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Chemical and organic fertilizer: The effect on apiin production by *Petroselinum crispum* var. *neapolitanum* Danert

Mylena Firmiano de Andrade[1], Luiziene Soares Alves[2], Bruna Cristina Borges Machado[1], Marco Andre Alves de Souza[5], Rosane Nora Castro[3], Douglas Siqueira de Almeida Chaves[4] and André Marques dos Santos[5]*

[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]Department of Organic Chemistry, Institute of Chemistry, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[4]Department of Pharmaceutical Sciences, Institute of Biological Sciences and Health, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.
[5]Laboratory of Plant Biochemistry, Department of Biochemistry, Institute of Chemistry, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil.

Parsley (*Petroselinum crispum* var. *neapolitanum* Danert) is a vegetable species with potential for pharmaceutical use because it contains apiin, a flavonoid that stands out for having biological activities, such as antioxidant. The aim of this study is to evaluate the influence of chemical or organic fertilizers on the nitrogen metabolism and production of flavonoids in parsley at different stages of development, aiming to establish the most appropriate management of this crop. The experiment was carried out in flower beds with three treatments: Without fertilizer, chemical fertilization and organic fertilization (Bokashi), with two harvest times, 28 and 56 days after germination (DAG). The harvest period significantly influenced the analyzed variables, but the fertilizer source used did not. In this sense, for the production of apiin, fertilization is unnecessary when the soil used has good fertility conditions. It is also recommended to harvest all the plants at 28 DAG, dispensing with late cultivation, thus resulting in reduced production costs.

Key words: Parsley, soluble fractions, fertilizer, chemical fertilization, Bokashi, flavonoids.

INTRODUCTION

Parsley (*Petroselinum crispum* var. *neapolitanum*, Apiaceae) is an aromatic species with medicinal, cosmetic, food and pharmaceutical uses (Linde et al., 2016), due to the presence of secondary metabolites such as flavonoids and their derivatives (Meyer et al., 2006), of which the majority substance is apiin, the

*Corresponding author. E-mail: amarques@ufrrj.br.

biglycosylated form of apigenin (Chaves et al., 2011; Epifanio et al., 2020). Among the biological activities related to parsley, its antioxidant, anti-inflammatory, antihemostatic and antimicrobial activities stand out (Chaves et al., 2011; Epifanio et al., 2020).

Flavonoids are natural substances that result from the secondary metabolism of plants, making up a large group of polyphenolic substances that are widely distributed in the plant kingdom and have biosynthetic origin in the chiquimate pathway (Ali et al., 2017). Flavonoids act by conferring antioxidant capacity (Apak et al., 2016) and also have a wide range of biological activities, including antibacterial, antithrombotic, vasodilator, anti-inflammatory and anticarcinogenic effects, mediated by different mechanisms (Zhang et al., 2006). Since the synthesis of flavonoids is strongly influenced by environmental factors and also by the supply of macronutrients, thorough knowledge of these effects should allow the prediction and selection of growth conditions to achieve the desired content of these secondary metabolites. Crops require different fertilizer doses to achieve optimum yields. The response of plants regarding the production of secondary metabolites is not always positive with increase of nutrients, and there may even be an inverse response, decreasing the levels of secondary metabolites (Souza et al., 2007; Xiaohong et al., 2017).

Fertilization is commonly used to supply nutrients to meet the nutritional demands of crops and thus achieve the highest yields. Chemical and/or organic fertilizers can be used. The use of chemical fertilizers and industrial formulations meets the nutritional demand of plants quickly, but is subject to losses and decreased physical and biological quality of the soil.

Organic fertilizers are obtained from the decomposition of dead plant material or animal excrement. Organic fertilization, in addition to making nutrients contained in waste available more slowly, also reduces losses. In addition, its use favors the increase of edaphic fauna and microorganisms responsible for beneficial biological processes of the soil (Resende et al., 2013; Frouz, 2018). From a physical point of view, organic matter improves soil structure, reduces plasticity, and increases water retention and aeration capacity, allowing greater penetration and distribution of roots (Céspedes-León, 2015). Among the organic fertilizers is Bokashi, obtained from organic substrates, usually bran; it is inoculated with a mixture of microorganisms submitted to controlled fermentation (Javaid, 2010). The different microorganisms used in the manufacture of Bokashi break down organic matter and provide nutrients in the form of amino acids, sugars and organic chelates. Chelates are organic structures that have the advantage of not being easily lost by volatilization or leaching after application. Bokashi does not have a standard formulation. The carbon source used in its preparation is usually wheat bran, and castor bean cake is also widely used in organic agriculture due to its richness in nitrogen and other nutrients (Siqueira and Siqueira, 2013).

