Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
ISSN: 1807-863X
actabiol@uem.br
Universidade Estadual de Maringá
Brasil

Lima, Milene Conceição; da-Silva, Cristiane Jovelina; Mariot, Marcio Paim; Freitag, Rogério Antônio; Serpa, Rosana; Ribeiro, Gladis Aver; Amarante, Luciano do

Effect of shading and nitrogen fertilization on nitrogen metabolism, essential oil content and antimicrobial activity of *Achillea millefolium*

Acta Scientiarum. Biological Sciences, vol. 42, 2020, pp. 1-12
Universidade Estadual de Maringá
Maringá, Brasil

DOI: https://doi.org/10.4025/actascibiolsci.v42i1.46412

Disponible en: https://www.redalyc.org/articulo.oa?id=187163790001

Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v42i1.46412

Effect of shading and nitrogen fertilization on nitrogen metabolism, essential oil content and antimicrobial activity of *Achillea millefolium*

Milene Conceição Lima[1†], Cristiane Jovelina da-Silva[1†*], Marcio Paim Mariot[2], Rogério Antônio Freitag[1], Rosana Serpa[1], Gladis Aver Ribeiro[1] and Luciano do Amarante[1]

[1]Universidade Federal de Pelotas, Campus Universitário, s/n, 96160-000, Capão do Leão, Rio Grande do Sul, Brazil. [2]Instituto Federal de Educação, Ciência e Tecnologia Sul-Rio-Grandense, Pelotas, Rio Grande do Sul, Brazil. *Author for correspondence. E-mail: cristianejovs@yahoo.com.br

ABSTRACT. The *Achillea millefolium* L. is a perennial herb with important antibacterial, antifungal, anti-inflammatory, antitumoral, and antioxidant properties. This research aimed to investigate the effect of shading (75%; black net) and nitrogen fertilization (0, 75 and 150 kg urea ha^{-1}) on the nitrogen metabolism, essential oil yield and antimicrobial activity of *A. millefolium* at vegetative- and reproductive-stage. The evaluated parameters varied depending on the organ and the phenological stage of the plant considered. Overall, our findings indicated that shading decreased nitrogen assimilation. Decreased activities of nitrate reductase and glutamine synthetase were observed on shaded plants during reproductive and vegetative stages, respectively. Nitrate and total amino acid levels increased in shaded plants at the vegetative stage. Regarding nitrogen supply, the improved nitrogen metabolism and essential oil yield values were accompanied by intermediate concentrations of urea (75 kg ha^{-1}). Plants fertilized with 75 kg urea ha^{-1} produced the highest amino acids concentration (vegetative stage), ammonium concentration (vegetative stage) and essential oil yield (reproductive stage). Shading or nitrogen supply did not influence the microbial activity of *A. millefolium* essential oil. However, the essential oil of leaves and flowers were highly effective against fungi and bacteria, especially gram-positive bacteria. In conclusion, the current study showed that full light and 75 kg urea ha^{-1} enhanced the nitrogen metabolism of *A. millefolium* in both vegetative and reproductive stages.

Keywords: Light reduction; medicinal plants; minimum inhibitory concentration; nitrogen assimilation; yarrow.

Received on July 25, 2019.
Accepted on December 4, 2019.

Introduction

Achillea millefolium L. (yarrow) is a perennial herb from the *Asteraceae* family common in the arid and semi-arid regions of subtropical and lower temperate latitudes. It develops and grows best in full light, but tolerates partial shading (Martins, Castro, Castellani, & Dias, 2003). *A. millefolium* is an important medicinal plant, with antibacterial, antifungal, anti-inflammatory and antitumoral properties (Ali, Gopalakrishnan, & Venkatesalu, 2017). These medicinal properties are due to its essential oil, which is mainly composed of sabinene, β-pinene, eucalyptol, camphor, borneol, 1,8-cineol, α-terpineol, bisabolol oxide, chamazulene, caryophyllene, linalool and eugenol (Mohammadhosseini, Sarker, & Akbarzadeh, 2017).

The concentration and composition of the yarrow essential oil can be influenced by its stage of development; environmental conditions, storage period and plant subtype (Verzár-Petri & Cuong, 1977). The use of different types of protection nets in the growth of a range of plants is increasingly becoming popular. Nets are used to improve the microclimate, protect plants from hail, pests, and excessive sunshine, and modify plant morphology and metabolism. The use of nets for shading is a widely used method for growing medicinal plants (Costa, Chagas, Bertolucci, & Pinto, 2014; Russo & Hornermeier, 2017; Ribeiro et al., 2018). Indeed, the content of chloroplastic pigments increased with shading in *A. millefolium* plants grown under conditions similar to those used in this study (Lima, Amarante, Mariot, & Serpa, 2011). Similarly, greater survival and rooting of *A. millefolium* seedlings cultivated *in vitro* with reduced radiation were observed (Alvarenga, Pacheco, Silva, Bertolucci, & Pinto, 2015)

† These authors contributed equally to this work

Adequate nitrogen fertilization can also play a key role in growth, development and the production of secondary metabolites in plant species (Tavarini, Sgherri, Ranieri, & Angelini, 2015; Urlić et al., 2017). Responses to nitrogen fertilization in plants can vary with respect to their capacity to assimilate nitrogen as ammonium (NH_4^+), nitrate (NO_3^-), urea and amino acids, as well as from atmospheric nitrogen *via* symbiotic associations (Han, Okamoto, Beatty, Rothstein, & Good, 2015). In agriculture, urea is intensively used as a nitrogen fertilizer. Nitrogen from urea either enters the plant directly or in the form of ammonium or nitrate after urea has been degraded by soil microbes (Witte, 2011). Absorbed nitrate can be metabolized directly in the root, stored in vacuoles, or transferred to the plant shoot. Regardless of plant tissue, nitrate is first reduced by cytosolic nitrate reductase (NR) to nitrite, which is then imported into the chloroplast and reduced further by nitrite reductase in order to produce ammonium (Krapp, 2015).

Ammonium, whether taken directly from the soil or converted from nitrate, is assimilated via the glutamine synthetase (GS)/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle. GS is the key enzyme involved in the transformation of inorganic nitrogen to assimilable organic forms in higher plants (Krapp, 2015). This enzyme catalyzes the ATP-dependent condensation of ammonium and glutamate to produce glutamine, which provides nitrogen groups directly or *via* glutamate for the biosynthesis of all nitrogen-containing compounds within the plant (Forde & Cullimore, 1989). Conversion of glutamine to other amino acids initially involves glutamate synthase, which catalyzes the transfer of the δ-amino group from glutamine to the α-keto position of α-ketoglutarate, producing two glutamate molecules. The reaction occurs at the expense of reducing power, which is provided by NADH or ferredoxin (Lea & Morot-Gaudry, 2001). The assimilation of ammonium into glutamate and glutamine occurs throughout plant metabolism since these amino acids are nitrogen donors needed for the biosynthesis of other amino acids, nucleic acids, and other nitrogen-containing compounds. After ammonium has been assimilated, glutamate can be rapidly converted into other amino acids by the action of transaminases (Krapp, 2015).

