Macronutrient Omission Changes *Lippia gracilis* Schauer, a Threatened Medicinal Plant, Growth and Volatile Chemical Composition

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**Abstract.** The effect of macronutrient omission on the growth and volatile chemical composition of *Lippia gracilis* was evaluated. The “minus one element” technique was employed by using a complete (Hoagland and Arnon, 1950) nutrient solution and solutions with macronutrient omission for N, P, K, Ca, Mg, and S. Macronutrient deficiency significantly influenced *L. gracilis* growth and volatile chemical composition. Leaf dry weight decreased in order of importance of the macronutrients as follows: Ca = K = N > P > Mg > S. The amount and composition of volatile compounds varied according to macronutrient omission. The major constituents were characterized by p-cymene (ranging from not detected to 43.41%), thymol (3.86% to 7.95%), carvacrol (44.09% to 76.69%), and caryophyllene (0.52% to 6.00%), the contents of which were dependent on the omitted macronutrient. Lack of Ca, Mg, and S increased the contents of cymene and decreased the thymol and carvacrol compared with control. Complete solution and N, P, and K omission retained the same thymol and carvacrol content. In summary, macronutrient availability effectively controlled plant growth and volatile chemical composition of *L. gracilis*.

*Lippia gracilis* Schauer (Verbenaceae), synonymy *Lippia grata* Schauer, is a branched shrub capable of reaching up to 2 m in height. It is a typical plant of the semiarid Brazilian northeast, known locally as Alecrim-de-Tabuleiro. Its leaves are rich in essential oil with significant antimicrobial activity due to the high content of phenolic monoterpenes, such as carvacrol and thymol (Albuquerque et al., 2006). Studies have indicated that the agrochemical potential of essential oils of *L. gracilis*. Santos et al. (2014) reported inhibition of *Xanthomonas campestris pv. viticola*, a causative agent of grapevine canker disease, and Fernandes et al. (2015) showed promising results against the fungus *Monosporascus cannonballus* Polack & Uecker, one of the main diseases in melon.

Mineral nutrition is one of the most important factors in the content and composition of essential oils in aromatic plants (Dudai, 2005). Research on essential oils was recently conducted in *Rosmarinus officinalis* L. (Tounekti et al., 2011), *Lippia origanoides* H.B.K. (Teles et al., 2014), *Melissa officinalis* L. (Yadegari and Shakerian, 2014), *Achillea millefolium* L. (Alvarenga et al., 2015), and *Mentha arvensis* (Carvalho et al., 2016). In *L. gracilis*, it has been reported that a combination of mineral fertilizers and organic manures (Souza et al., 2012), seasonality and hydric deficit (Cruz et al., 2014; Santos et al., 2016), salt stress (Ragagnin et al., 2014), and harvest phase (Bitu et al., 2015) influenced growth, yield, and volatile chemical composition.

Mineral nutrients play an essential role in the growth and development of plants, as well as in numerous physiological processes, including respiration, photosynthesis, and formation of the cell wall. The availability or absence of an element may induce changes in the biosynthetic pathways of plants (Alvarenga et al., 2015). Plants may also manifest symptoms that are characteristic of each nutrient, depending on the species studied and environmental factors (Daflon et al., 2014). The production of plants in a nutrient solution involves the cultivation of a species without the use of soil, using water instead, which transports the macro- and micronutrients to the plants. One technique adopted to identify nutritional requirements of crops is the “minus one element” technique (MOET), which compares individual omission of nutrients with a complete solution. By using MOET, we assessed nutritional requirements of plant species (Munguambe et al., 2017).

There are no reports in the literature on the effect of macronutrient omission on the growth and volatile constituents of *L. gracilis*. The aim of the present study was to evaluate the effects of macronutrient omission using MOET in hydroponic cultivation on the growth and chemical analyses of *L. gracilis*.

**Material and Methods**

Installation of the experiment and preparation of scion. Voucher specimens were deposited in PAMG Herbarium (no. 57859) of the agricultural research agency, Belo Horizonte, state of Minas Gerais, Brazil (EPAMIG). Scions of *L. gracilis* were produced from vegetative propagation by apical cuttings (4.5 cm) collected from mother plants. Cuttings were rooted in commercial substrate Trostrato HA (128-cell Styrofoam seedling trays). At 60 d, plants were transferred to a plastic tray containing 20 L of Hoagland and Arnon solution (1950) at 25% concentration in the first week and 50% in the subsequent week as an adaptation period. From the third week until the end of the experiment, undiluted solution was used.