In this scenario of dispute over energy resources and organic structures, involving the metabolism of carbohydrates, aromatic amino acids and flavonoids, it is opportune to study different forms of fertilization to verify the response of parsley in relation to the production of apiin. The objective of this study is to verify the influence of 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 of Rio de Janeiro Federal Rural University (UFRRJ), located in the city of Seropédica, RJ. Parsley seeds (*Petroselinum crispum* var. *neapolitanum* Darnet) were purchased from the company ISLA Sementes.

Three treatments were evaluated by cultivating parsley in beds: without fertilization (control); chemical fertilization at planting using 10 kg of N ha^{-1} in the form of calcium nitrate and 60 kg ha^{-1} of potassium in the form of potassium chloride; and organic fertilization at planting using 250 g m^{-2} of Bokashi. The soil used in preparing the beds was analyzed by the Chemical Fertility Analysis Laboratory of PESAGRO-RIO, revealing sandy texture, pH = 6.1; P = 139 mg dm^{-3}; K = 74 mg dm^{-3}; V(%) = 76.0 and the following concentrations (Cmol$_c$.dm^{-3} soil): H+Al = 1.4; Ca^{2+} = 3.1; Mg^{2+} = 1.1; SB = 4.5 and CEC at pH 7.0 = 5.9.

Alcoholic extraction of fresh material and determination of soluble fractions

On the day of collection, samples of the aerial part of the parsley plants were weighed to obtain the fresh mass, from which a homogeneous sample of 1 g was taken and subjected to alcoholic extraction (80% ethanol). This was followed by partition with chloroform (1:1) to obtain the soluble fraction (Fernandes, 1984). The soluble fraction was stored and used later to determine the levels of free N-amino (Yemm and Cocking, 1955), soluble sugars (Yemm and Willis 1957), N-NH$_4^+$ (Mitchel, 1972; Felker, 1977) and N-NO$_3^-$ (Cataldo et al., 1975).

Extraction and purification process for analysis of secondary metabolites

Extraction and purification of the aerial part of parsley were performed according to Chaves et al. (2011) using the decoction method at 10% (w/v), for 15 min; fresh material was fractionated in an industrial processor. Then the decoction was filtered and submitted to lyophilization to quantify the crude extract.

Quantification of total flavonoids

The total flavonoid content was determined using the method adapted from Epifanio et al. (2020). A 400 mL aliquot of each of these extracts 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 flavonoid content was determined using a standard quercetin curve with ten concentration points (0.5 - 5.0 µg ml^{-1}). The results were expressed in mg of quercetin equivalents per 100 mg of crude extract.

Quantification of apiin

The quantitative analysis of apiin was performed using a liquid chromatograph (Shimadzu LC-20AT) coupled to a SPD-20A photodiode array detector (PDA) (CBM-20A module), according to the method described by Chaves et al. (2011). A C18 reverse phase column was used (25 cm, 4.6 mm, 5 µm) with 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 initially 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, in concentrations of 0.02 - 1.0 mg mL^{-1}.

Statistical analysis

The experiment was conducted in a completely randomized design, in a factorial scheme (3 treatments x 2 collections x 3 repetitions). The means and standard deviations were calculated and the graphs plotted using GraphPad Prism version 8. The analysis of variance and the Tukey test ($p \leq 0.05$) for the averages was performed using the ExpDes.pt package (Ferreira et al., 2018) in Software R (Team R Core, 2018). Pearson's correlation analysis was performed in PAST 4.02 (Hammer et al., 2001). The original values of fresh weight, N-NO$_3^-$, N-NH$_4^+$, N-Amino, total flavonoids, apiin and soluble sugars were transformed into the "MAD scale" function (mean absolute deviation) to perform the analysis of main components based on in the singular value decomposition algorithm (SVD), using The Unscrambler Software®.

