Changes of Tropane Alkaloids in Black Henbane (*Hyoscyamus niger* L.) in Response to Different Types of Nitrogenous Fertilizers

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Abstract

Background: Since alkaloids are nitrogenous compounds, the availability of nitrogen (N) is expected to play an important role in the biosynthesis and accumulation of alkaloids in plants. Nitrogen has been found to increase the content of alkaloids in some of the medicinal plants.

Objective: The aim of this study was to compare the effects of nitrogen bio-fertilizers, conventional nitrogen fertilizer, and nitrogen nano-chelate fertilizer on the content of tropane alkaloids in *Hyoscyamus niger* L.

Methods: This experiment was conducted in a randomized complete block design (RCBD) with 8 treatments, and 3 replications. The experimental treatments included 2 $L.h^{-1}$ nitroxin, 1 $kg.h^{-1}$ *Azotobacter*, and 10 $t.h^{-1}$ vermicompost each as bio-fertilizer, 150 $kg.h^{-1}$ urea as a conventional chemical fertilizer of nitrogen, and nitrogen nano-chelate fertilizer with three amounts of 3, 5, and 7 percent, and control treatment (without nitrogen fertilizer).

Results: The results showed that the effects of bio-fertilizers, conventional nitrogen fertilizer, and nitrogen nano-chelate fertilizer were significant on the scopolamine and hyoscyamine content in black henbane at the level of 1%. The highest content of hyoscyamine (HYO) was related to the treatments of 5% per hectare nitrogen nano-chelate, and the maximum amount of scopolamine (SCO) was observed in the treatments of 3 and 5% nitrogen nano-chelate fertilizers. On the other hand, the lowest values of hyoscyamine and scopolamine were related to the treatment of 7% nitrogen nano-chelate fertilizer, and nitroxin treatment, respectively.

Conclusion: Generally, the highest yield of alkaloids content in black henbane were obtained by application of 3 and 5% nitrogen nano-chelate fertilizers.

Keywords: *Hyoscyamus niger* L., Conventional nitrogen fertilizer, Hyoscyamine, Nitrogen bio-fertilizer, Nitrogen nano-chelate, Scopolamine

Introduction

Medicinal plants are rich sources of secondary metabolites. Among the secondary metabolites of plants, alkaloids form the most important part of the large group of nitrogeneous secondary metabolites. Alkaloids are a group of nitrogen-containing bases and most of them are drugs. Alkaloids figure as a very prominent class of defense compounds show great variety in their botanical and biochemical origin, chemical structure and pharmacological action. A group of alkaloids called tropane alkaloids are mainly found in plants from the family of Solanaceae [1]. The first time, the word of alkaloid was used by a German pharmacologist with the meaning of quasi-alkali. It was later determined that their alkalinity is due to the presence of a base nitrogen atom [2]. Until now, more than 5000 different alkaloids are known in 15% of plants belonging to 150 plant families. Most of the alkaloid-producing plant species are from the family of Solanaceae, Papaveraceae, and Rubiaceae [3]. Tropane alkaloids include hyoscyamine or atropine with the formula of $C_{17}H_{23}NO_3$ and hyoscine or scopolamine with the formula of $C_{17}H_{21}NO_4$ and a small amount of atropine [4]. Hyoscyamine is widely used in the treatment of mental disorders, as well as in the preparation of anti-nausea and anti-seasickness [5]. These alkaloids are used to dilate the pupil of the eye [6]. Scopolamine has the property of relaxing the nervous system with very strong effect and it is used to relieve neurological disorders, parkinson, shakes in old age, and chorea and eliminate severe stimulations of insane. Scopolamine is hypnotic and the effect of morphine can be strengthened by using it. The alkaloid has the strong effect of dilating the pupil of the eye [7].

Hyoscyamus niger L. from the family of Solanaceae is a medicinal plant that it is a rich source of medicinal substances including tropane alkaloids. Hyoscyamine and scopolamine are two main tropane alkaloids in the Solanaceae family plants [8]. Delicate roots of the plant and the roots that do not have secondary growth are the biosynthetic places of tropane alkaloids and the main enzymes for make them are in this area [9]. A lot of the tropane alkaloids are transferred to shoot after synthesis and are concentrated in the vacuoles of various tissues. Tropane alkaloids hyoscyamine and scopolamine are the compounds with parasympatholytic property and are widely used in the treatment of a wide range of diseases [10].

Nitrogen is a main part of many chemical compounds such as proteins, nucleic acids, alkaloids and also forms a part of chlorophyll and is an element that in most cases, its reduction leads to reduce the growth of green plants. Nitrogen is not constantly available for the plant due to the processes such as denitrification, soil erosion and leaching [11]. It is also reported that nitrogen increases the content of alkaloids in some plants such as tobacco (*Nicotiana tabacum* L.), lupine (*Lupinus polyphyllus* L.), and barley (*Hordeum vulgare* L.) [12].

