

Effects of Nitrogen and Calcium Carbonate on Growth, Rosmarinic Acid Content and Yield of *Satureja hortensis* L.

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Abstract

Because of the presence of free phenolic acids, especially rosmarinic acid, extracts of *Satureja hortensis* L. possess antioxidant and antimicrobial activity. The production of rosmarinic acid by plant cells can be modified by many factors. In the present study, the changes induced by calcium carbonate and nitrogen application on rosmarinic acid content, plant growth, and yield of *S. hortensis* cv. Saturn were investigated. Nitrogen fertilization significantly increased plant height, fresh and dry weights of herb as well as rosmarinic acid content and yield. CaCO_3 treatments did not affect rosmarinic acid content or yield, while the fresh and dry weights of herb were increased by applying $5 \text{ t ha}^{-1} \text{ CaCO}_3$.

Keywords: *Satureja hortensis*, Rosmarinic acid, Nitrogen, Calcium carbonate

1. Introduction

Satureja hortensis L. (family Lamiaceae and subfamily Nepetoideae) is a well-known medicinal and spice plant cultivated in several areas of the world (Hadian *et al.*, 2008). The aerial parts of this plant are frequently used as tea or additive in commercial spice mixtures for many foods to offer aroma and flavor (Sahin *et al.*, 2003). *S. hortensis* has also been used as folk remedy to treat various ailments such as cramps, muscle pains, nausea, indigestion, diarrhea and infectious diseases (Zargari, 1970; Sahin *et al.*, 2003).

The extracts of *S. hortensis* contain flavonoids and caffeic acid derivatives among them rosmarinic acid is present in higher concentration and it is mainly responsible for biological activity of the extracts (Madsen *et al.*, 1996). Rosmarinic acid (RA) is an ester of caffeic acid and 3,4 dihydroxyphenyllactic acid. It is commonly found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae (Petersen and Simmonds, 2003). RA possesses a multitude of biological activities, e.g. antiviral, antibacterial, anti-inflammatory and antioxidant, which makes it a valuable product for the pharmaceutical and cosmetic industries (Li *et al.*, 2005). The presence of RA in medicinal plants, herbs and spices has beneficial and health promoting effects. In plants, RA is supposed to act as a performed constitutively accumulated defense compound (Petersen and Simmonds, 2003).

It is a common understanding that secondary metabolites production of medicinal plants is influenced by the genetics and cultivation conditions such as climate, plant density and use of fertilizers (Baranauskiene *et al.*, 2003). Nitrogen is one of the most important nutrients for crop production and influences both the amount and quality of plant secondary metabolites (Sifola and Barbieri, 2006). Also, plants need calcium to continue normal function of their membranes, to growth of the meristematic tissues and the youngest leaves and to send signals in response to internal and external cues (Dordas, 2009). The effects of nitrogen fertilizer on growth and secondary

metabolites content and yield of different plants have been shown in many studies (Ayanoglu *et al.*, 2002; Ashraf *et al.*, 2005; Sifola and Barbieri, 2006; Ozguven *et al.*, 2008; and Strissel *et al.*, 2005). On the other hand, many researchers have studied the effects of Ca on plant growth and production of secondary metabolites of several medicinal plants (Lee and Yang, 2005; Supanjani *et al.*, 2005; Karaivazoglu *et al.*, 2007; and Dordas, 2009).

There is no information available in the literature on the calcium and nitrogen effects on the RA production of *S. hortensis*. Thus, the present study aimed to investigate the influences of nitrogen and calcium carbonate application rates on plant growth, rosmarinic acid content and yield of *S. hortensis* cv. Saturn.