Either the availability of light can affect the uptake and assimilation of nitrogen, through the regulation of enzymes such as NR, or providing an environment with a higher level of nitrate or ammonium distributed in the soil (Stagnari, Galieni, & Pisante, 2015). Both factors also influence the production of secondary compounds in medicinal plants. However, there is a lack of information regarding the influence of light and nitrogen availability on the metabolism of *A. millefolium*. Our understanding of these features has the potential to provide growing conditions that are optimal for enhancing biological activity. Therefore, this research aimed to investigate nitrogen metabolism and the essential oil of *A. millefolium* grown under different levels of shading and concentrations of urea.

Material and methods

Plant material and experimental conditions

Plant cultivation was carried out in the experimental area *Conjunto Agrotécnico Visconte da Graça* (CAVG) located in *Instituto Federal Sul-rio-grandense*, Pelotas, RS (31º42'44.52" S and 52º18'46.70" W). Yarrow plants (*A. millefolium*) were obtained from a single clump to ensure the uniformity of data (Martins et al., 2003). Seedlings were grown in a greenhouse for four month, from which they were transferred to the experimental area (planosol soil). Phosphorus and potassium content was previously adjusted based on a soil analysis. Throughout the experiment, plants were irrigated using a drip system.

Forty-day-old plants of uniform size were grown using three different concentrations of nitrogen fertilizer (0, 75 and 150 kg ha^{-1}) in the form of urea. In addition, in reproductive-stage plants (full flowering), two levels of shading including 0% shading (full light) and 75% shading (covered with a black net) were assessed. After 56 day under these conditions, plants were harvested. Then, the black net was removed, and nitrogen fertilization was performed (as described above). Four months later, the vegetative stage plants were again shaded (0 and 75%) and they were grown under these conditions for 46 day when the second harvest was performed. A herbarium specimen was prepared and deposited at the PEL Herbarium (24.600).

Activity of nitrate reductase and glutamine synthetase

The activity of NR (EC 1.6.6.1) was previously evaluated using plant materials harvested at different periods of the day. Researchers were able to determine that 8:30 am was the best time to collect

materials for the analysis of NR activity (data not shown). The activity of NR was assessed *in vivo* according to Klepper, Flesher, and Hageman (1971) with some modifications. Fresh leaves and roots (0.5 g) were incubated in 0.2 M phosphate buffer (pH 7.5) containing 0.1 M KNO_3 and 1% (v v^{-1}) propanol. Afterward, plant material was vacuum-infiltrated three times (300 mmHg for 1 min.) and incubated in a shaking water bath at 30ºC in the dark. Then, aliquots from the samples were collected and incubated with 1% (w v^{-1}) sulfanilamide and 0.02% (w v^{-1}) naphthyl ethylene-diamine dihydrochloride at 30ºC for 15 min. and posteriorly analyzed at 540 nm. A standard curve with sodium nitrite was performed for determining nitrite formation.

The activity of GS (EC 6.3.1.2) in fresh plants was evaluated according to Cullimore, Lara, Lea, and Miflin (1983). Briefly, 2.0 g of leaves and roots were homogenized with 100 mM Tris-HCl (pH 7.8) containing 1 mM dithiothreitol, 5 mM glutamic acid, 10 mM $MgCl_2$, 10% (v v^{-1}) glycerol and 20% (w w^{-1}) polivinilpolipirrolidone. The homogenate was centrifuged at 20,000 × *g* and 4ºC for 20 min. and the supernatant was desalted using a Sephadex G-25 column. The reaction was initiated by adding resulting supernatants (100 µL) to media containing 100 mM Tris-HCl (pH 7.8), 100 mM glutamic acid, 80 mM hydroxylamine, 80 mM ATP and 160 mM $MgCl_2$ for 30 min., followed by the addition of stop solution (0.37 $FeCl_3$, 0.2 M TCA and 0.67M HCl). After, the contents were centrifuged at 14,000 × *g* at 25ºC to precipitate proteins. *y*-glutamyl hydroxamate content within supernatants was determined by measuring fluorescence at 535 nm and quantified using a standard curve.

Determination of total free amino acids, nitrate, and ammonium

Total free amino acids, nitrate, and ammonium were extracted according to Bieleski and Turner (1966). Frozen leaves and roots (1.0 g) were ground into a powder using liquid N_2 and metabolites were extracted for 24 hours with a 10 mL methanol:chloroform:water (12 5 3^{-1}; v v v^{-1}) solution. After centrifugation at 600 × *g* for 10 min., one volume of chloroform and 1.5 volumes of water were added to each of the 4 volumes of supernatants obtained. The aqueous phase was collected after 24 hours and used for the analysis of amino acids, nitrate, and ammonium. Total levels of soluble amino acids were measured using the ninhydrin method of Yemm, Cocking, and Ricketts (1955) and measured via spectrophotometric analyses at 570 nm. Nitrate was determined spectrophotometrically at 410 nm using the salicylic acid method as described by Cataldo, Maroon, Schrader, and Youngs (1975). The ammonium was analyzed using Berthelot reactions at 625 nm (McCullough, 1967).

Essential oil yield and determination of the minimum inhibitory concentration

Shade-dried, powdered, leaf or flower materials (70 g) were hydro-distilled in a Clevenger-type apparatus for 4 hours. The extracted volatile oils were dried over anhydrous sodium sulfate and the yield of the oils was calculated based on dry the weight of plants (Santos, Alves, Figueirêdo, & Neto, 2004).

The antimicrobial activity of the essential oil of *A. millefolium* was tested against reference strains (American Type Culture Collection) including *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Candida albicans* (ATCC 18804). The same test was performed for the isolates strains *Bacilus cereus*, *Enterobacter aerogenes*, *Shigella flexneri*, *Klebsiella* sp., *Salmonela* sp., *E. coli*, *S. aureus*, *S. epidermidis,* and *C. albicans*. The essential oil was tested in the concentration ranges of 12.5–100 mg L^{-1}. The minimal inhibitory concentration (MIC) of each sample was determined after incubating 24 hours at 37ºC and adding 20 µL of 0.5% of 2,3,5-triphenyl tetrazolium chloride. Then, a new incubation was performed at 37ºC for 30 min. until the lowest sample concentration prevented a color change in the medium and exhibited complete inhibition of microbial growth.

