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Ornithine Induced the Hyoscyamine Production more than Arginine Precursor in *Hyoscyamus muticus* L. *In vitro*

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IMPACT of different concentrations (0.25, 0.5 and 1mmol) of both alkaloids precursors, arginine (Arg) and ornithine (Orn) on production of hyoscyamine and its physiological attributes in 30 days old-callus of Egyptian henbane (*Hyoscyamus muticus* L.) was studied. Data of HPTLC revealed that *in vivo* wild leaves had high concentration of hyoscyamine but callus treated with 0.25mmol of Orn had more 3.2 and 1.4 time of total alkaloids and hyoscyamine than control, respectively. The same level of Orn induced the highest concentration of glutathione and reduced the H_2O_2 to the lowest rate. Although callus treated with 0.25mmol of Orn had only 6 visualized protein bands separated with SDS-PAGE, protein bands with low molecular weight 38, 33, 21, 15 and 9kDa were highly expressed. Callus cells exposed to low level of Orn had irregular amoeboid shape and lysiogenous intercellular spaces compared to regular ovate and schizogenous intercellular spaces in control. However, fresh and dry weight were increased under all levels of both precursors compared to control but water content % was maintained. Photosynthetic pigments and total carbohydrates were increased with increment of both Orn or Arg levels then decreased under high levels of each. Application of Orn at any levels decreased the content of free amino acids in contrary to Arg. Arg with high level induced the highest concentration of free phenolics and the activity of peroxidase. It was concluded that, addition of Orn with low concentration (0.25mmol) was efficient for hyoscyamine enhancement from Egyptian henbane callus than Arg.

Keywords: Alkaloids precursors, Biochemical compounds, Callus, Egyptian henbane, HPTLC, SDS-PAGE.

Inrtoduction

Hyoscyamineis a tropane alkaloid, synthesized in Solanaecous plants such as Egyptian henbane (*Hyoscyamus mutics* L.) which is a wild plants of Egypt, especially in Saint Catherine peninsula (Täckholm, 1974). Tropane alkaloids-containing plants have been used for medicinal purposes due to their powerful anticholinergic (as scopolamine) and hallucinogenic effects (as hyoscyamine and atropine), causing constipation, photophobia, pupil dilatation, vision disturbance and dryness of upper digestive and respiratory tract mucosa (Koleva et al., 2012).

The concentrations of tropane alkaloids were significantly varied among wild plants, *in vitro* plants and callus or root cultures. In this respect, the highest content of hyoscyamine and tropane was detected in callus, but the highest content of scopolamine was found in the wild plants (Besher et al., 2014).

Biological precursors of most alkaloids are amino acids, such as ornithine (Orn), arginine (Arg) and phenylalanine (Phe). Hyoscyamineis biosynthesized from Orn via Orn decarboxylase or Arg by Arg decarboxylase with synthesis of putrescine (polyamines) as immediate compound.

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The latter finally transformed to Tropine which condensed with phenyllactic acid (from Phe) to produce hyoscyamine (Facchini, 2001).

Amino acids precursors had different effects on secondary metabolites productionin different plants according to its type and concentration. In this respect, 300mg L^{-1} of each proteinaceous amino acids, L-glutamine or L-typtophane increased the total indole alkaloids (vinblastine and vincristine) compared with other amino acids (L-asparagine; L-cystine and L-arginine) in cell cultures of *Catharanthus roseus* (Taha et al., 2009). Also, glutamine and phenylalanine at 1 and 3mg L^{-1}, respectively enhanced the concentration of flavonoid in *Hydrocotyle bonariensis* callus butproline at 4mg L^{-1} had non-significant effect on its concentration (Masoumian et al., 2011). The expression of phenylalanine ammonia lyase (*PAL*) and stilbene synthase (*STS*) genes was increased after application of phenylalanine which enhanced the production of resveratrol by 8.5 times in *Vitis amurensis* cell cultures (Kiselev et al., 2013). The application of leucine increased the asiaticoside-triterpenes in leaf-derived callus and cell suspension cultures of *Centella asiatica* (Kiong et al., 2005). Shoot cultures fed with 2mM of isoleucine induced the hyperforin by 3-7-fold in *Hypericum perforatum* as reported by Karppinen et al. (2007). Moreover, Aljibouri et al. (2012) found that addition of 50 or 100ppm of proline to callus culture of *Hyoscyamus niger* increased the hyoscyamine concentration by 58.03 and 21.37%, respectively compared with the control. Also, scopolamine concentration was increased by 205.51 and 149.20%, respectively compared with the control. In contrary, phenylalanine, leucine and valine at 50, 100 and 150mg L^{-1} had negative effect on both callus fresh weight and callus dry weight of *Zingiber officinale* compared to control (El-Nabarawy et al., 2015).

Detailed understanding of alkaloid biosynthesis and mechanisms of action is essential to improve production of alkaloids of interest, or to discover new bioactive molecules and to sustainably exploit them against targets of interest, such as herbivores, pathogens, cancer cells, or unwanted physiological conditions (Matsuura & Fett-Neto,, 2017). Also, which effective in alkaloids production, proteinaceous amino acid such as Argor non-protein amino acid as Orn still an important question. Therefore, this work aimed to view the effect of different concentrations (0.25, 0.5 and 1mmol) of both Orn and Arg on alkaloids and hyoscyamine in wild Egyptian henbane callus and investigate the physiological and histological parameters participation during this stage.

Materials and Methods

Seeds preparation and in vitro cultivation

Wild seeds of *Hyoscyamus muticus* L. were collected from Saint Catherine peninsula and identified according to Täckholm (1974). Seeds were dipped in 250mg L^{-1} of gibberellic acid (GA3) for 24h at lab temperature (25±0.5˚C) to break dormancy (Alaghemand et al., 2013). Seeds were washed with tap water and surface-sterilized with 70% ethanol for 2min, then immersed in 25% commercial bleach (containing 5.25% sodium hypochlorite) with a drops of tween-80 for 20min and finally rinsed 3 times with double distilled sterilized water. The sterilized seeds were cultured in hormone-free MS medium (Murashige and Skoog), supplemented with 7g L^{-1} agar and 15g L^{-1} sucrose and maintained at 25±1°C under light condition (1, 500 lux, 16h/day) for germination. After germination, plantlets have been obtained for explants preparation.

