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Effect of water stress and nitrogen fertilization on the content of hyoscyamine and scopolamine in the roots of deadly nightshade (*Atropa belladonna*)

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Abstract

The study intended to elaborate the optimal environmental conditions of water supply and nitrogen fertilization for maximum content of hyoscyamine (% dw) and scopolamine (% dw). Plants grown from seeds of Slovene autochthonous population of deadly nightshade (*Atropa belladonna*), were treated with different water regimes (35–95% depletion of available soil water) together with enhanced nitrogen supply (0.37–1.60 g/pot N) in a greenhouse experiment. Dry plant extracts from 32-week old roots were analysed with capillary electrophoresis (CE) for the presence of tropane alkaloids (hyosciamyne, scopolamine). The results of the plant treatment responses showed that the maximal yield of tropane alkaloids (hyoscyamine: 54 mg/plant; scopolamine: 7 mg/plant) was achieved in plants grown under an optimal irrigation regime (35% depletion of available soil water) accompanied with total nitrogen supply of 0.37 g/pot. By contrast, the maximal content of alkaloids was achieved with 95% depletion of available soil water and a nitrogen supply of 1.60 g/pot.

Keywords: *Atropa belladonna*; Capillary electrophoresis; Nitrogen supply; Tropane alkaloids; Water stress

1. Introduction

Despite the very extensive studies on the possibilities for producing tropane alkaloids with plant cell cultures and from biotechnologically transformed transgenic plants (Aoki et al., 1997; Yoshimatsu et al., 1997), the conventionally cultivated roots of deadly nightshade (*Atropa belladonna*) are still believed to be one of the most important sources of natural hyoscyamine and scopolamine. The contents of tropane alkaloids in cultivated plants can be considerably influenced by environmental and agrotechnical parameters. In medicinal plants' agrosystems, the principles

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characterizing primary plant production (growth, development and yield) cannot be simply applied to the production of secondary metabolites, which are important factors in the quality control of drug plants. On the plant species level, there exists a well known relationship between primary production (yield) and the production of secondary metabolites. This relationship may be expressed as a linear, a power function or as an orthogonal polynomial relation, depending on the genotype–environment interactions of particular species (Bernath, 1986). The literature data, although sometimes contradictory, indicate that water stress (besides the temperature) as well as nutrient supplies considerably affect the alkaloid production in alkaloid-yielding species (Bernath and Tetenyi, 1978; Yadav et al., 1982). It is generally assumed that increasing the nitrogen supply to a plant will increase the alkaloid production (Khan and Harborne, 1990; Khan, 1991). A 10-fold increase in nitrogen fertilization of *Atropa acuminata* resulted in an increase in the content of hyoscyamine and of scopolamine in the whole plant and in stems, while their contents in leaves and in roots remained unchanged. The genotype as well as the stage of development significantly affect the content of tropane alkaloids in *A. belladonna* plants. According to Dhar and Bhat (1982) a great variability in alkaloid concentration was observed during different ontogenetic stages of the plant. The lowest percentage of alkaloids (0.38% dw) in individual genotypes of *A. belladonna* was found in the developmental stage 3 (50% of flowering, basal leaves fully mature, 40 days after the appearance of first flower), while in stage 9 (appearance of new foliage, fresh tillers and flowers, 130 days after the appearance of first flower) plants contained the highest percentage of alkaloids (0.67% dw).

Our hypothesis presumes that water stress decreases and enhanced nitrogen supply increases the content of tropane alkaloids in roots of deadly nightshade. The maximal root yields and their maximal content of tropane alkaloids are expected to be achieved by optimal water supply together with highest nitrogen dosage. The objective of our study was to verify this hypothesis in controlled greenhouse experiments.

2. Materials and methods

2.1. Plant material used and growing conditions

Seeds of *A. belladonna* L. were collected from the autochthonous population at Podzavrh, Slovenia (46°01′ N, 15°23′ W). After germination, the seedlings were grown at a nursery. Well developed, 2-month-old seedlings were transplanted to experimental pots ($V = 12.46$ l; 1 plant per pot) in a glasshouse (23 ± 2°C, daylight, 80 ± 5% relative humidity). The pots were filled with alluvial sandy loam soil (Cambisol; the mechanical composition of the soil was as follows: 40% sand, 40% silt, 20% clay; the apparent specific gravity of the soil was 1.24, pH 7.3). The basic soil macronutrient (N–P–K) supply was as follows: total available N, 0.37 g/pot; P_2O_5, 1.39 g/pot and K_2O, 2.57 g/pot.