Treatments consisted of complete nutrient solution (Hoagland and Arnon, 1950) and individual omission of the macronutrients based on MOET. Table 1 shows the chemical composition of nutrient solutions (mL·L⁻¹) used in MOET. Nutrient solutions were prepared with salts (analytical standard), and complete solution concentration was 210.1 mg·L⁻¹ of N; 31 mg·L⁻¹ of P; 234.6 mg·L⁻¹ of K; 200.4 mg·L⁻¹ of Ca; 48.6 mg·L⁻¹ of Mg; 64.2 mg·L⁻¹ of S; 500 μg·L⁻¹ of B; 20 μg·L⁻¹ of Cu; 648 μg·L⁻¹ of Fe; 502 μg·L⁻¹ of Mn; 11 μg·L⁻¹ of Mo, and

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**Abstract.** The effect of macronutrient omission on the growth and volatile chemical composition of *Lippia gracilis* was evaluated. The “minus one element” technique was employed by using a complete (Hoagland and Arnon, 1950) nutrient solution and solutions with macronutrient omission for N, P, K, Ca, Mg, and S. Macronutrient deficiency significantly influenced *L. gracilis* growth and volatile chemical composition. Leaf dry weight decreased in order of importance of the macronutrients as follows: Ca = K = N > P > Mg > S. The amount and composition of volatile compounds varied according to macronutrient omission. The major constituents were characterized by p-cymene (ranging from not detected to 43.41%), thymol (3.86% to 7.95%), carvacrol (44.09% to 76.69%), and caryophyllene (0.52% to 6.00%), the contents of which were dependent on the omitted macronutrient. Lack of Ca, Mg, and S increased the contents of cymene and decreased the thymol and carvacrol compared with control. Complete solution and N, P, and K omission retained the same thymol and carvacrol content. In summary, macronutrient availability effectively controlled plant growth and volatile chemical composition of *L. gracilis*.
The experiment was monitored daily, and the plants were harvested after 60 d; separated into root, shoot, and leaf; and individually dehydrated in an oven with forced ventilation at 40 °C until a constant weight was reached. Dry weights of plant material were measured as dry weights of root (RDW), shoot (SDW), leaf (LDW), and total (TDW) for the plant expressed in grams. The root and shoot ratio (R:S) represents root dry weight (RDW) divided by the shoot dry weight (SDW) and leaf dry weight (LDW).

Volatile fraction analysis through headspace—gas chromatography—mass spectrometry: Chemical analysis by headspace gas chromatography—mass spectrometry (GC-MS) was performed on an Agilent 7890A gas chromatograph coupled with a mass selective detector Agilent 5975C Series MSD (Agilent Technologies, CA), operated by electron impact ionization and equipped with a headspace extractor Combi PAL autosampler system (CTC Analytic AG, Switzerland). Samples consisted of 100 mg of dried L. gracilis leaves, packed in 20-mL vials, sealed with silicone septum/polytetrafluoroethylene, which were incubated individually in the automatic sampler for 60 min. After this period, a volume of 500 μL of the vapor phase was injected in split mode at 50:1 ratio with a gas-tight syringe at a temperature of 110 °C for 60 min. The initial oven temperature was 50 °C, followed by a temperature ramp of 3 °C per min up to 240 °C, followed by a ramp of 10 °C per min up to 280 °C. The constituent concentrations of the chemical analysis were expressed by the percentage of relative area of the total chromatogram of ions ± SD (n = 3).

Chemical constituents were identified by comparing their retention indices relative to the co-injection of a standard solution of n-alkanes (C₈-C₂₀; Sigma-Aldrich, St. Louis, MO) and by comparing mass spectra from the NIST/EPA/NHI library database (National Institute of Standards and Technology, 2008) and of the literature (Adams, 2007). The retention indices were calculated using the method of van den Dool and Kratz (1963), and the literature retention indices were consulted for the assignments (Adams, 2007).