Explant culture and callus induction

Shoot tip (18 days-old) were excised and cultured in MS media supplemented with different concentrations of benzyl amino purine (BAP) (0.5mg L^{-1}) and naphthalene acetic acid (NAA) (0.5, 1 and 2mg L^{-1}), pH (5.7-5.8) for callus induction with 10 replicate. The cultures were incubated at (25±1°C) with (16/8) light/dark cycle. After 4 weeks more suitable callus were selected for further work.

Precursors treatments

After four weeks, callus were removed from vessels under aseptic condition and cut a suitable weight (500mg) and subculture on the same medium for further proliferation. Arginine and Ornithine as a hyoscyamine precursor were added to the medium at level of 0.25, 0.5 and 1mmol. Callus cultures were incubated at 25±1°C under light condition (1, 500 lux, 16h/day). After 30 days treatment callus were harvested and washed with distilled water to remove remains agar and dried by filter paper.

Vegetative measurements

Forty pieces of callus fresh weight (FW) and dry weight (DW) after drying at 70°C until

constant weight was recorded. Water content (WC) of callus was calculated using FW and DW values according to Henson et al. (1981).

Biochemical determinations

Photosynthetic pigments (mg 100 g^{-1} FW) incallus (chl. a, b and carotenoids) were determined spectro photometrically at 662, 644 and 440.5nm (Lichenthaler & Wellburn, 1983). Anthocyanins concentration (mg g^{-1} FW) was determined after digesting callus with 1% of HCl (v/v) in methanol and kept overnight at 4ºC (Lange et al., 1971). Then centrifuged at 3000rpm for 10min at 5°C. The anthocyaninvalues are calculated as E_{535}- 0.25 (E_{650}) /g FW. Total carbohydrates (mg g^{-1}DW) were determined after digestion by 5ml of 2.5N HCl for 3h at 100ºC. Then neutralized with sodium carbonate. Sample was made to volume of 10ml and then filtrated. 50µl was diluted to 1ml with distilled water. To each tube, 1ml of 5% phenol and 5ml of 96% H_2SO_4 were added. After 10min, tubes were shaken at 25-30^{0}C for 20min. Optical density was measured at 490nm as reported by Hedge & Hofreiter (1962). Total protein (mg/g FW) was determined by Bradford method (Bradford, 1976) at 595nm. Proline as mg g^{-1} FW was estimated with ninhydrin reagent as described by Bates et al. (1973), the red color intensity was measured at 520nm against the toluene blank. Total glutathione (µmol/mg protein) was determined at 412nm by 5-5'-Dithiobis (2-nitrobenzoic acid) (DTNB) reagent (Griffith, 1980). For determination of total free amino acids and free phenols alcohol extraction of callus was prepared as Abdel-Rahman et al. (1975). Free phenolics (mg g^{-1} FW) were determined by a modified Folin-Ciocalteu method and measured at 650nm according to Horwitz et al. (1970). Total free amino acids (mg g^{-1} FW) was estimated using the method of Rosen (1957) with ninhydrin reagent. The blue colored were measured against blank sample at 570nm. Hydrogen peroxide (mmol/g FW) was determined at 390nm by the modified method according to Shi et al. (2007) from Sun et al. (2007). Malondialdehyde (µmol.g^{-1} FW) determined by the thiobarbituric acid (TBA) reaction as described by Gallego et al. (1996) from Heath & Packer (1968). All spectrophotometric analyses were done using UV/VIS spectrophotometer, PG instrument Ltd, USA.

Antioxidant enzymes activity

Enzymes extract was prepared according to Urbanek et al. (1991). Catalase (CAT, E.C.:1.11.1.6) activity was determined by measuring the oxidation of H_2O_2 at 240nm (Urbanek et al., 1991). The unit of CAT activity was defined as the amount of enzyme, which decomposes 1mM H_2O_2 per mg^{-1} protein. minute. Peroxidase (POD, E.C.: 1.11.1.7) activity estimated with 0.1% O-dianisidine and 0.2M hydrogen peroxide at 430nm (Urbanek et al., 1991). One unit of peroxidase activity was taken as the change of 1.0 unit of optical density per mg^{-1} protein.minute. Superoxide dismutase (SOD, E.C.: 1.15.1.1) activity was assayed by measuring its ability to inhibit reduction of nitro blue tetrazolium at 560nm as described with (Beauchamp & Fridovich, 1971). One unit of enzyme activity represents the amount of enzyme required for 50% inhibition of NBT reduction.

SDS-PAGE of soluble proteins

One dimensional SDS-PAGE gel electrophoresis based on the method of Laemmli (1970) was used to fractionate the soluble proteins in callus. Twenty milligrams of callus were dispersed in 1ml SDS 10% with 100µl β-mercaptoethanol for 15min, then centrifuged at 11000rpm for 10min. Twenty µl of extraction were mixed with 20µl of SDS-loading sample buffer (SDS 4%, β-mercaptoethanol 3%, glycerol 20%, TrisHCl 50mM pH 6.8 and bromophenol blue traces), heated at 96°C for 3min and 10µl aliquot was electrophoresed (10µl of protein/ lane). The resolving and stacking gels were prepared according to the standard procedure of Davis (1964). The electrode buffer contained 50mM TRIS, glycine 0.384M and SDS 0.1%. The protein bands were developed with Commassie Brilliant Blue R-250 dye (0.2% solution, freshly prepared in 45% methanol, 10% glacial acetic acid and 45% distilled water) at room temperature overnight. The gel was photographed and made by scan apparatus as densitometric (optical density) analysis at 600nm using standard maker protein (Pharmacia). Similarity % (= number of similar bands/ total number of bands x 100) of both positive and negative data was calculated according to Ladizinsky & Waines (1982).

Histological investigations

For longitudinal sections (15µm thick), callus was fixed in formalin acetic acid (FAA), then dehydrated with ethanol series and cleared with ethanol-xylene. Then samples were embedded in paraffin wax at 45-55ºC. Sections were cut

with steel blade on rotary microtome. The fixed sections were stained with Safranin O-Fast-green double stain. After staining, sections mounted in Canada balsam (Willey, 1971). Observation and photomicrographs were achieved using research microscope (LEICA DM500) fitted with digital camera (LEICA ICC50). For staining alkaloid, callus was macerated and stained with Dragendorff's reagent.