The soil water holding characteristics were determined through the development of a soil desorption curve (field capacity being at 38% on weight basis, and wilting point being at 15% on weight basis) and thereafter the quantity of daily water supply was determined. In order to study the impact of water stress on the alkaloid content in plant roots and to evaluate the possible interaction with nitrogen fertilization, four levels of water treatment (irrigation) were used (35, 55, 65 and 95% depletion of available soil water) and also four levels of treatment with nitrogen (additional supplies to the basic soil nitrogen level were 0, 0.41, 0.82 and 1.23 g/pot) were applied. A commercially available fertilizer (nitrochalk, KAN-Kutina) containing equal amounts of nitrogen in nitrate and in ammonium forms (soluble mineral forms, available to plants) was used for nitrogen treatments.

The total nitrogen amount per treatment was supplied three times over a 3-week period, the first application was given 14 days after transplantation of the seedlings to experimental pots.

The highest amount of applied nitrogen in our experiment (1.23 g/pot) corresponded to a dosage of 300 kg N/ha. This amount of nitrogen heavily exceeds the maximal permitted amount of nitrogen fertilization according to Slovenian legislation (210 kg/ha), and has been used to evaluate the impact of a high dosage of nitrogen on the yield and on the accumulation of tropane alkaloids, as well as to determine how this overdose of nitrogen interacts with water deficiency.

Each treatment was replicated four times. After 10 days of growth under water stress conditions the plants (32 weeks old) were harvested. The cleaned roots were dried at 60°C for 36 h, until the constant weight was obtained. The plant material was pulverized (sieve No. 0.75) in a mortar grinder (Waring model 34 BL 65) and preserved in dark glass until the extraction procedure.

2.2. Chemicals

The following chemicals of analytical grade (p.a.) were used in the extraction procedure: chloroform (Kemika Zagreb), ammonium hydroxide (Zorka Sabac), methanol (Merck Darmstadt), sodium hydroxide 1 M (Kemika Zagreb). In the quantitative chemical analysis, CE buffers (50 mM buffer phosphate, Hewlett Packard GmbH, Germany), CE sodium hydroxide 1 M (Hewlett Packard GmbH, Germany) and CE water (Hewlett Packard GmbH, Germany) were used. Commercially available standards (atropine sulfate salt—Sigma, St. Luis; scopolamine hydrobromide—Dolder & Co., Basel) were used as references.

2.3. Extraction of alkaloids

Because of the well known high biological variability of plant material, we had to select a precise and sensitive analytical technique for detecting accurate chemical signals for measurement of appropriate plant responses to the treatments used in the study. The GC (Wilms et al., 1977; Ylinen et al., 1986) or HPLC (Kamada et al., 1986) were proved to be very precise and sensitive chromatographic techniques in the quantitative determination of hyoscyamine and/or scopolamine in plant raw material; however, in our experience, they prove to be time consuming. Therefore, the capillary electrophoresis (CE) technique, which is believed to be one of the most precise and rapid analytical methods for determination of tropane alkaloids (Cherkaoui et al., 1997; Eeva et al., 1998), was applied in our study.

The modified method of Kamada et al. (1986) was used for the extraction of dry roots of deadly nightshade. A solvent mixture of chloroform, methanol and 25 v/v% aqueous solution of ammonium hydroxide ($CHCl_3 : MeOH : NH_4OH$, 15 : 5 : 1) was added to the pulverized plant samples (200 mg of dried roots, 10 ml of solvent mixture/100 mg dry weight). The plant–solvent mixture was then ultrasonicated (Ultrasound chamber Iskra, model U2–2R) for 10 min. After further maceration at room temperature (25°C) for 1 h., the extract was filtered and rinsed twice with 1 ml of chloroform. To obtain the dry plant extracts for the CE analysis of tropane alkaloids, the combined filtrates were evaporated under reduced pressure (Büchi Rotavapor®, Switzerland) at 40°C.

