The effect of ammonium nitrogen rates on bioactive compounds of *Cichorium spinosum* plants

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Abstract

In the present study, the effect of ammonium nitrogen rates on bioactive compounds of *Cichorium spinosum* L. was examined. For this purpose, five fertilizer treatments were applied with different percentages of ammonium nitrogen in relation to total nitrogen, namely (1) 14%, (2) 24%, (3) 34%, (4) 43%, and (5) 53% of total nitrogen applied in the form of ammonium nitrogen, while total nitrogen was the same for all treatments. *C. spinosum* were grown from seeds after putting them in seed trays containing peat. Young seedlings were transplanted in 2L pots containing peat and perlite in a ratio 1:1. Plants were harvested at the stage of flower stalk elongation and when plants started to form spiny bushes, while samples of both leaves and flower stalks were collected for fatty acids and phenolic compounds composition and content analysis. The results showed significant differences in chemical composition between the ammonium nitrogen rates, as well as between plant parts. In particular, the main fatty acids in all treatments and plant parts were α-linolenic, linoleic and palmitic acid; however, linoleic acid was higher in flower stems comparing to leaves harvested at the same time, whereas α-linolenic acid was the most abundant fatty acid, being higher in leaves, ranging from 52.02 to 59.67%. Palmitic acid was detected in similar amounts in both flower stems and leaves, except for treatment 3 where leaves content was higher than that in flower stems. Moreover, α-linolenic and linoleic acids were higher in treatment 1 and 5, respectively, while palmitic acid was higher in treatment 3. The main detected phenolic compounds were chicoric and 5-*O*-caffeoylquinic acid, followed by two kaempferol-*O*-glucuronide, 3,5-*O*-dicaffeoylquinic acid and quercetin-3-*O*-glucuronide, which were detected in lower amounts. Moreover, *p*-coumaroylquinic and 5-*O*-feruolyquinic acids were detected in flower stems of treatment 1 in significantly higher amounts in comparison to the other treatments, while significant differences were also observed between plant parts and fertilizer treatments for the other main phenolic compounds with no specific trends being observed. In conclusion, ammonium nitrogen rates and plant parts have a significant effect on chemical composition of *C. spinosum* at the flowering stage, while the high content of flower stems in phenolic compounds and fatty acids could be further exploited with alternative uses of these plant parts, such as pickled products and decoctions.

Keywords: ammonium nitrogen; chemical composition; fatty acids; phenolic compounds; chicoric acid; 5-*O*-caffeoylquinic acid; flavonoids

INTRODUCTION

Cichorium spinosum L. is a spiny shrub, more or less erected, depending on the ecotype, which forms rosettes of edible leaves. It is considered as a basic ingredient of the Mediterranean diet, consumed throughout the centuries from people of rural communities who used to hand pick the edible leaves and use them in various traditional dishes (Melliou et al., 2003; Petropoulos et al., 2016b).

Although the species has been introduced in commercial farming, so far no studies are available regarding plant requirements in macronutrients, especially nitrogen which is considered an essential element for leafy vegetables quality (Conesa et al., 2009). Apart from nitrogen amounts, nitrogen form may also affect leafy vegetables quality, since this element is essential in the biosynthesis pathways of various phytonutrients (Fontana et al., 2006; Szalai et al., 2010). Palaniswamy et al. (2000, 2004) have also reported that nitrogen form (nitrate: ammonium nitrogen ratios) may also affect fatty acids composition and content in purslane leaves, especially α-linolenic acid content which is the most abundant fatty acids in this species. Moreover, the combination of nitrogen form and photosynthetic active radiation (PAR) has been reported to significantly affect the content of various phytochemicals in leafy species of the Brassica genus (Fallovo et al., 2011).