Alkaloids determination

To extract alkaloids, powdered dry callus was percolated overnight in methanol till complete exhaustion, then methanol was removed by distillation under pressure. Residue was stirred with 0.1N HCl and extracted with CH_3CL. Chloroformic layer was washed with 0.1N HCL and discarded. The combined acidic layer was then rendered alkaline with NH_4OH and extracted with CH_3CL. Chloroformic layer was collected, then evaporated and the final residue was dissolved in desired chloroform (Karawya et al., 1975). Total alkaloids concentration was determined by adding 5ml of bromocresol green solution (prepared by heating 69.8mg bromocresol green with 3ml of 2N NaOH and 5ml distilled water until completely dissolved and the solution was diluted to 1000ml with distilled water) and 5ml of phosphate buffer solution (pH 4.7) (prepared by adjusting the pH of 2M sodium phosphate (71.6g Na_2HPO_4 in 1L distilled water) to 4.7 with 0.2M citric acid (42.02g citric acid in 1L distilled water)) to a part of chloroform solution. The mixture was shaken and complex extracted with 1, 2, 3 and 4ml chloroform by vigorous shaking, the extract was then collected in a 10ml volumetric flask and diluted with chloroform. Atropine standard solution was made by dissolving 1mg of Atropine (Sigma) in 10ml distilled water (Ajanal et al., 2012). The absorbance of the complex in chloroform was measured at spectrum of 470nm in UV-Spectrophotometer against the blank prepared as above but without Atropine.

Determination of hyoscyamine concentration

High- Performance Thin - Layer Chromatography (HPTLC) was performed on 20cm ×10cm HPTLC silica gel 60 F_{254} plates (Merck) with a mobile phase consisting of chloroform: methanol: acetone: aqueous ammonia (25%) 75: 15: 10: 1.6 (v/v/v/v) (Jaremicz et al., 2014). Hyoscyamine standard (Sigma) was diluted by chloroform to final concentration 20, 40, 60, 80, 120, 160µg/ml. All samples and standards were applied to the plates by means of CAMAG Linomat 5 with dosing syringe 100µL as 7mm bands with 10.5mm distance between tracks, application X 15mm and 13mm application Y edges of plate and the application volume was 1–20µL for samples and 2-8µL for standard. Loaded HPTLC plates were developed to a distance of 50mm in Camag Automatic Developing Chamber CADC 2 at room temperature. The development occurring in a two-steps (preconditioning with 10ml mobile phase for 5min and development with 25ml mobile phase for 20min). The plate was developed to a distance of 50mm and dried for 5min by a stream of warm air, then the plates derivatized with 200ml Dragendorff's reagent using Chromatogram Immersion Device. Then the plate was scanned and examined densitometry at l= 550nm by means of CAMAGTLC Scanner 4 with slit dimension of 6 x 0.30mm.

Statistical analysis

All data were statistically analyzed as randomized complete blocks design (Steel et al., 1997). Analysis of variance (one-way analysis; ANOVA) and means comparisons (Duncan's multiple range tests, 5%) were performed using the MSTAT-C statistical pack-age (M-STAT, 1990).

Results

Effect of Arg and Orn on callus histology and alkaloid crystals

Cell shapes of callus were differed according to Arginine (Arg) or Ornithine (Orn) precursors or its concentration. Cells had regular ovate shape in 0.25mmol of Arg fed-callus as well as control but it irregular ovate under both 0.5mmol of Arg and 1mmol of Orn fed-one. Callus cells were irregular amoeboid in 0.25 and 0.5mmol of Orn and 1mmol of Arg fed-callus. The highest length and width of cell was (91.7 and 60µm) were detected in callus exposed to 0.25mmol of Arg. High level of Orn and Arg induced the asymmetry and lysiogenous intercellular space in callus as senesces symptoms (Fig. 1B, Table 1).

Effect of Arg and Orn on FW, DW , WC and total carbohydrates of callus

All used concentrations of both Arg or Orn increased the FW and DW of callus after 30 days

than control. Non-significant differences were detected among both precursors on FW of callus (Fig. 2A). The maximum FW was recorded in 0.5mmol of Arg-nourished callus (3.385g/ 0.5g explant) with 2.2 times of increment compared to control. The same concentration of Arg accumulated the highest significant biomass of DW (0.149g/ 0.5g explant) with 1.8 times of increment than control (Fig. 2B). Callus maintained high percent of water content (94.9 to 95.2 %) without any significant differences among all treatments and control (Fig. 2C). The maximum concentration of total carbohydrates (155.11mg g^{-1} DW) was determined in callus nourished with 0.5mmol of Orn (Fig. 2D).

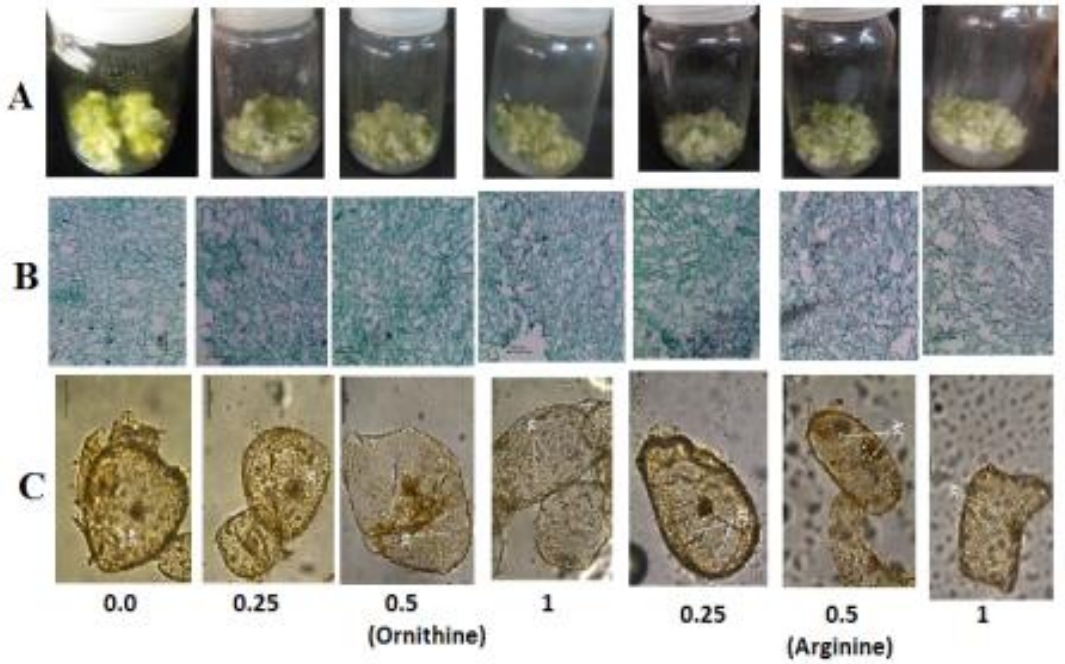

Fig. 1. Effect of different concentrations of alkaloid precursor (Orn. and Arg.) on growth (A), histology (B) and orange-stained alkaloid crystals (C) in callus of *Hyoscyamus muticus* L., after 30 days from cultivation (AC, Alkaloid crystals).