2.4. CE analysis of tropane alkaloids

The CE method similar to the one described by Eeva et al. (1998) was used in this research. In order to shorten the analysis time, we used a shorter capillary and also simplified the preconditioning of the capillary. The matrix of the extracted plant samples used in our study did not affect the quality of separation of hyoscyamine and/or scopolamine. The samples were dissolved in 2 ml of 50 mM phosphate buffer pH 8.3 and analysed by capillary electrophoresis (Hewlett Packard CE, 48.5 cm long capillary with inner diameter 50 mm, electrophoretic buffer: 50 mM phosphate buffer pH 8.3, capillary temperature 30°C, capillary rinsing time 3 min, hydrodynamic injection 5 s at 50 mbar, voltage 30 kV, electric current 300 mA). The signal was measured at 192 nm. The calibration curves for hyoscyamine and scopolamine were measured by analysing the standard solutions (1, 0.2, 0.04 and 0.008 mg/ml), six times each standard. The signal was linear in the whole range of the measured concentrations (regression coefficients > 0.999; standard errors for hyoscyamine and scopolamine were 0.499 and 1.032, respectively). The relative standard deviations of the peak areas were 1.37 and 1.30% for hyoscyamine and scopolamine, respectively. Detection limits were around 8 and 26 mg/ml for hyoscyamine and scopolamine, respectively.

The results of plant treatment responses/measurements in the greenhouse study were evaluated by ANOVA, and the significance of the differences between treatment groups was tested by t-test at $\alpha = 0.05$ (Statgraphics 5.0).

3. Results

At high soil water content (35% water depletion) a moderate nitrogen fertilization (Fig. 1a) resulted in the hyoscyamine content of the *A.*

