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Harvest timing and nitrogen fertilization alter the production of biomass and antioxidant compound in the parsley

Mylena Firmiano de Andrade[1], Luiziene Soares Alves[2], Evandro Silva P. Costa[3], Marco Andre Alves de Souza[4], Rosane Nora Castro[5], Douglas Siqueira de Almeida Chaves[6] and André Marques dos Santos[4]

[1]Pharmaceutical Sciences, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[2]Department of Chemistry (PPGQ), Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[3]Institute of Agronomy, Department of Phytotechnics, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[4]Laboratory of Plant Biochemistry, Department of Biochemistry, Institute of Chemistry, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[5]Department of Organic Chemistry, Institute of Chemistry, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[6]Department of Pharmaceutical Science, Institute of Biological Sciences and Health, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.

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Parsley is a plant widely used in cooking as a condiment and it has potential for pharmaceutical use due to flavonoids production, including the apiin, which has antioxidant activity. The current study investigates the influence of N fertilization on the biomass production, soluble fractions, total flavonoids and apiin in the parsley plants at 28 and 56 days after germination (DAG). The experiment was carried out in the greenhouse under different N levels: nitrogen free (control), 10 kg N ha^{-1} (10 kg N ha^{-1} at planting, without cover application of N) and 70 kg N ha^{-1} (10 kg of N ha^{-1} at planting and 60 kg of N ha^{-1} in cover). The biomass production and soluble fractions was influenced by harvest timing and nitrogen fertilization. The total flavonoid levels were lower at 56 DAG under the N fertilization effect. The highest levels of apiin were detected at 28 DAG in treatments with N fertilization. Thus, the harvest time at 28 DAG and N fertilization with 10 kg ha^{-1} yielded the highest apiin concentration and, therefore, less production time and costs with N fertilization.

Key words: *Petroselinum crispum*, soluble fractions, nitrate, flavonoids, Apiin, Apiaceae

INTRODUCTION

Parsley (*Petroselinum crispum* var *neapolitanum*, Apiaceae) is an aromatic, medicinal and seasoning

*Corresponding author. E-mail: amarques@ufrrj.br. Tel: +55 21 97284 1455.

specie native to Europe but now cultivated globally. Its leaves are sources of vitamins (A and C), minerals (calcium, potassium, phosphorus, iron, and magnesium) and carotenoids (Hossein, 2010; Mangels et al., 1993). Parsley is also rich in flavonoids, among which the majority substance is apiin, which is the biglycosylated form of apigenin (Chaves et al., 2011; Epifanio et al., 2020; Frattani et al., 2020). Among the biological activities related to parsley, its antioxidant, anti-inflammatory, antihemostatic and antimicrobial actions stand out (Al-Yousofy et al., 2017; Epifanio et al., 2020; Frattani et al., 2020). Parts of the biological activities of parsley are related to the bioavailability of apigenin after the metabolic action of apiin (Chaves et al., 2011; Meyer et al., 2006). Although nitrogen fertilization is often carried out indiscriminately in the cultivation of vegetables in order to increase productivity, excessive N-nitrate amounts applied can lead to soil and water pollution (Petropoulos et al., 2008). In addition, aromatic herbs and vegetables tend to accumulate nitrate in their foliage, especially in conditions of low light and high availability of nitrate (Colla et al., 2018; Luz et al., 2008; Maynard et al., 1976), thus posing a possible risk to human health (Sanchez-Echaniz et al., 2001; Sanatamaria, 2006). Economically important crops generally have adequate doses of nitrogen fertilizer to achieve optimum productivity. However, plants' production of secondary metabolites is not always positive to the increase in nutrients, which may lead to a decrease in the content of secondary metabolites (Souza et al., 2007). Plants' response to stress or favorable conditions for growth produces a scenario of competition between primary and secondary metabolism for energy resources and organic structures, with consequences specific to each species. In addition, the biosynthesis of flavonoids in parsley involves decarboxylation and deamination steps, leading to decreased efficiency of nitrogen and carbon assimilation (Heldt, 2005). In this context, this study aimed to verify the influence of nitrogen fertilization on some aspects of metabolism and production of biomass, total flavonoids and apiin.