TABLE 1.Callus cells description of *Hyoscyamus muticus* L. callus after 30 days from cultivation as affected by different concentrations of alkaloid precursors, ornithine and arginine (0.0, 0.25, 0.5, 1.0mmol.).

	Precursor conc. (mmol)	Cell			Symmetric of cells	Intercellular space
		Shape	Length (µm)	Width (µm)		
	0.0 (control)	Regular ovate	73^{d} ±0.58	54^{b} ±0.41	Symmetric	Schizogenous
Ornithine	0.25	Irregular amoeboid	70^{d} 0.42±	46^{c} ±0.32	Asymmetric	Lysigenous
	0.5	Irregular amoeboid	83.6^{b} ±0.65	52.7^{b} ±0.57	Asymmetric	Lysigenous
	1	Irregular ovate	77.5^{c} ±0.63	49.2^{b} ±0.44	Asymmetric	Lysigenous
Arginine	0.25	Regular ovate	91.7^{a} ±0.54	60^{a} ±0.67	Symmetric	Schizogenous
	0.5	Irregular ovate	78^{c} ±0.57	59^{a} ±0.65	Asymmetric	Schizogenous
	1	Irregular amoeboid	56^{e} ±0.32	41.7^{c} ±0.21	Asymmetric	Lysigenous

Mean values ± SD followed by the same letter show non-significant different at the P≤ 0.05 probability level.

Effect of Arg and Orn on pigments

Photosynthetic pigments (chl. a, b and carotenoids) and total carbohydrates had similar trend. Its concentration was increased gradually with rising of both precursors levels in the growth medium to maximum values at 0.5mmol then decreased at high level (1mmol). Chl. a increased by 9.6 and 18.3% in callus treated with 0.5mmol of both Orn and Arg than control, respectively (Fig. 3A). Moderate level (0.5mmol) of Arg and Orn gave the highest values of chl. b but without significant differences with control (Fig. 3B). The highest significant value of carotenoids (1.612mg $100g^{-1}$ FW) was found in callus exposed to 0.5mmol of Arg with 31.3% of increment compared to control (Fig. 3C). Anthocyanin concentration was decreased in callus under all Arg and Orn levels compared to control (Fig. 3 D). The highly reduction (6.1 times) was observed in callus treated with 1mmol of Arg.

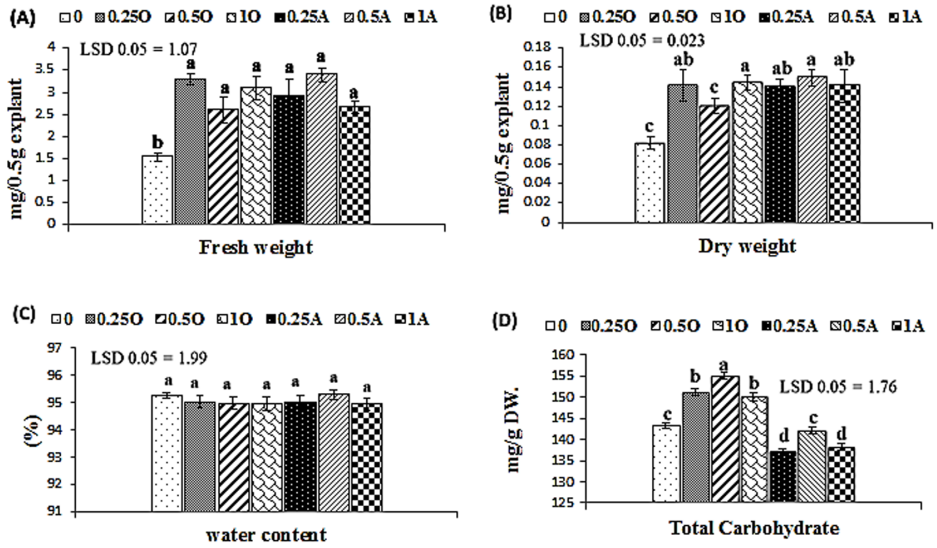

Fig. 2. Effect of different concentrations of alkaloid precursors, ornithine and arginine (0.0, 0.25, 0.5, 1.0mmol.) on fresh (A), dry weight (B), water content (C) and total carbohydrate (D) of *Hyoscyamus muticus* L. callus, after 30 days from cultivation [Data represented as mean of 3-replica, mean values± SD followed by the same letter in each bar show non-significant different at the P≤ 0.05 probability level].

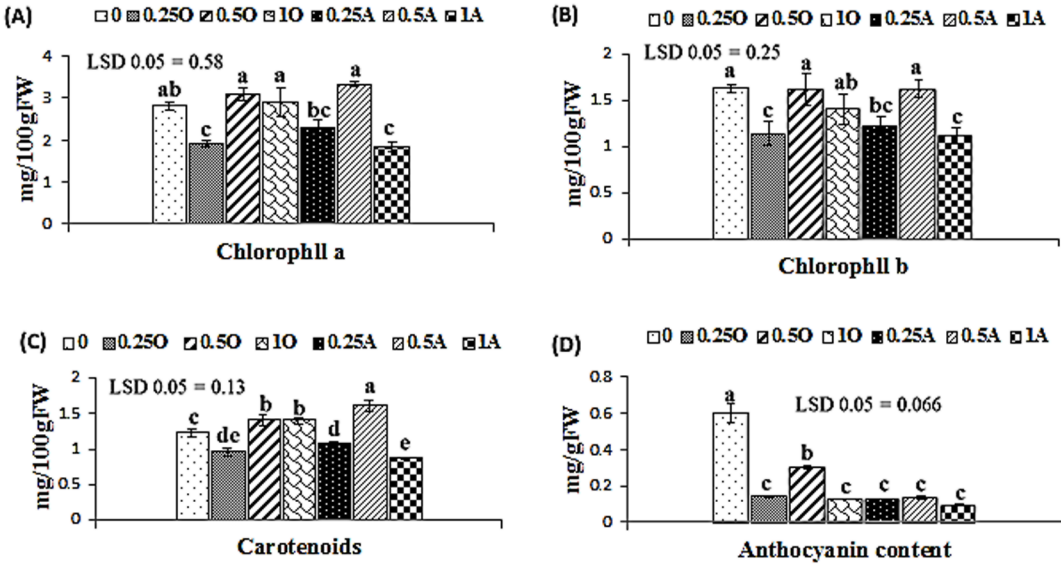

Fig. 3. Effect of different concentrations of alkaloid precursors, ornithine and arginine (0.0, 0.25, 0.5, 1.0mmol.) on pigments (Chl. a, Chl. b, carotenoid and anthocyanin) content of *Hyoscyamus muticus* L. callus, after 30 days from cultivation [Data represented as mean of 3-replica, mean values± SD followed by the same letter in each bar show non-significant different at the P≤ 0.05 probability level].