Today, nano-science can affect all areas of science and the agricultural science is no exception. One of the applications of nano-science in agriculture is plant nutrition management. By using nano-fertilizers, the nutrients are released slowly and with an appropriate speed throughout the plant growth season plants, and so the plants will be able to attract the largest amount of foods due to a sever reduction of leaching elements. The aim of this study was to compare the effects of nitrogen bio-fertilizers, conventional nitrogen fertilizer, and nitrogen nano-chelate fertilizer at different concentrations on the content of tropane alkaloids (HYO, SCO) in black henbane.

Materials and Methods

This study was conducted in 2014-2015 at the research farm of Shahre-Ray branch of Islamic Azad University (IAU). After selecting the desired field, a deep plowing was performed in the fall of 2014 and then a medium plowing was done in the spring of 2015. In the following, the disk and leveler was used to uniformly level the field surface. To determine the physical and chemical soil properties, the experiment place was sampled and the samples were sent to laboratory (Table 1).

The research was done on the base of randomized complete block design (RCBD) with three replications and eight treatments. The experimental treatments included 2 $L.h^{-1}$ nitroxin, 1 $kg.h^{-1}$ *Azotobacter*, and 10 $t.h^{-1}$ vermicompost each as bio-fertilizer, 150 $kg.h^{-1}$ urea as a conventional chemical fertilizer of nitrogen, and nitrogen nano-chelate fertilizer with three amounts of 3, 5, and 7 percent, and control treatment (without nitrogen fertilizer). Seeds with a suitable quality of germination were prepared from the seed bank (1218-MPISB) of Medicinal Plants Institute (MPI, ACECR). Seeds were sown in the rows with 50 cm distance from each other, and 30 cm inter-row spacing in April of 2015. Plots were constructed in a uniform shape with 2 m width and 4 m length. The space between replications was 1.5 m and distance from each plot side was considered 1 m. The irrigation and other field practices were done as needed.

Nitroxin bio-fertilizer had a set of the most effective nitrogen-fixing bacteria from the genus of *Azospirillum*, *Azotobacter*, and phosphate-solubilizing bacteria from the genus of *Pseudomonas*. The number of each bacterium per milliliter of nitroxin (CFU) was 10^8 living cells [13]. For mixing and inoculating the seeds, the soaked seeds were inoculated with 2 liters per hectare of liquid nitroxin fertilizer. The soaked black henbane seeds were dipped Arabic gum and then inoculated with the amount of 1 kg per hectare of *Azotobacter*, and also dried in the shade for sowing on the farm [14]. The urea fertilizer was used for black henbane with the amount of 150 kg per hectare in three stages (before planting, 2 to 3 leaf stage, and one month before harvest). Used vermicompost was also prepared by using the animal manure and a kind of earthworm named *Eisenia foetida* with the amount of 10 tons per hectare and was poured between two rows of stacks of each plot at a depth of 2 cm of soil before planting and then was covered by soil. Nitrogen nano-chelate fertilizer (26% N) with the amounts of 3, 5, and 7 percent during three growth stages (8, 10, and 12 weeks) was sprayed on the leaves of black henbane by using a sprayer. To increase the absorption of solutions by plants, foliar application of nitrogen nano-chelate fertilizers were performed under without wind or rain conditions and before sunrise when

Table 1- Soil properties of the experimental field

Properties	EC ($dS.m^{-1}$)	pH	OC (%)	N (%)	P (ppm)	K (ppm)	Clay (%)	Silt (%)	Sand (%)	Texture (%)	S (ppm)	CEC (C.mol.kg)
Experimental method	Conducto meter	pH meter	Titration	Kjeldahl	Olsen	Film-photometer	Hydrometer	Hydrometer	Hydro meter	-	Turbidity metery	Film-photometer
Rate	3.41	8.0	0.84	0.076	18.2	355.4	34	41.3	24.6	Clay-loam	108.3	62.12

plant stomata are open. After doing the farm operations and plant physiological maturity, phytochemical traits included content of hyoscyamine, and scopolamine (hyoscine), and yield of shoot dry weight (SDW), scopolamine, and hyoscyamine were measured.