Experimental design and statistical analysis

Experiments were arranged in a completely randomized block, 3 x 2 factorial design (nitrogen concentrations and shading levels). All analyses were carried out using four repetitions, and each biological sample consisted of five plants. Data were first submitted to a Shapiro-Wilk test to assess the normality of data and to a Levene's test to verify homoscedasticity, followed by ANOVA. The post hoc comparisons were tested using a Tukey or *t*-test at the significance level of $p < 0.05$ with the statistical program SAS (2002).

Results

There was no interaction between shading and nitrogen levels when NR and GS were evaluated. Shaded yarrow leaves from reproductive-stage plants exhibited decreased (1.7-fold) NR activity (Table 1). However, NR was not affected by shading in leaves from vegetative-stage plants and roots from plants at both stages ($p < 0.05$). Regarding the influence of nitrogen fertilization, NR activity increased (1.5-fold) in yarrow roots from vegetative-stage plants treated with 75 kg urea ha^{-1}, and did not change in leaves of the same stage or in leaves and roots from reproductive-stage plants (Table 2). In plants at the vegetative stage, the activity of GS decreased 2.2- and 1.6-fold in shaded leaves and roots, respectively (Table 3). However, GS activity increased in leaves of plants at the vegetative stage fertilized with 75 kg urea ha^{-1}. The GS enzyme was not influenced by the concentrations of nitrogen or shading in reproductive-stage plants (Table 4; $p < 0.05$).

Table 1. Nitrate reductase activity in leaves and roots of *Achillea millefolium* L. plants submitted to shading at reproductive and vegetative stages.

Levels of shading	Nitrate reductase activity (μmol g^{-1} FW)			
	Reproductive stage		Vegetative stage	
	Leaves	Roots	Leaves	Roots
Black net (75%)	2.58 b	2.58 a	4.19 a	2.34 a
Full light (0%)	4.34 a	2.35 a	3.86 a	2.52 a

Distinct letters indicate significant differences between treatments (*t*-test; $p < 0.05$).

There was an interaction between shading and nitrogen levels with respect to levels of total free amino acids in vegetative-stage plants. In reproductive-stage plants, leaf and root amino acid content was unaffected by shading (Table 3; $p < 0.05$), but decreased 1.3-fold in plants treated with 75 and 150 kg urea ha^{-1} (Table 5). In contrast, in shaded plants at the vegetative stage, amino acid content increased 1.5-fold with 75 and 150 kg urea ha^{-1} treatments, respectively, when compared to levels in plants that did not receive urea treatment (Table 6). The free amino acid content in plants grown under full light increased (1.9-fold) post-treatment with 150 kg urea ha^{-1}. Other concentrations of urea treatment did not significantly affect free amino acid content. The total amino acid content in plants treated with 75 and 150 kg urea ha^{-1} was greater (2.8- and 1.5-fold, respectively) when plants were shade-grown, rather than grown under full light (Table 6).

Table 2. Nitrate reductase activity in leaves and roots of *Achillea millefolium* L. plants cultivated under different nitrogen concentrations at reproductive and vegetative stages.

Nitrogen concentrations (Kg urea ha^{-1})	Nitrate reductase activity (μmol g^{-1} FW)			
	Reproductive stage		Vegetative stage	
	Leaves	Roots	Leaves	Roots
0	3.54 a	2.68 a	4.55 a	2.27 b
75	3.03 a	2.49 a	3.74 a	3.12 a
150	3.83 a	2.22 a	3.77 a	1.90 b

Distinct letters indicate significant differences among treatments (Tukey; $p < 0.05$).

Overall, in the two studied stages, nitrate content was higher in roots than in leaves of *A. millefolium* (Table 7, Figure 1). There was an interaction between shading and nitrogen levels with respect to nitrate content in yarrow plants at the reproductive stage (Table 7). In shaded leaves, nitrate content was increased in plants treated with 150 kg urea ha^{-1} (17.55 μmol g^{-1} FW) and 0 kg urea ha^{-1} (14.80 μmol g^{-1} FW), respectively, compared with plants treated with 75 kg urea ha^{-1} (11.72 μmol g^{-1} FW). In the roots of shaded plants, there were no differences observed between nitrogen supplementation. In unshaded plants, the highest levels of nitrate content in leaves occurred in the privation of nitrogen and in roots in the privation of urea and when the plants were treated with 150 kg urea ha^{-1} (Table 7). When the plants were treated with 150 kg urea ha^{-1}, the highest levels of nitrate (1.3-fold) in leaves were found in shaded plants compared with non-shaded plants. In roots, shading decreased nitrate content 1.5-, 1.2- and 1.4-fold, respectively when plants were treated with 0, 75 and 150 kg urea ha^{-1}. On the other hand, the nitrate content in leaves and roots of shaded plants increased (2.6- and 1.8-fold, respectively) when the plants were shaded at the vegetative stage (Figure 1). The nitrogen supply did not affect nitrate content in vegetative-stage plants ($p < 0.05$; data not shown).

Table 3. Glutamine synthetase activity and total amino acids levels in leaves and roots of *Achillea millefolium* L. plants submitted to shading at the vegetative stage.

Levels of shading	Glutamine synthetase (μmol g^{-1} FW)		Total amino acids (μmol g^{-1} FW)	
	Leaves	Roots	Leaves	Roots
Black net (75%)	4.83 b*	1.79 b	3.41 a	18.21 a*
Full light (0%)	10.46 a*	2.88 a	3.67 a	17.35 a*

Distinct letters indicate significant differences between treatments (*t*-test; $p < 0.05$); Asterisks indicate significant differences between leaves and roots (*t*-test; $p < 0.05$)

There was no interaction between shading or nitrogen dosage on ammonium content in *A. millefolium* reproductive-stage leaves. In contrast, in the vegetative stage, ammonium content was increased when the plants were shaded and treated with 0 kg urea ha^{-1} (3.3 fold) and 150 kg urea ha^{-1} (2.0 fold), respectively, compared with plants under shading and treated with 75 kg urea ha^{-1}. Under full light conditions, the highest level of ammonium content was found in plants fertilized with 150 kg urea ha^{-1}. However, in roots and leaves of shaded and unshaded plants, ammonium levels in response to nitrogen fertilization did not change (Table 8). Shading decreased ammonium levels in the leaves and roots of plants fertilized with nitrogen. This reduction was 3.1- and 1.6-fold for leaves and 2.3- and 7.3-fold for roots treated with 75 and 150 kg urea ha^{-1} respectively (Table 8). Ammonium levels were also decreased (4.8-fold) in the roots of shaded plants that did not receive nitrogen fertilization.

Table 4. Glutamine synthetase activity in leaves and roots of *Achillea millefolium* L. plants at the vegetative stage cultivated under different nitrogen concentrations.