Effect of Arg and Orn on non-enzymatic compounds and H_2O_2

Addition of both precursors in MS medium significantly increased the glutathione (GSH), free phenolics and proline concentrations in callus than control. Callus exposed to 0.25mmol of Orn gave the highest concentration (4.262µmol mg^{-1} protein) of GSH with 35.7% of increment than control (Fig. 4A). Although values of proline concentrations were very low, callus exposed to 1mmol of Arg synthesized the highest significant values (0.082mg g^{-1} FW) (Fig. 4C). Un-nourished callus with Arg or Orn contained the highest significant amount of H_2O_2 and application of both precursors decreased it as shown in Fig. 4D. The maximum reduction (34.5%) of H_2O_2 was recorded in callus treated with 0.25mmol of Orn compared to control. Callus treated with 1mmol of Arg recorded the highest value of free phenolics concentration (1.519mg g^{-1} FW) with 39.1% of increment than control but without significant differences with moderate or high level of Orn (Fig. 4B).

Effect of Arg and Orn on enzymatic antioxidant activity and MDA

In general, the activity of POD was higher in both treated or untreated callus, followed by SOD then CAT (Fig. 5). High level of both Arg and Orn (1mmol) induced the highest activity of POD (0.671 and 0.653 unit mg^{-1} protein min, respectively) with 42.5 and 38.6% of increment compared to control (Fig. 5A). Application of both precursors at all concentrations decreased the activity of SOD. The highest activity of SOD (0.116 unit mg^{-1} protein min) which detected in callus treated with 1mmol of Orn wasn't differed from control (Fig. 5B). All values of CAT activity were very low especially with addition of two precursors. Un-nourished callus with Arg or Orn had the highest activity of CAT activity (Fig. 5C). The maximum values of MDA was detected in callus of control or that exposed to 0.5mmol of Arg (2.934 and 2.667µmol g^{-1} FW, respectively), but most of treatments had similar values (Fig. 5D).

Effect of Arg and Orn on nitrogenous compounds

Callus under all Arg or Orn concentrations had low amount of protein except that exposed to 0.5mmol of Orn which non-significantly differed from control (Fig. 6B). Application of Orn decreased the amount of free amino acids, in contrary to Arg at all concentrations (Fig. 6A). The maximum significant values of free amino acids (2.717mg g^{-1} FW) was determined in callus exposed to 1mmol of Arg with 74.6% of increment compared to control. The same amount of total alkaloids was found in both callus (as control) and leaves of wild plant but the latter had 3.4 foldof hyoscyamine amount (2.806mg g^{-1} DW) (Fig. 6D, 7). Add of both precursors increased alkaloids concentration several times. Callus treated with 0.25mmol of Orn gave the highest value of alkaloids (Fig. 6C) and hyoscyamine concentrations (51.91 and 1.11mg g^{-1} DW, respectively) with 3.2 and 1.4 times of increment than control, respectively (Fig. 6C, 7).

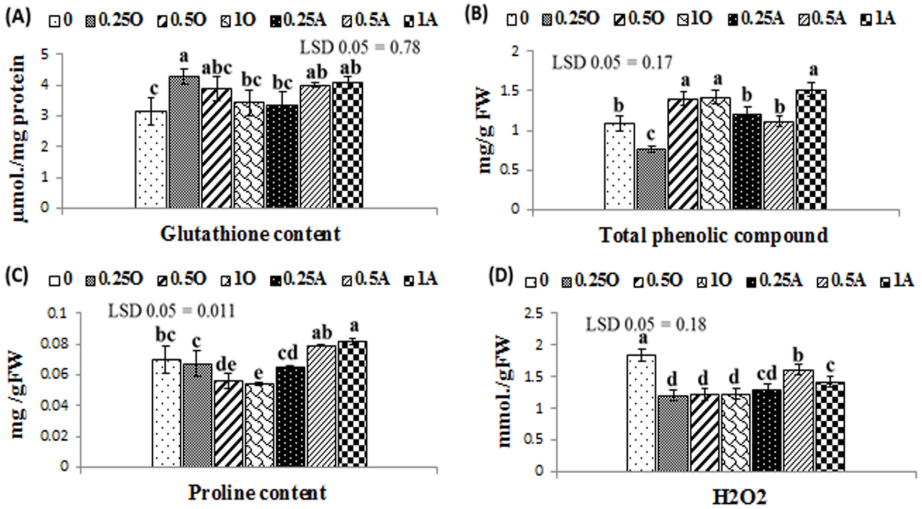

Fig.4. Effect of different concentrations of alkaloid precursors, ornithine and arginine (0.0, 0.25, 0.5, 1.0mmol.) on glutathione content (A), total phenolic compound (B), proline concentration (C) and H_2O_2 (D) of *Hyoscyamus muticus* L. callus, after 30 days from cultivation [Data represented as mean of 3-replica, mean values ± SD followed by the same letter in each bar show non-significant different at the P≤ 0.05 probability level].

