Seed Alkaloids Content and Antioxidant Enzymes Activity in Black Henbane as Influenced by Ammonium Nitrate Application and Water Deficit Stress

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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.

Objective: This study intended to investigate the nitrogen (N) fertilization and water deficit stress (WDS) effects on seed tropane alkaloids elicitation including hyoscyamine (HYO) and scopolamine (SCO), and also antioxidant enzymes activities variations including superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) in black henbane.

Methods: Plants were treated with different nitrogen (0, 0.14, 0.28 and 0.56 g N pot^{-1} as ammonium nitrate, N0-N3, respectively) and WDS treatments (30, 60 and 90% depletion of water from field capacity, W1-W3). Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis.

Results: Results showed that the highest alkaloid content values in seeds (HYO: 0.145% dw; and SCO: 0.271% dw) achieved in plants grown under sever (W3) and moderate (W2) WDS accompanied with nitrogen supply of 0.28 g N pot^{-1} (N2), respectively. The maximum and minimum (2.112 and 0.114 $g.plant^{-1}$) total alkaloids yield were obtained in N3W1 and N3W3 treatments, respectively. Furthermore, SOD activity increased with increasing N fertilization in all WDS treatments. CAT activity increased with WDS up to 60% FC, and then decreased with WDS increase. The POX activity was the opposite to that of CAT activity with N application under WDS levels.

Conclusion: Our results suggest that N in appropriate level may act as a modifier for seed physiological responses and as an elicitor for tropane alkaloids biosynthesis pathway in black henbane (*hyoscyamus niger*) plants.

Keywords: ***Hyoscyamus niger*, Alkaloids, Antioxidant enzymes, Seed, Nitrogen, Water stress.**

Introduction

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds found in about 20% of plant species. Solanaceous plants are regarded as rich sources of alkaloids, namely the pharmaceutical by interesting tropane derivatives. Tropane alkaloids, especially hyoscyamine (HYO) and scopolamine (SCO) are widely used in medicine for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties [1]. SCO, which is the 6,7-epoxide of HYO, is the most valued of the two tropane alkaloids (due to fewer side effects on nervous system), its worldwide demand being 10 times higher than that for HYO and its racemic form, atropine [2]. The synthetic production of these alkaloids is more expensive than their extraction from plant materials and they are, therefore, currently industrially extracted from various solanaceous plants belonging to the genera *Atropa*, *Duboisia*, *Datura*, *Scopolia* and *Hyoscyamus*.

Black henbane (*Hyoscyamus niger*) has a very long history of use as a medicinal plant. A cosmopolitan, strong-scented annual or biennial herb, which all it's parts (root, leaf, and seed) contain tropane alkaloids such as HYO and SCO [3]. These metabolites are synthesized in roots and then transported to the aerial parts of the plant [4].

Water deficit stress (WDS) is one among the several environmental stresses causing drastic changes in the growth, physiology and metabolism of plants. Although, drought stress negatively affect the growth and development of field crops, the contents of secondary metabolites are mostly increased through the positive effects on the metabolic pathways of active compounds synthesis in medicinal plants [5]. This type of abiotic stress, affect the plant water status at cellular and whole plant level causing specific as well as unspecific reactions and damages [6]. Environmental stresses impair electron transport system leading to the generation of reactive oxygen species (ROS) such as H_2O_2, O^{-2} and OH^- causes rapid cell damage [7]. These compounds initiate damage in the chloroplast and cause a cascade of damaging effect including chlorophyll destruction [8]. Also, root meristem activity is very sensitive to ROSs; these compounds were accumulated in the differentiation zone, where root hairs grow [9]. In plants metabolism of ROSs is kept in dynamic balance. Under water deficit stress, the balance is broken and antioxidant systems such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) along with the antioxidant ascorbic acid and glutathione are needed to scavenge the reactive free radicals [10]. Agrochemical factors, such as fertilizers influence the alkaloid content in lupin seeds, e.g. the content of alkaloids is significantly increased by 9–17 % by the application of nitrogenous fertilizers without magnesium [11]. The same was found in L. albus L. in which the protein, alkaloid and a-galactoside content in seeds is strongly influenced by the nitrogen fertilizer used, e.g. the cultivar 'Butan' got the highest total alkaloid content when nitrogen was supplied as NO_3) [12]. The content of alkaloids in plants could be increased through genetic and or environmental manipulations. 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 as well as