MATERIALS AND METHODS

Cultivation conditions

The experiment was carried out in the experimental area of the Horticulture Sector of the Department of Phytotechnology (IA, UFRRJ), located in the city of Seropédica, Rio de Janeiro. The parsley seeds (*Petroselinum crispum* var. *neapolitanum* Darnet), produced by the company ISLA Sementes, and was purchased from agricultural product stores. The substrate used for the production of parsley in the greenhouse was obtained from the first 30 cm of a planossol, ground and sieved through 4 mm mesh. The analysis of the substrate was carried out in the Chemical Fertility Analysis Laboratory of PESAGRO-RIO and the characteristics were sandy texture, pH = 6.2; P = 32 mg dm^{-3}; K = 68 mg dm^{-3}; V (%) = 80.0, with the following concentrations (Cmol$_c$.dm^{-3} soil): H+Al = 1.2; Ca^{2+} = 3.4; Mg^{2+} = 1.2; BS = 4.9 and CEC at pH 7.0 = 6.1.

Experimental conditions

The seeds were sown in trays containing autoclaved washed sand. Irrigation was carried out manually during germination, until the plants developed two to three leaves, when they were transplanted to plastic boxes (experimental units) with a surface area of 0.09 m^2. Three treatments were evaluated, with different N levels using the mixed mineral fertilizer 4-14-8 (Vitaplan®): nitrogen free (control), 10 kg N ha^{-1} (10 kg N ha^{-1} at planting, without cover application of N) and 70 kg N ha^{-1} (10 kg of N ha^{-1} at planting and 60 kg of N ha^{-1} in cover, provided after the first harvest at 28 days after germination (DAG)). Two harvests were performed with the shoots of the aerial part of the plants being harvested. The harvest took place at 28 and 56 DAG, comprising the start and end of the crop cycle. Irrigation was performed daily until the field capacity of the substrate.

Extraction and determination of soluble fractions

At harvest, homogeneous samples containing 1 g of fresh aerial parts were subjected to ethanol extraction (80%), followed by partition with chloroform (1:1), to obtain the soluble fraction (Fernandes, 1983), which was later used to determine the total levels of free N-amino (Yemm and Cocking, 1955), soluble sugars (Yemm and Willis, 1954), N-NH$_4^+$ (Felker, 1977; Mitchell, 1972) and N-NO$_3^-$ Cataldo et al., 1975).

Extraction and purification of secondary metabolites

The extraction and purification processes were carried out according to Chaves et al. (2011) using smooth aerial parts of parsley using the decoction method at 10% (w/v), for 15 min, with the fresh material fractionated in an industrial processor. Then the decoction was filtered and dried by lyophilization to quantify the crude extract.

Total flavonoids quantification

The total content of flavonoids was determined using the Epifanio method (Epifanio et al., 2020). A 400 mL aliquot of each of extract was transferred to a 10 mL volumetric flask. Then, 200 mL of a methanol solution of aluminum chloride (2%) was added and the volume was completed with methanol. After 30 min, the absorbance was read at 425 nm against the blank (methanol) in order to quantify flavonoids. The total flavonoids content was determined using a standard quercetin curve with ten concentrations (0.5 - 5.0 µg mL^{-1}). The results were expressed in quercetin equivalents mg per 100 mg of crude extract.

Apiin quantification

Quantitative analysis of apiin was performed using a liquid chromatograph (Shimadzu LC-20AT) coupled to an SPD-20A photodiode detector (CBM-20A module), according to the method described by Chaves et al. (2011). A reverse phase C18 column (25 cm, 4.6 mm, 5 µm) was used with a mobile phase consisting of water acidified with 1% acetic acid (pump A) and methanol (pump B), with injection volume of 20 µL. The elution flow was 1 mL min^{-1} and absorbance was measured between 200 - 450 nm. The gradient used was 0 - 15 min (35 - 70% B), 15 - 17 min (70 - 80% B) and 17 - 18 min (80 - 35% B). The compounds were quantified from a calibration curve using an external standard of apiin, at concentrations of 0.02 -1.0 mg mL^{-1}.