The aim of the present study was to evaluate the effect of the ammonium nitrogen percentage of total applied nitrogen on fatty acids composition and phenolic compounds content of *C. spinosum* plant parts (flower stalks and leaves). For this purpose, five fertilizer treatments were applied, namely (1) 10%, (2) 20%, (3) 30%, (4) 40%, and (5) 50% of total nitrogen applied in the form of ammonium nitrogen.

MATERIALS AND METHODS

Plant Material

Seeds of *Cichorium spinosum* L. (Asteraceae) were put in seed trays on December 1st 2015 containing peat and transplanted at the stage of 3 leaves on February 15th 2016, in 2 L pots containing peat (Klassman-Deilmann KTS2, 1.0 L) and perlite (1.0 L), as previously described by Anesti et al. (2016). Plants were fertilized with the same amount of nitrogen (300 mg/L) through the irrigation water. Five fertilizer treatments (1-5) were applied, namely (1) 14%, (2) 24%, (3) 34%, (4) 43%, and (5) 53% of total nitrogen applied in the form of ammonium nitrogen by using the following fertilizers: a) 20-20-20 (N-P-K), b) ammonium nitrate, c) calcium nitrate, d) urea, e) ammonium sulphate.

Plants were harvested at the stage of flower stalk elongation and when plants started to form spiny bushes. At the day of harvest, samples of leaves and flower stalks were collected, subjected to freeze-drying and kept under deep freezing conditions (-80 °C) until further analysis.

Fatty acids analysis

Fatty acids were analysed with a DANI 1000 gas chromatographer (GC, Milan, Italy) coupled to a flame ionization detector (FID), after a transesterification procedure described by Guimarães et al., (2013). The FAMEs were identified by comparing their retention time with authentic standards and the results were recorded and processed using Clarity 4.0.1.7 Software (DataApex, Podohradska, Czech Republic). The results were expressed as relative percentage (%) of each fatty acid.

Phenolic compounds analysis

For methanolic/water (80:20, v/v) extraction, one gram of lyophilized material was extracted twice for 1 h in a magnetic stirrer plate (25 ºC at 150 rpm), with 30 mL of methanol/water (80:20, v/v), filtered through a Whatman No. 4 paper and vacuum-dried in a rotary evaporator (rotary evaporator Büchi R-210, Flawil, Switzerland) at

40°C to remove the methanol. The extracts were further frozen and lyophilized. Afterwards the extracts were re-dissolved in methanol/water (80:20, *v/v*) at a final concentration of 30 mg/mL and filtered through a 0.45 µm Whatman syringe filter, transferred to amber color HPLC vial for phenolic compound analysis. The phenolic profile was determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), as previously described by Bessada et al. (2016). For the double online detection, 280, 330 and 370 nm were used as preferred wavelengths for DAD and in a mass spectrometer (MS) connected to HPLC system. The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Phenolic compounds identification was performed using standard compounds, when available, by comparison with their retention times, UV-vis and mass spectra; and also, comparing the obtained information with available data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds with not available commercial standards, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg/g of extract.

Statistical analysis

For all the analyses, three samples were analysed for each treatment, while all the assays were carried out in triplicate. The results are expressed as mean values and standard deviations (SD), and analysed using one-way analysis of variance (ANOVA) for the main effects, followed by Tukey's HSD Test ($p = 0.05$) for means comparison. Statistical analysis was carried out with Statgraphics 5.1.plus (Statistical Graphics Corporation).