non-medicinal plants, such as, tobacco, lupines, barley, *Datura*, *Atropa*, *Papaver* [13]. However, not much information is available on the effect of N fertilization with water deficit stress on the content of leaf and root alkaloids in *Hyoscyamus niger*. Low or appropriate nitrogen supply might alleviate drought stress by altering plant adaptation strategies in dry conditions [14]. This study focuses on the effects of N fertilizer in combination with WDS on *Hyoscyamus niger* seed weight, seed antioxidant enzymes (SOD, POX, CAT) activity and tropane alkaloids (HYO, SCO) variatinos.

Materials and Methods

Plant growth conditions and treatments

The experiment was conducted in greenhouse conditions. During the experiment the temperature ranged from 25 °C to 28 °C, and the relative humidity was between 65% and 70%. Henbane seeds generally have low germination rate under normal laboratory conditions. Therefore, seeds were treated with 250 mg.l gibberellic acid (GA_3) for 48 h at room temperature (25 ± 0.5 °C) for breaking dormancy and accelerating germination. After that seeds were surface-sterilized in 70% ethanol for 2 min and then in 25% commercial bleach (containing 6% sodium hypochlorite) for 10 min and finally rinsed with sterile distilled water. Subsequently, seeds were placed in petri dishes on two layers of filter paper (Whatman No.1) moistened with 4 ml distilled water. After 3 days, 90% of seeds germinated steadily. After germination, the seedlings (when they had three true leaves) individual healthy, uniform plants were transplanted into experimental pots (25 cm diameter and 30 cm deep, containing 8 kg soil). The physical and chemical composition of the soil was as follows: 58.4% sand, 18.8% silt, 22.8 % clay; total available N; 0.12%, P; 8.14 ppm, K; 175 ppm. The study was set up as randomized complete block design (RCBD) with factorial treatments of three watering frequencies (30, 60, and 90% depletion of FC) and four nitrogen levels as 0 kg N ha^{-1} (N0, control), 75 kg N ha^{-1} (N1), 150 kg N ha^{-1} (N2) and 225 kg N ha^{-1} (N3), equivalent to 0, 0.14, 0.28 and 0.56 g N pot^{-1}, respectively. Nitrogen fertilization was split into three equal applications as ammonium nitrate (33% N) and applied as solution before planting, at the 25 and 50 days after planting. Plants were subjected to WDS treatments from 50 days after planting for 45 days (i.e. from beginning of plant flowering to the full seed ripping stage). The field capacity (FC) of soil was determined 12% using the pressure plate apparatus. The pot surface was covered by sterile plastic pot beads to minimize evaporation and avoid contamination. Estimation of water depletion levels was done through weighing pots daily (morning hours). Monitoring of soil moisture levels was conducted using gravimetric method. All pots were kept to the field capacity up to 50 days after planting. After 45 days of WDS induction, all pots were harvested at seed maturity stage. Then seeds were separated from capsules and weighted with a precision of 0.0001 g scale. Then seeds were finely powdered in an electronic blender for enzymes assays and alkaloids extraction.

Antioxidant enzymes assays

A crude enzyme extract was prepared by homogenizing 0.5 gram of powdered seeds in extraction buffer containing 0.5% Triton X-100 and 1% polyvinyl pyrrolidone in 100 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate

was centrifuged and the supernatant was used for the following enzyme assays.

