Antioxidant activity and chemical composition of *Cichorium spinosum* L. in relation to nitrogen rate

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Abstract

In the present study, the effect of nitrogen (N) application rate on phenolic compounds content and antioxidant activity of *Cichorium spinosum* L. leaves was examined. Seeds were sown in seed trays containing peat and young seedlings were transplanted in 2-L plastic pots containing soil. Four nitrogen rates were applied, namely: a) control (no added N), b) 200 mg L^{-1} of total N, c) 400 mg L^{-1} of total N, and d) 600 mg L^{-1} of total N. Plants were harvested when rosettes reached marketable size. The results showed that the most abundant phenolic compounds of leaves extracts were chlorogenic acid, chicoric acid and kaempferol-3-*O*-glucuronide, while increasing nitrogen rates resulted in a significant decrease of the main phenolic compounds content. Similarly, the application of 600 mg L^{-1} of total nitrogen resulted in significant decrease of antioxidant potency of leaf extracts comparing to control, regardless of the performed assay, whereas for all the assays except for DPPH the application of 200 mg L^{-1} showed the best antioxidant properties. In conclusion, although the application of high nitrogen rates is tempting in order to achieve higher yields, rates higher than 200 mg L^{-1} are not recommended for *C. spinosum* cultivation, since a significant decrease of the bioactive compounds is observed that could compromise the quality of the final product.

Keywords: nitrates, phenolic compounds, 5-*O*-caffeoylquinic acid, chicoric acid, flavonoids

INTRODUCTION

The application of fertilizers is essential in commercial agriculture in order to obtain high yields of marketable quality. Especially for nitrogen fertilizers, their use is among the highest among various agrochemicals and they are considered as one of the key factors for increasing yields. However, this usually results in irrational use with excessive amounts of fertilizers being used aiming toward yields that exceed the crop potential. Apart from the unnecessary production cost increase, excessive use of fertilizers may have detrimental effects on the environment and the quality of the final product by increasing nitrates content in plant tissues. According to Hord et al. (2009), leafy vegetables tend to accumulate nitrates in amounts which depend mostly on application rates and the species. In addition, the various forms of nitrogen may affect leafy vegetables quality in other ways than increase nitrates content, since it is involved in many biosynthetic pathways, such as polyphenols (Lombardo et al., 2017), including flavonoids (Fallovo et al., 2011; Chrysargyris et al., 2017). Moreover, antioxidant activity has also been reported to be affected by nitrogen fertilization in various species, such as kale (Łata, 2014), spearmint (Chrysargyris et al., 2017), and pumpkin (Oloyede et al., 2012, 2014).

Therefore, the aim of the present study was to evaluate the effect of nitrogen rates on the antioxidant activity and phenolic compounds content of *C. spinosum* leaves.

MATERIALS AND METHODS

Plant material

Plants were grown from seeds as previously described by Anesti et al. (2016). More specifically, seeds were sown in seed trays on December 15, 2015 containing peat by Vianame

S.A. (Timpaki, Greece). Seedlings of *Cichorium spinosum* L. were transplanted at the 3rd-leaf stage on February 15, 2016 in 2-L pots containing peat (Klassman-Deilmann KTS2, 1.0 L) and perlite (1.0 L). Plants were fertilized through the irrigation water with four nitrogen rates, namely: a) Control (no added N), b) 200 mg L^{-1} of total N, c) 400 mg L^{-1} of total N, and d) 600 mg L^{-1} of total N. All fertilization treatments (200, 400, and 600 mg L^{-1} of nitrogen) were prepared by adding the same amount of a water soluble synthetic fertilizer (Atlas 20-20-20 + TE; Gavriel S.A., Greece) in order to have 200 mg L^{-1} of N-P-K in all the treatments, while for the treatments of 400 and 600 ppm, the rest of the nitrogen (200 and 400 mg L^{-1}, respectively) was added in the form of ammonium nitrate (34.5-0-0; N-P-K). Plants were harvested when rosettes reached a marketable size. After harvest samples of raw leaves were stored at -80°C and lyophilized for further chemical composition analyses.

Antioxidant activity assays

One gram of lyophilized material was extracted twice for 1 h in a magnetic stirrer plate (25°C 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 (Büchi R-210, Switzerland) at 40°C to remove the methanol. The extracts were further frozen and lyophilized, re-dissolved in methanol/water (80:20, v/v) for in vitro antioxidant activity assays at a final concentration of 20 mg mL^{-1}, and further diluted to different concentrations, as previously described by Petropoulos et al. (2015).