RESULTS

The analysis of the soil of the beds revealed adequate cultivation conditions, without toxic aluminum and adequate pH, and levels of other nutrients were within the expected concentration ranges.
Chemical fertilization resulted in a significant increase in N-NO$_3^-$ concentrations at the end of the culture cycle (56 DAG), on the order of 286.93 mg kg^{-1} of fresh weight, when compared to collection at 28 DAG (Table 1). The application of fertilizer, whether organic or chemical, did not result in a significant increase in the levels of N-NO$_3^-$ in the leaf tissues when collected at 28 DAG. This did not also differ from the treatment without nitrogen.There was an increase in the concentrations of free N-amino in the aerial part of the parsley, regardless of the type of fertilization used, both at 28 and 56 DAG, compared to the control without N.
Furthermore, the levels of N-NH$_4^+$ did not differ significantly in the leaf tissues according to the treatments, and there was only a small increase in plants collected at 56 DAG (Table 1). Organic fertilization with Bokashi in the early stages of cultivation of parsley stimulated the carbohydrate metabolism, resulting in

elevation of the soluble sugar contents in the aerial part at 28 DAG (Table 1). At 56 DAG, reduction in the levels of soluble sugars in the parsley leaf tissues was observed when subjected to organic fertilization.
Organic and chemical fertilization in the beds did not increase the production of total flavonoids or apiin. The highest level of this secondary metabolite was found in the treatment without addition of N (without fertilization) when the parsley was collected at 28 DAG (Figure 1A-B). There was no positive correlation between the production of biomass (fresh weight) and the levels of apiin when the parsley was collected at 28 DAG (Figure 2A). On the other hand, at 56 DAG, a negative correlation was observed between the levels of N-NO$_3^-$ and soluble sugars, as well as between the fresh mass and the levels of soluble sugars. The levels of N-NO$_3^-$ in the tissues correlated positively with the levels of apiin (Figure 2B).
Fresh weight data, soluble fractions and secondary metabolites obtained at 28 and 58 DAG were submitted to principal component analysis and showed the dispersion of the data according to the age of the plant and the type of soil fertilization. Principal components 1 and 2 (PC1 and PC2) added 74% of variability observed in the Bi-plot graph (Figure 3). The samples obtained at 56 DAG were positioned mainly to the right of PC1, except for the control treatment, while the samples obtained at 28 DAG grouped to the left of PC1. The values of the apiin content and N-NO$_3^-$ variables, in turn, were the ones that most contributed to the dispersion of the samples due to PC2 (Figure 3).

DISCUSSION

The use of chemical fertilizers resulted in a significant increase in N-NO$_3^-$ concentrations, of 88.95% (from 151.85 to 286.93 mg kg^{-1} of fresh mass) at the end of the culture cycle (56 DAG). The same was not observed in the earlier period (collection at 28 DAG), regardless of the source of fertilizer used. The significant accumulation of nitrate due to fertilization at the end of the crop cycle may be related to the lower metabolic activity present in older leaves, which act as nitrate drains, thus changing the source-drain relationship. Similar behavior was found previously in lettuce (Demsar et al., 2004; Siomos et al., 2002) and parsley (Petropoulos et al., 2011). This accumulation of nitrate can also be attributed to the good fertility of the soil used in the beds, even before the treatments were applied.
The accumulation of nitrate in leaf tissues due to the application of chemical fertilizers is potentially harmful and should be monitored in food crops. The consumption of foods with high levels of nitrate is associated with diseases such as methemoglobinemia and some types of cancer (Manassaram et al., 2006). Nitrate alone is not considered toxic, but when present in the body it is endogenously transformed into nitrite, which can react with amines and amides to produce N-nitrous compounds

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 organic and chemical fertilizer, in beds, with two harvest times: 28 and 56 DAG (days after germination).

Treatments	Days after germination (DAG)			
	28		56	
	Fresh weight (g)			
Without N	178.57	Aa	135.94	Bb
Bokashi	84.90	Bb	156.75	Ab
Chemical fertilizer	49.74	Bb	252.91	Aa
Average	104.40	b	181,87	a
CV (%)		11.61		
	N-NO$_3^-$ (mg kg^{-1} fresh weight)			
Without N	158.52	Aa	101.31	Bb
Bokashi	192.59	Aa	79.13	Bb
Chemical fertilizer	151.85	Ab	282.60	Aa
Average	167.65	a	154.35	a
CV (%)		14.84		
	N-amino (µmoles g^{-1} fresh weight)			
Without N	3.86	Aa	4.11	Ba
Bokashi	3.94	Ab	7.16	Aa
Chemical fertilizer	3.42	Ab	6.93	Aa
Average	3.74	b	6.07	a
CV (%)		12.65		
	N-NH$_4^+$ (µmoles g^{-1} fresh weight)			
Without N	0.37	Ab	0.51	Aa
Bokashi	0.32	Ab	0.48	Aa
Chemical fertilizer	0.30	Ab	0.58	Aa
Average	0.33	b	0.52	a
CV (%)		12.78		
	Soluble sugars (mg g^{-1} fresh weight)			
Without N	8.18	Ba	8.56	Aa
Bokashi	10.21	Aa	8.00	Ab
Chemical fertilizer	5.49	Ca	5.82	Ba
Average	7.96	a	7.46	a
CV (%)		8.89		

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).

