilisers were applied:

1. Control-MT (mineral fertilisation – NPK)
2. Control – not fertilised
3. Italpollina – granular organic fertiliser
4. Arkobaleno – granular organic fertiliser
5. Lombricompost – granular bio-fertiliser
6. EKOprop NX – liquid bio-fertiliser

The granulated fertilisers were imported according to a pre-developed scheme as basic fertilisation with pre-seed tillage in the following norms: N: 0.0125 kg m^{-2}, P_2O_5: 0.00125 kg m^{-2}, K_2O: 0.00475 kg m^{-2}, Italpollina: 0.025 kg m^{-2}, Arkobaleno: 0.1 kg m^{-2}, and Lombricompost: 0.4 L m^{-2}. The bio-fertiliser Ekoprop NX dissolved in water was introduced by four times pouring at a dose of 0.1 g m^{-2} from the phase of 5 leaves at 14-day intervals.

Ultrasound-assisted extraction of polyphenols. An aliquot of 0.3 g powder of dry lettuce samples was weighed. Five parallel determinations of the same sample

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were prepared. The polyphenols were analysed in their glycoside form (and therefore no hydrolysed plant extracts were prepared) by extracting the samples with 10 mL of 70% (v/v) aqueous methanol in an ultrasound bath for 40 min at room temperature (25 °C). The extracts were filtrated under reduced pressure. The volume of the samples was adjusted to 10 mL and passed through a membrane filter 0.45 µm prior to HPLC analysis.

HPLC-PDA profiling of polyphenols. The instrumentation used for HPLC analysis consisted of Smartline Manager 5000 quaternary mixer, Smartline 1000 pump and PDA 2800 detector (Knauer, Germany). Separation of polyphenol components was performed on Synergi™ 4 µm Fusion-RP 80 Å, LC column 150 × 4.6 mm (Phenomenex, USA).

The chromatography was carried out using the mixture from 90 parts of 2% formic acid in water and 10 parts of 2% formic acid in acetonitrile as mobile phase A. The mixture from 10 parts of 2% formic acid in water and 90 parts of 2% formic acid in acetonitrile was used as solvent B. The polyphenols were eluted with a gradient system, as shown in Table 1.

The mobile phase flow rate was set by 1.0 mL min^{-1}; sample volume was 20 µL. The polyphenols were monitored at 320 nm, 370 nm, 352 nm and 280 nm.

Identification of polyphenol components. The spectral characteristics of eluting peaks of each sample, scanned with a PDA detector (λ = 200–400 nm), were compared with those of authentic analytical standards 5-*O*-caffeoylquinic acid (5-CQA) (C3878), 2,3-dicaffeoyltartaric acid (2,3-diCTA) (C7243), caffeic acid (CA) (C0625) and quercetin (Q4951), all supplied by Sigma-Aldrich Chemie GmbH (Germany). The identification of compounds was done by summarising the data for retention times, UV spectra of standards and the peaks in the samples, and previously published information (Materska et al. 2019). Caffeoyltartaric acid (CTA), quercetin-3›-*O*-glucuronide and quercetin-3-*O*-*(6"-O*-malonyl)- glucoside were tentatively identified by comparison of the retention time and spectra with those published previously. Certified *Echinacea purpurea* aerial powdered extract (Cas 90028-20-9; Select Botanical, Spain) was used as a surrogate standard for confirmation of CTA (Brown et al. 2011; Dagnon et al. 2019).

Table 1. The polyphenols elution with a gradient system

Time (min)	Phase A (%)	Phase B (%)
Initial	100	0
30	75	25
35	50	50
40	0	100

Quantification of polyphenols. Quantification of main polyphenolic components was performed by using the data from the fingerprint profiles obtained from HPLC-PDA analysis.

The calibration curves were prepared from stock solutions of analytical standards at a concentration of 1 000 mg L^{-1} in methanol by successive dilution until the optimal range of application for each compound (r = 0.999). The calibration standards and the samples were injected in duplicate. The content of caffeoyltartaric acid (CTA) was calculated as 2,3-diCTA, the content of quercetin glycosides (quercetin 3-*O*-glucuronide, peak 6 – quercetin 3-*O*-6-malonylglucoside) as quercetin and the caffeic acid derivatives as caffeic acid.