2. Materials and Methods

2.1 Field experiment

A field experiment was conducted in 2008 at the experimental garden of the Research Center of Horticultural Sciences Department, University of Tehran, Karaj, Iran (altitude 1320 m, longitude 51° E, latitude 35° 48' N). The average day and night temperatures during experiment were 26.9 and 20.7° C, respectively. At the beginning of the study, some physico-chemical characteristics of the soil at the depth of 30 cm were analyzed (Table 1). The seeds of *S. hortensis* cv. Saturn, a bred cultivar with high rosmarinic acid content, were sown in the greenhouse on July 5, 2008 in small pots containing sand, clay and humus (1: 1: 1). After four weeks, the plantlets with 7-10 cm height were transplanted to the field at a density of 6.35 plants m⁻² (0.45 × 0.35 m distance). The length and width of each plot were 2.1 and 1.8 m, respectively (total area = 3.78 m²). A factorial experiment based on the randomized complete block design with three replications was used. N fertilizer was applied at different levels of 0 (unfertilized), 50, 100 and 150 Kg N ha⁻¹, as NH₄(NO₃). These fertilizers were divided into two equal doses and added at first and fifth weeks after transplanting. A few months prior to transplanting, CaCO₃ treatment was carried out as slaked lime at different levels of 0 (control), 5 and 10 t ha⁻¹ by mixing properly with soil (up to 30 cm depth). During the growing period, the plants were weekly irrigated and kept free of weeds by hand hoeing.

2.2 Sampling and traits analysis

Eight plants were sampled from the inner part of each plot on October 12th (71 days after transplanting) at full bloom. Plant height and fresh weight of the herb were measured for each sample. Plants were then dried in the shade and dry weight was recorded.

2.3 Preparation of crude extract

The aerial parts of plants were grounded to fine powder out of which 400 mg was suspended in 10 ml of MeOH and mixed vigorously. Extraction was performed by sonicating the sample for 30 min (Power Sonic 450, Hwasing, Korea). The extract was then filtered, and stored at 4° C until analysis.

2.4 Chromatographic condition

2.4.1 Instrumentation

Camag TLC (Thin Layer Chromatography) system composed of Camag linomat 5 autosampler, TLC scanner 3, and Wincats 1.2.2 software (CAMAG, Muttens, Switzerland) was used. Samples applied in 6-mm length at 10 mm intervals under N₂ stream. The experiment was performed on TLC aluminum sheets silica gel 60 F254 plates (10 × 10 cm), which was developed to a distance of 85 mm in a CAMAG® twin trough chamber previously equilibrated with the mobile phase: acetone-toluene-formic acid (4:5:1) for RA. The developed TLC plate was dried using a hair dryer for 5 min with cool air. Position of the first track was 15 mm. Vertical development to a distance of 85 mm was performed on 10 × 10 cm CAMAG Twin Trough Chamber. Quantitative evolution of the plate was performed in absorption-reflection mode at 329 nm for RA, slit width of 6 × 0.45 mm and data resolution 100 µm step and scanning speed 20 mm. Quantification of RA in the extract of *S. hortensis* was performed by external standard method, using pure compound. RA appeared as brightly yellow spot at R_f ~ 0.42.

2.4.2 Calibration curve for rosmarinic acid

Stock solutions of RA (100 µg mL⁻¹) were prepared in methanol and different amounts (50 to 1000 ng spot⁻¹ for RA) were applied on a TLC plate, using Linomat V for preparing six point calibration graph of peak area versus concentration.

2.5 Data analysis

Data of all measured parameters were subjected to variance analysis using MSTAT-C statistical software. When the analysis of variance showed significant treatment effects, Duncan's multiple range test was used to compare treatment means at a probability level of 0.05.

3. Results

3.1 Growth and yield parameters

Results of the study showed that nitrogen fertilization up to 100 Kg ha⁻¹ significantly increased plant height of *S. hortensis* (37.64 ± 0.66 cm), but at 150 Kg ha⁻¹ it was slightly decreased, but statistically not significant (Fig 1). On the other hand, the effect of CaCO₃ treatments on the plant height was not significant.

Nitrogen fertilization significantly affected fresh weight of herb as well as dry weight. Fresh weight was greater in the plants fertilized with 100 Kg ha⁻¹ (198.0 ± 6.8 g plant⁻¹) than the control (171.1 ± 6.4 g plant⁻¹), while no further increase was observed in plots fertilized with 150 Kg ha⁻¹ (Fig. 2). Dry weight was also significantly increased by nitrogen fertilization as it was highest (48.53 ± 1.63 g plant⁻¹) at 150 kg ha⁻¹ (Fig. 3). There was a significant effect of CaCO₃ treatment on fresh and dry weights of the herb. The highest plant fresh and dry weights (200.2 ± 6.4 and 46.7 ± 1.7 g plant⁻¹, respectively) was obtained from adding 5 t ha⁻¹ CaCO₃ (Fig's. 4 & 5). Furthermore, the interaction between CaCO₃ and N treatments was not significant for any trait.