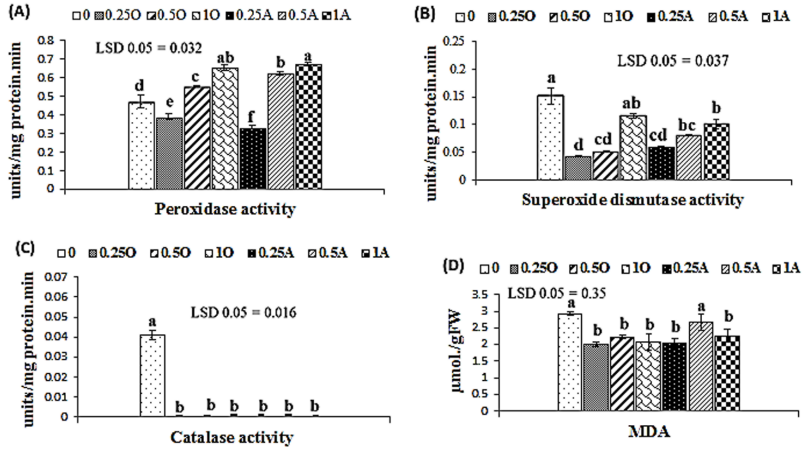

Fig. 5. Effect of different concentrations of alkaloid precursors, ornithine and arginine (0.0, 0.25, 0.5, 1.0mmol.) on activity of POD (A), SOD (B), CAT (C) and MDA concentration (D) of *Hyoscyamus muticus* L. callus, after 30 days from cultivation [Data represented as mean of 3-replica, mean values ±SD followed by the same letter in each bar show non-significant different at the P≤ 0.05 probability level].

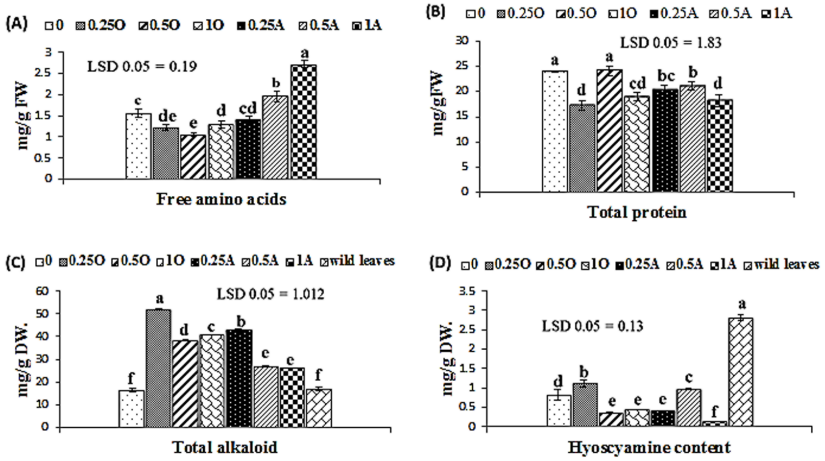

Fig. 6. Effect of different concentrations of alkaloid precursor, ornithine and arginine (0.0, 0.25, 0.5, 1.0mmol.) on free amino acids (A), protein (B), total alkaloids content (C) and hyoscyamine concentration (D) of *Hyoscyamus muticus* L. callus, after 30 days from cultivation [Data represented as mean of 3-replica, mean values ±SD followed by the same letter in each bar show non-significant different at the P≤ 0.05 probability level].

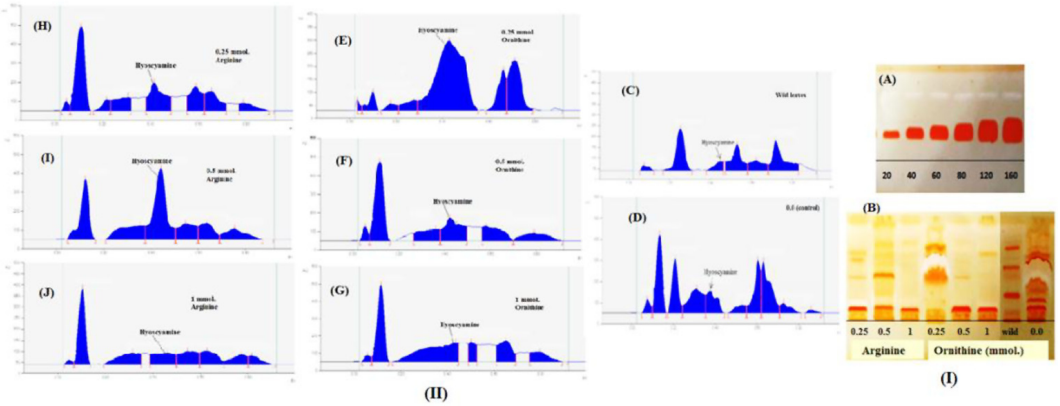

Fig.7. (I). Chromatogram of alkaloid fraction from (A) hyoscyamine standard solutions (µg/ml) and (B) Hyoscyamus muticus callus. (II) Densitogram of alkaloid fraction from Hyoscyamus muticus callus recorded at λ=550nm after derivatisation with Dragendorff's reagent, (C) wild leaves, (D) untreated callus, (E) 0.25 mmol Orn, (F) 0.5 mmol Orn, (G) 1mmol Orn, (H) 0.25 mmol Arg,(I) 0.5 mmol Arg and (J) 1mmol Arg.

Effect of Arg and Orn on protein profile

Ten protein bands ranged from molecular weight (MW), 277 to 9kDa were separated from callus treated with different concentrations of Arg and Orn (Fig. 8). Arginine fed-callus expressed new two protein bands with MW 65 and 21kDa compared to control. Under moderate or high level of Arg the ten protein bands was visualized compared to only five bands in control or low level of Arg (Fig. 8A). Protein bands with low MW, 43, 33 and 13kDa were found in both moderate and high level of Arg. High expression (as optical density) of protein bands with MW, 65, 43, 38, 33, 32, 21 and 9kDa was detected in callus exposed to 0.5mmol of Arg but Bands with MW, 277, 15 and 13kDa in 1mmol of Arg were highly expressed. Protein band with MW 13kDa was absent in callus treated with all concentrations of Orn. Callus exposed to 0.25, 0.5 and 1mmol of Orn had 6, 8 and 9 protein bands, respectively. The high expression of protein bands with MW 38, 33, 21, 15 and 9kDa was found in callus treated with 0.25mmol and bands with MW 277, 65, 43 and 32kDa in 0.5mmol of Orn (Fig. 8B). Protein bands were similar by 100% in low or high level of Arg fed-callus, then similarity % was decreased with other treatments. Protein bands was differed in precursors treatments by 27% with control (Fig. 8C).

Discussion

Results reported herein cleared that, application of both alkaloid precursors, arginine (Arg) or ornithine (Orn) enhanced the total alkaloids in Egyptian henbane callus more than control and wild plants. The results also revealed that wild leaves had similar amount of total alkaloids in non-fed callus with Orn or Arg but it had the highest concentration of hyoscyamine as shown by HPTLC (Fig. 7). Present results were coordinated with De Luca & St Pierre (2000) who reported thatbiosynthesis of alkaloid is highly attributed with stages of plant growth and development. Results were agreed with Taiz & Zeiger (2002) who obvious that alkaloids can act as defense compounds in plants, being efficient against biotic agents due to their toxicity.

