

Calcium fertilization effects on hyoscyamine and scopolamine accumulation in henbane (*Hyoscyamus niger* L.) under hydroponic culture

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ABSTRACT

Tropane alkaloids, hyoscyamine and scopolamine, are well-known secondary metabolites produced by many solanaceous species such as *hyoscyamus niger*. These compounds are widely used for scopolamine hydrobromide and hyoscyamine sulfate tablets production as anticholinergic, antispasmodic and sedative agents. The objective of current study was to investigate the effects of various calcium concentrations (0, 16.6, 33.3 and 50 mg/L) on black henbane roots and leaves dry weight and their alkaloids content under hydroponic culture. Results showed that the plants root dry weight increased with increasing the amount of calcium fertilization from 0 to 33.3 mg Ca/L, but decreased at 50 mg Ca/L. However, the total foliage dry weight was recorded at the lowest calcium level. Results also revealed that the maximum content of hyoscyamine and scopolamine in leaf was observed in 50 mg Ca/L, while the highest content of scopolamine and hyoscyamine were found in control and in 33.3 mg Ca/L levels, respectively. It seems that a calcium fertilizer is essential nutrient elements necessary for henbane growth and metabolism.

Keywords: *Hyoscyamus niger*, Hyoscyamine, Scopolamine, Calcium, Hydroponic.

INTRODUCTION

Plant alkaloids constitute the largest groups of natural products, providing many pharmacologically active compounds. Tropane alkaloids such as hyoscyamine and scopolamine are among the most valuable drugs in medicine due to its higher pharmacological activities and fewer side effects, which are produced by many solanaceous species such as *hyoscyamus niger* [3]. These secondary metabolites are used in medicine as anticholinergic, antispasmodic, hypnotic, and sedative [10]. Hyoscyamine is used in the treatment of gastrointestinal disorders and Parkinson's disease, while scopolamine is employed against naupathia [9]. Although, plant heredity controls the biosynthesis of alkaloids, some environmental and agrotechnical parameters (e.g., biological and abiological factors and nutrition) could enhance or inhibit tropane alkaloids production [4]. It is reported that mineral nutrition is one of the most obvious environmental factors to manipulate. In fact, a secondary metabolite pathway depends on numerous factors but nutritional status leads to major effects, major minerals such as nitrates, calcium, potassium, sulfur and also carbon hydrates, are among the most essential ingredients of the nutrient medium known to affect the growth and metabolism, such as alkaloids. [1] stated that the availability of essential nutrient elements necessary for *Datura* growth and metabolism cause vigorous vegetation and high chemical production. Also, [7] reported that the fertilization of medicinal plants causes an increase in the yield of bioactive compounds. Data on the effect of the ion-balance on yield and alkaloid content in *Datura stramonium* were showed differences in alkaloid accumulation in the upper vegetative plant parts when potassium or calcium were the dominant cations within the interionic balance of the six major elements [2]. It is well established that that cell wall

strength and thickness were increased by calcium addition. Calcium is one the most important essential nutrient element in plants, which affects all levels of plant function from metabolism to resource allocation, growth and development. However, not much information is available on the effect of calcium fertilization on the content of leaf and root alkaloids in *Hyoscyamus niger* plants under hydroponic culture conditions. Therefore, purpose of the present study was to investigate the effect of various concentrations of calcium fertilizer on black henbane root and leaf dry weight and their alkaloids content under hydroponic culture.

MATERIALS AND METHODS

2.1. Seed preparation and germination

Generally, black henbane seeds have little germination rate even under normal laboratory conditions. Thus, seeds were treated with gibberellic acid (GA₃, 250 mg/L) for 48 h at room temperature (22±2 °C). Then, seeds were surface-sterilized with 70% ethanol for 2 min and then in 25% commercial bleach for 10 min and finally rinsed three times with sterile distilled water. Subsequently, seeds were placed in petri dishes on two layers of filter paper moistened with 4 ml distilled water. After three days, seeds were germinated (with 1-2 mm radicle length). After two weeks later, seedling (with double leaves) were planted in plastic pots and irrigated immediately for better establishment. During the whole experiment period, they received 16-h light/8-h dark (day/night light cycle) under greenhouse conditions. The plants were cultivated on cocopeat and perlite.