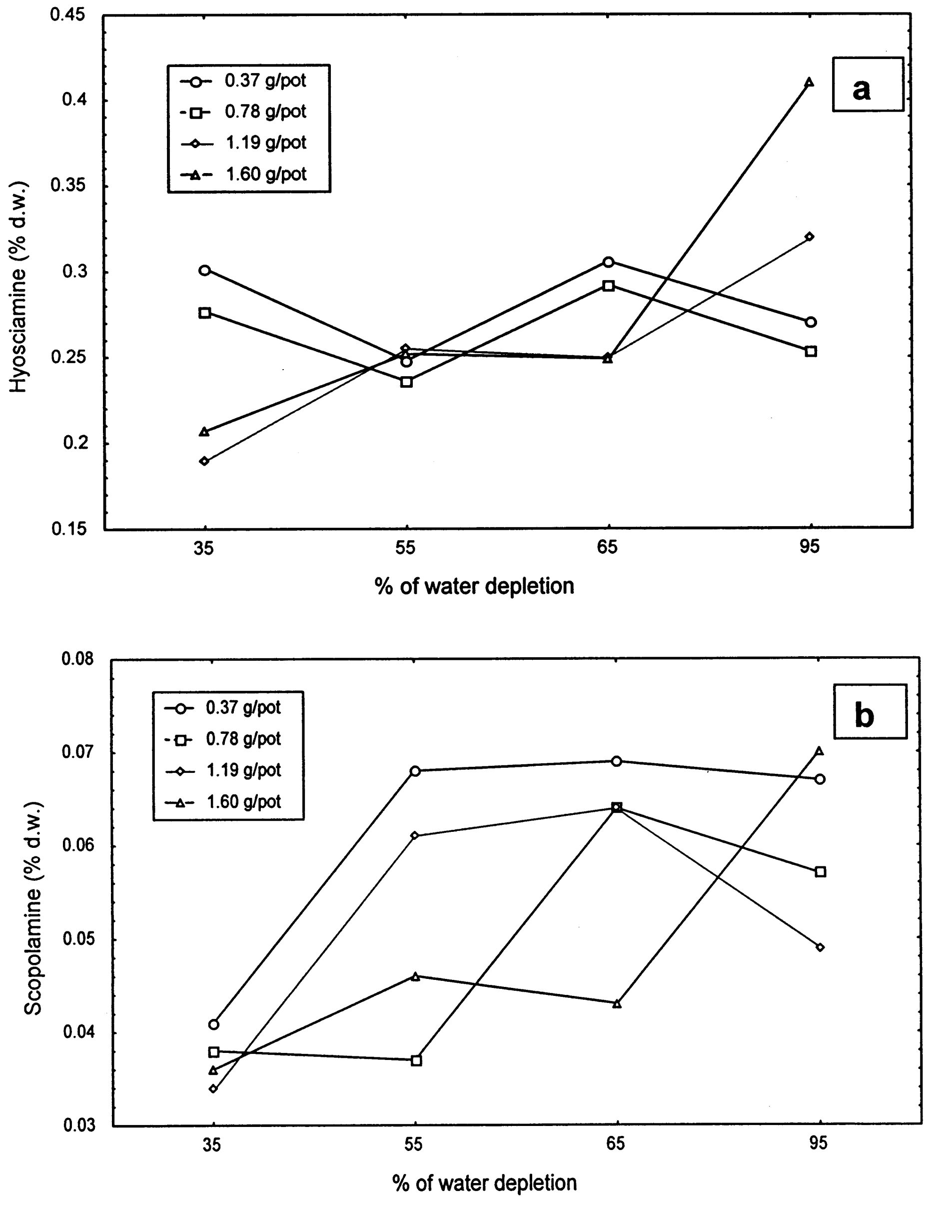

Fig. 1. The interaction between irrigation regime and nitrogen supply on the concentration of hyoscyamine (a) and scopolamine (b) in the roots of *A. belladonna*.

Table 1
The average values (x) of hyoscyamine or scopolamine contents (%), corresponding SD, and the significance of the differences between treatment groups, considering the interaction of irrigation regime × nitrogen supply in the roots of deadly nightshade at $\alpha = 0.05$ (t-test)

Description of interaction		Hyoscyamine contents (%)		Scopolamine contents (%)	
(% of w.d.)	(g/pot N)	x	SD	x	SD
35	0.37	0.302 aehi[a]	0.081	0.041 ac	0.016
35	0.78	0.227 bf	0.028	0.038 ae	0.016
35	1.19	0.190 c	0.045	0.034 ae	0.022
35	1.60	0.207 bc	0.054	0.036 a	0.011
55	0.37	0.248 abfg	0.065	0.068 b	0.028
55	0.78	0.236 bcd	0.069	0.037 a	0.012
55	1.19	0.255 abfgh	0.085	0.061 abc	0.041
55	1.60	0.252 abfgh	0.088	0.046 ac	0.018
65	0.37	0.306 ahi	0.020	0.069 b	0.014
65	0.78	0.292 adhi	0.100	0.064 bcd	0.038
65	1.19	0.250 bde	0.048	0.064 b	0.018
65	1.60	0.249 df	0.025	0.043 ad	0.013
95	0.37	0.270 adefh	0.074	0.067 b	0.011
95	0.78	0.253 deg	0.012	0.057 bcd	0.027
95	1.19	0.320 hi	0.088	0.049 cde	0.012
95	1.60	0.410 i	0.175	0.070 b	0.014

[a] Observation group values, assigned by different letters differ significantly at $\alpha = 0.05$.

belladonna roots being significantly ($P < 0.001$) higher than at high nitrogen fertilization. At moderate soil water levels (55 and 65% of water depletion) the nitrogen fertilization had no impact on the tropane alkaloid, whereas at very low soil water the situation was inverse to that of high soil water content.

In the case of scopolamine, the nitrogen status of the soil did not affect the synthesis, as long as the soil water was high (35% of water depletion) (Fig. 1b). At low water content, low and very high N-fertilization resulted in a high scopolamine content (0.067% dw and 0.070% dw, respectively). By contrast, a high N-fertilization at moderate soil water stress (55 and 65% of water depletion) influenced the low scopolamine content of roots of *A. belladonna*.

The results of the ANOVA showed that the irrigation regime significantly affected the hyoscyamine ($P < 0.001$) and scopolamine ($P < 0.001$) contents in the roots of deadly nightshade. The root scopolamine content also depended on the N-fertilization level ($\alpha = 0.05$).

Also, the interaction between the irrigation regime and the nitrogen supply significantly affected the contents of both alkaloids (hyoscyamine: $P < 0.001$; scopolamine: $P = 0.015$). In order to evaluate the significance of the differences between treatment groups, taking into the account the interaction influence, the average values of hyoscyamine/scopolamine contents (% dw) were compared by t-test ($\alpha = 0.05$).