Superoxide dismutase (SOD, EC 1.15.1.1)

SOD activity was determined according to Beauchamp and Fridovich [15]. The reaction mixture contained 1.17×10^{-6} mol.L^{-1} riboflavin, 0.1 mol.L^{-1} methionine, 2×10^{-5} mol.L^{-1} KCN and 5.6×10^{-5} mol.L^{-1} nitroblue tetrazolium (NBT) salt dissolved in 3 ml of 0.05 mol.L^{-1} sodium phosphate buffer (pH 7.8). 3 ml of the reaction medium was added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes in a single row. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity is expressed in U mg^{-1} protein. (U = change in 0.1 absorbance h^{-1} mg^{-1} protein under assay conditions).

Catalase (CAT, EC 1.11.1.6)

CAT activity was assayed according to the method of Chandlee and Scandalios [16]. The assay mixture contained 2.6 ml of 50 mmol.L^{-1} potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mmol.L^{-1} H_2O_2 and 0.04 ml of enzyme extract. Changes in the absorbance were read at 240 nm. The enzyme activity was expressed in U mg^{-1} protein (U=1mM of H_2O_2 reduction min^{-1} mg^{-1} protein). The enzyme protein was estimated by the method of Bradford [17].

Peroxidase (POX, EC 1.11.1.7)

POX activity was determined by the method of Kumar and Khan [18]. Assay mixture of POX contained 2 ml of 0.1 mol.L^{-1} phosphate buffer (pH 6.8), 1 ml of 0.01mol.L^{-1} pyrogallol, 1 ml of 0.005 mol.L^{-1} H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5 mol.L^{-1} H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 mol.L^{-1} H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One U is defined as the change in the absorbance 0.1 min^{-1} mg^{-1} protein.

Alkaloid extraction

Seed samples were air dried, grinded into fine powder and sieved with laboratory mesh (size 30, mesh opening 545 µm). A subsample of one gram from each samples was added to appropriate volume of $CHCl_3$: MeOH: NH_4OH 25%, (15:5: 1), sonicated for 20 min and then kept at water bath (40◦C) for one hour. Subsequent sample preparation and alkaloids extraction were based essentially on the method described by Kamada [19].

Alkaloid analysis and quantification

Alkaloids extracted were identified by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis according to Drager [20]. GC analysis was performed using a GC system equipped with a flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Injector and detector temperatures were set at 220 and 290 °C, respectively. The column temperature was initially kept at 50 °C for 5 min, then gradually increased to 300 ◦C at a rate of 3 ºC/min and maintained for 3 min. The flow rate of gas helium was 0.8 mL/min. Then 1 µL of extract was directly injected into the gas chromatograph. Each extraction was replicated three times and the compound percentages are the means of the three replicates. GC-MS analysis was carried out on an Agilent 6890 gas chromatograph (Agilent Technologies,

Palo Alto, USA) fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). Oven temperature was programmed from 50ºC to 285 ºC at 3 ºC/min, and helium was used as carrier gas (0.8 mL/min), Mass spectra were obtained in an Agilent 5973 system operating in electron impact mode (EIMS) at 70 eV, coupled to an GC system. The identification of alkaloids was based on the comparison of their GC retention time and mass spectra (MS) data with their standards substances (HYO. HCl and SCO. HBr, Merck). The total tropane alkaloids (HYO + SCO) yield was quantified by both alkaloid content and seed dry weight; Total alkaloid yield (g.plant^{-1}) = Alkaloid content (% d.w) × Plant dry weight (g.plant^{-1}).

Statistical analysis

All analyses were performed based on a randomized complete block design (RCBD) with factorial experiment. The data were subjected to ANOVA and were analyzed by using SAS and MSTAT-C program, and probabilities of significance were used to test for significance among treatments and interactions, and the Duncan's Multiple Range Test ($p \leq 0.05$) was used to compare means. Values obtained were expressed as mean ± SD (standard deviation) from three replications (n=3) of each treatment.