RESULTS AND DISCUSSION

Fatty acids composition is presented in **Table 1**. The main detected fatty acids were α-linolenic acid (C18:3n3), followed by linoleic acid (C18:2n6c) and palmitic acid (C16:0), with significant differences between ammonium nitrogen rates and plant parts. The same fatty acids have been previously reported by the authors (Petropoulos et al., 2016a, 2016b) for *C. spinosum* leaves; however, to the best of our knowledge, this is the first repot regarding fatty acids composition of the flower stalks of the species. However, α-linolenic and linoleic acid contents differed from our previous reports, due to differences in genotype and fertilizer treatments. Linoleic acid was higher in flower stems comparing to leaves, whereas a-linolenic acid was higher in the leaves, in amounts ranging from 52.02 to 59.67%. Palmitic acid was detected in similar amounts in both flower stems and leaves, except for treatment 3 where leaves contained higher amounts than flower stems. Moreover, α-linolenic and linoleic acids were higher in treatment 1 and 5, respectively, while palmitic acid was higher in treatment 3. Palaniswamy et al. (2000) and Fontana et al. (2006) have reported that nitrogen source may affect fatty acid content in purslane leaves, especially α-linolenic acid which is the main fatty acid. In contrast, Szalai et al. (2010) did not report significant changes of fatty acids profile in purslane leaves when nutrient solutions with different ratios of nitrate: ammonium nitrogen were used.

Table 1. Composition in fatty acids of the studied *Cichorium spinosum* plant parts (%; mean ± SD).

	Treatment (T)*									
	1		2		3		4		5	
	FS**	L	FS**	L	FS**	L	FS**	L	FS**	L
C6:0	0.084±0.002	0.052±0.001	0.069±0.001	0.044±0.001	0.058±0.001	0.077±0.005	0.062±0.001	0.040±0.001	0.048±0.001	0.058±0.005
C8:0	0.045±0.002	0.025±0.001	0.089±0.001	0.012±0.001	0.15±0.01	0.059±0.003	0.085±0.008	0.026±0.001	0.058±0.001	0.028±0.001
C10:0	0.060±0.001	0.026±0.002	0.066±0.004	0.018±0.001	0.065±0.004	0.101±0.003	0.050±0.001	0.019±0.001	0.056±0.002	0.019±0.001
C12:0	0.097±0.001	0.068±0.001	0.179±0.01	0.064±0.001	0.300±0.007	0.121±0.001	0.197±0.005	0.090±0.005	0.130±0.004	0.104±0.006
C14:0	0.53±0.02	0.97±0.03	0.704±0.001	1.28±0.01	0.58±0.02	1.61±0.01	0.653±0.001	1.008±0.029	0.60±0.01	1.226±0.004
C15:0	0.272±0.004	0.72±0.03	0.407±0.001	1.25±0.02	0.41±0.06	1.23±0.01	0.50±0.04	1.09±0.05	0.53±0.02	1.35±0.02
C16:0	14.88±0.16	15.43±0.13	16.74±0.05	17.65±0.18	15.33±0.05	18.15±0.01	16.95±0.01	15.67±0.08	16.61±0.08	17.24±0.02
C16:1	0.220±0.001	0.692±0.006	0.25±0.01	1.170±0.001	0.385±0.008	1.15±0.01	0.38±0.01	1.12±0.01	0.383±0.001	1.26±0.02
C17:0	0.194±0.004	0.31±0.01	0.32±0.01	0.385±0.004	0.334±0.002	0.490±0.009	0.339±0.001	0.308±0.006	0.35±0.01	0.464±0.009
C18:0	1.54±0.01	1.73±0.01	1.91±0.04	1.88±0.01	1.43±0.01	2.15±0.01	2.43±0.02	2.337±0.001	2.42±0.06	2.87±0.04
C18:1n9c	1.41±0.04	1.03±0.01	1.55±0.02	0.740±0.002	1.03±0.05	0.88±0.03	0.931±0.002	0.707±0.002	1.20±0.03	0.958±0.006
C18:2n6c	24.58±0.02	16.52±0.04	25.65±0.03	14.28±0.02	24.59±0.06	14.27±0.03	26.53±0.01	15.74±0.01	28.52±0.08	18.16±0.03
C18:3n3	52.90±0.06	59.67±0.10	48.29±0.08	57.70±0.16	51.90±0.04	56.17±0.03	46.61±0.05	58.74±0.12	45.33±0.14	52.02±0.10
C20:0	0.385±0.003	0.61±0.04	0.58±0.03	1.00±0.01	0.512±0.001	0.98±0.02	0.62±0.02	0.72±0.02	0.56±0.02	1.11±0.03
C20:1	0.038±0.003	0.017±0.001	0.047±0.001	0.032±0.001	0.060±0.001	0.055±0.001	0.039±0.001	0.040±0.002	0.044±0.001	0.053±0.001
C20:2	0.074±0.001	0.073±0.001	0.104±0.008	0.087±0.002	0.110±0.006	0.155±0.009	0.099±0.006	0.111±0.004	0.093±0.003	0.072±0.004
C20:3n3	0.088±0.001	0.148±0.004	0.106±0.003	0.136±0.001	0.095±0.002	0.129±0.009	0.102±0.005	0.123±0.006	0.097±0.001	0.12±0.01
C21:0	0.074±0.007	0.086±0.001	0.144±0.005	0.142±0.003	0.062±0.002	0.20±0.01	0.191±0.001	0.128±0.005	0.19±0.02	0.23±0.02
C20:5n3	0.210±0.009	0.218±0.005	0.315±0.006	0.267±0.009	0.265±0.010	0.196±0.008	0.15±0.04	0.124±0.001	0.20±0.01	0.313±0.005
C22:0	0.93±0.5	0.54±0.03	1.08±0.02	0.73±0.01	0.96±0.05	0.70±0.01	1.30±0.01	0.71±0.04	1.10±0.01	1.10±0.01
C23:0	0.203±0.001	0.242±0.003	0.22±0.02	0.232±0.001	0.31±0.02	0.25±0.01	0.34±0.01	0.23±0.02	0.37±0.03	0.30±0.01
C24:0	1.18±0.01	0.83±0.03	1.19±0.05	0.91±0.02	1.09±0.03	0.88±0.04	1.45±0.01	0.93±0.04	1.13±0.01	0.96±0.03
Total SFA (% of total FA)	20.48±0.12h	21.64±0.15g	23.69±0.09e	25.59±0.15b	21.57±0.16g	27.00±0.03a	25.16±0.02c	23.31±0.15f	24.13±0.20d	27.05±0.10a
Total MUFA (% of total FA)	1.67±0.04f	1.74±0.02e	1.84±0.00d	1.94±0.01c	1.47±0.04h	2.08±0.02b	1.35±0.01i	1.86±0.01d	1.62±0.03g	2.27±0.03a
Total PUFA (% of total FA	77.85±0.09a	76.63±0.14c	74.47±0.10e	72.47±0.15h	76.96±0.12b	70.92±0.01i	73.49±0.01g	74.83±0.15d	74.24±0.23f	70.68±0.07j