DPPH radical-scavenging activity was evaluated with an ELX800 microplate reader (Bio-Tek Instruments, Inc., USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of sample solution at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} to Fe^{2+}, measuring the absorbance at 690 nm in the abovementioned microplate reader. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2 h of assay/initial absorbance)×100. Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A]×100%, where A and B were the absorbance of the control and the sample solution, respectively.[16] The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity and Trolox was used as a positive control.

Phenolic compounds analysis

The above-mentioned extracts were re-dissolved in methanol/water (80:20, v/v) at a final concentration of 30 mg mL^{-1} 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, USA), following a procedure previously described by Bessada et al. (2016). For 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, 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 by 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. When commercial standards were not available, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg g^{-1} of extract.

Statistical analyses

For chemical composition analyses, three samples were analyzed for each treatment, while all the assays were carried out in triplicate. The results are expressed as mean values and standard deviations (SD), and analyzed 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 (Statpoint Technologies, Inc., USA).

RESULTS AND DISCUSSION

The antioxidant properties of *C. spinosum* leaves in relation to nitrogen application rates are presented in Table 1. The results show that the highest antioxidant activity of leaf extracts was observed when 200 mg L^{-1} of total nitrogen were applied for all the studied assays, except for DPPH scavenging activity assay where control treatment showed the best results. Therefore, high nitrogen rates, although they may increase fresh weight and yield, they also have a negative effect on antioxidant properties. Similar results have been reported for other vegetables species, where high nitrogen application rates decreased significantly antioxidant activity (Rodríguez-Hidalgo et al., 2010; Łata, 2014; Oloyede et al., 2014; Chrysargyris et al., 2017). According to a previous study of our team (Petropoulos et al., 2016), nitrogen rates up to 400 mg L^{-1} result in high yields without compromising nutritional value of the final product. However, in order to retain their antioxidant properties, plants should be fertilized with nutrient solutions with concentrations of 200 ppm L^{-1} of total nitrogen.

Table 1. Antioxidant properties of *Cichorium spinosum* leaves in relation to nitrogen application rates.

	Reducing power	Radical scavenging activity		Lipid peroxidation inhibition
	Ferricyanide/ Prussian blue (EC_{50}; mg mL^{-1})	DPPH scavenging activity (EC_{50}; mg mL^{-1})	β-carotene/ linoleate (EC_{50}; mg mL^{-1})	TBARS (EC_{50}; mg mL^{-1})
Control	0.835±0.003b	1.39±0.07d	0.79±0.04b	0.46±0.02b
200 mg L^{-1}	0.728±0.020c	1.62±0.06c	0.75±0.01c	0.35±0.008d
400 mg L^{-1}	0.839±0.006ab	2.23±0.10b	0.79±0.02b	0.38±0.02c
600 mg L^{-1}	0.849±0.008a	2.93±0.07a	1.00±0.03a	0.54±0.02a

Means in the same column followed by different letters are significantly different according to Tukey HSD test at p<0.05.

The detected phenolic compounds and their identification data are presented in Table 2. Identification of phenolic compounds was carried out and cross checked through their mass spectrum and UV absorption. The main phenolic compounds were chicoric acid and kaempferol-3-*O*-glucuronide, followed by 5-*O*-caffeoylquinic acid, isorhamnetin-3-*O*-glucuronide and quercetin-3-*O*-glucuronide (Table 3). According to the results, the application of nitrogen through the irrigation water has a negative effect on phenolic compounds content. Moreover, phenolic composition was also affected, with increasing nitrogen rates resulting in lower contents of both phenolic acids and flavonoids. In particular, chicoric acid, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-glucuronide and isorhamnetin-3-*O*-glucuronide decreased, whereas kaempferol-*O*-glucuronide increased and 5-*O*-caffeoylquinic acid presented fluctuating trends. Therefore, in general, increasing nitrogen application rates results in a significant decrease of phenolic compounds content, which could have a negative effect on bioactivity of *C. spinosum* leaves. Similar results have been reported by Petropoulos et al. (2017), who also identified chicoric acid and 5-*O*-caffeoylquinic acid as the main phenolic compounds of *C. spinosum* leaves, while Lombardo et al. (2017) and Oloyede et al. (2012) reported that increasing nitrogen rates resulted in the decrease of total phenolic compounds content in globe artichoke and pumpkin, respectively.

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* (mg g^{-1} extract; mean ± SD) in relation to nitrogen application rates.