Table 1. Soluble fractions content (fresh weight, N-NO$_3^-$, free N-amino, N-NH$_4^+$ and soluble sugars) in the aerial part of parsley cultivated with supply of different mineral fertilizer doses, with two harvest times: 28 and 56 DAG (days after germination).

Treatments	**Days after germination (DAG)**	
	28	**56**
Fresh weight (g plastic box^{-1})		
Without N	20.03^{aA}	29.37^{aB}
10	27.78^{aA}	32.21^{aB}
70	21.82^{bA}	54.19^{aA}
Average	23.21^{b}	38.59^{a}
CV (%)	17.87	
N-NO$_3^-$ (mg kg^{-1} fresh weight)		
Without N	108.15^{aB}	81.70^{aA}
10	141.48^{aAB}	92.81^{aA}
70	208.15^{aA}	83.66^{bA}
Average	152.60^{a}	86.05^{b}
CV (%)	27.55	
Free N-amino (µmoles g^{-1} fresh weight)		
Without N	3.24^{bA}	2.26^{bB}
10	2.41^{bB}	3.92^{aA}
70	3.07^{bA}	3.83^{aA}
Average	2.91^{b}	3.34^{a}
CV (%)	7.68	
N-NH$_4^+$ (µmoles g^{-1} fresh weight)		
Without N	0.38^{aA}	0.24^{bB}
10	0.24^{bB}	0.52^{aA}
70	0.36^{bA}	0.59^{aA}
Average	0.36^{b}	0.45^{a}
CV (%)	9.50	
Soluble sugars (mg de glucose g^{-1} fresh weight)		
Without N	18.77^{aA}	7.63^{bA}
10	10.65^{aB}	7.48^{bA}
70	6.26^{aC}	6.63^{aA}
Average	11.89^{a}	7.25^{b}
CV (%)	10.14	

Averages followed by the same lowercase letter in the row and the same uppercase letter in the column, for each harvest time, do not differ significantly (Tukey test, $p < 0.05$).
Source: Author

Statistical analysis

The experiment was conducted in a completely randomized design, in a factorial scheme (3 treatments x 2 collections x 3 repetitions). Analysis of variance followed by the Tukey test ($p \leq 0.05$) to compare the means, means and standard deviations and plotting of graphs was carried out using GraphPad Prism version 7. Principal component analysis and Pearson's correlation analyses were performed with PAST 4.01 (Hammer et al., 2001).

RESULTS

The soil analysis revealed adequate conditions for cultivation, without toxic aluminum, with adequate pH, and other nutrients within the expected range. Nitrogen fertilization increased the fresh weight with harvest at 56 DAG and application of 70 kg N ha^{-1} producing the largest fresh weight of the aerial part, 54.19 g box^{-1}, while the lowest production, 23.21 g box^{-1}, occurred at 28 DAG, with no difference between treatments (Table 1). As for the soluble fractions contents, the highest value of N-NO$_3^-$ was found in the first harvest, at 28 DAG, with an average of 152.6 mg kg^{-1} of fresh weight (fw). The highest N-NO$_3^-$ content, 208.15 mg kg^{-1} fw, was observed at 28 DAG and treatment with 70 kg N ha^{-1}. The N-NH$_4^+$