2.2. Calcium treatments application

Calcium was applied at the concentrations of 0 (control), 16.6, 33.3, 50 mg/L. The plants were fertilized six times with six equal portions of the proposed fertilizer dose in once every five days intervals, starting after 40 days from germination time. The experiment was arranged in a randomized complete block design with six replications (n=6). Also, calculation of the weight of chemical nutrients used to prepare stock solution under open hydroponic system is given in table 1. During the experimental period, all normal agricultural practices were performed (no pesticide or herbicide was applied to the plants). After 4 month, the plants were harvested and their parts were separated into leaf and root, and then dried at ambient temperature in the shad. Thereafter, root and leaf dry weight and alkaloids content were evaluated.

Table 1: Stock solution

Chemical materials	mg/L
P	40
K	50
Fe	250
Zn	200
Cu	150
Mg	100
Br	5
Mo	5

2.3. Alkaloids extraction

After harvesting, plant samples (root and shoot) were air dried, grinded into fine powder and sieved with laboratory mesh (size 30, opening 545 µm). Then, two grams of each samples was added to appropriate volume of CHCl₃: MeOH: NH₄OH 25%, (15:5:1), sonicated for 10 min. Alkaloids extraction were based essentially on the procedure described by [6] as following:

- Solution was kept at room temperature (22±2 °C) for 1 hour.
- Then filtered through paper and washed two times with 1 ml CHCl₃ and then evaporated
- CHCl₃ (5 ml) and NH₂SO₄ (2 ml) was added and then mixed thoroughly.
- CHCl₃ fraction was removed and adjusted to pH 10 with 28% NH₄OH on ice.
- Alkaloids were extracted once with 2 ml CHCl₃ and twice with 1ml CHCl₃.
- Extract was filtered after the addition of anhydrous Na₂SO₄ and washed the residue with 1-2 ml CHCl₃.
- Samples were evaporated and dissolved in 1-2 ml MeOH.

2.4. Determination of tropane alkaloids

Alkaloids extracted were identified by gas chromatography (GC) analysis. The chromatographic column for the analysis was a Chrompack WC OT-Fused Silica CP-Sil 5CB capillary column (30 m × 0.25 mm I.D., film thickness 0.25 µm). The carrier gas used was helium at a flow rate of 1 mL/min. Then 1-µL crude alkaloid fractions were injected and analyzed with the column held initially at 125 °C for 1 min and then increased to 250 °C with a 10 °C /min heating ramp and subsequently kept at 250 °C for 5 min. The injection was performed in split less mode at 280

°C. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas. Hyoscyamine and scopolamine were measured according to [8].

2.5. Statistical analysis

Data were processed by the analysis of variance (ANOVA) on the basis of completely randomized design (CRD) with 3 replications. The data were analyzed using computer SAS software (version 9.1; CoHort Software), and the means were compared by Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

3.1. Leaf and root dry weight

The effect of different calcium fertilization doses on leaf and root dry weight of henbane plants were shown in figure 1 and 2. Results revealed that calcium treatments had significant ($P < 0.05$) influence on henbane leaf dry weight. The lowest (1.4 gr) and the highest (1.9 gr) leaf dry weight were observed at the control and 16.6 mg/L calcium treatment, respectively (Fig1). The results also showed that calcium treatments significantly ($P < 0.05$) influenced plant root dry weight. The minimum (1.2 gr) and maximum (1.7 gr) root dry weight were obtained at the 50 and 13.3 mg/L calcium concentration, respectively (Fig 2).

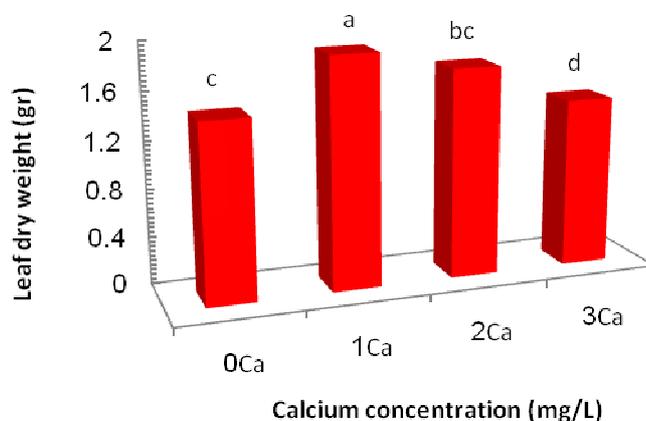


Fig. 1. Effect of calcium fertilization on henbane leaf dry weight (n=6). Values with different letter(s) were significantly different at $P < 0.05$ (Duncan test). Ca0, Ca1, Ca2 and Ca3 refer to 0, 16.6, 33.3 and 50 mg/L calcium, respectively