Table 1. Chemical composition of nutrient solutions (mL·L⁻¹) used in hydroponic cultivation of Lippia gracilis under macronutrient omission.

| Stock solution | Complete* | -N | -P | -K | -Ca | -Mg | -S |
|----------------|-----------|----|----|----|-----|-----|----|
| NH₄H₂PO₄       | 1 M       | 1  | 0.1| 0.1| 1   | 1   | 1  |
| KNO₃           | 1 M       | 6  | 0.6| 0.6| 6   | 6   | 6  |
| Ca(NO₃)₂·4H₂O  | 1 M       | 4  | 0.4| 0.4| 0.4 | 4   | 4  |
| MgSO₄·7H₂O     | 1 M       | 2  | 2  | 2  | 2   | 2   | 0.1|
| K₂SO₄          | 0.5 M     | 3  |    |    |     |     |    |
| Ca(H₂PO₄)₂·H₂O | 0.5 M     | 10 |    |    |     |     |    |
| CaSO₄·2H₂O     | 0.01 M    | 200|    |    |     |     |    |
| Mg(NO₃)₂·6H₂O  | 1 M       | 2  |    |    |     |     |    |
| NH₄NO₃         | 1 M       | 3  | 4  |    |     |     |    |
| Micronutrient solution |     | 1  | 1  | 1  | 1   | 1   | 1  |
| Fe-EDTA        |           | 1  | 1  | 1  | 1   | 1   | 1  |
|                |           | 7  | 3  | 4  | 4   | 4   | 4  |
|                |           | 1  | 1  | 1  | 1   | 1   | 1  |

Treatments (mL·L⁻¹)

|                | Complete | -N | -P | -K | -Ca | -Mg | -S |
|----------------|----------|----|----|----|-----|-----|----|
| NH₄H₂PO₄       | 1 M      | 1  | 0.1| 0.1| 1   | 1   | 1  |
| KNO₃           | 1 M      | 6  | 0.6| 0.6| 6   | 6   | 6  |
| Ca(NO₃)₂·4H₂O  | 1 M      | 4  | 0.4| 0.4| 0.4 | 4   | 4  |
| MgSO₄·7H₂O     | 1 M      | 2  | 2  | 2  | 2   | 2   | 0.1|
| K₂SO₄          | 0.5 M    | 3  |    |    |     |     |    |
| Ca(H₂PO₄)₂·H₂O | 0.5 M    | 10 |    |    |     |     |    |
| CaSO₄·2H₂O     | 0.01 M   | 200|    |    |     |     |    |
| Mg(NO₃)₂·6H₂O  | 1 M      | 2  |    |    |     |     |    |
| NH₄NO₃         | 1 M      | 3  | 4  |    |     |     |    |
| Micronutrient solution |    | 1  | 1  | 1  | 1   | 1   | 1  |
| Fe-EDTA        |          | 1  | 1  | 1  | 1   | 1   | 1  |
|                |          | 7  | 3  | 4  | 4   | 4   | 4  |
|                |          | 1  | 1  | 1  | 1   | 1   | 1  |

*Hoagland and Arnon (1950).
7.86 g·L⁻¹ H₂BO₃; 2.43 g·L⁻¹ MnSO₄·H₂O; 0.22 g·L⁻¹ ZnSO₄·7H₂O; 0.08 g·L⁻¹ CuSO₄·5H₂O; 0.02 g·L⁻¹ H₂MoO₄·H₂O.
EDTA = ethylenediaminetetraacetic acid.
**Statistical analysis.** The experimental design was completely randomized with seven treatments and four replications. The experimental unit comprised three plants per pot. Growth data were submitted to analysis of variance and average Tukey test at the 5% probability level. Principal component analysis (PCA) was used to study the major compounds of essential oil in relation to macronutrients omission. Each variable (i.e., percentage of an identified compound of total oil composition) was subtracted by the variable mean; this process ensured that all results would be interpretable in terms of variation from the mean. Statistica software, version 13.3 (StatSoft, Tulsa, OK) was used for the statistical analysis.