In this research, content of alkaloids was determined in the plant dry matter of black henbane by HPLC (High performance liquid chromatography) method. Plant samples (leaf) were sieved with laboratory mesh (size 30, mesh opening 545 µm). Extracting the alkaloids was conducted by different solvents [15]. In this way, 2 g of plant dry matter with the combination of chloroform ($CHCl_3$): methanol (MeOH): 25% ammonia (NH_4OH) with the ratio of 15:5:1 were exposed to ultrasound for 15 minutes. Then, the sample was transferred to hot water bath (40 °C) for one hour. Subsequent sample preparation and alkaloids extraction were based essentially on the method described by Kamada et al. [15]. The extract obtained was passed through the filter paper and filter was washed twice with 10 ml of chloroform. The chloroform phase was dried by using a rotary evaporator system. 25 ml of chloroform and 10 mL of sulfuric acid (1 N) were added and stirred well. The chloroform phase was separated and discarded and the aqueous phase containing alkaloids was adjusted to pH between 10 and 11 with 25% ammonia solution. Alkaloids were extracted once with 25 ml and twice with 10 ml of chloroform. The solution obtained was dehydrated by sodium sulfate anhydride and filtrated. On the filter was washed with 10-20 ml of chloroform and the samples obtained were dissolved in 1-2 ml of methanol. HPLC analysis was performed by using a Knauer K2600A liquid chromatography (Germany) set with KNAUER-K1001 pump, solvent vacuum degasser, Teknokroma-C18 column (ODS; 250 × 4.6 mm, 5µm particle size), a variable wavelength detector, and an auto sampler with a 10 µL injection loop. Peak detection was made at 210 nm at the room temp. of 25 °C by UV detector [KNAUER-UV (K2501)] from the above mentioned company. Separation was performed with a water and acetonitrile solvent system. Mobile phase was prepared from HPLC grade water (80%; A) and acetonitrile (ACN) (20%; B), respectively. The flow rate was 1.0 $ml.min^{-1}$ and the injection volume was 10 µL. The standard was run on HPLC system and retention time for alkaloids were determined. Identification of alkaloids in different treatments were calculated by comparing the retention time of samples with standard. Alkaloids standard was purchased from Sigma Aldrich Ltd., USA (95 % purity). All the other chemicals used were of analytical grade. The stock and standard solution were diluted with water-acetonitrile (8:2) accordingly and were used for the confirmation of retention times.

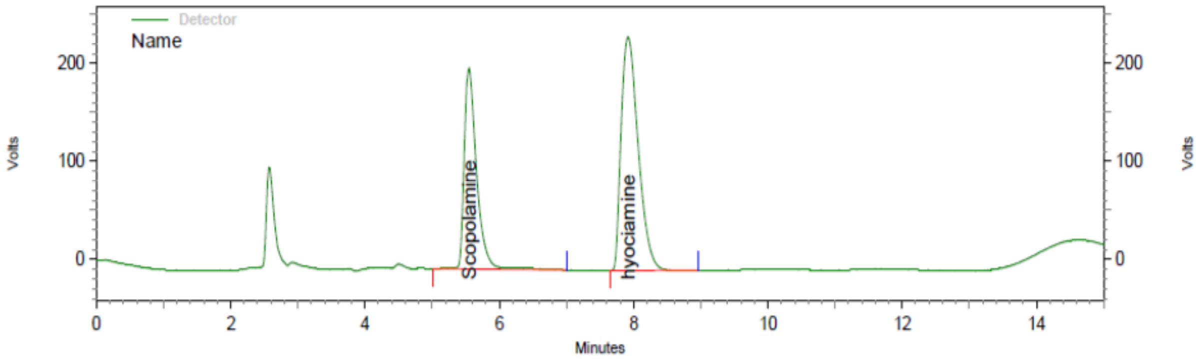
All data were subjected to the statistical analysis based on a randomized complete block design (RCBD) with eight treatments and three replications. Analysis of variance of the results was done using the SAS software (ver. 9.3), and the treatments means were compared using a Duncan's multiple range test at a probability level of 0.05.

Results

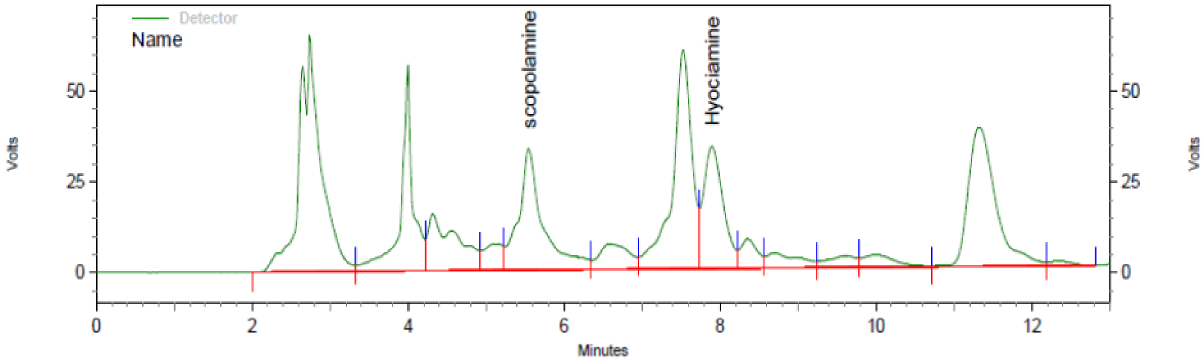
In the present experiment, identification and quantification of alkaloids content in the samples was done by comparing the retention time, UV spectra and peak area of sample with that of the standard. Figure 1 shows some selected peaks on HPLC chromatograms and fingerprinting of scopolamine and hyoscyamine in black henbane (*Hyoscyamus niger* L.) with similar retention time, such as standard, 5% nitrogen nano-chelate, and nitroxin treatment. The retention time (RT) of alkaloids standard were found to be 5.54 to 5.61 min for scopolamine, and 7.91 to 8.07 min for hyoscyamine.