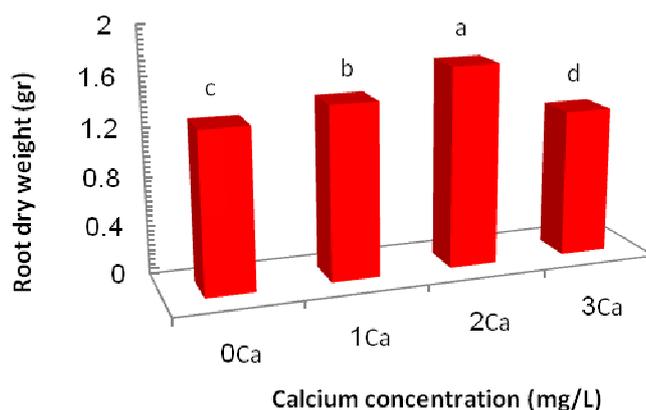


Fig.2. Effect of calcium fertilization on henbane root dry weight (n=6). Values with different letter(s) were significantly different at $P < 0.05$ (Duncan test). Ca0, Ca1, Ca2 and Ca3 refer to 0, 16.6, 33.3 and 50 mg/L calcium, respectively

It is reported that calcium is directly essential for root growth [8]. According to [1] the excess amount of fertilizers caused burning and death of the root hairs effecting negatively the root growth by inhibiting the elongation and enlargement of roots in the soil as they became weak, short and fluffy. Consequently, the burning and death of root hairs resulting from high calcium fertilizer supply led to a lower effectiveness of calcium absorption that causes a decrease in the yield of henbane. Results recorded in this study confirm these findings since, the highest dose of calcium fertilization caused substantial reductions in the dry weight of leaves and roots.

3.2. Hyoscyamine and scopolamine production

The influences of various calcium concentrations of leaf and root scopolamine content are given in fig 3 and 4. Calcium treatments had significant ($P < 0.05$) effect on scopolamine content of both root and leaf parts. The scopolamine content increased by increasing calcium rate of application and reached a maximum values at 50 mg/L. Scopolamine content at four various calcium treatments (0, 16.6, 33.3 and 50 mg/l) comprised 8.2, 11.25, 11.5 and 13 gr/gr $\times 10^{-2}$ in terms of leaf dry weight, respectively. Also, scopolamine content under different employed four calcium treatments comprised 4.1, 4.7, 5.2 and 6 gr/gr $\times 10^{-2}$ in terms of root dry weight, respectively.

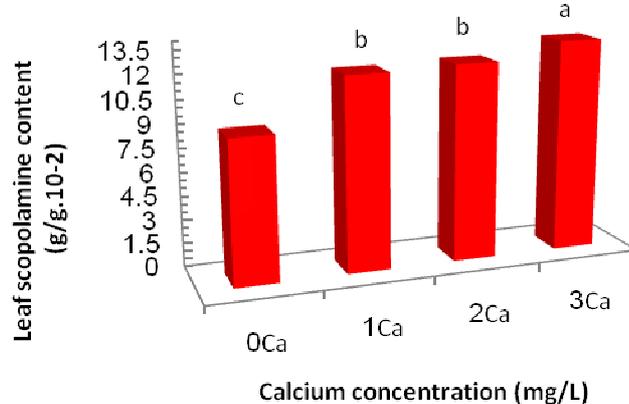


Fig.3. Effect of calcium fertilization on leaf scopolamine content of henbane (n=6). Values with different letter(s) were significantly different at $P < 0.05$ (Duncan test). Ca0, Ca1, Ca2 and Ca3 refer to 0, 16.6, 33.3 and 50 mg/L calcium, respectively

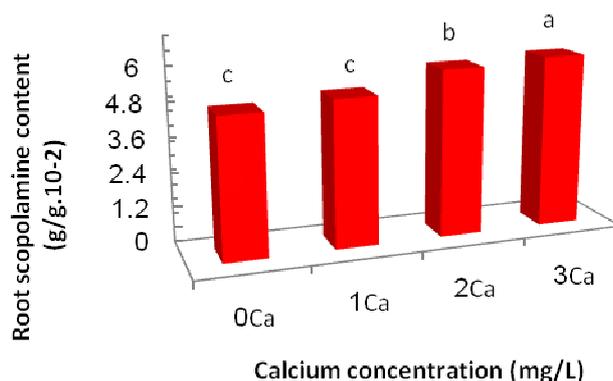


Fig.4. Effect of calcium fertilization on root scopolamine content of henbane (n=6). Values with different letter(s) were significantly different at $P < 0.05$ (Duncan test). Ca0, Ca1, Ca2 and Ca3 refer to 0, 16.6, 33.3 and 50 mg/L calcium, respectively