3.2 Rosmarinic acid content and yield

TLC analysis of RA content of *S. hortensis* methanolic extract showed that nitrogen fertilization had a significant effect on RA content. The highest RA content (1.15 ± 0.07 mg g⁻² DW) was obtained from the treatment of 150 kg ha⁻¹ (Fig. 6). Rosmarinic acid yield was also significantly increased by nitrogen fertilization and it was highest (352 ± 19.7 mg m⁻²) at 150 kg ha⁻¹ (Fig. 7). But CaCO₃ application had no effect on RA content and yield of methanolic extract. Interaction between nitrogen and CaCO₃ application was not significant for both RA content and yield.

4. Discussion

As was mentioned above, plant height and fresh and dry weights of herb were significantly increased by nitrogen fertilization up to 100 kg ha⁻¹, while no further increase was observed in plots fertilized with 150 Kg ha⁻¹. This agreed with the results of Ram *et al.* (1995), Ayanoglu *et al.* (2002), Baranauskiene *et al.* (2003) and Ashraf *et al.* (2005). Up to 75% of leaf N content is found in chloroplasts mainly as a part of ribulose biphosphate carboxylase (Rubisco) (Dordas and Sioulas, 2008). When there was adequate N supply in the soil, photosynthesis rate increase and enabled the plant to grow rapidly and produce considerable biomass. In addition, the application of nitrogen fertilizer increased the uptake and accumulation of other nutrients such as phosphorus (P) and potassium (K) (Baranauskiene *et al.*, 2003). Hence, nitrogen plays an important role in vegetative growth and basic metabolism of plant, which might be directly or indirectly involved in production of secondary metabolites (Baricevic and Zupancic, 2002). Under nitrogen deficiency, due to the reduction of chlorophyll content and Rubisco activity, growth and development is inhibited and plant yield decrease (Dordas and Sioulas, 2008). High nitrogen fertilization may also causes a decrease in activities of phosphoenolpyruvate carboxylase (PEP) and Rubisco thereby reducing the photosynthesis rate, plant growth and accumulation of secondary products (Ashraf *et al.*, 2006), although in the present study no decrease in plant growth was observed with high nitrogen fertilization (150 kg ha⁻¹). Moreover, nitrogen fertilization significantly increased RA content and yield of extract. It is well known that biosynthesis of RA starts with the amino acids L-phenylalanine and L-tyrosine. Also, eight enzymes are involved in the RA biosynthesis (Petersen and Simmonds, 2003). Since N is present in the structure of amino acids and enzymes, therefore increase in RA content by nitrogen fertilization could be explained by the increase in biosynthesis of RA precursors (L-phenylalanine and L-tyrosine) and catalyze enzymes. Coronado *et al.* (1995) reported that root flavonoid and isoflavonoid production of *Medicago sativa* L. increased under nitrogen limiting conditions due to the enhancement of expression of the flavonoid biosynthesis genes. Ozguven *et al.* (2008) showed that nitrogen fertilizer had a significant effect on artemisinin content of *Artemisia annua* and maximum of artemisinin was obtained at 80 kg ha⁻¹. Also, the increase in RA yield by enhanced level of nitrogen depends on an increase in both plant biomass and RA content.

Concerning the effect of CaCO₃ application, plant growth and performance were increased with the application of CaCO₃ up to 5 t ha⁻¹, while further increase in CaCO₃ reduced the growth. Similar result was reported by Supanjani *et al.* (2005), who mentioned that plant growth and yield of *Chrysanthemum coronarium* L. was increased by applying of CaCO₃ up to 2 t ha⁻¹ and more amounts decreased yield. Also Lee and Yang (2005) reported that an increase in plant growth and yield of *Ch. boreale* M. was observed with increasing CaCO₃ levels up to 1.5 t ha⁻¹. Since *S. hortensis* L. is characterized as calcicole species and in natural habitats it is mainly found in calcareous soils (Ghahraman, 1996), this plant responded to higher levels of soil CaCO₃ content more positively than other species. Moreover, Quaggio *et al.* (2004) mentioned that the effect of CaCO₃ treatment on peanut yield depends on many factors including the initial Ca and Mo status of the soil, time of CaCO₃ application and the peanut cultivar. The increase in plant growth and yield at 5 t ha⁻¹ CaCO₃ could be explained