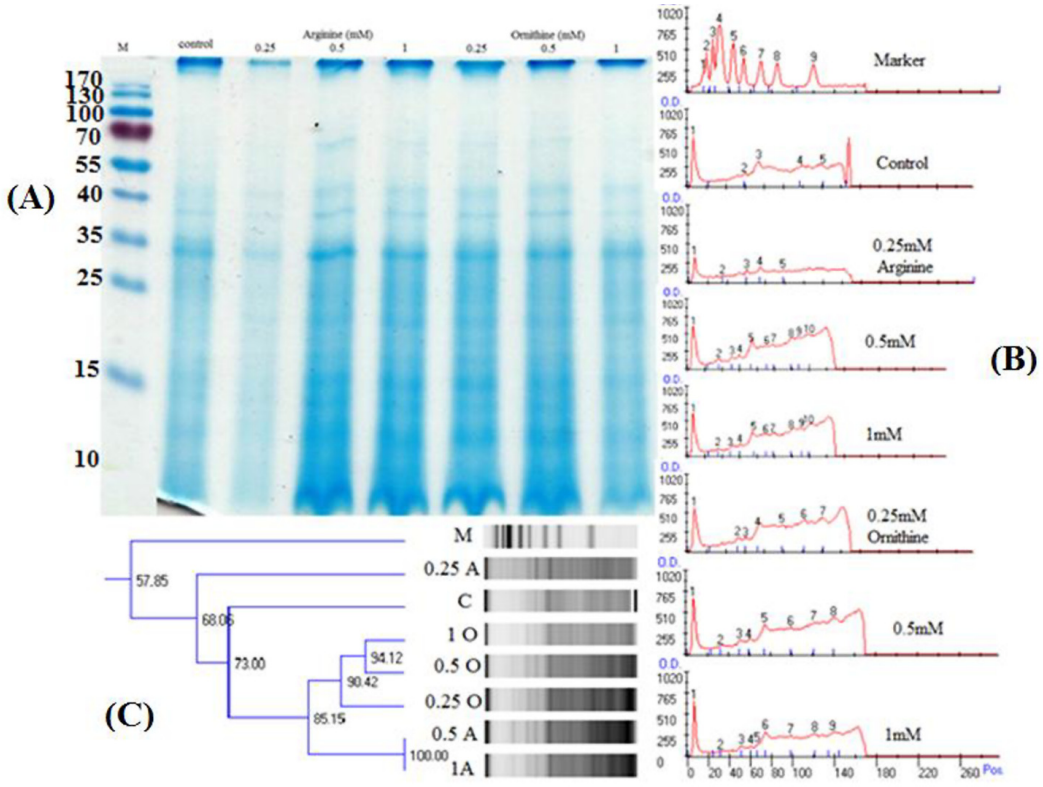

Fig. 8. SDS-PAGE of protein profile (A), optical densities of each protein band (B) and similarity % (C) of each concentration of alkaloid precursor (ornithine and arginine) of callus of *Hyoscyamus muticus* L., after 30 days.

However, application of Ornto cultural medium was more efficient than Arg on alkaloids and hyoscyamine production. In this respect, 0.25mmol of Orn increased both total alkaloids and hyoscyamine than unfed-callus. Results were agreed with the fact of Arg is protein amino acid but Orn non-protein amino acid can give rise to the amines cadaverine and putrescine (Matsuura & Fett-Neto, 2017). Also, amino acids can transform to each other such as Orn transformed toArg or proline in glutamate pathway (Facchini, 2001). Results were in line with Sevón et al. (1998) who found that hairy root culture of *Hyoscyamus muticus* L. had 12.2mg g^{-1} DW of hyoscyamine. In contrary, Besher et al. (2014) found that callus had the highest content of hyoscyamine and tropane alkaloids but the wild plants had the highest content of scopolamine.

The results reported herein showed that application of Arg and Orn to callus medium improved the fresh (FW) and dry (DW) weight of callus of Egyptian henbane as shown in Fig. 2. Increment of FW and DW attributed with histological investment which obvious that callus cells exposed to low level of Orn (0.25mmol) had long irregular amoeboid shape and more lysiogenous intercellular spaces compared to small regular ovate and schizogenous intercellular spaces in control as shown in Fig 1A, B and Table 1. These results may explain as the indirect effect of alkaloids precursors, Arg and Orn, which synthesized polyamines. It was known that putrescine polyamine is intermediate compound in biosynthesize pathway of hyoscyamine alkaloid from Orn or Arg (Facchini, 2001). Also, previous literatures obvious that polyamines had protective role on plant cell under normal or stress conditions via preserving the functional biomolecules by joining its positive charges with negative charges of chlorophyllase and membrane phospholipids, proteins and ribonucleic acids (Evans & Malmberg, 1989). These results agreed with previous reports as Gholami et al. (2014) who found that aplication of L-and D-ornithine increased tobacco cell suspension culture. Also, addition of L-arginine enhanced the indole alkaloids and various cell growth parameters (cell number, fresh and dry weight) of *Catharanthus roseus* (Taha et al., 2009). In addition, Added of tropic acid increased callus growth of *Hyoscyamus muticus* (Ibrahim et al., 2009). In contrary, Moreno et al. (1993) reported that application of terpenoid precursors as mevalonic acid, loganin, loganic acid or secologanin to suspension cultures of *Catharanthus roseus* did not affect the culture growth, as determined by dry weight. Also, mevalonic acid, phenylalanine, leucine and valine showed negative effect on both callus fresh dry weight of *Zingiber officinale* compared to control (El-Nabarawy et al., 2015).

Results reported herein showed the increment of both photosynthetic pigments (Fig. 3) and total carbohydrates concentrations (Fig. 2D) with rising of both precursors levels to 0.5mmol. Results were agreed with the fact of chlorophyll synthesized in higher plants from the immediate precursor glycine amino acid after condensation with succinyl Co.A (Taiz & Zeiger, 2002). High level of each precursor (1mmol) may have inhibition effect on pigments synthesis. This result was in line with theresults of von Abrams, (1974), who found that Arg had little effect on chlorophyll and protein catabolism in leaf of *Avena sativa* than L-ornithine, L-lysine and L-2,4-diamino-n-butyric acid.