(Sanatamaria, 2006). These compounds have been linked to an increased risk of various diseases (Choi et al., 2007; Sanatamaria, 2006).

The European Commission, through European Commission Regulation No. 563/2000, adopted as maximum limits the presence of 3000 and 4500 mg NO$_3^-$ kg^{-1}, in spinach and lettuce, respectively (Sanatamaria, 2006). China, in turn, adopted a maximum limit for nitrate consumption of 3100 mg kg^{-1} per day (Zhou et al., 2000). In both jurisdictions, there are no maximum limits considered specifically for parsley. In Brazil, there is still no definition of these limits, so the European limits are adopted. Therefore, although considering the higher N-

NO$_3^-$ content (286.93 mg kg^{-1} of fresh mass) found in the aerial part of the parsley, the nitrate concentration was significantly lower than the maximum limits recommended by the European Commission. In Brazil, environmental conditions are also an additional advantage in this regard, since 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).

Regarding the analyzed soluble fractions, the increase in the levels of free N-amino at 56 DAG with consequent reduction in the levels of soluble sugars evidenced the increase in the metabolic activity of the plants at the end
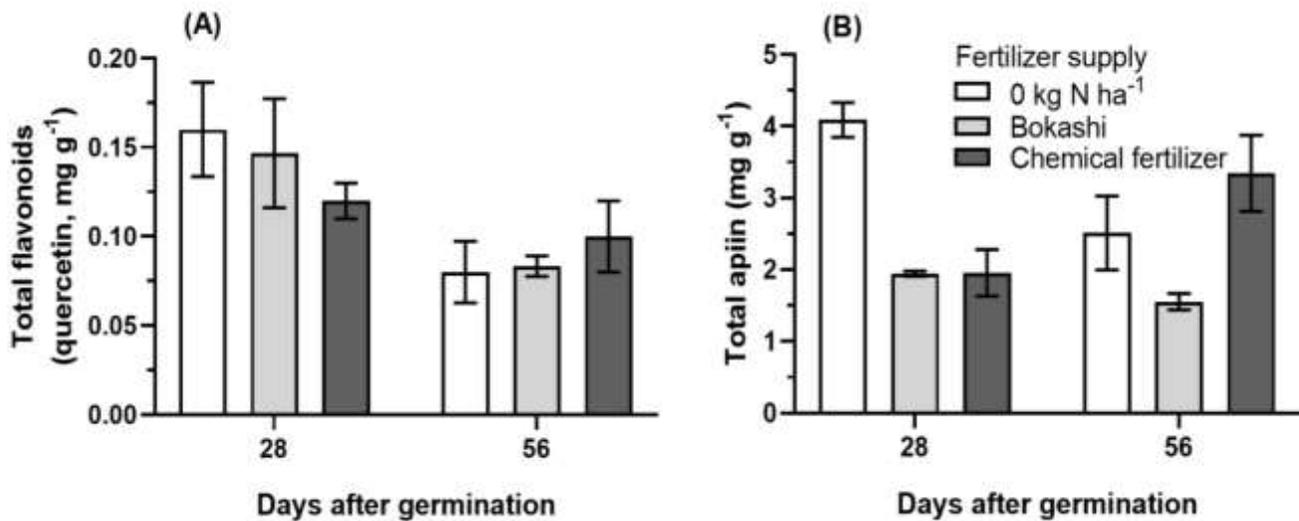
Figure 1. Total flavonoids (A) and apiin (B) content obtained from the crude extract of the aerial parts of parsley cultivated with supply of organic and chemical fertilizer, in beds, with two harvest times, 28 and 56 DAG (days after germination). The vertical bars correspond to ± the standard error of the mean (n = 3).
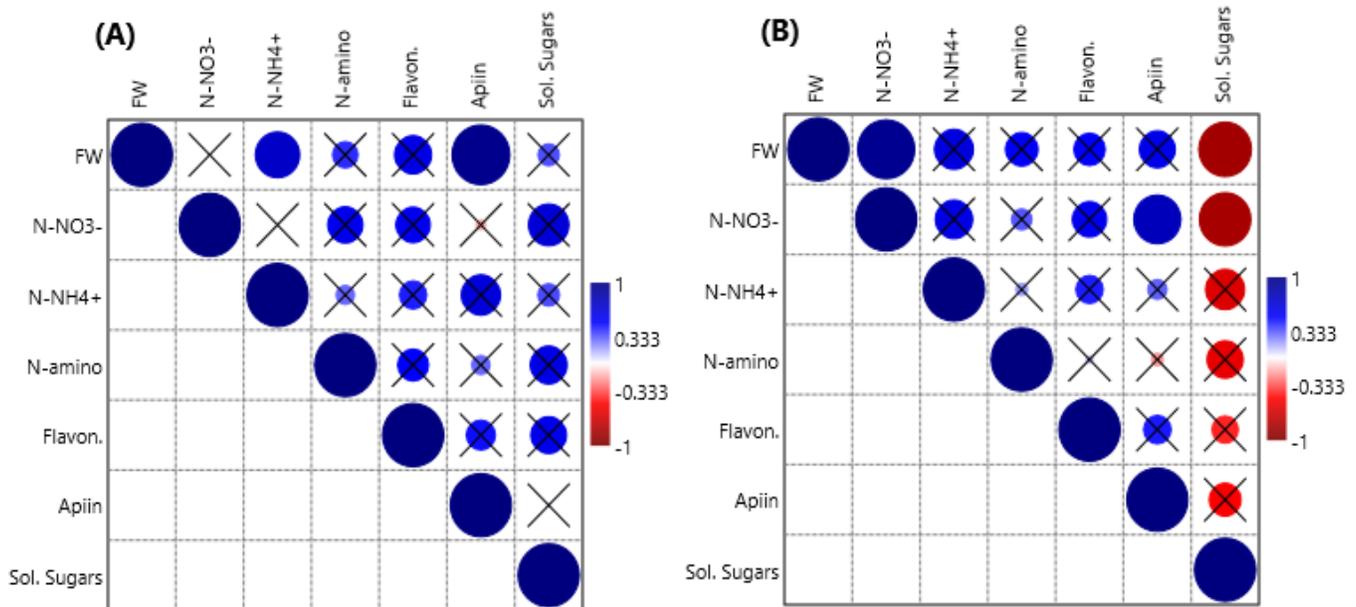

