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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (8): 1236-1242, 2007
ISSN 1028-8880

Growth and Production Optimization of Tropane Alkaloids in *Datura stramonium* Cell Suspension Culture

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Abstract: A number of physicochemical conditions such different concentration of glucose, sucrose, potassium nitrate, ammonium nitrate, calcium chloride and temperatures were tested to optimize growth and production of tropane alkaloids from *Datura stramonium* (Solanaceae) plants. Cell suspension from semi-clear calli of leave explants developed in MS medium containing kinetin (0.5 mg L^{-1}) and NAA (2 mg L^{-1}) hormones was used to measure biomass and total alkaloids and comparison of treatments. The results showed that 30 and 40 g L^{-1} glucose led to the highest level of alkaloids and biomass productions, respectively. 20 and 40 g L^{-1} sucrose concentrations resulted in order the most rates of alkaloids and biomass productions. The results showed that increasing of nitrate concentration led to the reduction of the alkaloids. The best concentration of potassium nitrate for the production of tropane alkaloids and biomass were in order 9.4 and 3.76 mM. Also it was evinced that the optimized concentration of ammonium nitrate for alkaloids production was 10.3 mM and for the biomass was 41.22 mM. The best concentration of calcium chloride for growth and production of the alkaloids was 7.92 mM. Testing different temperature specified that the best condition for production of the alkaloids was 20°C whereas it was 25°C for biomass production. The results of this study could be recommended to farmers involved in production of *D. stramonium* for tropain alkaloids at industrial and semi-industrial scales.

Key words: *Datura stramonium*, tropane alkaloids, physicochemical condition

INTRODUCTION

Datura stramonium (Solanaceae) is a plant distributed throughout most parts of temperate regions of the world (Berkov *et al*., 2006) and is a rich source for numerous medicinal substances (Wagner *et al*., 1989). The alkaloids are the most numerous group of active herbal substances, there are known to be over 10000 alkaloids (Leete, 1990). One of these groups is called tropane alkaloids. Tropane alkaloids have significant medicinal importance as they are compounds with a variety of pharmacological effects. They have healing and useful effects on some human organs such eyes, nerve system, heart, blood circulation and respirations (Martindale, 1996).

Despite the fact that the number of synthetic medicinal substances on the drug market has grown significantly during the last decade, 25% of prescription medicines still contain one or more herb-derived substances, as their main constituent. In order to simplify processing and enhance production yield, many medicinal substances are currently being produced synthetically. However, in the case of certain medicinal substances, as with the majority of alkaloids, chemical synthesis has been found to be prohibitively expensive and natural material is therefore the only economical source for such compounds (Oksman-Caldentey and Hilturen, 1996).

Tropane alkaloids are mainly produced by extraction from cultivated plant materials. Breeding of plants in a field culture enables direct, control and improve their growth more effectively. However, there are several reasons for considering plant tissue cultures as an alternative to field cultures; they allow for a shortened growth period, eliminate the need for herbicides and pesticides as well as maintaining constant growth conditions and product quality (Oksman-Caldentey and Hilturen, 1996).

The production of tropane alkaloids in the tissue cultures depends to a large degree on media composition (Dixon, 1985). The effect of different factors, such as a different source of plant nutritional elements, plant growth regulators and different growth conditions on the production of tropane alkaloids has been studied using tissue cultures (Oksman-Caldentey *et al*., 1987;

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Biondi *et al*., 1993; Tone *et al*., 1997). Plant nutritional elements are necessary for plant growth and development and could not be substituted with any other element due to their specific functions. Carbon, hydrogen, oxygen plus a further 17 elements such nitrogen, phosphorus, sulphur, potassium, calcium, magnesium, iron, manganese, copper, zinc and molybdenum are primarily required in plant development. The elements are necessary for the normal functioning of the protoplasm of plant cells, formation of the cell structures and cell development, also for the regulation of the metabolic processes in the cells (Loneragan, 1997).