Enhancement of total carbohydrates as final products of photosynthesis process due to application of each precursors (Orn and Arg) increased the fresh and dry weight of callus during 30 day. Result was agreed with Ebeed et al. (2017), who reported that, application of polyamines significantly increased the soluble sugars and free amino acids in wheat. Maintaining the water content % in callus may due to the higher content of amino acids especially Arg as shown in Fig. 6A. Results were coordinated with Taiz & Zeiger (2002) who demonstrated that amino acids participate in regulation of osmotic balance and protection of membrane and protein.

The low level (0.25mmol.) of Orn induced the highest concentration of glutathione followed by high level of Arg (1mmol.) and the lowest value at non-fed callus. Application of Arg or Orn reduced the H_2O_2 and MDA concentration more than non-fed callus. Results were agreed with Noctor & Foyer (1998) who reported that, glutatione is a pivotal component of the glutathione-ascorbate cycle, a system that reduces poisonous H_2O_2 in plant cells. In addition to its protective role in plant cell, it had medical importance such as preventing oxidative damage of human skin therefore it used in cosmetics production. Reduction of H_2O_2 may be due to

the enhancement of enzymatic or nonenzymatic antioxidants agents or due to the alkaloids itself which have a major role in plants as antioxidants rather than as toxins as reported by Porto et al. (2014). Our results were also in line with that obtained by Nasibi et al. (2011) who showed that Arg pretreatment reduced the lipid peroxidation ratein tomato plant under oxidative stress.

Arg with high level (1mmol) induced the highest concentration of free phenolics, proline and the activity of Peroxidase in callus. Positive relation between alkaloids and phenolics was found due to the fact of hyoscyamine synthesized from tropine after condensing with phenyllactic acid (Taiz & Zeiger, 2002). Phenolic as defense molecules in plants compounds participate with glutathione peroxidase to quench the H_2O_2. Also, it formed lignin from phenylalanine which support the structure of plant cells (Noctor & Foyer, 1998). These results coordinated with previous report which illustrated that pretreatment of tomato plant with arginine, the activity of CAT and POD was decreased while SOD activity was increased (Nasibi et al., 2011). However, application of high concentration of Arg reduced proline content in Fenugreek (*Trigonella foenum-graecum* L.) (Ahadi et al., 2018).

Under all levels of Arg and Orn the anthocyanins content was decreased. Decreasing of anthocyanin concentration may be due to its oxidation after participate as antioxidant in quenching the reactive oxygen species especially it is a group of phenolic substances (Steyn et al., 2002; Winkel-Shirley, 2002).

However, protein bands with low molecular weights, 38, 33, 21, 15 and 9kDa was highly expressed in callus treated with 0.25mmol of Orn.These findings were in line with Park & Seo (2015) who reported that low molecular weight proteins had a protective role for membrane or functional biomolecules as heat shock proteins. Moreover, Arg fed-callus expressed new two protein bands with MW 65 and 21kDa compared to control. Result was agreed with Sghaier et al. (2009) who reported that, Arg treated callus of date palm formed new four protein bands with MW 21, 70, 85 and 97.4kDa which disappear in control callus. Also, 12 protein bands with MW, 92, 90, 80, 71.5, 60, 32.5, 30.3, 25, 23, 16.5, 14.5 and 8kDa were found in mung bean seeds after sprayed with Arg compared with only 8 protein bands in control (Qados, 2010). In addition, El–Bassiouny et al. (2008) reported that, Arg induced the appearance of new protein bands with MW 222.0, 214.6, 131.8, 93.1, 78.7, 50.7, 34.6 and 14.1kDa in wheat plants.

Conclusion

Non-protein amino acid ornithine at low concentration was effective in alkaloids or hyoscyamine accumulation in Egyptian henbane callus more than arginine. Ornithine enhanced the vegetative growth of callus and alkaloids concentration via enhancement of antioxidants compounds as glutathione, free phenolics and the activity of peroxidase. Ornithine increased the expression of low molecular weight proteins which have protective role.

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الاورنيثين يستحث إنتاج الهيوسيامين فى السكران المصرى النامى معمليا مقارنة بالأرجنين

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تم دراسة تاثير التركيزات المختلفة (0.25 و 0.5 و 1 ملليمول) من كلا من بادئى تخليق القلويدات : الأرجنين والأورنثين على انتاج الهيوسيامين ودورهما الفسيولوجى فى كالس نبات السكرات المصرى حتى عمر 30 يوم. أوضحت نتائج التحليل الكروموتوجرافى ذو الطبقة الرقيقة عالى الضغط ارتفاع تركيز الهيوسيامين فى النبات البرى مع ارتفاع تركيز كلا من القلويدات والهيوسيامين من 3.2 و1.4 مرة على التوالى فى الكالس المعامل ب 0.25 ملليمول من الأورنيثين مقارنة بالكنترول. ادى نفس التركيز إلى ارتفاع تركيز الجلوتاثيون وانخفاض تركيز فوق أوكسيد الهيدروجين لأقل معدل. رغم أن تركيز الأورنيثين 0.25 ملليمول اظهر 6 حزم بروتينية فقط عن طريق التحليل الكهربى للبروتين إلا أن البروتينات ذات الوزن الجزيئى المنخفض 38 و 33 و 21 و 15 و 9 كيلودالتون كانت اعلى تعبيرا. ظهرت الخلايا فى الكالس المعامل بالتركيز المنخفض من الأورنيثين غير منتظمة اميبية الشكل ومسافات بينية انقراضية مقارنة بشكل الخلايا البيضية المنتظمة والمسافات البينية الأنفصالية فى خلايا الكنترول. زاد الوزن الطازج والجاف فى جميع التركيزات من كلا البادئين مقارنة بالكنترول بينما ظل المحتوى المائى ثابت. زادت الصبغات التمثيلية والكربوهيدرات الكلية مع زيادة تركيز البادئين ثم انخفضت فى التركيز المرتفع من كلاهما. ادى الأورنيثين فى جميع مستوياته إلى انخفاض تركيز الأحماض الأمينية مقارنة بالأرجينين. التركيز العالى من الأرجنين ادى إلى أعلى زيادة فى تركيزالفينولات الحرة ونشاط انزيم البيروكسيديز. يمكن استنتاج أن إضافة الأورنيثين بتركيز منخفض 0.25 ملليمول وسيلة فعالة فى زيادة تركيز الهيوسيامين فى السكران المصري مقارنة بالأرجنين .