Results

Seed weight and seed antioxidant enzymes activity

Analysis of variance of data showed that the measured traits have been significantly ($p \leq 001$) affected by WDS and N fertilization levels. Mean comparison of data revealed that irrespective of N fertilization level, increasing WDS significantly diminished the seed weight. Application of N fertilization significantly enhanced seed dry weight. In low WDS conditions (W1, 30% FC) seed weight increased with increasing N fertilization. However, there was no significant difference between N2 and N3 levels (Table 1). Seed weight in both medium (W2) and sever (W3) WDS conditions increased with application of N fertilization up to N2 level, and then decreased with N fertilization increase. On the other hand, the highest seed weight per plant was related to 30% FC with 0.56 g N pot^{-1}. Application of high N with high WDS reduced seed weight per plant probably because of competition for use of water content in soil. Low or appropriate N supply might alleviate drought stress by altering plant adaptation strategies in dry conditions.

Table 1- Mean values for seed weight, seed alkaloids (HYO and SCO) content and yield (mean ± S.D., n=3) of black henbane treated with nitrogen fertilizer at different water deficit stress levels

Treatment	Seed weight (g. plant^{-1})	Seed alkaloids content (%dw)		Seed alkaloids yield (g.plant^{-1})		Total alkaloids yield (g.plant^{-1})
		HYO	SCO	HYO	SCO	
N0 W1	3.212±0.26	0.075 ± 0.012	0.177±0.007	0.240±0.015	0.568±0.022	0.808±0.042
N1 W1	4.082±0.19	0.112±0.004	0.226±0.003	0.457±0.011	0.922±0.014	1.379±0.015
N2 W1	5.563±0.21	0.119±0.004	0.239±0.004	0.661±0.016	1.329±0.012	1.990±0.016
N3 W1	5.634±0.25	0.124±0.003	0.251±0.004	0.698±0.014	1.414±0.013	2.112±0.013
N0 W2	2.956±0.22	0.081±0.014	0.182±0.012	0.239±0.011	0.537±0.014	0.776±0.019

Table 1- Continued

Treatment	Seed weight (g. plant^{-1})	Seed alkaloids content (%dw)		Seed alkaloids yield (g.plant^{-1})		Total alkaloids yield (g.plant^{-1})
		HYO	SCO	HYO	SCO	
N1 W2	3.431±0.17	0.113±0.003	0.262±0.006	0.387±0.009	0.898±0.009	1.285±0.023
N2 W2	4.843±0.25	0.128±0.006	0.271±0.008	0.619±0.016	1.312±0.008	1.931±0.016
N3 W2	2.291±0.19	0.135±0.008	0.258±0.011	0.309±0.017	0.591±0.017	0.900±0.017
N0 W3	1.124±0.28	0.097±0.002	0.101±0.005	0.109±0.012	0.113±0.013	0.222±0.011
N1 W3	2.291±0.18	0.128±0.009	0.208±0.007	0.293±0.021	0.476±0.015	0.769±0.026
N2 W3	3.431±0.26	0.145±0.006	0.229±0.009	0.497±0.019	0.785±0.021	1.282±0.019
N3 W3	0.431±0.23	0.105±0.007	0.161±0.005	0.045±0.011	0.069±0.024	0.114±0.014

W1, W2 and W3: water deficit stress at 30, 60 and 90 % of water depletion from field capacity, respectively. N0, N1, N2 and N3: 0, 0.14, 0.28 and 0.56 g N pot^{-1}respectively.

There were differences in antioxidant enzymes activities among the treatments. SOD activity increased with WDS, and N fertilizer application played a significant role in adjusting the enzyme activity (Figure 1). SOD activity increased with increasing N fertilization in all WDS conditions. However, in severe WDS conditions N fertilization up to N2 level increased SOD activity. On the other hand, SOD activity decreased with the highest N supply under severe WDS treatment.