The results indicated that the application of nitrogenous fertilizers on the black henbane (*Hyoscyamus niger* L.) had a significant effect on phytochemical and physiological traits included content of hyoscyamine, and scopolamine, and yield of scopolamine and hyoscyamine ($P \leq 0.01$), and also, shoot dry weight ($P \leq 0.05$) (Table 2).

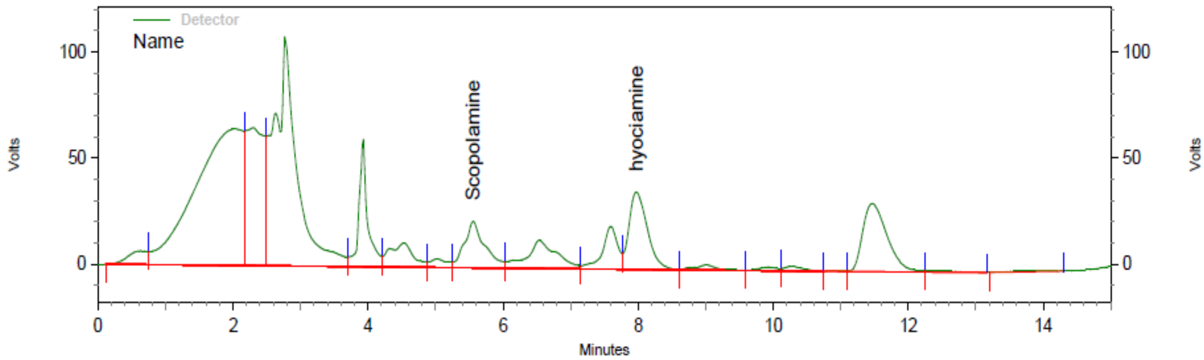
The mean comparisons of traits showed that the maximum content of hyoscyamine was observed in treatment of 5% nitrogen nano-chelate fertilizer with the mean of 0.112 $mg.g^{-1}$ DW (Figure 2), and the highest amount of scopolamine was obtained in treatments of 3 and 5% nitrogen nano-chelate fertilizer with the mean of 0.123 and 0.129 $mg.g^{-1}$ DW, respectively (Figure 3). Also, the lowest amounts of hyoscyamine and scopolamine were related to the treatments of 7% nitrogen nano-chelate fertilizer with the mean of 0.018 $mg.g^{-1}$ DW (Figure 2), and nitroxin treatment with the mean of 0.016 $mg.g^{-1}$ DW, respectively (Figure 3). The highest yield of hyoscyamine was attained in treatment of 5% nitrogen nano-chelate fertilizer with the amount of 0.038 $g.m^{-2}$ (Figure 4), and the maximum yield of scopolamine was belonged to the treatments of 3 and 5% nitrogen nano-chelate fertilizer with the amounts of 0.045 $g.m^{-2}$, and 0.044 $g.m^{-2}$, respectively (Figure 5). Also, the lowest yield of hyoscyamine (0.0058 $g.m^{-2}$) and scopolamine (0.0054 $g.m^{-2}$) were related to the treatment of 7% nitrogen nano-chelate fertilizer and nitroxin, respectively (Figure 4 and 5). The highest amount of shoot dry weight was recorded to the treatment of 3% nitrogen nano-chelate fertilizer with the mean of 366.66 $g.m^{-2}$, and the lowest amount of shoot dry weight was related to the treatment of vermicompost fertilizer with the mean of 276.66 $g.m^{-2}$ (Figure 6).



(*a*)



(*b*)



(*c*)

Figure 1- Some selected peaks on HPLC chromatograms and fingerprinting of scopolamine and hyociamine in black henbane (*Hyoscyamus niger* L.) with similar retention time (*a-c*), *a*; standard, *b*; 5% nitrogen nano-chelate, and *c*; nitroxin treatment.

Table 2- Analysis of variance for effects of nitrogenous fertilizers on phytochemical traits and shoot dry weight of black henbane (*Hyoscyamus niger* L.)