Statistical analysis. Five replications of each sample were analysed. Data are expressed as means ± standard deviation (SD), n = 5 to process the statistics. Statistical program SPSS 19.0 software was used for data analysis by ANOVA followed by Duncan's test (SPSS Inc., USA). The differences in the polyphenol components between cultivars were analysed and the effect of mineral and organic fertilisation was assessed.

RESULTS AND DISCUSSION

Three lettuce cultivars (Batavia cv. Maritima, Lolo rosa cv. Tuska and cv. Winter Butterhead) were studied for the distribution and quantification of the main polyphenolic compounds. The plants were subjected to different fertilisation practices including mineral, organic and bio-fertilisers. The granular organic fertilisers (Italpollina and Arkobaleno) and the two bio-fertilisers (Lombricompost – granular and EKOprop NX – liquid) were applied for the first time in an experiment directed to study the polyphenol complex in lettuce cultivars. In the last decade the polyphenolic compounds of lettuce have been intensively investigated by means of HPLC-PDA-MS methods pointing at the chromatographic profiles and the main components (Llorach et al. 2008). As we used a new different column Synergi (Fusion RP 18), a new HPLC-PDA method needed to be developed for obtaining the fingerprint chromatographic profile of polyphenols in lettuce. The use of mobile phase containing water, formic acid and acetonitrile resulted in good separation of the components in 25 min (Figure 1). The method was characterised with RSD from 3.3 to 8.2% and average of LOQ = 3.2 µg mL^{-1}. The chromatographic profiles of the three lettuce cultivars (control samples,

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not fertilised) are quite the same as those obtained previously (Romani et al. 2002). Regarding the other chromatographic methods, the reversed elution order of 2,3-diCTA and quercetin glycosides must be mentioned, which depends on the column type (Llorach et al. 2008, Materska et al. 2019).

Main polyphenolic compounds in lettuce cultivars (unfertilised samples)

The chromatographic fingerprint profiles of the polyphenols in three lettuce cultivars (Batavia cv. Maritima, Lolo rosa cv. Tuska and cv. Winter Butterhead) (Samples 2 – unfertilised control) show identical peaks, predominantly belonging to caffeoyl derivatives (Figure 1). The main components of polyphenols are 2,3-diCTA (peak 7), 5-CQA (peak 2) and CTA (peak 1) and two quercetin glycosides (peaks 5 and 6) (Figure 1). Three caffeoyl derivatives (peaks 3, 4, 8) in smaller amount were also defined by means of their UV spectra and as previously published (Romani et al. 2002, Llorach et al. 2008, Materska et al. 2019). Hence, only quantitative differences in the polyphenols of the three cultivars were recorded. The main component was 2,3-diCTA, when its content varied from 1.28 to 5.6 mg g^{-1} DW