Nitrogen concentrations (Kg urea ha^{-1})	Glutamine synthetase (μmol g^{-1} FW)	
	Leaves	Roots
0	7.19 b*	2.09 a
75	10.05 a*	2.47 a
150	5.70 b*	2.44 a

Distinct letters indicate significant differences among treatments (Tukey; $p < 0.05$); Asterisks indicate significant differences between leaves and roots (*t*-test; $p < 0.05$)

The concentration of nitrogen fertilizer applied and the degree of shading had significant effects on the essential oil yields from leaves and flowers, but there was no interaction observed between the factors. Urea supply did alter yields of the essential oils in leaves (data not shown). However, in flowers, fertilization with 75 kg urea ha^{-1} increased the oil yields 1.2-fold compared to other treatments (Figure 1). In contrast, leaf oil yields decreased 1.2-fold with shading compared to control leaves (Figure 1), while this parameter did not alter flower essential content (data not shown).

Biological activities of essential oils of both yarrow leaves and flowers were similar among treatments (Table 9-10). Thus, the different concentrations of nitrogen and shading levels studied did not affect the inhibitory activity of the essential oil ($p < 0.05$). The MIC values of essential oils from yarrow leaves ranged from 12.5 to 25.0 mg mL^{-1} for *S. aureus* ATCC 25923, *B. cereus*, *S. aureus* and *S. epidermidis* bacterial strains (Table 9). Similarly, *C. albicans* ATCC 18804 and *C. albicans* were inhibited by 12.5 mg mL^{-1} essential oil (Table 9). On the other hand, MIC values for *E. coli* ATCC 8739, *Salmonella sp*. and *E. coli* bacterial strains ranged from 50 to 100 mg mL^{-1}. The essential oil of yarrow leaves did not inhibit the growth of *E. faecalis* ATCC19433, *P. aeruginosa* ATCC 9027, *E. aerogenes*, *S. flexneri*, and *Klebsiella sp*. bacterial strains at any of the concentrations tested.

Table 5. Total amino acids in leaves and roots of *Achillea millefolium* L. plants at the reproductive stage cultivated under different nitrogen concentrations.

Nitrogen concentrations (Kg urea ha^{-1})	Total amino acids (μmol g^{-1} FW)	
	Leaves	Roots
0	3.69 a	20.96 a*
75	3.26 a	16.19 b*
150	3.66 a	16.19 b*

Distinct letters indicate significant differences among treatments (Tukey; $p < 0.05$); Asterisks indicate significant differences between leaves and roots (*t*-test; $p < 0.05$)

Table 6. Total amino acids in roots of *Achillea millefolium* L. plants at vegetative stage cultivated under different nitrogen concentrations.

Levels of shading	Total amino acids (μmol g^{-1} FW)		
	0 Kg urea ha^{-1}	75 Kg urea ha^{-1}	150 Kg urea ha^{-1}
Black net (75%)	16.08 Ba	24.40 Aa	22.73 Aa
Full light (0%)	6.88 Ba	8.77 Bb	14.86 Ab

Distinct uppercase letters indicate significant differences in the line while distinct lowercase letters indicate significant differences in the column (Tukey; $p < 0.05$).

The antimicrobial activity of the essential oil of flowers was very similar to that of leaves (Table 10). The *B. cereus* bacterial strain and *C. albicans* ATCC 18804 and *C. albicans* fungal strains were inhibited by 12.5 mg mL^{-1} essential oil. While *S. aureus*, *E. faecalis* ATCC 19433, *S. aureus* and *S. epidermidis* bacterial strains were sensitive to essential oil at concentrations of 12.5 and 25 mg mL^{-1}. For strains, *E. coli* ATCC 8739, *Salmonella sp.*, *E. aerogenes,* and *E. coli,* MIC varied from 50 to 100 mg mL^{-1}. Finally, for *P. aeruginosa* ATCC 9027, *S. flexneri* and *Klebsiella sp.* strains, the essential oil showed no inhibitory activity (Table 10).

Table 7. Nitrate content in the leaves and roots of *Achillea millefolium* L. plants at the reproductive stage grown under different concentrations of nitrogen (Kg urea ha^{-1}) and subject to shading.

Levels of shading	Nitrate content (μmol g^{-1} FW)					
	Leaves			Roots		
	0	75	150	0	75	150
Black net (75%)	14.80 Ba	11.72 Ca	17.55 Aa	37.36 Ab*	40.87 Ab*	42.89 Ab*
Full light (0%)	16.74 Aa	12.60 Ca	13.18 Bb	57.04 Aa*	50.67 Ba*	58.16 Aa*

Distinct uppercase letters indicate significant differences in the line while distinct lowercase letters indicate significant differences in the column (Tukey; $p < 0.05$); Asterisks indicate significant differences between leaves and roots (t-test; $p < 0.05$)

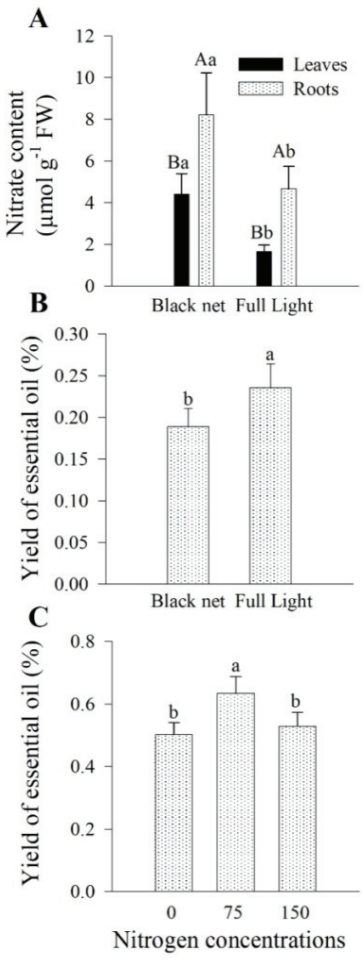

Figure 1. Nitrate content in leaves and roots (A) and yield of essential oil in leaves (B) and flowers (C) of plants of *Achillea millefolium* L. submitted to levels of shading (black net - 75%; full light - 0%) or different concentrations of nitrogen (kg urea ha^{-1}). Distinct uppercase letters indicate differences between roots and leaves, lowercase letters indicate differences between shading or among nitrogen supply treatments.

Table 8. Ammonium content in the leaves and roots of *Achillea millefolium* L. plants at the vegetative stage grown under different concentrations of nitrogen (Kg urea ha^{-1}) and subject to shading.