Concerning soil water availability and the nitrogen supply, the differences in tropane alkaloid contents between treatment groups are presented in Table 1.

The maximal contents of hyoscyamine (0.41%) and of scopolamine (0.07%) in roots were observed under severe water stress growth conditions (95% depletion of available soil water) at a nitrogen level of 1.60 g/pot. The same range of hyoscyamine or scopolamine contents in roots was present under less severe water stress (55 and 65% of soil water depletion) at lower N-fertilization levels.

Irrespective of the N-fertilization level, increasing water stress significantly diminished the root biomass of *A. belladonna* ($P < 0.001$). Maximal

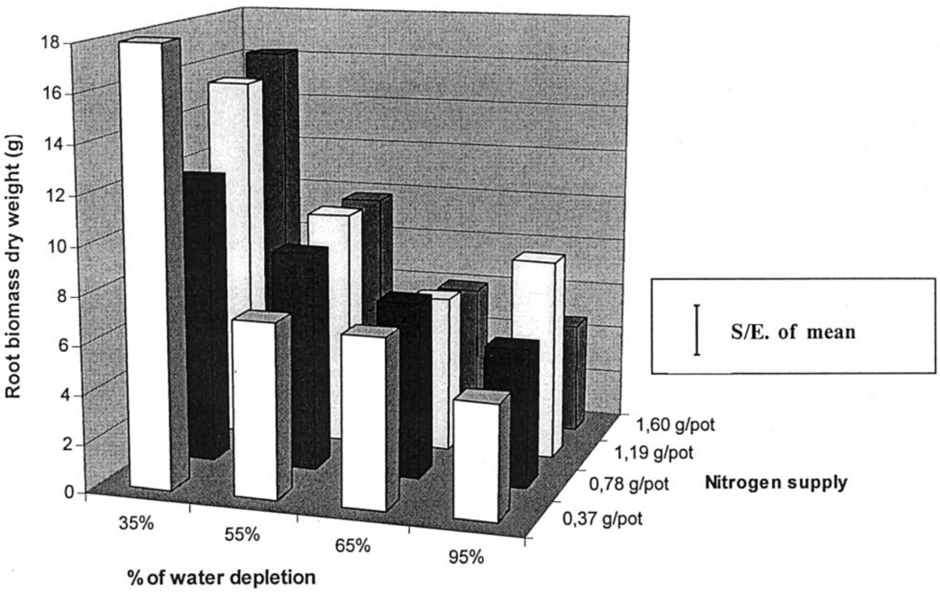

Fig. 2. Interaction between the irrigation regime and nitrogen supply on the dry weight of roots biomass (g/plant) of *A. belladonna* and SE = 2.05 g.

average yields of biomass of roots (18 g dw/plant) were measured in plants grown under the highest water supply (35% of water depletion) at a very low nitrogen level of 0.37 g/pot (Fig. 2).

A typical CE electropherogram in root extract analysis of deadly nightshade (*A. belladonna*) is presented in Fig. 3.

4. Discussion

Recent studies by Hartmann et al. (1986) and Aoki et al. (1997) have revealed more than 18 nitrogen-containing compounds in extracts of intact roots or of hairy root cultures of *A. belladonna*. The major compound in the alkaloid fraction both of intact roots and of root culture was proved to be a hyoscyamine. Other alkaloids, identified by current chromatographic techniques (GC and GC/MS, HPLC) were indentified as hygrine, pseudotropine, 6-hydroxyhyoscyamine, tropinone, tropine, cuscohygrine in intact plant roots (Hartmann et al., 1986) and littorine in hairy root cultures (Aoki et al., 1997). All these alkaloids were found in much lower amounts than hyoscyamine, some of these in a concentration range comparable to that of scopolamine (about 0.8% of total alkaloid fraction). Hyoscyamine was present in intact roots or in hairy roots culture in concentration ranging from 0.06 to 0.5% dw. However, the objective of our study was not to screen the total alkaloid pattern of roots of deadly nightshade; instead, we were concerned with determination of hyoscyamine and scopolamine, referred to as the two major anticholinergic alkaloids in *A. belladonna* in different Pharma-