by several reasons including enhancement of exchangeable Ca and Mg, increase of available Mo, decrease of Al, Mn and heavy metals toxicity due to increase of pH and improvement of soil characteristics (increase of pore volume and optimum moisture content) (Quaggio *et al.*, 2004; Bakker *et al.*, 2005; Rajasekaran, 2005). It seems that the decrease in growth by application of 10 t ha⁻¹ CaCO₃ could be related to excessive Ca content of soil solution, which causes precipitation of P, S and Zn (Wenming, 2001; Bakker *et al.*, 2005; Rajasekaran, 2005). Although the effect of CaCO₃ on other secondary metabolites has been shown in several studies (Lee and Yang, 2005 and Supanjani *et al.*, 2005), RA content and yield were not significantly affected by CaCO₃ treatment in the present study.

5. Conclusion

From the above-mentioned results, it could be concluded that nitrogen fertilizer has significant effect, not only on plant growth, but also on RA production. Since the commercial value of *S. hortensis* depends on the amount of phenolic acids mainly RA, the increase in RA yield induced by N fertilization has positive effects. Furthermore, CaCO₃ application significantly increased plant growth, but had no significant effect on RA production of *S. hortensis*. Finally, application of 150 kg N ha⁻¹ without CaCO₃ was identified as the best treatment for the production of RA.

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Table 1. Some physico-chemical properties of the soil of experimental field

Soil property	content
Total N (%)	0.09
Available K (ppm)	696
Available P (ppm)	104
Ca (meq lit ⁻¹)	20
pH	6.4
EC(ds m ⁻¹)	1.26
Organic C (%)	0.93
Organic matter (g Kg ⁻¹)	6.25
Textural class	Clay loamy
Active lime (%)	5.1

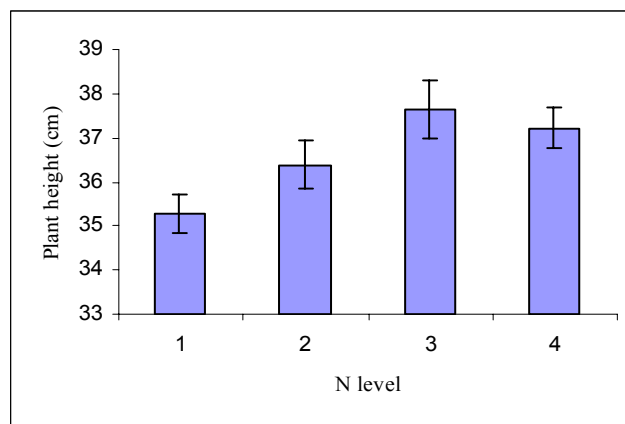


Figure 1. The effect of N fertilizer levels on plant height of *S. hortensis* cv. Saturn

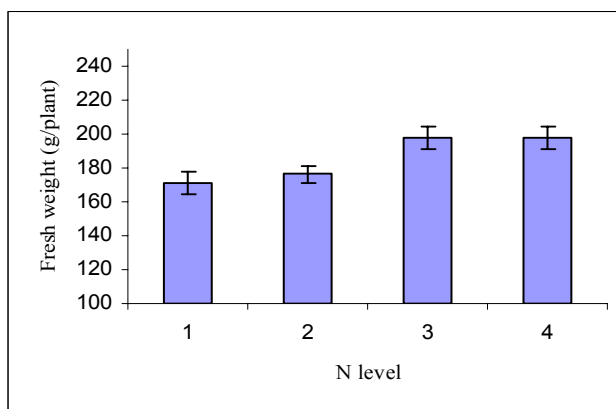


Figure 2. The effect of N fertilizer levels on fresh weight of *S. hortensis* cv. Saturn

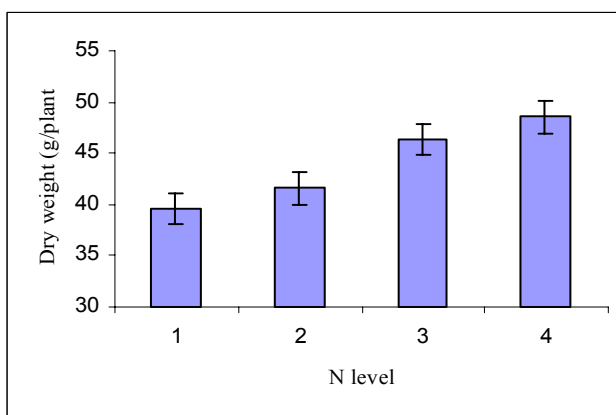


Figure 3. The effect of N fertilizer levels on dry weight of *S. hortensis* cv. Saturn