**Results and Discussion**

**Lippia gracilis growth parameters.** Omission of macronutrients affected the growth of *L. gracilis* (Fig. 1). Regarding, leaf production (LDW), the order of nutritional limitation was Ca = K = N > P > Mg > S (Fig. 1A). The leaf of this medicinal plant is the main organ of commercial interest, and increased dry weight of leaves is desirable. The macronutrients P, Mg, and S did not limit LDW, perhaps because they were sufficiently absorbed from the adaptation solution, supplying the plant’s initial demand. It was also observed that the omission of P (1.89 g/plant), Mg (2.91 g/plant), and S (5.15 g/plant) resulted in higher LDW production than the complete treatment (0.74 g/plant). This can be attributed to the type and concentration of salts prepared by MOET (Table 1). The nutrient solution used in this experiment presents differences in the provided forms between the complete treatment and nutrient omission treatment. In the complete solution, S is fully supplied as MgSO₄, whereas with omission of S, the magnesium is supplied as Mg(NO₃)₂, resulting in increased availability of N. In the absence of Mg, the MgSO₄ is replaced by K₂SO₄, and with omission of P, NH₄H₂PO₄ by NH₄NO₃. Both modifications resulted in increases in the macronutrients (K and N) that most limited the growth of *L. gracilis*.

Prado and Leal (2006) reported that *Helianthus annuus* growth in hydroponic cultivation was not limited by the omission of S. These authors attributed the uptake of S before transplanting to the nutrient solution, and particularly to the variety used, which showed low susceptibility to sulfur deficiency, possibly due to greater efficiency in the use of the nutrient. According to Vale et al. (2011), the omission of Ca, Mg, and S in a nutrient solution did not limit initial growth and production of the dry matter mass of *Saccharum* spp. due to the low extraction of these macronutrients by the crop and because the reserves in the stalks are sufficient for growth. Treatment with the omission of calcium resulted in better growth of *Brachia tria brizantha* even compared with the complete solution (Monteiro et al., 1995). The authors attributed this to the differences in N forms provided in the nutrient solution between the complete solution and the Ca- or S- omission solution. In the complete treatment, N was totally supplied as nitrate (NO₃⁻), whereas in treatment with Ca omitted, N is partly supplied as ammonium (NH₄⁺).

The highest (5.86 g/plant) and lowest (0.26 g/plant) SDW were observed with a deficiency of S and Ca, respectively (Fig. 1B). Lower RDW was also observed with the omission of Ca (0.27 g/plant) and N (0.49 g/plant) (Fig. 1C). Calcium plays an important role in plant growth and development, particularly with regard to cell wall formation and in the meristem regions (Veigas et al., 2013). Nitrogen is the nutrient most required by plants and is present in several compounds that are essential for plant growth and development, as well as the biosynthesis of amino acids, proteins, and enzymes; it is present in the structure of nucleic acids and proteins (Freitas et al., 2011). Phosphorus omission promoted higher RDW (3.40 g/plant) compared with the complete treatment (1.69 g/plant). According to Gram et al. (2001), plants demonstrate adaptation mechanisms to improve their access to P under deficient conditions of this element; one such mechanism is an increased root system, whereby lateral roots with abundant root hairs quickly develop. Therefore, *L. gracilis* appears to be nondemanding with respect to P, possibly because it is a typical plant of the semiarid Brazilian northeast, a region characterized by irregular rainfall distribution, shallow and rocky soils, and low P availability. It can be observed from the root-to-shoot ratio (R:S) that omission of P produced more RDW than SDW due to greater photosynthetic translocation from leaves to root (Fig. 1E).

The limiting order for total production (TDW) was Ca = K = N > P = Mg > S (Fig. 1D). The omission of N and Ca were also limiting for TDW for *Achillea hispidinervum* C. DC. (Veigas et al., 2013). The growth of *L. gracilis* under S omission increased the TDW by 75% compared with the complete treatment. Thus, analyzing biomass accumulation parameters indicated that...
N, P, and Ca were more limiting for the growth of *L. gracilis*, and omission of S promoted larger growth than did the complete solution.

*Lippia gracilis* visual symptoms. Visual symptoms of macronutrient deficiency in *L. gracilis* were observed during the 60-d cultivation. Calcium deficiency caused poor formation in young leaves, curving apices, and marginal chlorosis that evolved into necrosis, causing the leaf to die from the margin to the center. Mascarenhas et al. (2013) reported similar symptoms in soybean plants, highlighting the importance of this nutrient in the process of cell division, as C deficiency can cause gradual death of apices.