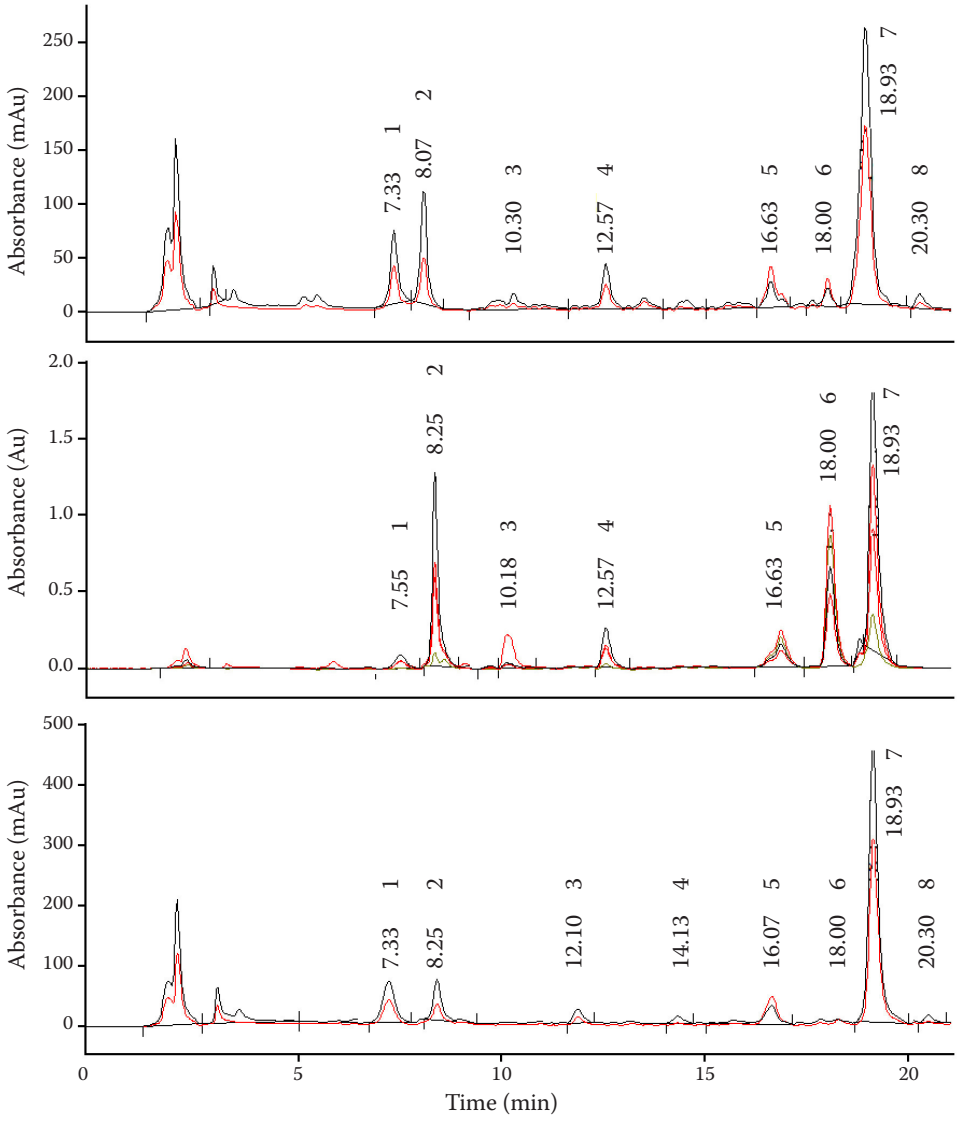

Figure 1. Chromatographic profiles of polyphenols in lettuce cultivars: Batavia cv. Maritima (A), Lolo rosa cv. Tuska (B), and cv. Winter Butterhead (C)

Peak 1 – caffeoyltartaric acid (CTA); peak 2 – 5-*O*-caffeoylquinic acid (5-CQA), peaks 3, 4, and 8 – caffeoyl derivatives, peak 5 – quercetin-*3'-O*-glucuronide, peak 6 – quercetin-*3-O-(6"-O*-malonyl)-glucoside, peak 7 – 2,3-dicaffeoyltartaric acid (2,3-diCTA); black line – absorption at 320 nm; red line – absorption at 352 nm

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Table 2. Content of main polyphenolic compounds (mg g^{-1} DW) in lettuce cultivars (Batavia cv. Maritima, Lolo rosa cv. Tuska and cv. Winter Butterhead)