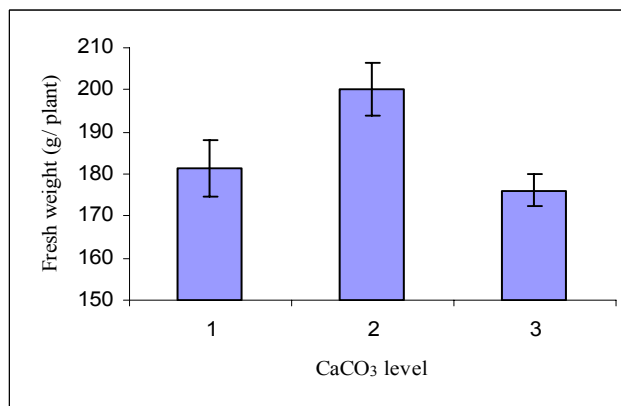


Figure 4. The effect of CaCO₃ levels on fresh weight of *S. hortensis* cv. Saturn

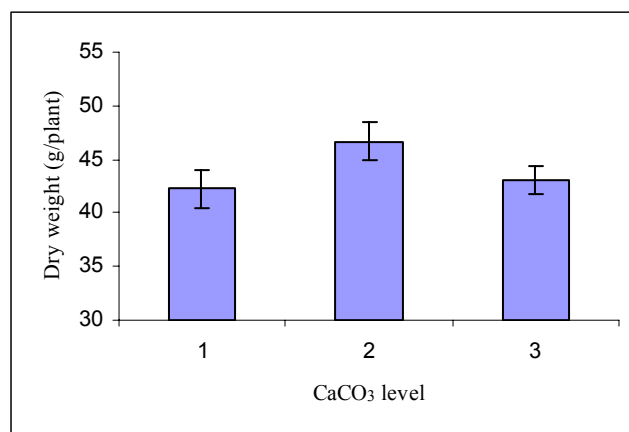


Figure 5. The effect of CaCO₃ levels on dry weight of *S. hortensis* cv. Saturn

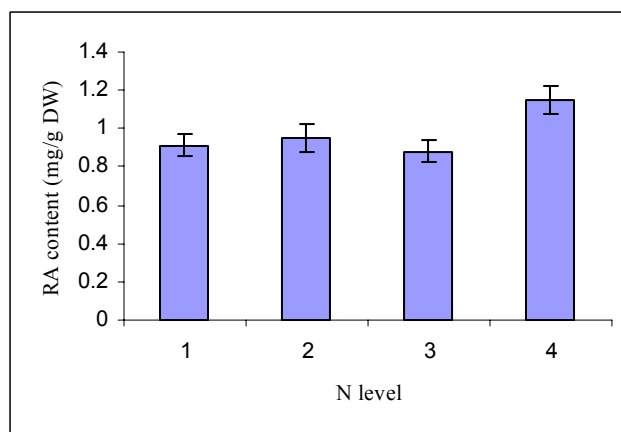


Figure 6. The effect of N fertilizer levels on RA content of methanolic extract of *S. hortensis* cv. Saturn

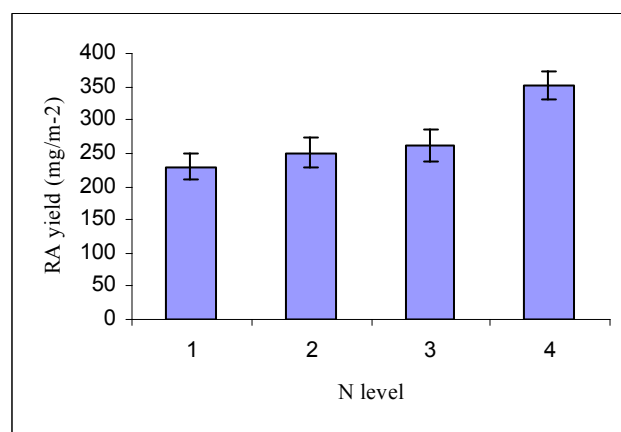


Figure 7. The effect of N fertilizer levels on RA yield of *S. hortensis* cv. Saturn