Levels of shading	Ammonium content (μmol g^{-1} FW)					
	Leaves			Roots		
	0	75	150	0	75	150
Black net (75%)	0.23 Aa	0.07 Cb	0.14 Bb	0.05 Bb	0.09 Ab	0.03 Bb
Full light (0%)	0.19 Ba	0.22 Aa	0.23 Aa	0.24 Aa	0.21 Aa	0.22 Aa

Distinct uppercase letters indicate significant differences in the line while distinct lowercase letters indicate significant differences in the column (Tukey; $p < 0.05$).

Table 9. Minimum inhibitory concentration (mg mL^{-1}) of the essential oil of leaves of *Achillea millefolium* L. plants grown under nitrogen concentrations (0, 75 and 150 kg urea ha^{-1}) and submitted to shade levels [full light (0%) and black net (70%)] for different microorganisms.

Microrganisms	Minimal Inhibitory Concentration (mg mL^{-1})					
	0 Kg urea ha^{-1}		75 Kg urea ha^{-1}		150 Kg urea ha^{-1}	
	Full light	Black net	Full light	Black net	Full light	Black net
Gram-positive bacteria						
Staphylococcus aureus ATCC 25923	12.5	12.5	12.5	25	12.5	12.5
Enterococcus faecalis ATCC 19433	>100	>100	>100	>100	>100	>100
Bacillus cereus	12.5	12.5	12.5	25	12.5	12.5
Staphylococcus aureus	12.5	12.5	12.5	25	12.5	12.5
Staphylococcus epidermidis	12.5	25	12.5	25	12.5	12.5
Gram-negative bacteria						
Pseudomonas aeruginosa ATCC 9027	>100	>100	>100	>100	>100	>100
Escherichia coli ATCC 8739	100	100	50	100	100	100
Salmonella sp.	100	100	100	100	100	50
Enterobacter aerogenes	>100	>100	>100	>100	>100	>100
Shigella flexneri	>100	>100	>100	>100	>100	>100
Klebsiella sp.	>100	>100	>100	>100	>100	>100
Escherichia coli	100	100	50	100	100	100
Fungi						
Candida albicans ATCC 18804	12.5	12.5	12.5	12.5	12.5	12.5
Candida albicans	12.5	12.5	12.5	12.5	12.5	12.5

Table 10. Minimum inhibitory concentration (mg mL^{-1}) of the essential oil of flowers of *Achillea millefolium* L. plants grown under nitrogen concentrations (0, 75 and 150 kg urea ha^{-1}) and submitted to shade levels [full light (0%) and black net (70%)] for different microorganisms.

Microrganisms	Minimal Inhibitory Concentration (mg mL^{-1})					
	0 Kg urea ha^{-1}		75 Kg urea ha^{-1}		150 Kg urea ha^{-1}	
	Full light	Black net	Full light	Full light	Black net	Full light
Gram-positive bacteria						
Staphylococcus aureus ATCC 25923	25	12.5	12.5	12.5	12.5	12.5
Enterococcus faecalis ATCC 19433	12.5	25	12.5	12.5	12.5	12.5
Bacillus cereus	12.5	12.5	12.5	12.5	12.5	12.5
Staphylococcus aureus	25	12.5	12.5	12.5	12.5	12.5
Staphylococcus epidermidis	12.5	12.5	25	12.5	25	25
Gram-negative bacteria						
Pseudomonas aeruginosa ATCC 9027	>100	>100	>100	>100	>100	>100
Escherichia coli ATCC 8739	50	100	50	50	50	50
Salmonella sp.	100	50	50	50	100	50
Enterobacter aerogenes	100	50	100	50	100	100
Shigella flexneri	>100	>100	>100	>100	>100	>100
Klebsiella sp.	>100	>100	>100	>100	>100	>100
Escherichia coli	50	100	50	50	50	50
Fungi						
Candida albicans ATCC 18804	12.5	12.5	12.5	12.5	12.5	12.5
Candida albicans	12.5	12.5	12.5	12.5	12.5	12.5

Discussion

Nitrogen is an essential element for plants that is available in agricultural soils mainly in the form of nitrate and ammonium (Pinton, Tomasi, & Zanin, 2016). In the leaves of shaded plants treated with the highest concentration of nitrogen, enhanced nitrate accumulation was observed. This increase in nitrate

levels is due to the low light intensity required for ion reduction and the increased availability of nitrate supplied by nitrogen fertilizer. Further, the increased levels of nitrate found in the roots of unshaded plants compared to those that were not shaded may be a result of enhanced nitrification of the soil. Under these conditions, decreased humidity and increased temperatures may have favored microbial activity in comparison to shady conditions, thus allowing more nitrate to be accessed via the roots of plants. In contrast, nitrogen supply in soil treated with 75 kg of urea ha^{-1} for reproductive-stage plants seems to have improved nitrate metabolism, thus reducing its accumulation in the leaves of shaded and unshaded plants and the roots of control plants.

After uptake, nitrate can be stored in vacuoles or metabolized by NR in both the plant root and shoot. Subtropical and perennial plants such as *A. millefolium* carry out a substantial proportion of their nitrate assimilation in the shoot, which occurs independently of the external concentration of nitrate (Andrews, 1986). In fact, lower concentrations of nitrate were found in leaves than roots, which may have been due to the relatively higher nitrate reduction activity that occurs within leaves. Moreover, NR requires reducing compounds, such as NAD(P)H, which are derived from photosynthesis. Thus, enzyme activity in leaves is favored, since roots and other subterranean organs must translocate and oxidize carbohydrates to provide the reducing power for NR activity (Guo et al., 2017).

Light is also important for the regulation of NR. It is well known that NR is positively regulated by light at the transcriptional level (Hageman & Flesher, 1960; Creighton et al., 2017). Furthermore, NR activity is inhibited by phosphorylation at a conserved serine residue when plants are transferred from light to darkness (Nemie-Feyissa et al., 2013). Indeed, NR activity is enhanced drastically by light exposure in reproductive-stage yarrow plants; and in the vegetative stage, shaded plants accumulate nitrate in leaves and roots. In addition to light, nitrate also regulates NR activity in plants. However, nitrogen fertilization did not alter NR activity in yarrow leaves at either stage of development. These findings may have been a result of the nitrogen source supplied, which provided more ammonium than nitrate. Ammonium can be converted to nitrate in the soil via the nitrification process, which varies depending on soil microbial activity, precipitation, oxygen availability and pH (Pinton et al., 2016; Hernández-Martínez, Prado, Cayetano-Salazar, Bischoff, & Siebe, 2018). Thus, the nitrate may have been supplied in varying amounts to the plants and in concentrations insufficient to modify NR activity. The increase in NR activity in roots was only observed in vegetative-stage plants treated with 75 kg of urea ha^{-1}. The increase in nitrogen supply in the form of urea (150 kg of urea ha^{-1}) did not increase NR activity, probably because increased levels of ammonium, rather than nitrate, were made available to the plants.