Lettuce cultivars	Samples	CTA	5-CQA	2,3-diCTA	Sum of CA derivatives	Quercetin -3'-O- glucuronide	Quercetin-3-O- (6"-O-malonyl)- glucoside	Total polyphenols
Batavia cv. Maritima	1	0.28 ± 0.01	0.60 ± 0.03	1.61 ± 0.09	0.22 ± 0.01	0.44 ± 0.02	0.23 ± 0.01	3.38 ± 0.02
	2	0.31 ± 0.02	0.79 ± 0.03	2.22 ± 0.09	0.30 ± 0.01	0.97 ± 0.05	0.09 ± 0.01	4.6 ± 0.3
	3	0.12 ± 0.01	0.19 ± 0.01	0.37 ± 0.02	0.20 ± 0.01	0.23 ± 0.01	–	1.11 ± 0.07
	4	0.40 ± 0.03	0.53 ± 0.02	1.50 ± 0.08	0.27 ± 0.01	0.45 ± 0.03	0.20 ± 0.01	3.35 ± 0.17
	5	0.21 ± 0.01	0.60 ± 0.03	1.39 ± 0.07	0.22 ± 0.01	0.58 ± 0.03	0.19 ± 0.01	3.18 ± 0.20
	6	0.38 ± 0.02	0.84 ± 0.04	2.17 ± 0.15	0.61 ± 0.03	0.57 ± 0.03	0.14 ± 0.01	4.7 ± 0.3
Lolo rosa cv. Tuska	1	0.46 ± 0.02	5.4 ± 0.4	6.5 ± 0.5	1.41 ± 0.07	2.90 ± 0.12	7.7 ± 0.6	24.4 ± 0.8
	2	0.30 ± 0.02	4.4 ± 0.3	5.6 ± 0.3	0.79 ± 0.04	1.71 ± 0.07	5.2 ± 0.2	18.0 ± 0.7
	3	0.33 ± 0.02	6.6 ± 0.4	7.4 ± 0.5	1.94 ± 0.10	2.77 ± 0.14	8.0 ± 0.6	27.1 ± 0.9
	4	0.33 ± 0.02	7.1 ± 0.5	6.8 ± 0.6	1.94 ± 0.11	3.35 ± 0.17	8.7 ± 0.7	28.2 ± 0.9
	5	0.35 ± 0.02	6.0 ± 0.4	7.0 ± 0.6	1.48 ± 0.07	3.29 ± 0.17	7.4 ± 0.5	25.4 ± 0.8
	6	0.34 ± 0.02	5.8 ± 0.4	6.6 ± 0.5	1.05 ± 0.05	3.05 ± 0.17	7.9 ± 0.6	24.7 ± 0.8
cv. Winter Butterhead	1	0.37 ± 0.02	0.37 ± 0.02	1.99 ± 0.13	0.18 ± 0.01	0.48 ± 0.03	0.062 ± 0.004	3.45 ± 0.17
	2	0.27 ± 0.02	0.93 ± 0.04	1.28 ± 0.07	0.16 ± 0.01	0.20 ± 0.01	0.12 ± 0.01	2.96 ± 0.15
	3	0.50 ± 0.02	0.66 ± 0.03	3.18 ± 0.20	0.19 ± 0.01	0.50 ± 0.02	0.36 ± 0.02	5.4 ± 0.3
	4	0.44 ± 0.02	1.01 ± 0.06	3.13 ± 0.21	0.27 ± 0.01	0.82 ± 0.04	0.10 ± 0.01	5.8 ± 0.4
	5	0.41 ± 0.02	0.61 ± 0.03	2.28 ± 0.16	0.24 ± 0.01	0.66 ± 0.03	–	4.2 ± 0.2
	6	0.57 ± 0.03	0.96 ± 0.05	3.54 ± 0.28	0.32 ± 0.02	1.11 ± 0.06	0.14 ± 0.01	6.6 ± 0.5

1 – Control-MT (mineral fertilisation – NPK); 2 – unfertilised control; 3 – Italpollina, granular organic fertiliser; 4 – Arkobaleno, granular organic fertiliser; 5 – Lombricompost, granular bio-fertiliser; 6 – EKOprop NX, liquid bio-fertiliser; data are means of five replicates ± SD; CA – caffeic acid

(Table 2). The content of 5-CQA differed also in a very wide range from 0.79 to 4.4 mg g^{-1} DW. The content of quercetin-3-*O-(6"-O*-malonyl)-glucoside (peak 6) varied from 0.09 to 5.2 mg g^{-1} DW being the highest in Lolo rosa cv. Tuska (Figure 1, Table 2). Recently the same statement, concerning the high content of quercetin-*3-O-(6"-O*-malonyl)-glucoside and its bioactivity, has been expressed in a study on the alteration of phenolic composition in red lettuce by reducing nitrogen supply which enhances its anti-proliferative effects on colorectal cancer cells (Zhou et al. 2019). Quercetin-3'-*O*-glucuronide (peak 5) (1.71 mg g^{-1} DW) and the sum of caffeic acid derivatives (CA) derivatives (0.79 mg g^{-1} DW) also showed the highest content in red lettuce, while the CTA content was nearly the same in the three cultivars (Table 2).