CAT activity increased with WDS up to 60% FC, and then decreased with WDS increase (Figure 2). Application of N fertilization enhanced CAT activity up to N2 level. The maximum CAT activity was observed in N2W2 treatment. With regard to the effects of N on adjusting CAT activity, low (N1) and medium (N2) N application significantly ($p \leq 0.01$) increased CAT activity at all WDS conditions.

Similar to CAT activity, POX activity increased under employed WDS up to 60% FC, and then decreased with WDS intensity (Figure 3). The POX activity was the opposite to that of CAT activity with N application under WDS levels. The high application of N significantly ($p \leq 001$) increased POX activity only at low WDS conditions, but at severe WDS the minimum value of N application showed the maximum rate of POX activity. It seems that in different treatments of N fertilization and WDS different antioxidant enzymes act as ROS scavenger. N is necessary for protein synthesis and the structure of antioxidant enzymes are protein.

Seed alkaloids production

The results of the ANOVA showed that the interaction of WDS and N fertilization affected the seed HYO and SCO content of black henbane plants ($p \leq 001$).

At low WDS (30% FC), very high N fertilization (N3) resulted in a high HYO and SCO content (0.124 and 0.251 % dw, respectively). By contrast, a moderate N fertilization (N2) at moderate WDS (60% FC) influenced the high SCO content of seeds.

The maximal content of HYO (0.145 % dw) in seeds were observed under moderate WDS conditions (W2) at a N level of 0.56 g.pot^{-1} (N3).

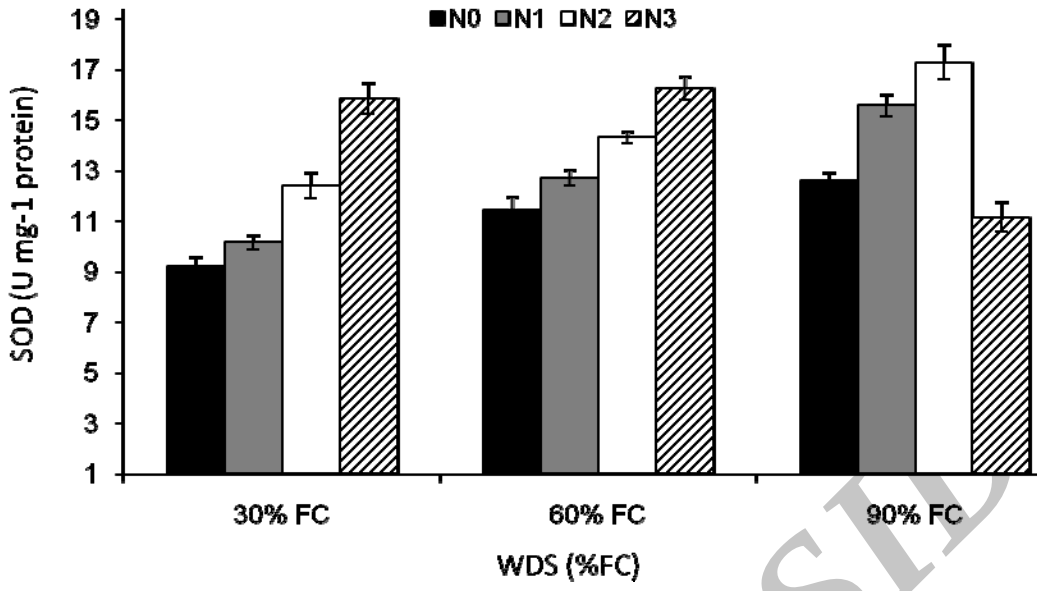

Figure 1- Effects of water deficit stress (WDS, 30, 60 and 90 % of water depletion from field capacity) and nitrogen (N0, N1, N2 and N3: 0, 0.14, 0.28 and 0.56 g N pot^{-1}, respectively) levels on superoxide dismutase (SOD) activity of black henbane seeds. Values are given as mean ± S.D., (n=3)