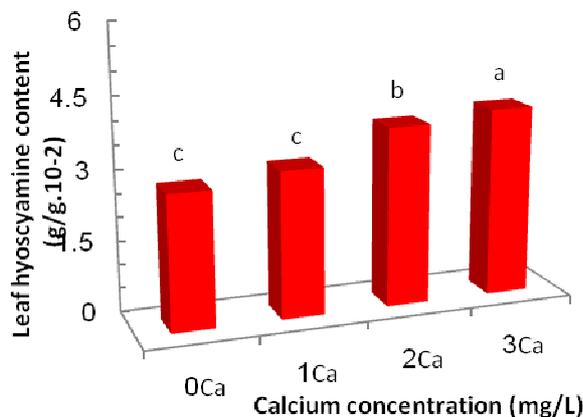


Fig.5. Effect of calcium fertilization on leaf hyoscyamine content of henbane (n=6). Values with different letter(s) were significantly different at $P < 0.05$ (Duncan test). Ca0, Ca1, Ca2 and Ca3 refer to 0, 16.6, 33.3 and 50 mg/L calcium, respectively

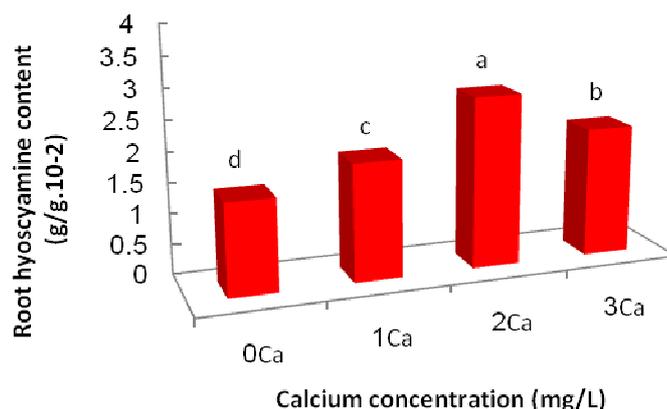


Fig.6. Effect of calcium fertilization on root hyoscyamine content of henbane (n=6). Values with different letter(s) were significantly different at $P < 0.05$ (Duncan test). Ca0, Ca1, Ca2 and Ca3 refer to 0, 16.6, 33.3 and 50 mg/L calcium, respectively

The effect of various levels of calcium on the hyoscyamine content of henbane leaf and root are shown in fig 5 and 6. The results showed that various doses of calcium had significant effect ($P < 0.05$) on the hyoscyamine content of leaves and roots. The plants leaf hyoscyamine content increased by increasing calcium rate (fig5). On the other hand maximum leaf hyoscyamine value was observed at the highest calcium treatment. The results also indicated that the root hyoscyamine content increased with increasing the amount of calcium fertilization from 0 to 33.3 mg Ca/L, but decreased at 50 mg/L calcium (Fig 6).

The content of alkaloids in plants could be increased through genetic and or environmental manipulations. In fact, a secondary metabolite pathway depends on numerous factors but nutritional status leads to major effects. A major mineral such as calcium is among the most essential ingredients of the nutrient medium known to affect the growth and metabolism [5]. It has been established that calcium acts as an intracellular messenger in coupling a wide range of extracellular signals to specific responses such as cell division, cell elongation or cell differentiation [11]. The availability of calcium is expected to play an important role in the biosynthesis and accumulation of alkaloids in plants. Not enough researches are available regarding the effect of calcium fertilization on hyoscyamine and scopolamine content of henbane particularly in greenhouse culture and management. From our results, it can be concluded that the content of hyoscyamine and scopolamine of leaf tissues in *Hyoscyamus niger* plants increased significantly with increasing calcium levels. However, the root and leaf biomass of plants increased only at the low or moderate calcium concentrations i.e., high calcium treatment had reverse effect on plant dry weight and alkaloids content for commercial production of henbane. This plant is a rich source of tropane alkaloids. Therefore, in order to obtain high bioactive compounds content calcium is necessary as main macro nutrient

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