Source of variation	Degree of freedom	Shoot dry weight	Content of hyocyamine	Content of scopolamine	Yield of hyocyamine	Content of scopolamine
Block	2	20055.79	0.000039	0.0002	0.00078	0.00401
Treatment	7	25284.4*	0.0022**	0.004**	0.05562**	0.10113**
Error	14	5228.64	0.000018	0.0002	0.00009	0.00104
Coefficient of variations	-	15.69	7.71	11.95	12.09	18.74

*: significant at $P \leq 0.05$; **: significant at $P \leq 0.01$

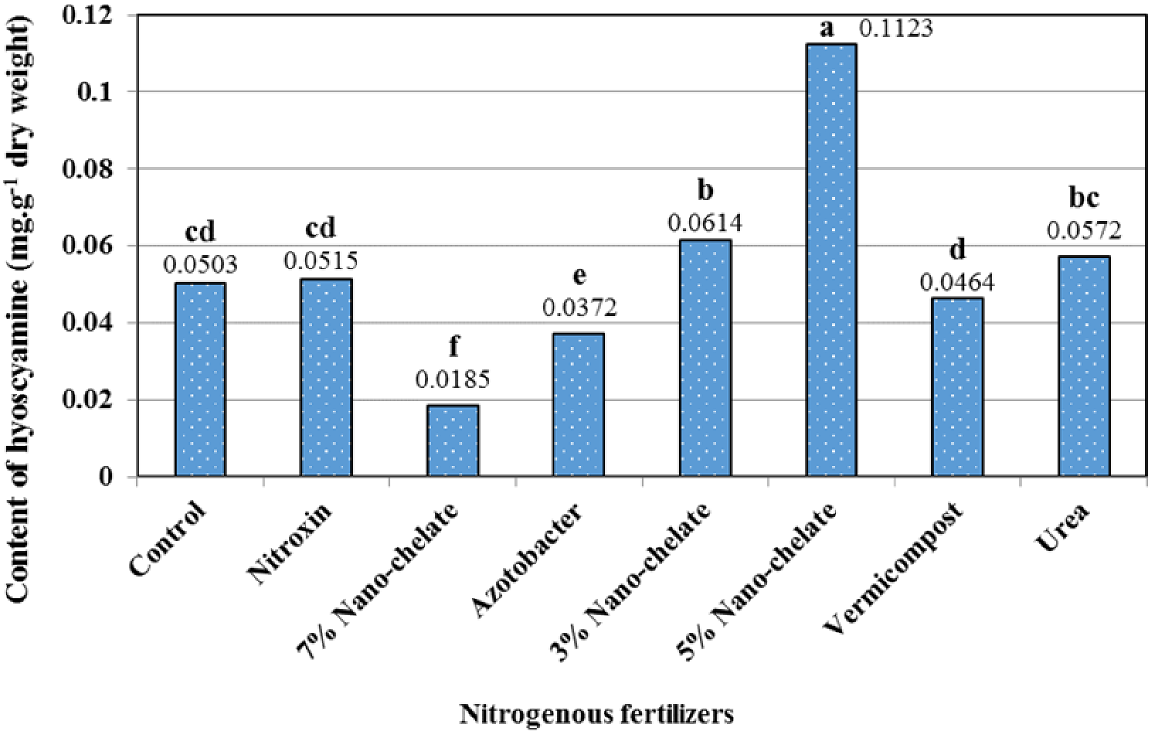


Figure 2- Mean comparison of nitrogenous fertilizers effect (P≤0.01) on content of hyoscyamine (mg.g^{-1} dry weight) in black henbane (*Hyoscyamus niger* L.).