Light positively affected the accumulation of ammonium in the leaves and roots of vegetative-stage yarrow. Metabolizing nitrate to amino acids involves the conversion of nitrate to nitrite by NR, followed by the conversion of nitrite to ammonium by nitrite reductase (Krapp, 2015). As NR is more responsive to light (Creighton et al., 2017), the accumulation of ammonium in unshaded plants may be due to nitrate reduction. Nitrate metabolism to ammonium may also have contributed to the accumulation of ammonium in plants treated with nitrogen since only leaves exposed to full light conditions had increased levels of ammonium when treated with both concentrations of urea. In addition, the ammonium produced within leaves by photorespiration may also have contributed to the values of ammonium observed. On the other hand, in shaded plants, ammonium accumulation differed between leaves and roots. In leaves, the lowest levels of ammonium accumulation occurred in plants treated with 75 kg of urea ha^{-1}, however, in roots the same treatment resulted in enhanced accumulation of ammonium. These effects may have been due to differing availabilities of ammonium, as a result of differences in moisture, pH and microbial activity (Hernández-Martínez et al., 2018). It may also have been a result of the rapid conversion of the molecule since it can cause toxicity in plants when present in excess (Krapp, 2015).

Ammonium is assimilated via the GS/GOGAT cycle (Krapp, 2015). Thus, the observed increases in GS activity appears to have occurred as a positive-feedback response to increased ammonium levels (Ishiyama et al., 2004). Thus, the activity of the enzyme was higher in roots and leaves of plants grown in full light than in plants under shading and matching with the observed increases in ammonium levels. In contrast, plants that were treated with 75 kg of urea ha^{-1} had lower ammonium content and higher GS activity, compared with the other nitrogen treatments. This may be a result of the transient depletion of ammonium since enzyme activity was high at the time of sample harvesting.

The performance of GS together with the GOGAT complex functions to incorporate the ammonium into glutamate, which can be metabolized in other amino acids as needed. The total amino acid content in roots

was approximately 5.0-fold higher than in the leaves. Taken together, these, along with the high levels of GS activity in the leaves observed, suggest that nitrogen metabolism varies between the two organs. Thus, the high content of amino acids in the roots can be attributed to the transfer of the assimilated nitrogen from leaves to roots *via* the phloem. Similar results were observed in *Coffea arabica* (Gonçalves, Gallo, & Favarim, 2007). In this work, shading had no influence on the concentration of total amino acids in leaves or roots of reproductive-stage yarrow. Furthermore, the addition of 75 and 150 kg of urea ha^{-1} decreased amino acid accumulation in roots. The ammonium ion should stimulate amino acid formation, by increasing levels of their precursors. However, the increased availability of nitrogen may have stimulated the production of other nitrogenous compounds, such as proteins and nucleic acids necessary for the growth, flowering and fruiting of the plant, thus reducing the free amino acid content.

At the vegetative stage, increased amino acid content occurred in parallel with nitrogen supply increases. The amino acid content also increased when shaded plants were provided with urea, which may be a characteristic of an adaptive shade. In other words, increased accumulation of amino acids may be associated with decreased utilization, since the low-light condition limits growth. Indeed, yarrow produced decreased quantities of dry matter when they were grown in the shade (Lima et al., 2011). Chen et al. (2017) showed that most enzymes involved in the biosynthesis of amino acids were down-regulated by dark treatment. In addition, the authors suggested that the proteolysis of chloroplast proteins contributed to amino acid accumulation in dark-treated leaves of *Camellia sinensis*. The differences in amino acid content found in plants at vegetative and reproductive stages are probably due to the allocation of nitrogen to reproductive organs in reproductive-stage plants since this phase demands a high level of energy and nutritive investment.

The yields of essential oils from shaded leaves were higher than those yields of unshaded plants. Similar results were observed in *Tetradenia riparia* grown with 50% shading (Araujo, Melo, Paula, Alves, & Portes, 2018). Essential oil production is usually associated with higher irradiance levels, as demonstrated in studies with peppermint (*Mentha piperita*; Costa et al., 2014) and basil (*Ocimum basilicum*; Paulus, Valmorbida, Ferreira, Zorzzi, & Nava, 2016). However, the excess in light can cause photoinhibition in some species of plants, and consequently, decrease essential oil yield (Araujo et al., 2018). This suggests that the effects of shading may be species-specific. On the other hand, the increased essential oil yield observed in flowers of plants fertilized with urea (75 kg ha^{-1}) was attributed to enhanced oil biosynthesis, since there was no observed increase in the biomass of treated flowers (Lima et al., 2011). In basil treated with three concentrations of ammonium nitrate, the highest yield of essential oil was found in plants treated with 300 kg of N ha^{-1}, demonstrating that nitrogen enhances essential oil yield in other medicinal species (Sifola & Barbieri, 2006). In our study, however, the highest concentration of urea (150 kg ha^{-1}) did not enhance oil yield, which indicates that oil yield is not a concentration-dependent parameter.

A MIC concentration of 12.5 mg mL^{-1} essential oil from either leaves or flowers of yarrow produced strong inhibition activity; moderate activity was associated with 25 mg mL-^{1} essential oil. The MIC values of 50, 100 and > 100 mg mL^{-1} were considered weak, very weak and without inhibition activity, respectively (Aligiannis, Kalpoutzakis, Mitaku, & Chinou, 2001; Sartoratto et al., 2004). In addition, for strains of *S. aureus* ATCC 25923, *B. cereus*, *S. aureus,* and *S. epidermidis* the inhibitory activity of essential oils from yarrow leaves was considered strong to moderate. Similarly, the inhibitory activity of yarrow oil was considered strong against *C. albicans* ATCC 18804 and *C. albicans*. On the other hand, the inhibitory activity of leaf oil was considered weak or very weak *E.coli* ATCC 8739, *Salmonella* sp. and *E. coli*. The essential oil of flowers strongly inhibited the bacterial strain *B. cereus*, as well *C. albicans* ATCC 18804 and *C. albicans* fungal strains. Inhibition was considered moderate for *S. aureus*, *E. faecalis* ATCC 19433, *S. aureus* and *S. epidermidis*, and weak to very weak for *E. coli* ATCC 8739, *Salmonella* sp., *E. aerogenes,* and *E. coli* strains.

The *E. faecalis* ATCC 19433 and *E. aerogenes* strains were inhibited by essential oils from flowers, but not leaves; a finding that indicated that only some compounds present in the flowers are effective against these species of microorganisms. The differences in the antimicrobial activity of the essential oils extracted from leaves and flowers of *A. millefolium* can be explained by variability in the chemical compositions of the oils, which contain monoterpenes, sesquiterpenes and phenolic compounds such as sabinene, β-phenyl alcohol, kaempferol, farnesol, caryophyllene, linalool and eugenol (Alvarenga et al., 2015; Mohammadhosseini et al., 2017).