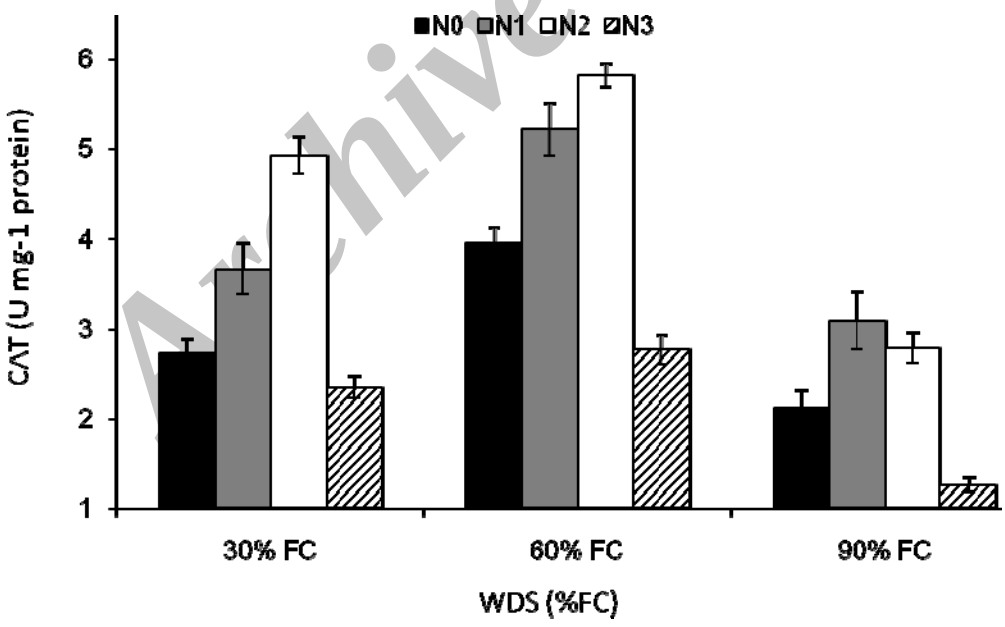

Figure 2- Effects of water deficit stress (WDS, 30, 60 and 90 % of water depletion from field capacity) and nitrogen (N0, N1, N2 and N3: 0, 0.14, 0.28 and 0.56 g N pot^{-1}, respectively) levels on catalase (CAT) activity of black henbane seeds. Values are given as mean ± S.D., (n=3)