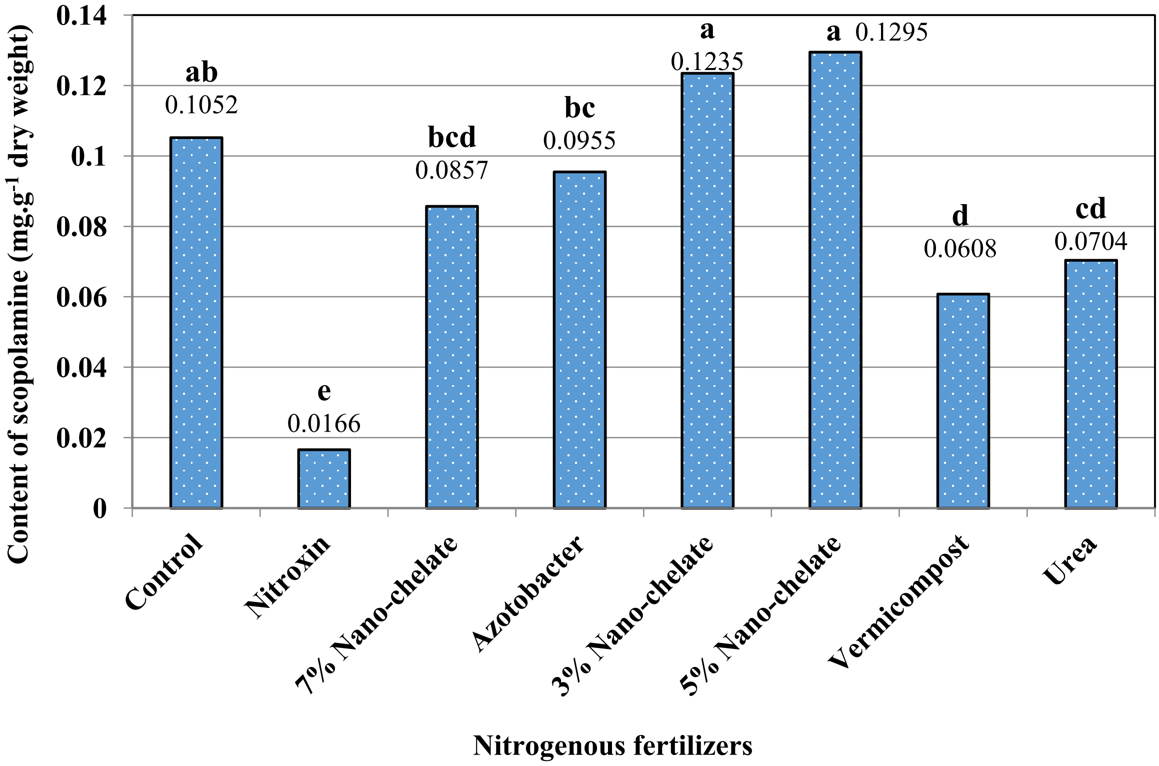


Figure 3- Mean comparison of nitrogenous fertilizers effect (P≤0.01) on content of scopolamine (mg.g^{-1} dry weight) in black henbane (*Hyoscyamus niger* L.)

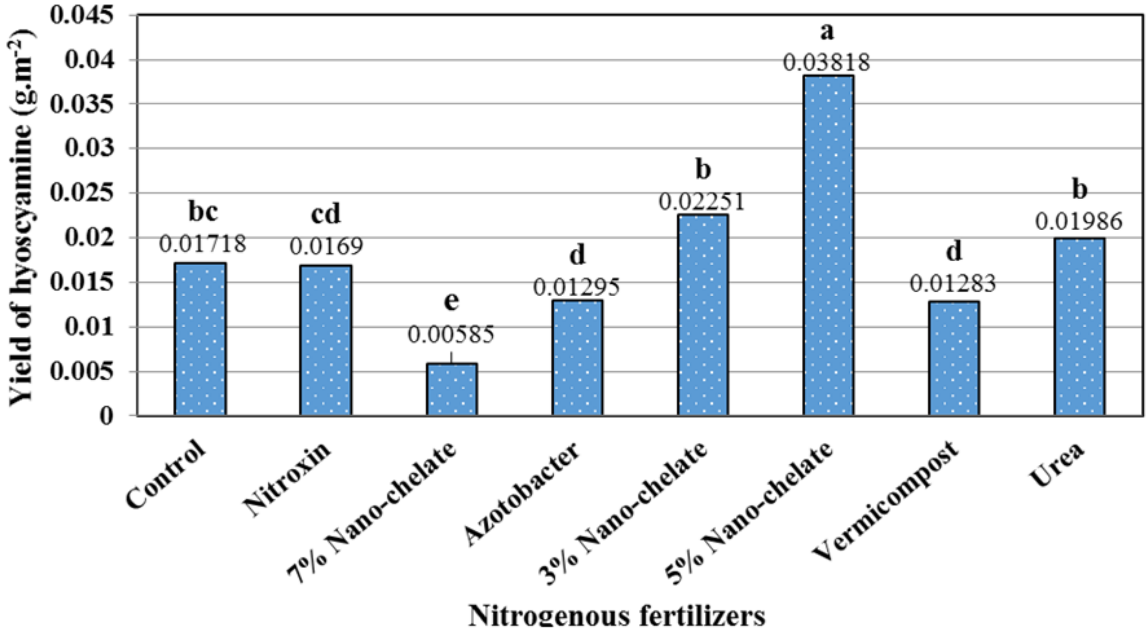


Figure 4- Mean comparison of nitrogenous fertilizers effect (P≤0.01) on yield of hyoscyamine ($g.m^{-2}$) in black henbane (*Hyoscyamus niger* L.)