Effect of mineral and bio-organic fertilisation. The effect of mineral, organic and bio-fertilisers on the content of polyphenols was assessed by applying Duncan's test to the data. It makes it possible to compare the data on fertilised samples with those of the control. Duncan's test allows estimating the differences between the components in the lettuce cultivars on the one hand, and on the other, to assess how the content of polyphenols changes depending on the type of fertilisation. The data showed that in fertilised green cultivars (Batavia cv. Maritima and cv. Winter Butterhead) the content of polyphenols changes drastically compared to the control, while in red lettuce (Lolo rosa cv. Tuska) smaller differences were observed (Figure 2). The two green lettuces responded differently to the treatment with the fertilisers, correspondingly, in cv. Maritima with decreasing the polyphenols and in cv. Winter Butterhead with increasing their content. The quantitative differences in quercetin glycosides resulted in qualitative differences, as quercetin-3-*O-(6"-O*-malonyl)-glucoside was absent in two samples (cv. Maritima-3 and cv. Winter Butterhead-5). Most affected due to fertilisation in cv. Maritima were 2,3-diCTA (0.37 ÷ 2.22 mg g^{-1} DW) and quercetin-3'-

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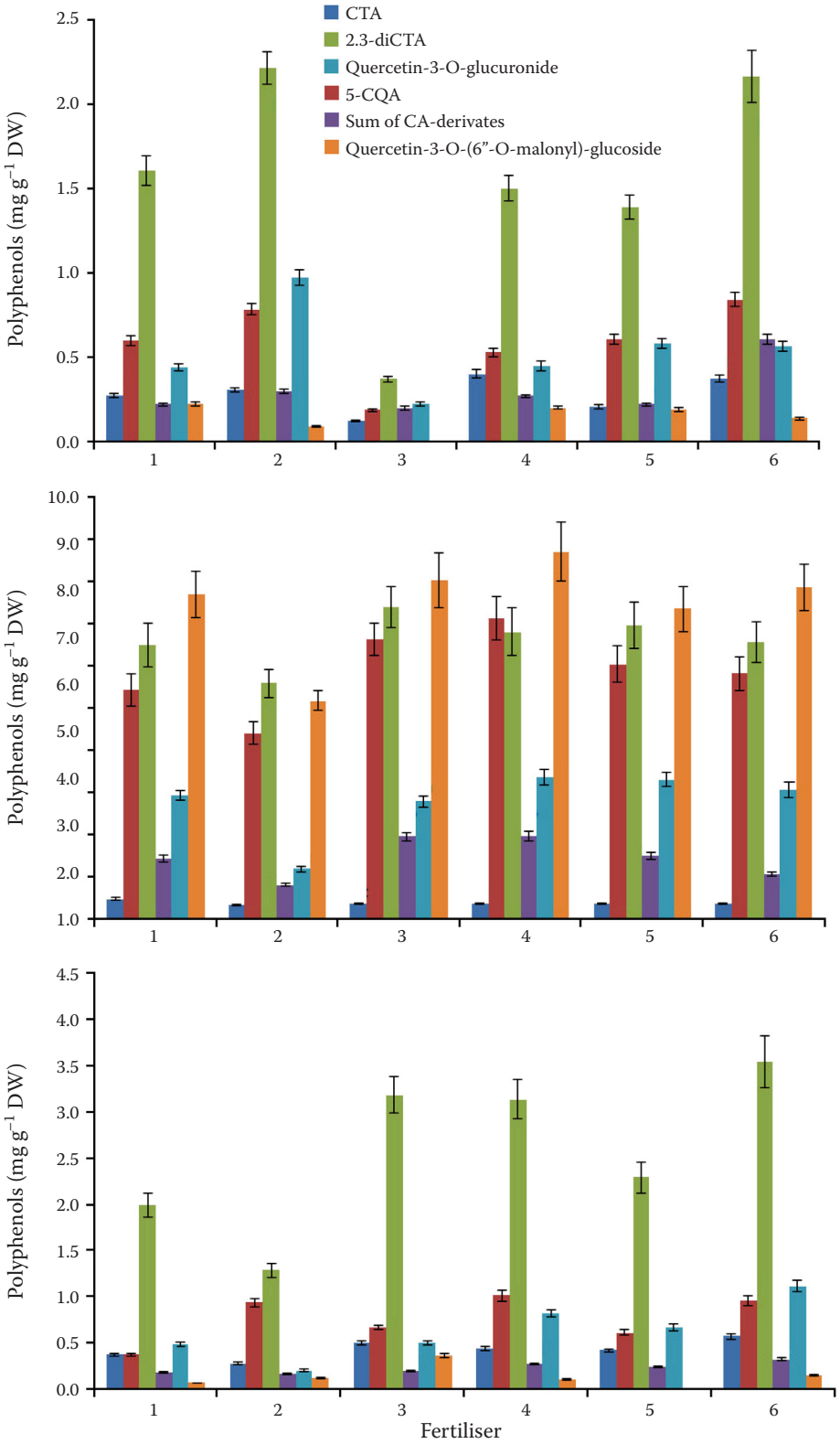