In vitro researches for efficient production of tropane alkaloids have led to the experience of some variables such exogenic phytohormones, micro and macro elements, glucoses, vitamins and other physical factors. It is shown that the production of callus was mainly stemmed from leaf explant rather than peduncle explant and the best environment for leaf explant is MS culture comprising NAA hormone at 0.5 g L^{-1} concentration (Iranbakhsh and Riazi, 2000). Yoshimatsu *et al*. (2004) and Zhang *et al*. (2004) recommended the culture of organized tissue for yield of tropane alkaloids. They showed a positive connection between the root tissues and tropane alkaloids biosynthesis and suggested *in vitro* root cultivation as an efficient biotechnology system for tropane alkaloid production.

Demeyer and Dejaegere (1988) reported that NO_3^- causes an increase in dry weight and hyocyamine production in *D. stramonium* in green-house conditions. Demeyer and Dejaegere (1989) reported positive effects of nitrogen treatment on raising hyocyamine in root culture in the plant. Amirjani (1993) reported that 2 and 3% concentration of sucrose raised the rate of atropine in *Atropa belladonna* (Solanaceae). They also reported that 10 mM calcium is needed for maximum atropine yield. Gontire *et al*. (1994) examined the effects of calcium, alginate and calcium alginate on growth and rate of tropane alkaloids in cell suspension lines of *D. innoxia*. They reported that 10 mM calcium causes an increase, about 10 times as much of alkaloids production efficiency as compared with control cells in MS medium. Rhoton and Bouteraouy (1994) reported that leaf and stem alkaloids in *D. stramonium* in 24°C were half of it in 14°C. Hilton and Rhodes (1994) reported that the hyocyamin production in tissue culture was much higher in 20-25°C than in 30°C. Hilton and Rhodes (1995) reported that in tissue culture of various species of *D. stramonium*, the NH_4^{1+} PO_4^{3-} would be absorbed entirely from medium and NH4 would probably be a substrate for the biosynthesis of alkaloids. Chalapian and Majd (2003) examined the biosynthesis of tropane alkaloids in *Hyoscyamus niger* and showed the effect of some elements and sucrose on the biosynthesis of tropane alkaloids. They reported the culture medium containing nitrate resulted in an increase in root growth and differentiation. However, this treatment led to decline in the alkaloid production. They also reported the optimized concentration of sucrose was 10 mM. The increase in sucrose led to the root growth and differentiation and tropane alkaloids biosynthesis.

This research has been conducted to optimize the conditions for production of tropane alkaloids in Iranian *D. stramonium* cultivar which could be recommended for production of tropane alkaloids at the semi- industrial and industrial scales. This research also intended to investigate the production of callus as the first substance of cell suspension to approach highest dosage of alkaloid production.

MATERIALS AND METHODS

Plants and leaf explant: Plants of *D. stramonium* collected from the roadside of Rasht-Fouman, north of Iran. The collected plants have been identified in Farabi Herbarium Centre, Tehran, Iran. Also some seeds of the plant were provided from seed bank of Grassland and Forest Research Institute, Tehran, Iran.

In order to produce explants, the leaves of plant were divided to three parts and placed into MS medium comprising 0.5 mg L^{-1} Kinetin (KIN) and 2 mg L^{-1} Naphthalene Acetic Acid (NAA) in a shaker was set at 120 rpm. Four types of callus (hyaline, semi hyaline, green and organogenesis) were examined by cellular and biochemical assays and semi hyaline callus containing idioblast cells and tropane alkaloids were identified using the methods already explained by Demeyer and Dejaegere (1993).

Physicochemical tests: The semi hyaline calluses were used in order to investigate the effects of physical and chemical conditions and to optimize cell suspension conditions for tropane alkaloids production. In order to know the role of chemical factors, concentrations of 10, 20, 30 and 40 g L^{-1} of either glucose or sucrose; 0, 9.4, 18.8, 37.6 mM of potassium nitrate; 0, 10.30, 20.61 and 41.22 mM of ammonium and 0, 1.98, 3.96 and 7.92 mM of $CaCl_2$ were tested. Also the effects of temperatures (20, 25 and 30°C) were examined. The parameters of the tropane alkaloids dosage, cell number and weight of fresh and dry biomass were measured for comparison of different treatments.