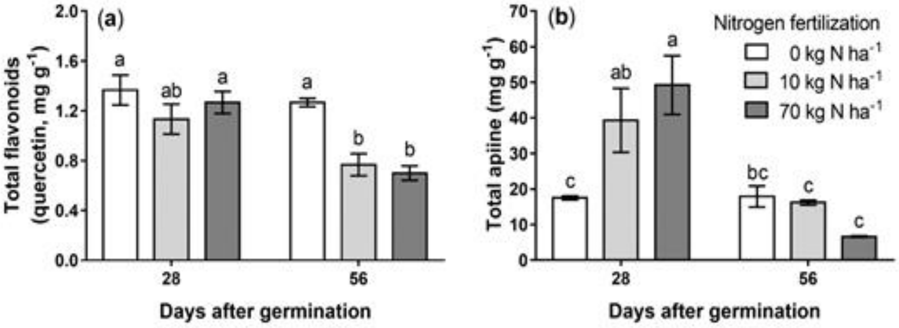

Figure 1. Total flavonoids (a) and apiin (b) content obtained from the crude extract of the aerial parts of parsley at two harvest times, 28 and 56 DAG, and under the effect of three fertilization regimes, without nitrogen (control), with 10 and 70 kg N ha^{-1}. The vertical bars correspond to ± the standard error of the mean (n = 3). Different letters between the columns indicate a significant result (Tukey test, p ≤ 0.05).
Source: Author

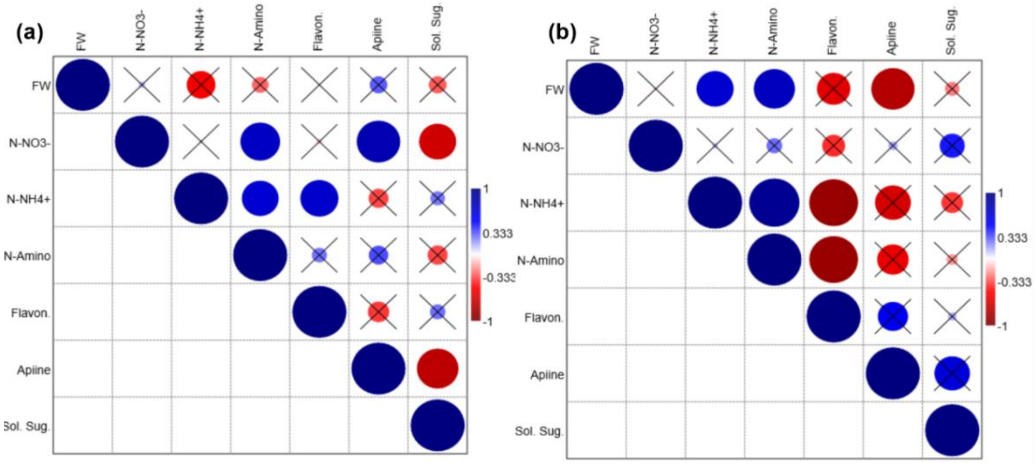

Figure 2. Pearson's correlation for the variables fresh weight, N-NO_3^-, N-NH_4^+, free N-amino, total flavonoids, apiin and soluble sugars from the aerial part of parsley collected at 28 (a) and 56 (b) DAG and under the effect of three fertilization regimes, without nitrogen (control), with 10 and 70 kg N ha^{-1}. Crossed cells (X) did not show significant correlation (p ≤ 0.05). Blue circles indicate positive correlation and red ones negative.
Source: Author

and N-amino levels were higher at 56 DAG, with averages of 0.45 and 3.34 µmoles g^{-1} fw, respectively. The highest levels of N-amino and N-NH_4^+ were observed at 56 DAG and 10 and 70 kg N ha^{-1}, with 3.92, 3.83, 0.52 and 0.59 µmoles g^{-1} fw, respectively. The highest content of soluble sugars was observed at 28 DAG and treatment without nitrogen fertilization, with 18.77 mg g^{-1} fw. There was no significant difference between treatments at 56 DAG, with the mean being 7.25 mg g^{-1} fw (Table 1). The total flavonoid levels were measured in the samples based on the quercetin standard and on average the total content of flavonoids at 28 DAG was higher than at 56 DAG, with concentrations respectively of 0.12 and 0.09 mg 100 mg^{-1}. At 56 DAG, treatments with 10 and 70 kg N ha^{-1} produced the lowest concentrations of total flavonoids, respectively, 0.08 and 0.07 mg 100 mg^{-1} (Figure 1a). Apiin contents were also measured, which on average were higher at 28 DAG (3.53 mg 100 mg^{-1}) compared to 56 DAG (1.36 mg 100 mg^{-1}). The treatments with 10 and 70 kg N ha^{-1} produced the highest levels of apiin, with concentrations of 3.93 and 4.92 mg 100 mg^{-1}, respectively (Figure 1b). Pearson's correlation analysis was applied to the experimental variables, and the data observed at 28 DAG showed a positive correlation between the treatments of free N-amino, N-NO_3^- and N-NH_4^+ levels. They also pointed to a positive correlation between the levels of apiin and N-NO_3^- and between the levels of total flavonoids and N-NH_4^+. The soluble sugar levels showed a negative correlation with those of N-NO_3^- and apiin (Figure 2a). Pearson correlation analysis was