The omission of N and P caused chlorosis in old leaves, evolving to necrosis and falling leaves. Nitrogen-deficient plants readily redistribute the micronutrient via phloem, exhibiting yellow staining in older leaf parts (Malavolta, 2006), as observed in the present study. According to Malavolta, symptoms of N deficiency, such as light green staining are associated with lower chlorophyll production. With the omission of P, dark-green staining and reduced size of older leaves were observed. Potassium is a major component of essential structural molecules such as nucleic acids, proteins, lipids, sugars, and adenylate, and it is an important factor in the regulation of various enzymatic activities and energy transformation due to adenosine triphosphate molecules that contain P (Zhang et al., 2014).

Magnesium deficiency caused chlorosis followed by tanning in old leaves and inter-nerval chlorosis. This micronutrient plays a role in enzyme activation on the respiration process, photosynthesis, and synthesis of DNA and RNA; it is also a phosphorus carrier and generates magnesium porphyrins, more commonly known as chlorophylls (Malavolta, 2006). The omission of sulfur demonstrated visual symptoms at the end of the experimental period, including shrinkage of new leaves. According to Malavolta (2006), the symptomatology of sulfur deficiency results from the nutrient being absorbed by plants in the form SO₄⁻² and transported from the base of the plant upward in an acropetal direction with little mobility, being therefore observed first in younger organs.

*Volatile fraction analysis through headspace GC-MS.* Analysis by headspace GC-MS of *L. gracilis* leaves indicated quantitative and qualitative chemical differences (Table 2). In total, 90.55 to 97.22 constituents were identified. Qualitative chemical variations can be clearly observed through the total number of chemical constituents present in the samples, which ranged from 14 to 22 constituents. Plants cultivated with nitrogen and calcium omission, which had earlier visual symptoms and lower accumulations of biomass, had the least complex chemical profiles, with 16 and 14 constituents, respectively. On the other hand, plants cultivated with S omission, which showed the later visual symptoms and larger accumulations of shoot and total biomass, had the most complex chemical profile (22 chemical constituents identified), which was higher than the control treatment (19 constituents).

The chemical profile was quite different between treatments that omitted N compared with Ca. The constituents α-pinene, α-terpinene, p-cymene, limonene, γ-terpinene, and carvacrol acetate were present in the volatile chemical composition of plants cultivated with calcium omission and absent in plants cultivated with nitrogen omission. Whereas, reciprocally, *cis*- and *trans*-sabinene hydrates, linalool, ipsdieol, etc., were not detected in plants grown under nitrogen omission.

![Fig. 2. Principal component analysis of the averages of major compounds of the essential oil of *Lippia gracilis* grown under macronutrients omission.](image-url)
According to reports in the literature, in 76.69% and thymol (6.24% to 7.95%) concentrations were lower than 5.38%, whereas the omission of N, P, and K were (0.52 to 6.00). It can be observed that the volatile fraction of plants cultivated with nitro- gen omission and the omission of N, P, and K were significantly different from those with complete nutrient solution. In chemical analyses of volatile compounds, the plants cultivated in the complete solution and with omissions of N, P, and K had greater concentration of thymol, carvacrol, and E-carvophyllene. These results suggest that macronutrients affect the biosynthesis pathway flux or the specific path of monoterpene metabolism in L. gracilis. Our findings show that the content of volatile compounds can be significantly changed in plants using MOET in hydroponic cultivation. Furthermore, it is possible to induce biosynthesis of a compound of interest for commercial production in field-grown L. gracilis plants with supplementation or omission of macronutrients.

**Conclusion**

Vegetative growth and volatile chemical composition of L. gracilis are changed as a function of macronutrients, allowing for proper management of nutritional requirements and thus the accumulation of biomass to the desired contents of volatile constitu- ents. The DLW decreased in order of impor- tance of the macronutrients as follows: Ca = K = N > P > Mg > S. Nitrogen, potassium, and calcium were more limiting for the growth of L. gracilis, and sulphur omission promoted more growth than did the complete solution. In chemical analyses of volatile compounds, carvacrol was the major chemical constituent identified in all treatments. However, significant decreases in contents of this component were observed in treatments in which C, Mg, and S were omitted.

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