*T: treatment (1: 14% NH_4-N, 2: 24% NH_4-N, 3: 34% NH_4-N, 4: 43% NH_4-N, 5: 53% NH_4-N of total nitrogen).**FS: flower stalks; L: leaves.
Means in the same row followed by different letters are significantly different according to Tukey HSD test at $p<0.05$.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and tentative identification of phenolic compounds in *Cichorium spinosum*.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	7.1	325	353	191(100),179(5),173(3),161(3),135(3)	5-*O*-Caffeoylquinic acid
2	12.0	313	337	191(100),173(3),163(10),145(3),119(3)	*p*-Coumaroylquinic acid
3	12.4	328	473	311(100),293(94),219(3),179(7),149(3),135 (3)	Chicoric acid
4	13.6	327	367	193(10),191(100),173(5),143(3),134(3)	5-*O*-Feruolyquinic acid
5	16.5	328	193	178(20),134(100),117(8)	Ferulic acid
6	18.2	342	477	301(100)	Quercetin-3-*O*-glucuronide
7	18.7	348	461	285(100)	Kaempferol-*O*-glucuronide
8	19.2	353	463	301(100)	Quercetin-3-*O*-glucoside
9	20.5	360	505	463(26),301(100)	Quercetin-7-*O*-(6′′-*O*-acetyl)-glucoside
10	20.9	329	515	353(100),191(97),179(48),173(5),161(3),135(7)	3,5-*O*-Dicaffeoylquinic acid
11	22.2	345	461	285(100)	Kaempferol-3-*O*-glucuronide
12	23.4	337	447	269(100)	Apigenin-7-*O*-glucuronide
13	23.7	350	491	315(100)	Isorhamnetin-3-*O*-glucuronide
14	25.0	345	489	285(100)	Kaempferol-3-*O*-(6′′-*O*-acetyl)-glucoside
15	26.3	334	519	477(5),315(100)	Isorhamnetin-3-*O*-(6′′-O-acetyl)-glucoside