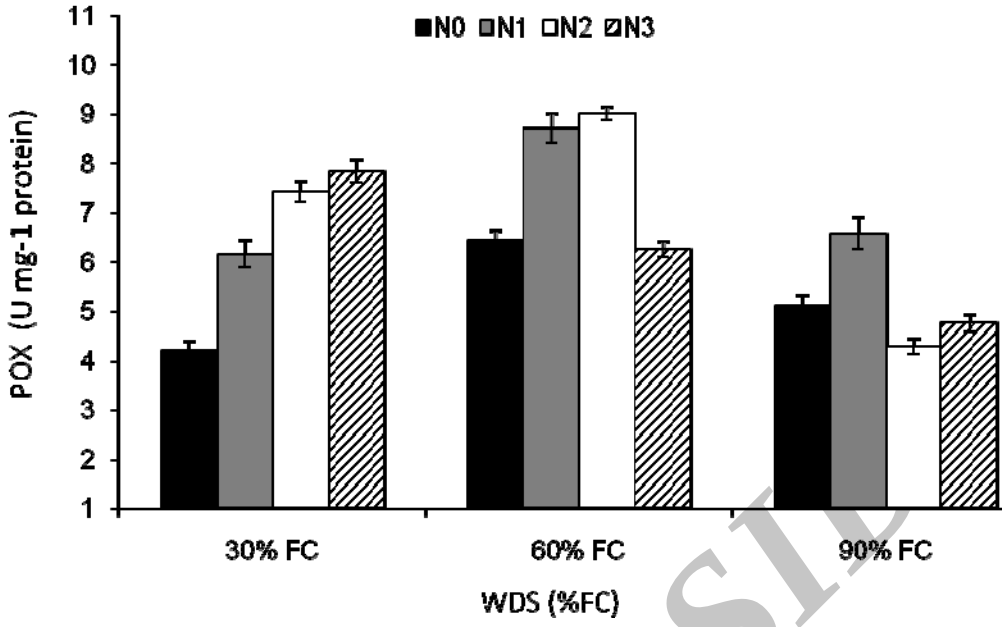

Figure 3- Effects of water deficit stress (WDS, 30, 60 and 90 % of water depletion from field capacity) and nitrogen (N0, N1, N2 and N3: 0, 0.14, 0.28 and 0.56 g N pot^{-1}, respectively) levels on peroxidase (POX) activity of black henbane seeds. Values are given as mean ± S.D., (n=3)

Seed HYO yield black henbane plants was decreased with the increase in WDS as described in table 1. N fertilizer at all levels significantly increased seed HYO and SCO yield under W1 conditions. These parameters were also increased up to N2 level under W2 and W3 conditions. On the other hand, the minimum seed HYO and SCO yield was recorded with application of the highest N value under W3 conditions.

The largest total alkaloids (HYO+ SCO) yield (2.112 g.plant^{-1}) per plant showed in high N and high water availability conditions (N3W1) mainly because of less seed dry weight reduction under this situation in comparison with the other treatments (Table 1).

Discussion

WDS is known to increase O^{-2} production in plants. SOD is an enzyme that catalyzes the conversion of the O^{-2} to O_2 and H_2O_2. Enhanced SOD activity of seeds under WDS conditions for N treated plants may be interpreted as a direct response to augmented O^{-2} generation.

CAT, which is localized in peroxisomes, dismutates H_2O_2 into H_2O and O_2 [21]. According to our results, CAT activities decreased under severe WDS treatment. The decline in CAT activity is regarded as a common response to many stresses. The reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It may also be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme [22].

POX showed an increase in activity in response to WDS and N application in seeds of black henbane plants. This enzyme is involved in scavenging of H_2O_2, growth and

developmental processes [23]. It is suggested that the overexpression of SOD, if accompanied by enhanced H_2O_2 scavenging mechanisms like CAT and POX activities, has been considered as an important anti-drought mechanism to cope with oxidative stress during WDS conditions [24].

The content of alkaloids in plants could be increased through genetic and or environmental manipulations. Since alkaloids are nitrogenous compounds, the availability of nitrogen 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 as well as non-medicinal plants, such as, tobacco, lupines, barley, *Datura*, *Atropa*, *Papaver* [13]. However, not much information is available on the effect of N fertilization with WDS on the content of seed alkaloids in *Hyoscyamus niger* plants.