Figure 2. Comparison (Duncan's test) of the content of main polyphenolic compounds in lettuce cultivars: Batavia cv. Maritima (A), Lolo rosa cv. Tuska (B), and cv. Winter Butterhead (C)

1 – Control-MT (mineral fertilisation NPK); 2 – unfertilised control; 3 – Italpollina, granular organic fertiliser; 4 – Arkobaleno, granular organic fertiliser; 5 – Lombricompost, granular bio-fertiliser; 6 – EKOprop NX, liquid bio-fertiliser; means with similar letters are not significantly different at Duncan's grouping at ($P < 0.05$)

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O-glucuronide (0.23 ÷ 0.97 mg g^{-1} DW), when their content drastically decreased after the use of Italpollina. Previously, a decrease in polyphenols due to different fertilisation practices was also observed in green lettuce Maravilla de Verano in open-field experiments (Sofo et al. 2016). When the bio-fertiliser EKOprop NX was applied, no significant changes in the polyphenol complex were recorded (Table 2, Figure 2). Therefore, it could be suggested that it is appropriate to use fields without fertilisation or fields fertilised with EKOprop NX for the cultivation of lettuce cv. Maritima with preserved bioactivity. The data show that the red lettuce Lolo rosa cv. Tuska was characterised by the highest content of polyphenols, which was less affected by the type of fertilisation. Hence, the use of mineral, organic and bio-fertilisers has rather a small influence on the content of the major polyphenolic components 5-CQA, 2,3-diCTA and quercetin-3-*O*-(6''-*O*-malonyl)-glucoside (Table 2, Figure 2). Striving for producing lettuce with high bioavailability and without chemical fertilisers, it is advisable to use organic and bio-fertilisers. In cv. Winter Butterhead, the response of the polyphenolic compounds due to organic and bio-fertilisation was directed to increasing the content in dependence on the type of fertiliser. The use of organic fertiliser Arkobaleno and bio-fertiliser EKOprop NX most significantly increased the content of the main polyphenols. The high content of polyphenols is in direct relation to the antioxidant and other biological activities. Recently a review has been published summarising these health benefit studies and the underlying mechanisms of 2,3-dicaffeoyltartaric acid (chicoric acid), which include antivirus, anti-inflammation, glucose and lipid homeostasis, neuroprotection, and antioxidation effects (Peng et al. 2019).

CONCLUSION

The data show that the polyphenol complex in three lettuce cultivars was quantitatively affected by fertilisation depending on the type of lettuce and fertilisers. The content of the main polyphenolic compounds in red lettuce type Lolo rosa cv. Tuska was less affected, while the polyphenols in both green lettuces (Batavia cv. Maritima and cv. Winter Butterhead) changed drastically in a different manner in dependence on the type of fertiliser. Hence, the choice of fertiliser is a very important task for producing high-quality lettuce with high biological activity without chemical processing, which is important for human health.

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