Table 3. Phenolic compounds quantification of *Cichorium spinosum* plant parts (mg/g extract, mean ± SD).

	Treatment (T)*									
	1		2		3		4		5	
	FS**	L	FS	L	FS	L	FS	L	FS	L
5-*O*-Caffeoylquinic acid[1]	4.93±0.07	9.681±0.003	9.73±0.03	9.5±0.1	10.5±0.2	8.28±0.05	9.5±0.3	10.59±0.09	9.45±0.32	10.1±0.3
p-Coumaroylquinic acid[2]	13.07±0.02	0.541±0.001	0.63±0.01	0.36±0.01	0.556±0.001	0.29±0.01	0.740±0.009	0.519±0.003	0.682±0.008	0.40±0.01
Chicoric acid[3]	1.99±0.02	18.00±0.09	12.20±0.17	23.44±0.08	11.5±0.2	13.1±0.1	19.27±0.03	23.3±0.2	13.27±0.14	17.3±0.2
5-*O*-Feruolyquinic acid[4]	2.564±0.004	nd	0.304±0.003	nd	0.134±0.001	nd	nd	0.322±0.001	nd	nd
Ferulic acid[4]	nd	0.166±0.002	nd	nd	nd	nd	nd	nd	nd	0.154±0.001
Quercetin-3-*O*-glucuronide[5]	1.245±0.007	3.130±0.009	1.675±0.004	2.592±0.004	1.38±0.05	1.867±0.001	1.252±0.001	2.290±0.005	0.879±0.002	1.35±0.02
Kaempferol-*O*-glucuronide[6]	4.07±0.06	2.71±0.04	5.77±0.02	2.46±0.04	1.436±0.005	2.198±0.005	3.39±0.09	1.47±0.01	3.741±0.009	1.00±0.02
Quercetin-3-*O*-glucoside[5]	nd	nd	nd	nd	0.483±0.003	nd	nd	nd	nd	nd
Quercetin-7-*O*-(6´´-*O*-acetyl)-glucoside[5]	0.464±0.004	0.461±0.001	0.672±0.001	0.757±0.004	0.48±0.01	0.615±0.003	0.586±0.002	0.671±0.006	0.398±0.001	0.44±0.01
3,5-*O*-Dicaffeoylquinic acid[1]	6.70±0.03	0.756±0.006	2.02±0.02	nd	1.78±0.03	1.24±0.07	4.198±0.005	0.81±0.01	2.864±0.022	0.72±0.04
Kaempferol-3-*O*-glucuronide[6]	1.401±0.001	2.409±0.005	2.040±0.001	2.255±0.004	1.78±0.04	2.314±0.005	1.880±0.001	2.79±0.01	0.936±0.001	1.08±0.01
Apigenin-7-*O*-glucuronide[7]	0.395±0.002	0.353±0.005	0.598±0.003	0.304±0.008	0.351±0.003	0.315±0.001	0.698±0.001	0.296±0.006	0.368±0.001	nd
Isorhamnetin-3-*O*-glucuronide[5]	0.371±0.003	0.562±0.001	0.643±0.001	0.663±0.003	0.473±0.001	0.695±0.002	0.548±0.002	0.669±0.003	0.348±0.003	0.368±0.008
Kaempferol-3-*O*-(6´´-*O*-acetyl)-glucoside[6]	0.696±0.005	0.505±0.004	1.304±0.006	0.818±0.002	0.91±0.03	0.754±0.003	1.309±0.005	0.952±0.005	0.484±0.005	0.292±0.007
Isorhamnetin-3-*O*-(6´´-O-acetyl)-glucoside[5]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total phenolic acids	**29.3±0.1d**	**29.14±0.08d**	**24.9±0.1g**	**33.3±0.2c**	**24.4±0.4h**	**22.88±0.07i**	**33.7±0.2b**	**35.6±0.3a**	**26.3±0.2f**	**28.7±0.4e**
Total flavonoids	**8.64±0.07g**	**10.12±0.03b**	**12.70±0.04a**	**9.85±0.04c**	**7.3±0.1i**	**8.757±0.008f**	**9.66±0.09d**	**9.143±0.002e**	**7.73±0.01h**	**4.53±0.02j**
Total phenolic compounds	**37.9±0.2d**	**39.3±0.1ce**	**37.6±0.1d**	**43.1±0.2b**	**31.7±0.5g**	**31.64±0.07g**	**43.37±0.32b**	**44.7±0.2a**	**34.0±0.2**	**33.2±0.5f**