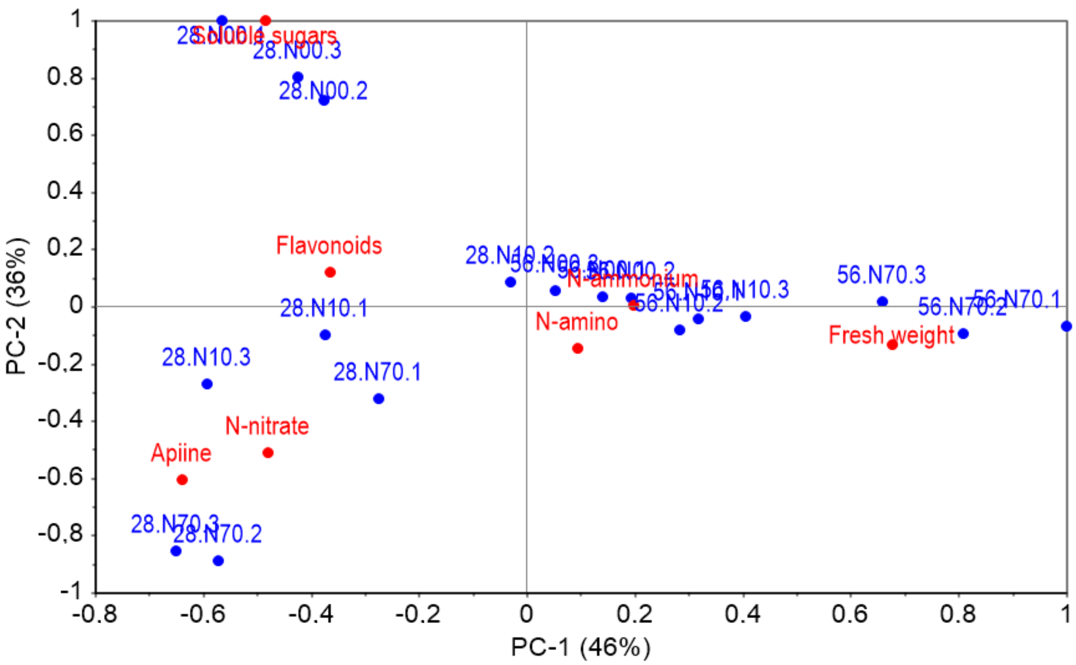

Figure 3. Bi-plot graph of the principal component analysis. Blue samples represent the parsley plants collected at 28 and 56 DAG and under the effect of three fertilization regimes, without nitrogen (control), with 10 and 70 kg N ha^{-1} (scores). Variables in red indicate the contribution of fresh weight, N-NO_3^-, N-NH_4^+, free N-amino, total flavonoids, apiin and soluble sugars for the dispersion of the samples (factor loadings).
Source: Author