Gram-negative bacterial species are generally most resistant to essential oils than gram-positive organisms, due to the composition of their peptidoglycan lipid bilayer (Semeniuc, Pop, & Rotar, 2017). This

barrier is responsible for restricting diffusion of hydrophobic compounds, prevents their direct exposure to the oil and its bioactive components. Many substances, which are qualitatively and quantitatively different in each oil, have specific capacities to break or penetrate bacterial membranes. In order to determine the mode of action of the oils, it is necessary to evaluate the effects of each active ingredient, both in combination and separately. Only after these analyses are performed will researchers be able to determine whether they have bactericidal or bacteriostatic action individually or in combination with other compounds (Mahmoud, El-Baky, Ahmed, & Gad, 2016).

Conclusion

Full light exposure enhanced nitrogen metabolism in *A. millefolium*. However, the species adapts well to shading being able to produce increased yields of essential oils compared to plants grown under full light. Regarding nitrogen supply, intermediate concentrations of urea (75 kg ha^{-1}) resulted in optimal nitrogen metabolism and essential oil yields. In addition, the antimicrobial activity demonstrated that the essential oil of leaves and flowers of *A. millefolium* is of great interest to the pharmaceutical industry, since knowledge regarding the components of this oil may enhance research facilitating the production of bioactive products that act predominantly against gram-positive bacteria.

Acknowledgements

The authors are thankful to Dr. Maria Antonieta Décio da Costa (*Universidade Católica de Pelotas*, Brazil) for species identification. This study was financed in part by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - Brazil (CAPES) – Finance Code 001, *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul* (FAPERGS).

References

Ali, S. I., Gopalakrishnan, B., & Venkatesalu, V. (2017). Pharmacognosy, phytochemistry and pharmacological properties of Achillea millefolium L.: a review. Phytotherapy Research, 31(8), 1140-1161. doi: 10.1002/ptr.5840

Aligiannis, N., Kalpoutzakis, E., Mitaku, S., & Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two Origanum species. Journal of Agricultural and Food Chemistry, 49(9), 4168-4170. doi: 10.1021/jf001494m

Alvarenga, I. C. A., Pacheco, F. V., Silva, S. T., Bertolucci, S. K. V., & Pinto, J. E. B. P. (2015). In vitro culture of *Achillea millefolium* L.: quality and intensity of light on growth and production of volatiles. *Plant Cell, Tissue and Organ Culture*, *122*(2), 299-308. doi: 10.1007/s11240-015-0766-7

Andrews, M. (1986). The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant, Cell & Environment*, *9*(7), 511-519. doi: 10.1111/1365-3040.ep11616228

Araujo, L. L. N., Melo, H. C., Paula, J. R., Alves, F. R. R., & Portes, T. A. (2018). Yield and composition of the essential oil of *Tetradenia riparia* (Hochst) Codd (Lamiaceae) cultivated under different shading levels. *Planta Daninha*, *36*, 1-9. doi: 10.1590/s0100-83582018360100066

Bieleski, R. L., & Turner, N. A. (1966). Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Analytical Biochemistry*, *17*(2), 278-293. doi: 10.1016/0003-2697(66)90206-5

Cataldo, D. A., Maroon, M., Schrader, L. E., & Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in Soil Science and Plant Analysis*, *6*(1), 71-80. doi: 10.1080/00103627509366547

Chen, Y., Fu, X., Mei, X., Zhou, Y., Cheng, S., Zeng, L., ... Yang, Z. (2017). Proteolysis of chloroplast proteins is responsible for accumulation of free amino acids in dark-treated tea (*Camellia sinensis*) leaves. *Journal of Proteomics*, *157*, 10-17. doi: 10.1016/j.jprot.2017.01.017

Costa, A. G., Chagas, J. H., Bertolucci, S. K. V., & Pinto, J. E. B. (2014). Shading levels and net type on vegetative growth and essential oil production of peppermint. *Horticultura Brasileira*, *32*(2), 194-199. doi: 10.1590/S0102-05362014000200013

Creighton, M. T., Sanmartín, M., Kataya, A. R. A., Averkina, I. O., Heidari, B., Nemie-Feyissa, D., ... Lillo, C. (2017). Light regulation of nitrate reductase by catalytic subunits of protein phosphatase 2A. *Planta*, *246*(4), 701-710. doi: 10.1007/s00425-017-2726-4

Cullimore, J. V., Lara, M., Lea, P. J., & Miflin, B. J. (1983). Purification and properties of two forms of glutamine synthetase from the plant fraction of *Phaseolus* root nodules. *Planta*, *157*(3), 245-253. doi: 10.1007/BF00405189

Forde, B. G., & Cullimore, J. V. (1989). The molecular biology of glutamine synthetase in higher plants. In B. J., Miflin (ed.). *Oxford surveys of plant molecular and cell biology* (p. 246–296). Oxford. UK: Oxford University Press.

Gonçalves, G. C., Gallo, L.A., & Favarim, J. L. (2007). Assimilação do carbono por plantas de cafeeiro (*Coffea arabica* L var. obatã) crescendo a pleno sol e com sombreamento parcial. *Revista de Agricultura*, *82*(1), 35-46.

Guo, Q., Turnbull, M. H., Song, J., Roche, J., Novak, O., Späth, J., ... Love, J. (2017). Depletion of carbohydrate reserves limits nitrate uptake during early regrowth in *Lolium perenne* L. *Journal of Experimental Botany*, *68*(7), 1569-1583. doi: 10.1093/jxb/erx056

Hageman, R. H., & Flesher, D. (1960). Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. *Plant Physiology*, *35*(5), 700-708. doi: 10.1104/pp.35.5.700

Han, M., Okamoto, M., Beatty, P. H., Rothstein, S. J., & Good, A. G. (2015). The genetics of nitrogen use efficiency in crop plants. *Annual Review of Genetics*, *49*, 269-289. doi: 10.1146/annurev-genet-112414-055037

Hernández-Martínez, J. L., Prado, B., Cayetano-Salazar, M., Bischoff, W.A., & Siebe, C. (2018). Ammonium-nitrate dynamics in the critical zone during single irrigation events with untreated sewage effluents. *Journal of Soils and Sediments*, *18*(2), 467-480. doi: 10.1007/s11368-016-1506-2

Ishiyama, K., Inoue, E., Watanabe-Takahashi, A., Obara, M., Yamaya, T., & Takahashi, H. (2004). Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in *Arabidopsis*. The *Journal of Biological Chemistry*, *279*(16), 16598-16605. doi: 10.1074/jbc.M313710200

Klepper, L., Flesher, D., & Hageman, R. H. (1971). Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiology*, *48*(5), 580-590. doi: 10.1104/pp.48.5.580

Krapp, A. (2015). Plant nitrogen assimilation and its regulation: a complex puzzle with missing pieces. *Current Opinion in Plant Biology*, *25*, 115-122. doi: 10.1016/j.pbi.2015.05.010

Lea, P. J., & Morot-Gaudry, J.-F. (2001). *Plant nitrogen*. Berlin, DE: Springer Verlag.