Figure 2. Pearson's correlation for the variables fresh weight, $N-NO_3^-$, $N-NH_4^+$, free N amino, total flavonoids, apiin and soluble sugars from the aerial part of parsley cultivated with supply of organic and chemical fertilizer, in beds, with two harvest times, 28 (A) and 56 (B) DAG (days after germination). Crossed cells (X) did not show significant correlation ($p \leq 0.05$). Blue circles indicate positive correlation and red ones negative.

of the cycle. This results in an increase in biomass. This behavior shows that the levels of free N-amino in the aerial part of parsley were influenced by the age of the crop, showing an increase with the greater age of the plants.

Interestingly, fertilization, whether with chemical or organic fertilizer (Bokashi), did not influence either the synthesis of total flavonoids or that of apiin. On the contrary, the highest levels of total flavonoids and apiin were found in the control treatment (without N) in the
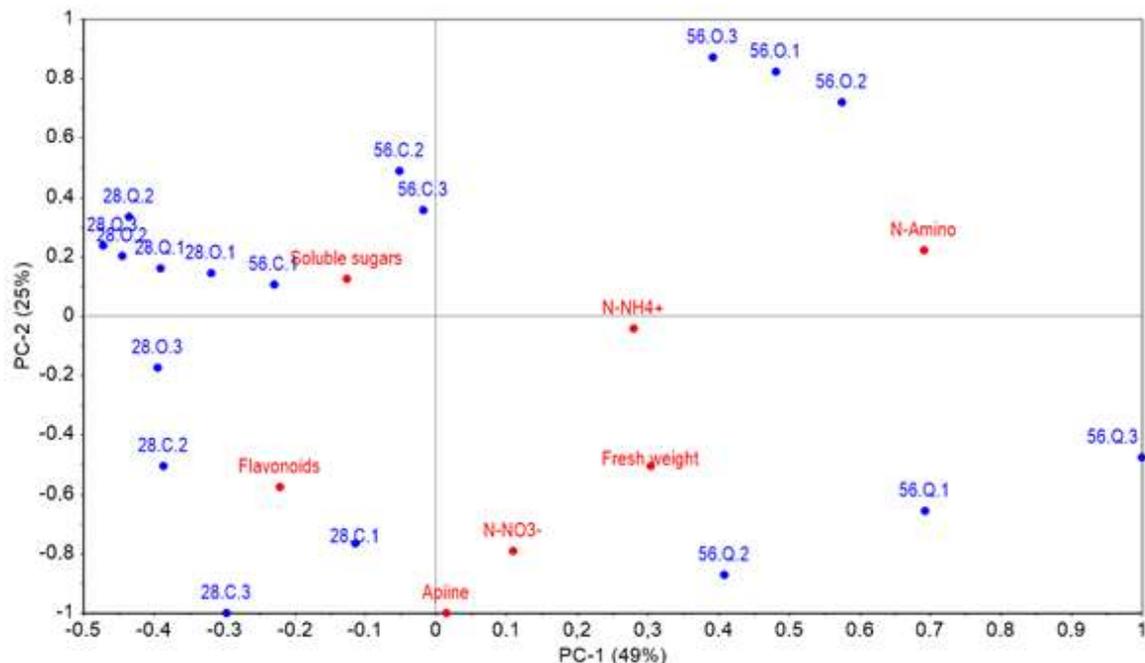
Figure 3. Bi-plot graph of the principal component analysis. Blue samples represent the parsley plants collected at 28 and 56 days after germination (DAG) supply of three fertilization level: without nitrogen (control), organic and chemical fertilizer (scores). Variables in red indicate the contribution 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).

collection performed at 28 DAG. Therefore, fertilization did not influence either the production of total flavonoids or that of apiin, with the harvest season being the limiting factor for its synthesis in our experimental conditions.

The influence of the growing period was also reported by Valares et al. (2016), who found higher levels of thoracic flavonoids and apigenin in young leaves of *Cistus ladanifer* L., in different seasons. The authors attributed this to the fact that young leaves have higher metabolic rates, which decrease over the growing period. It should also be noted that the metabolism of flavonoids is also influenced by the plant's response to light and oxidative stress (Brunetti et al., 2018; Zhang et al., 2017), or a protective response to the attack of microorganisms (Górniak et al., 2019). Thus, under cultivation conditions in flower beds, collection at 28 DAG without the need for nitrogen fertilization favored the production of total flavonoids as well as apiin. Similar results were found in another study, in which the synthesis of flavonoids was strongly influenced by the depletion of nitrogen and phosphorus (Lillo et al., 2008).