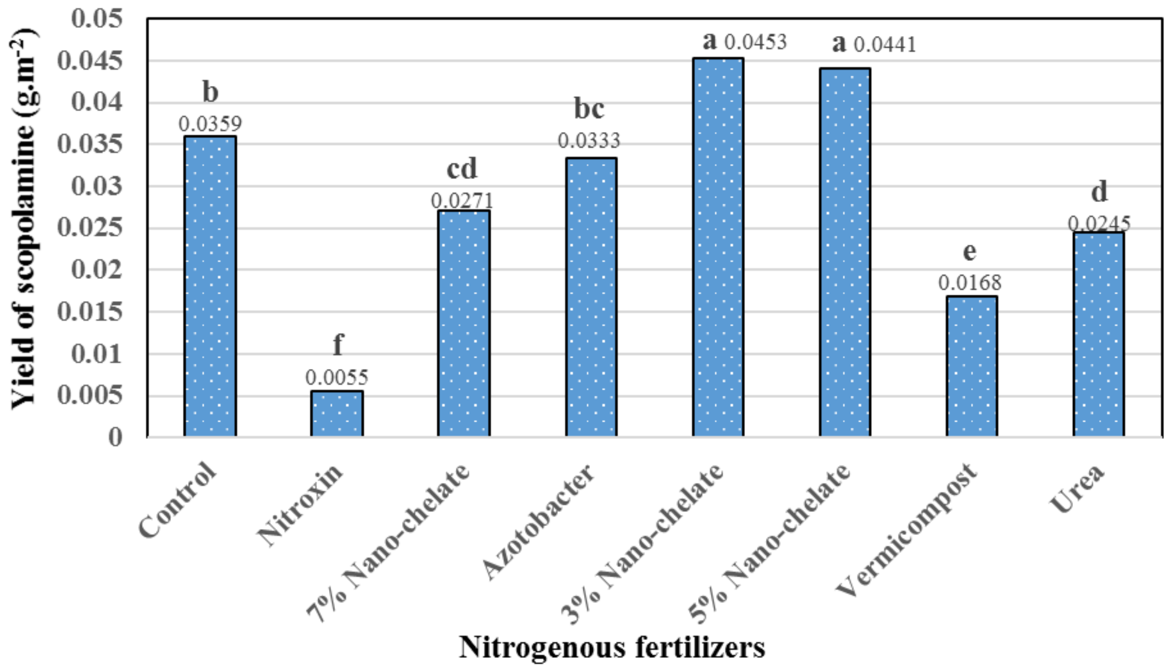


Figure 5- Mean comparison of nitrogenous fertilizers effect (P≤0.01) on yield of scopolamine ($g.m^{-2}$) in black henbane (*Hyoscyamus niger* L.)

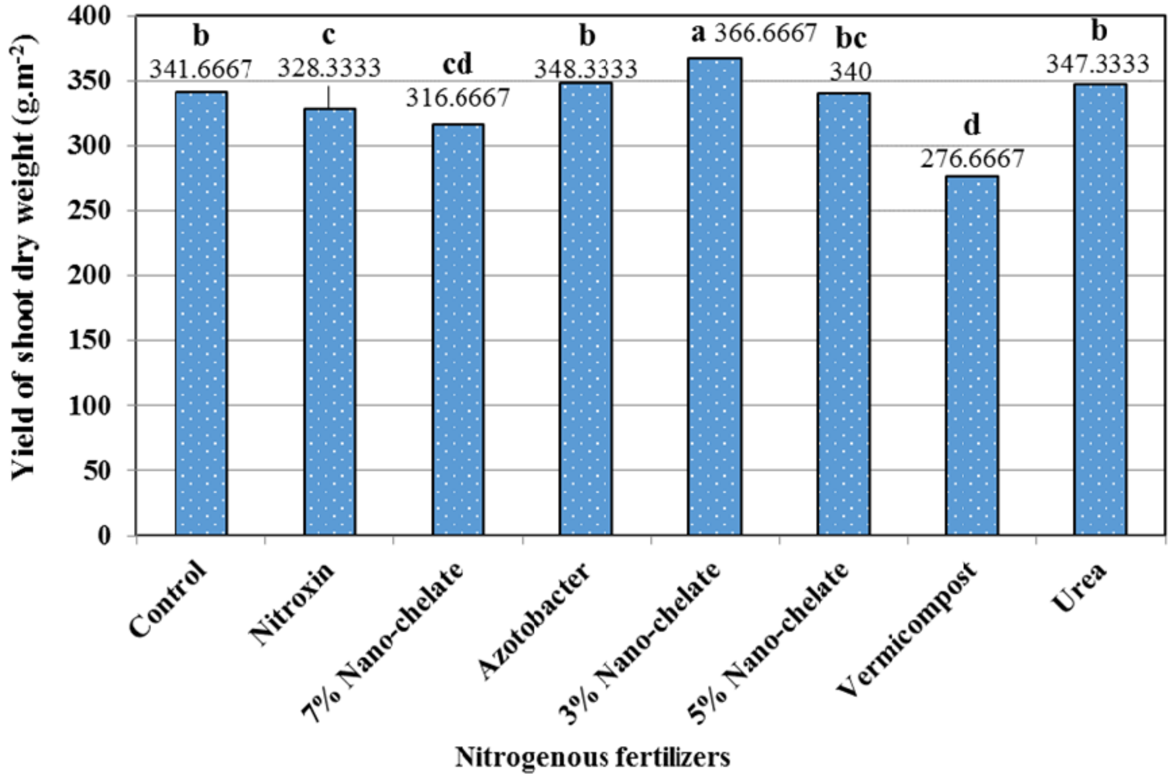


Figure 6- Mean comparison of nitrogenous fertilizers effect ($P \leq 0.05$) on yield of shoot dry weight ($g.m^{-2}$) in black henbane (*Hyoscyamus niger* L.)