also applied to the plants harvested at 56 DAG, which showed a positive correlation between fresh weight and N-NO_3^-, N-NH_4^+ levels, and a negative correlation with apiin content. The N-NH_4^+ levels showed a positive correlation with the N-amino levels and negative with total flavonoids, which in turn, presented a negative correlation with free N-amino (Figure 2b). Fresh weight data of soluble fractions and secondary metabolites obtained at 28 and 58 DAG were subjected to principal component analysis, revealing the dispersion of the data mainly as a function of plant age. The samples obtained at 56 DAG were positioned in the positive quadrant of PC1, highlighting the group formed by plants submitted to treatment with 70 kg N ha^{-1}, which produced more fresh weight. In the negative quadrant of PC1, samples collected at 28 DAG were mainly clustered, which dispersed as a function of PC2 to form two distinct groups, the first in the positive quadrant formed by plants without nitrogen fertilization and with a higher content of soluble sugars and total flavonoids, and the second, in the negative quadrant, composed mainly of plants submitted to nitrogen fertilization, which had higher content of apiin and total flavonoids (Figure 3).

DISCUSSION

The results pointed to the effect of age in the production of apiin and total flavonoids (Figures 1 and 3), but nitrogen fertilization modulated the production of these metabolites, stimulating apiin in treatments with 10 and 70 kg N ha^{-1} at 28 DAG and inhibiting the production of total flavonoids at 56 DAG (Figures 1 and 3). Similar results were reported by Masa et al. (2016), who found that young leaves had a higher content of total flavonoids and apigenin than adult leaves of *Cistus ladanifer* L., in different seasons. The authors attributed this to the fact that younger leaves have a higher rate of metabolite secretion/degradation and that this rate decreases in older leaves. It is also possible that the higher level of apiin in the young leaves is a plant response to the greater availability of nitrate and soluble sugars (Table 1), that are the energy and structure sources for the biosynthesis and can indicate a favorable condition for the flavonoids and apiin synthesis, manly the second, that is a glycosylated form (Figure 1). In addition, light and oxidative stress is higher in young leaves and may be one of the reasons associated with higher apigenin production, as reported by Brunetti et al. (2018) and Zhang et al. (2017), or a response to the microorganism protection processes, as described by Górniak et al. (2019). Also, the metabolic pathways can be modulated according to priority. That is, the adult plant can prioritize energy resources to biomass production and/or to start the reproductive period, decreasing the synthesis of total flavonoids and glycosylated forms (such as apiin), and at the same time catabolizing them for conversion of biomass and energy. Contributing to this hypothesis are the decrease in the levels of soluble sugars at 56 DAG (Table 1) and the negative correlation of apiin with fresh weight (Figure 2). In addition, this hypothesis is also supported by the decrease in N-NO_3^-, increase in N-NH_4^+ and free N-amino and biomass gain at 56 DAG, indicating protein synthesis that consumes many energy resources. The highest N-NO_3^- content found in the aerial part of the parsley was 208.15 mg kg^{-1} fw (Table 1). This nitrate concentration was 14 or 21 times lower than the maximum limits recommended by the European Commission Regulation Nº 563/2000, 3,000 and 4,500 mg NO_3^- kg^{-1} in spinach and lettuce, respectively (Sanatamaria, 2006). China, in turn, adopts a maximum limit for nitrate consumption of 3,100 mg kg^{-1} per day (Zhou et al., 2000). In both cases, there are no maximum limits specific for parsley. In Brazil, environmental conditions are also an additional advantage in this regard, as the high availability of radiation favors the assimilation of nitrate, preventing its accumulation at high levels in plants, as evidenced by data gathered in a review prepared by Luz et al. (2008). In general, nitrogen fertilization influenced the levels of nitrogen fractions and apiin in the aerial part of parsley compared to the condition without N. However, the harvest time predominantly influenced all the parameters evaluated. In addition, the supply of N more than doubled the production of apiin compared to the control group (1.75%), at 28 DAG.

Conclusion

In the experimental conditions evaluated, the shortest harvest time (28 DAG) and nitrogen fertilization with 10 kg ha^{-1} yielded the highest apiin concentration. Therefore, when the objective of cultivation is the production of total flavonoids and apiin, it is best to harvest at 28 DAG without applying N doses higher than 10 kg ha^{-1}.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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