Supply of N from beginning is essential for optimal alkaloid formation. The critical period of N application is 40 days after germination [25]. Nitrogen availability commonly regulates biomass production as well as alkaloid synthesis. Many studies have noticed that increased N application could improve water-use efficiency, alleviate drought stress effects on plant growth by preventing cell membrane damage and enhancing osmoregulation [14]. It was clear that additional amounts of N did not always play a positive role in alleviating the adverse effects of drought on plant growth [26]. Nitrogen fertilizer increased dry matter, grain production and water use efficiency of wheat under drought stress, and the effect was due to increased leaf area index and the maintenance of leaf area duration [27]. Unexpectedly, scopolamine was found as a main alkaloid in the seeds of black henbane plants. The biosynthesis of tropane alkaloids is often tissue specific, occurs in most plants in their roots, and the produced alkaloids are exported from roots to other organs [28]. HYO and SCO, derived from phenylalanine, ornithine and arginine, are synthesized almost exclusively in the plant roots. SCO is synthesized from HYO via 6b-hydroxyhyoscyamine. The hyoscyamine 6b-hydroxylase catalyzes the two-step epoxidation of HYO to SCO (Figure 4). In general HYO and SCO are synthesized in the pericycle of branch roots and accumulated in the aerial plants [29]. The phenomenon of the influence of an excess of N fertilizers at very low soil water availability on the raised accumulation of HYO and SCO can be explained by the N 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, which can be further incorporated in N-containing secondary metabolites in intact plants. Moreover, plants exposed to stress growth conditions accelerate the nitrate accumulation in plant tissue and slow down the protein synthesis. It is possible, that alkaloid plants under these conditions shift the metabolism towards accelerated synthesis of alkaloids, which might represent the form of nitrogen storage in stressed plants. Some experimental evidence exists for the increase in tropane alkaloid level in *Hyoscyamus niger* or in *H. muticus* [30], when plants suffered from various stress conditions. Since epoxilation rates of HYO decline as plants develop, the increased HYO to SCO ratio associated with higher plant N content could result from an influence of N on plant development. The N content may also directly affect the epoxydation of HYO to SCO.

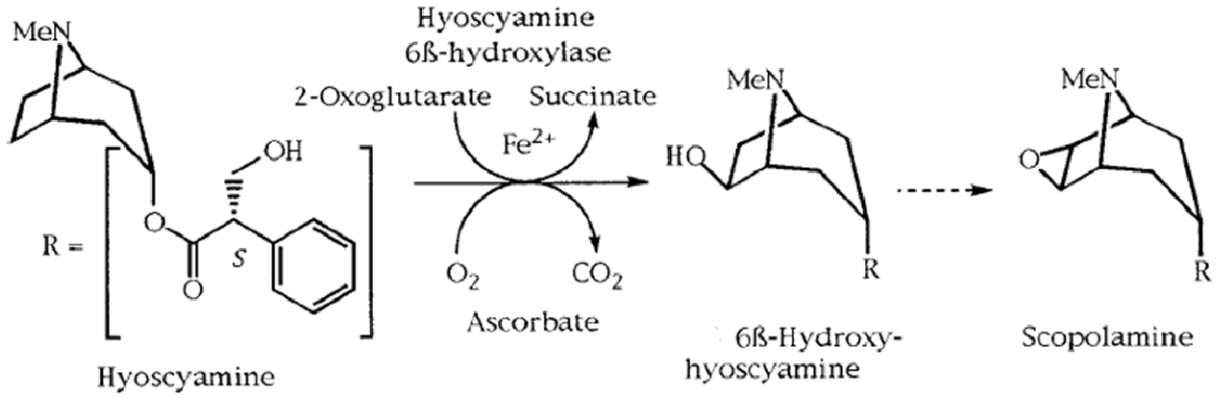

Figure 4- The final step in the pathway for the biosynthesis of scopolamine from hyoscyamine

Though not incorporated into SCO, molecular oxygen (O_2) is required for the conversion HYO to SCO. Net O_2 release is determined by the activity of Rubisco carboxylase and oxygenase and a positive effect of N on Rubisco oxygenase has been reported previously. Also, rates of dark respiration have been demonstrated to increase when N levels are increased. Conceptually, a higher N level in the growing medium could thus reduce the quantity of O_2 needed for SCO production by increasing respiration [31].

Conclusion

Our results suggest that N in appropriate level may act as a modifier for seed physiological responses and as an elicitor for tropane alkaloid biosynthesis pathway in *Hyoscyamus niger* plants. In addition, moderate WDS (30% of water depletion from FC) showed enhancement on the production of seed SCO content. Also, SCO was found as a main alkaloid in *H. niger* seeds.

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