Phenolic compound	Nitrogen rates			
	Control	200 mg L^{-1}	400 mg L^{-1}	600 mg L^{-1}
5-*O*-Caffeoylquinic acid[a]	3.13±0.05a	1.97±0.09c	1.71±0.03d	2.862±0.008b
p-Coumaroylquinic acid[b]	0.330±0.002c	0.360±0.002b	0.473±0.001a	0.272±0.002d
Chicoric acid[c]	13.96±0.07a	8.66±0.07b	8.33±0.04c	3.25±0.02d
5-*O*-Feruolyquinic acid[d]	0.83±0.01b	0.752±0.001c	0.92±0.03a	0.530±0.001d
Ferulic acid[d]	0.41±0.02a	0.128±0.002b	0.138±0.003b	0.090±0.001c
Quercetin-3-*O*-glucuronide[e]	2.21±0.03a	1.76±0.02b	1.81±0.01b	1.164±0.001c
Kaempferol-*O*-glucuronide[f]	1.651±0.004d	2.47±0.02b	3.16±0.02a	2.26±0.03c
Quercetin-3-*O*-glucoside[e]	0.71±0.03a	nd	0.39±0.01b	nd
Quercetin-7-*O*-(6”-*O*-acetyl)-glucoside[e]	0.761±0.005a	0.328±0.001b	0.321±0.004b	0.255±0.001c
3,5-*O*-Dicaffeoylquinic acid[a]	0.79±0.05a	0.298±0.006b	0.307±0.006b	0.256±0.004b
Kaempferol-3-*O*-glucuronide[f]	5.69±0.01a	2.70±0.02b	2.50±0.01c	1.776±0.007d
Apigenin-7-*O*-glucuronide[g]	0.322±0.003c	0.217±0.002d	0.493±0.001a	0.408±0.002b
Isorhamnetin-3-*O*-glucuronide[e]	1.860±0.003a	0.558±0.002c	0.612±0.005b	0.518±0.003d
Kaempferol-3-*O*-(6”-*O*-acetyl)-glucoside[f]	1.17±0.03a	0.35±0.01b	0.388±0.006b	0.296±0.003c
Isorhamnetin-3-*O*-(6”-*O*-acetyl)-glucoside[e]	0.35±0.01a	0.218±0.006b	0.224±0.004b	0.209±0.002b
Total phenolic acids	**19.4±0.2a**	**12.8±0.2b**	**11.88±0.03b**	**7.26±0.02c**
Total flavonoids	**14.723±0.003a**	**8.60±0.05c**	**9.902±0.001b**	**6.89±0.03d**
Total phenolic compounds	**34.2±0.2a**	**20.8±0.2c**	**21.78±0.04b**	**14.15±0.01d**

Means in the same row followed by different letters are significantly different according to Tukey HSD test at $p<0.05$. nd – not detected.

Calibration curves used: [a]chlorogenic acid (y=168823x – 161172; R^2=0.999); [b]*p*-coumaric acid (y = 301950x + 6966.7; R^2=0.999); [c]caffeic acid (y = 388345x + 406369; R^2=0.994); [d]ferulic acid (y = 633126x - 185462; R^2=0.999); [e]quercetin-3-*O*-glucoside (y = 34843x – 160173; R^2=0.999); [f]kaempferol-3-*O*-rutinoside (y = 11117x + 30861; R^2=0.999); [g]apigenin-7-*O*-glucoside (y = 10683x - 45794; R^2=0.996).

In addition, Brieudes et al. (2016) reported chicoric acid as the main phenolic compound in water decoctions of *C. spinosum* and *C. intybus* leaves, while Jaiswal and Kuhnert (2011) also identified this phenolic acid in *C. intybus*. 5-O-Caffeoylquinic acid has also been previously reported in *C. intybus* leaves in other studies (Rossetto et al., 2005; Jaiswal et al., 2011; Carazzone et al., 2013; Ferioli et al., 2015). Antioxidant properties of *C. spinosum* has been associated with phenolic compounds, especially chicoric acid, which has been suggested as the main antioxidant compound of *C. spinosum* (Brieudes et al., 2016). In another study, with wild edible greens, Pereira et al. (2011) also demonstrated a positive linear correlation of phenolic compounds content and antioxidant activity, indicating however selectivity between phenolic compound classes and antioxidant activity assays.

CONCLUSIONS

Considering that *C. spinosum* is a species recently introduced for commercial cultivation, most farmers apply fertilization regimes, based on their experience from other leafy vegetables or by empirical information. Although high nitrogen rates may increase fresh weight and total yield, they also have a negative effect on bioactive compounds content and antioxidant properties of *C. spinosum* leaves. Therefore, there must be a compromise between yield and phytochemicals content. Therefore, in the case of *C. spinosum* leaves, low medium nitrogen rates (200 mg L^{-1}) should be applied in order to achieve the best balance between yield, antioxidant capacity and/or phytonutrient content.

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