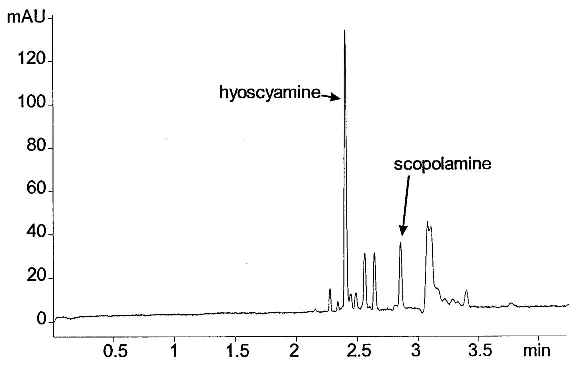

Fig. 3. The CE electropherogram of an *A. belladonna* root extract was analysed at $\lambda = 192$ nm.

copeas (Ph. Eur. III, DAB 10), grown under different soil water and nitrogen regimes. Moreover, because hyoscyamine presents about 70% of the total alkaloids of the drug plant we assume that the proportions of individual minor alkaloids in the total alkaloid content did not play an essential role in assessment of the impact of water stress and nitrogen fertilization on alkaloid production in the roots of *A. belladonna*.

The simplified/shortened and rapid extraction procedure, as used in this study, proved to be suitable for CE analysis of hyoscyamine and scopolamine in roots of deadly nightshade. In our preliminary study, when extraction was done according to the extraction protocol for HPLC quantitative determination of tropane alkaloids (Kamada et al., 1986), a loss of alkaloids was registered.

Hyoscyamine and scopolamine were well separated from each other (resolution >4.1) by CE under the conditions used in the study and the variability of the signal ($RSD_{peak\ area}$, $RSD_{peak\ height}$ and $RSD_{retention\ time}$) was estimated to be less than 2%.

The results of the study showed that the hyoscyamine or scopolamine contents in roots of *A. belladonna* depended on soil water and nitrogen availability, the hyoscyamine content ranging from 0.19 to 0.41% dw and scopolamine ranging from 0.034 to 0.070% dw. The concentration range of hyoscyamine is consistent with previous reports by Kamada et al. (1986), Hartmann et al. (1986), Aoki et al. (1997), while scopolamine was present in roots in higher amounts than reported by Geßner (1953), Hartmann et al. (1986). The plants in our study were harvested in the developmental stage, where a high alkaloid content of plants was expected (approximately 130 days after the appearance of the first flower). This might be the reason why the scopolamine contents in our study are higher than that found by Geßner (1953), Hartmann et al. (1986), in which no harvest time was indicated.

The reverse relationship between root biomass and alkaloid accumulation in response to water stress at high N level observed in our study was consistent with observations of Saker et al. (1997). They found that external constraints, which limit the rate of dry matter production, may elicit the secondary metabolism in *Hyosciamus muticus*, *A. belladona* and *Datura stramonium* suspension cultures.

The phenomenon of the influence of an excess of nitrogen fertilizers at very low soil water availability on the raised accumulation of hyoscyamine and scopolamine can be explained by the nitrogen turnover and by the response in stressed plants. It is well known that excess nitrate nitrogen in soils, deriving from intensive nitrogen fertilization, leads to an increase of nitrate in plants, a soluble form of nitrogen, that can be further incorporated in N-containing secondary metabolites in intact plants. Moreover, plants exposed to stress growth conditions (all factors which obstruct normal biochemical processes in plant metabolism) accelerate the nitrate accumulation in plant tissue and slow down the protein synthesis (Larcher, 1995; Maticic, 1997). It is possible, that alkaloid plants under these conditions shift the metabolism towards accelerated synthesis of nontoxic secondary products—alkaloids, which might represent the form of nitrogen storage in stressed plants (Nowacki et al., 1976; Bernath, 1986; Saker et al., 1997). Some experimental evidence exists for the increase in tropane alkaloid level in *Hyoscyamus niger* or in *H. muticus* (Strauss, 1988), when plants suffered from various stress conditions (infection, treatment with insecticides). Similar results were reported by Saker et al. (1997), who found that osmotic stress enchanced the tropane alkaloid content in suspension cultures of *Hyoscyamus*, *Datura* and *Atropa* spp.

Thus, the increased content of hyoscyamine and scopolamine in the case of high nitrogen fertilization at high water deficit might represent a stress response by *A. belladonna* plants. Further studies are needed to verify these assumptions.

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