Discussion

The content of alkaloids in the plants can be improved by genetic manipulation or environmental conditions. Since, alkaloids are nitrogenous compounds, it is expected that nitrogen availability plays a very important role in the biosynthesis and accumulation of alkaloid in the plants. It is reported that nitrogen increases the content of alkaloids in some medicinal plants such as datura (*Datura stramonium* L.), deadly nightshade (*Atropa belladonna* L.), and opium poppy (*Papaver somniferum* L.) [12]. Payne *et al*. [16] stated that the nitrogen concentration and the ratio of carbon to nitrogen in the cultivation area affect the alkaloid synthesis. The results obtained by Bensaddek *et al*. [17] on *Atropa belladonna* showed that in the nitrate treatment, the root and radicle (alkaloid biosynthesis area) have more growth, and it affects the alkaloid content. Increasing the nitrate concentration in the medium of hairy roots improved the content of HYO and SCO in the studied plant. Also, increasing the nitrogen as nitrate in the soil leads to increase the ion in plants that is the cause of more production of tropane alkaloids in the healthy plant. Khavari Nejad and Mohammadi [18] showed that increasing ammonium concentration decreased the alkaloid contents compared with the control treatment without affecting the ratio of scopolamine to hyoscyamine. The highest amount of alkaloid was related to the time when there were both forms of nitrogen in the environment.

Transforming materials into nano-scale will change their physical, chemical, biological characteristics and catalytic activities. Specific surface area of most of materials at nano-scale will increase their chemical and biological activities. Thus, new properties will appear in nano-particles such as, more solubility, chemical activities and the ability of penetration into cell membrane. The nano-fertilizers are easily taken up by plant and are more efficient than conventional chemical fertilizers [19]. Several studies are concerned with the synthesis of nano-materials using biological routes. Only limited studies have been reported on the promotory effects of nano-particles on plants in low concentrations [19, 20]. Amirjani *et al.* [20] stated that amount of total alkaloid concentration, enzymatic and non-enzymatic antioxidants, and plant physiology indices has increased by increasing the concentration of nano-fertilizer and this increase in the concentrations more than 4 $m.mol^{-1}$ zinc oxide was significant at the level of 1% compared to non-nano fertilizer.

Providing nitrogen for the plant from the beginning of the growth is necessary for achieving the optimal formation of alkaloids because the availability of nitrogen regulates alkaloid synthesis in addition to the production of biomass. Usually, critical time of using nitrogen has been reported 40 days after the germination [21, 22]. Ghorbanpour *et al.* [22] showed that the content of hyoscyamine and scopolamine of the root increases by increasing the nitrogen and water shortage stress that one part can be related to decreasing the biomass of organs under these circumstances and another part is due to a direct effect of nitrogen and stress on the construction of these metabolites. The studies conducted by researchers show that if nutritious fertilizers are available for the plant nutrients with the appropriate amounts and in the appropriate time, it will have a main role in increasing the vegetative body and the amount of its alkaloids [23]. In another experiment, the effects of nitrogen fertilizer levels (0, 50, 150 kg per hectare) on the alkaloid content of two mutants containing high alkaloid of periwinkle compared to parent cultivar were studied to determine the possibility of more increases in their alkaloid content. The results showed that nitrogen fertilizer (150 kg per hectare) significantly increased the content of alkaloids in the leaves (42%) and roots (32%) of all genotypes [24]. Since alkaloids are nitrogenous compounds, it is expected that the availability of the element plays an important role in the biosynthesis and accumulation of alkaloids in the plant [22]. Quantitatively, nitrogen is the second quantitative constituent of plant tissue that forms 1.5% of the dry matter after carbon. Nitrogen is one of the molecules that play a central role in the metabolism of plants, for example it is directly involved in the seed germination and tissue structure (wall membrane), protein molecules, nucleic acid and pigment [25, 26].

Conclusion

In general, the highest content of tropane alkaloids (HYO, SCO) in black henbane (*Hyoscyamus niger* L.) were obtained by using the treatment of 5% nitrogen nano-chelate fertilizer as an external elicitor for alkaloids production. The use of nitrogen nano-chelate fertilizer as the most effective and simplest way is recommended to reduce the loss of nitrogen, increase the efficiency of fertilizer use, save the cost of production and prevent from the environmental problems. Therefore, this study could be recommended to farmers involved in sustainable production of black henbane (*Hyoscyamus niger* L.) for increasing of tropain alkaloids with least environmental and economic cost.

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