Lima, M. C., Amarante, L., Mariot, M. P., & Serpa, R. (2011). Growth and production of photosynthetic pigments in *Achillea millefolium* L. grown under different levels of shading and nitrogen. *Ciência Rural*, *41*(1), 45-50. doi: 10.1590/S0103-84782011000100008

Mahmoud, A. M., El-Baky, R. M. A., Ahmed, A. B. F., & Gad, G. F. M. (2016). Antibacterial activity of essential oils and in combination with some standard antimicrobials against different pathogens isolated from some clinical specimens. *American Journal of Microbiological Research*, *4*(1), 16-25. doi: 10.12691/ajmr-4-1-2

Martins, E. R., Castro, D. M., Castellani, D. C., & Dias, J. E. (2003). *Plantas medicinais*. Viçosa, MG: UFV.

McCullough, H. (1967). The determination of ammonia in whole blood by a direct colorimetric method. *Clinica Chimica Acta*, *17*(2), 297-304. doi: 10.1016/0009-8981(67)90133-7

Mohammadhosseini, M., Sarker, S. D., & Akbarzadeh, A. (2017). Chemical composition of the essential oils and extracts of *Achillea* species and their biological activities. *Journal of Ethnopharmacology*, *199*, 257-315. doi: 10.1016/j.jep.2017.02.010

Nemie-Feyissa, D., Królicka, A., Førland, N., Hansen, M., Heidari, B., & Lillo, C. (2013). Post-translational control of nitrate reductase activity responding to light and photosynthesis evolved already in the early vascular plants. *Journal of Plant Physiology*, *170*(7), 662-667. doi: 10.1016/j.jplph.2012.12.010

Paulus, D., Valmorbida, R., Ferreira, S. B., Zorzzi, I. C., & Nava, G. A. (2016). Biomass production and essential oil composition of basil grown under shading nets and harvested at different times. *Horticultura Brasileira*, *34*(1), 46-53. doi: 10.1590/S0102-053620160000100007

Pinton, R., Tomasi, N., & Zanin, L. (2016). Molecular and physiological interactions of urea and nitrate uptake in plants. *Plant Signaling & Behavior*, *11*(1), e1076603. doi: 10.1080/15592324.2015.1076603

Ribeiro, A. S., Ribeiro, M. S., Bertolucci, S. K. V., Bittencourt, W. J. M., Carvalho, A. A., Tostes, W. N., ... Pinto, J. E. B. P. (2018). Colored shade nets induced changes in growth, anatomy and essential oil of *Pogostemon cablin*. *Anais da Academia Brasileira de Ciências*, *90*(2), 1823-1835. doi: 10.1590/0001-3765201820170299

Russo, M., & Honermeier, B. (2017). Effect of shading on leaf yield, plant parameters, and essential oil content of lemon balm (*Melissa officinalis* L.). *Journal of Applied Research on Medicinal and Aromatic Plants*, *7*, 27-34. doi: 10.1016/j.jarmap.2017.04.003

Santos, A. S., Alves, S. M., Figueirêdo, F. J. C., & Neto, O. G. R. (2004). *Descrição de sistema e métodos de extração de óleos essenciais e determinação de umidade de biomassa em laboratório*. Belém, PA: MAPA.

Sartoratto, A., Machado, A. L. M., Delarmelina, C., Figueira, G. M., Duarte, M. C. T., & Rehder, V. L. G. (2004). Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Brazilian Journal of Microbiology*, *35*(4), 275-280. doi: 10.1590/S1517-83822004000300001

Statistical Analysis System [SAS]. (2002). *SAS/STAT User's guide, Version 9.1*. Cary, NC: SAS Institute Inc.

Semeniuc, C. A., Pop, C. R., & Rotar, A. M. (2017). Antibacterial activity and interactions of plant essential oil combinations against Gram-positive and Gram-negative bacteria. *Journal of Food and Drug Analysis*, *25*(2), 403-408. doi: 10.1016/j.jfda.2016.06.002

Sifola, M. I., & Barbieri, G. (2006). Growth, yield and essential oil content of three cultivars of basil grown under different levels of nitrogen in the field. *Scientia Horticulturae*, *108*(4), 408-413. doi: 10.1016/j.scienta.2006.02.002

Stagnari, F., Galieni, A., & Pisante, M. (2015). Shading and nitrogen management affect quality, safety and yield of greenhouse-grown leaf lettuce. *Scientia Horticulturae*, *192*, 70-79. doi: 10.1016/j.scienta.2015.05.003

Tavarini, S., Sgherri, C., Ranieri, A. M., & Angelini, L.G. (2015). Effect of nitrogen fertilization and harvest time on steviol glycosides, flavonoid composition, and antioxidant properties in *Stevia rebaudiana* Bertoni. *Journal of Agricultural and Food Chemistry*, *63*(31), 7041-7050. doi: 10.1021/acs.jafc.5b02147

Urlić, B., Jukić-Špika, M., Becker, C., Kläring, H. P., Krumbein, A., Goreta-Ban, S., & Schwarz, D. (2017). Effect of NO_3 and NH_4 concentrations in nutrient solution on yield and nitrate concentration in seasonally grown leaf lettuce. *Acta Agriculturae Scandinavica Section B-Soil and Plant Science*, *67*(8), 748-757. doi: 10.1080/09064710.2017.1347704

Verzár-Petri, G., & Cuong, B. N. (1977). On the quantitative determination of chamazulene and prochamazulenes in essential oils and crude drugs from yarrow (*Achillea sp.*-Compositae). II.: a new colorimetric method of high sensitivity for determination of chamazulene in the essential oils. *Acta Pharmaceutica Hungarica*, *47*(3), 134-141. doi: 10.5505/gida.00710

Witte, C.-P. (2011). Urea metabolism in plants. *Plant Science*, *180*(3), 431-438. doi: 10.1016/j.plantsci.2010.11.010

Yemm, E. W., Cocking, E. C., & Ricketts, R. E. (1955). The determination of amino-acids with ninhydrin. *Analyst*, *80*(948), 209-214. doi: 10.1039/AN9558000209