The positive correlation observed between the fresh weight and the levels of apiin and N-NH$_4^+$, at 28 DAG demonstrated that in the beginning of the cultivation, the contents of these metabolites depended exclusively on the production of plant biomass. On the other hand, in the later collection (56 DAG), there was a negative correlation between the levels of N-NO$_3^-$, fresh weight and soluble sugars. In contrast, apiin levels correlated positively with the levels of N-NO$_3^-$. Thus, there is an indication that soluble sugars provide carbon skeletons used in the assimilation of nitrogen, to the detriment of flavonoid synthesis.

The principal component analysis showed that treatments with organic and chemical fertilizers collected at 28 DAG showed the highest values of soluble sugars, contributing to the formation of group 1. Higher levels of flavonoids were found in the collection control samples at 28 DAG, contributing to the formation of group 2. The highest levels of apiin were observed in the control treatments collected at 28 DAG and chemical at 56 DAG; on the other hand, the lowest levels of apiin were found in the treatments with organic fertilization at 56 DAG. Taken together, the data reinforce the hypothesis of a negative correlation between nutrient supply and apiin production. It has been observed that nutritional stress results in an increase in the concentration of secondary metabolites (Lattanzio et al., 2009). In other words, in soils that are poor in nutrients, generally there is higher production of secondary metabolites, particularly phenolic derivatives (Yang et al., 2018).

Conclusion

The production of flavonoids and apiin was not influenced by fertilization, either chemical or organic, when the parsley was grown in beds. On the other hand, the time

of collection was decisive to produce these metabolites in the cultivation conditions studied. In this sense, when parsley is grown in beds for the production of apiin, fertilization can be omitted when the soil used has good fertility conditions, thus contributing to the reduction of production costs. Likewise, the harvest can also be carried out at 28 DAG, since longer growing periods do not have a beneficial effect.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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