Means in the same row followed by different letters are significantly different according to Tukey HSD test at $p<0.05$. *T: treatment (1: 14% NH_4-N, 2: 24% NH_4-N, 3: 34% NH_4-N, 4: 43% NH_4-N, 5: 53% NH_4-N of total nitrogen).**FS: flower stalks; L: leaves.

nd-not detected. Calibration curves used: 1- chlorogenic acid (y = 168823x – 161172; R^2=0.999); 2- *p*-coumaric acid (y = 301950x + 6966.7; R^2=0.999); 3- caffeic acid (y = 388345x + 406369; R^2=0.994); 4-ferulic acid (y = 633126x - 185462; R^2=0.999); 5- quercetin-3-*O*-glucoside (y = 34843x – 160173; R^2=0.999); 6- kaempferol-3-*O*-rutinoside (y = 11117x + 30861; R^2=0.999); 7- apigenin-7-*O*-glucoside (y = 10683x - 45794; R^2=0.996).

The detected phenolic compounds in *C. spinosum* plant parts are presented in **Table 2**. The same compounds have been previously reported for leaves of *Cichorium* species in other studies (Carazzone et al., 2013; Ferioli et al., 2015; Heimler et al., 2009; Lee and Scagel, 2013), however, to the best of our knowledge this is the first report regarding phenolic compounds composition of flower stalks of *C. spinosum*. The main phenolic compounds were chicoric and 5-*O*-caffeoylquinic acid, followed by two kaempferol-*O*-glucuronide, 3,5-*O*-dicaffeoylquinic acid and quercetin-3-*O*-glucuronide, which were detected in lower amounts (**Table 3**). Moreover, *p*-coumaroylquinic and 5-*O*-feruolyquinic acid were detected in flower stems of treatment 1 in significantly higher amounts in comparison to the other treatments, while significant differences were also observed between plant parts and fertilizer treatments for the other main phenolic compounds, with no specific trends being observed. The abundance of phenolic acids in relation to flavonoids has also been confirmed for *C. endivia* and *C. intybus* (D'Antuono et al., 2016; Ferioli et al., 2015), as well as for *C. spinosum* (Petropoulos et al., 2017).

Conclusions

In conclusion, ammonium nitrogen rates and plant part have a significant effect on chemical composition of *C. spinosum* at the flowering stage, with profound changes in both fatty acids and phenolic compounds content. Moreover, the high content of flower stems in phenolic compounds and fatty acids could be further exploited by introducing alternative uses of these plant parts, such as pickled products and decoctions.

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