Tropane alkaloids extraction: For tropane alkaloids extraction, the samples were dried and then 300 mL 90%

ethanol was added to 50 g dry plant and kept in 25°C for 48 h. The ethanol was vaporized in an evaporator. Two hundred milliliter sulfuric acid (5%) was added to the sample and slowly spun about eight hours in 25°C. Then 50 mL chloroform was added to the solution and chloroform pellet removed. The acidic supernatant was filtered and the pH of solution reached 10. Chloroform was added to the solution four times (each time 50 mL) and the pellet containing alkaloids was detached. The solution was vaporized and methanol was added to the remaining alkaloids.

Identification of tropane alkaloids: Thin Layer Chromatography (TLC) method was used for qualitative identification where stable phase contained silica gel and the mobile phase was a mixture of chloroform and methanol (9:1). When the mobile phase moved on stable phase about 10 cm, it was separated from the tank and sprayed by dregendrov indicator. In case of having alkaloids, the orange color represents alkaloid and the background will change to cream color.

Spectrophotometer, High Performance Liquid Chromatography (HPLC) and Gas Chromatography/Mass Spectrophotometer (GC/MS) were used for quantitative analysis. Spectrophotometry was performed on methanol extract where the machine was set on 245-265 nm. The obtained spectrums supplied from different samples of cellular and plant organs were compared with standard spectrum and the dosage of their alkaloids was measured. HPLC was applied using a C18 column (150 4.6 mm Id) and flow rate of 0.7. The mobile phase of Iso chratic contained acetonitril and water. The graphs were compared and measured with atropine sulfate and scopolamine chloride. In GC/MS method the final identification were done based on the comparison of obscure mass spectrums with standard alkaloids compounds.

RESULTS

It was shown that 30 g L^{-1} glucose resulted in most quantity of alkaloid production. The highest production of alkaloids occurred in sixth week and the maximum dosage occurred in fifth week (Fig. 1). The most numbers of cells and weight of fresh or dry biomass were observed in the medium containing 40 g L^{-1} glucose in the fifth week.

Sucrose was used with concentrations of 10, 20, 30 and 40 g L^{-1}. The result showed that maximum production rate of alkaloid was in the medium with 20 g L^{-1} sucrose in the fifth week after culture (Fig. 2). The highest number of cells, weight of fresh and dry biomass was observed in the medium containing 40 g L^{-1} sucrose in the seventh week.

Experiments with concentration of 0, 9.4, 18.8, 37.6 mM of KNO_3 revealed that the concentration of 9.4 mM is the most favorable condition for the alkaloid production (Fig. 3). The maximum of cells were related to the medium with 37.6 mM KNO_3 in the sixth week after culture. The most amount of fresh or dry weight was related to the medium with 37.6 mM KNO_3 in the fifth week.

NH_4 NO_3 was used with the 0, 10.30, 20.61 and 41.22 mM. The maximum alkaloid production was seen with 10.30 mM NH_4 NO_3 in the third week (Fig. 4). This concentration of NH_4 NO_3 resulted in the maximum numbers of cells in the sixth week and fresh and dry weight of biomass in the fifth week.

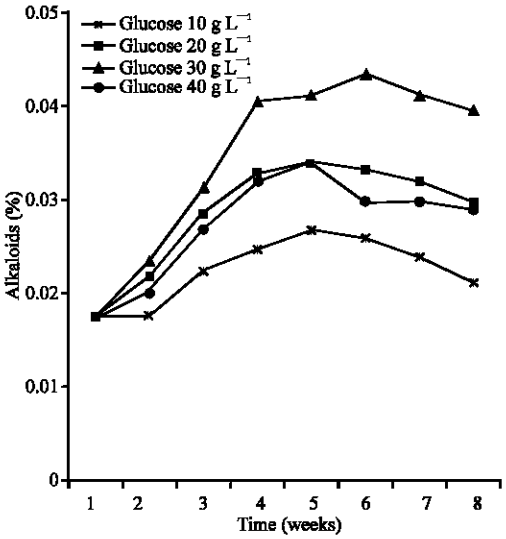

Fig. 1: The role of different quantities of glucose on the tropane alkaloid production in *D. strananium* planted in MS medium with hormone treatment of Kin = 0.5 and NAA = 2

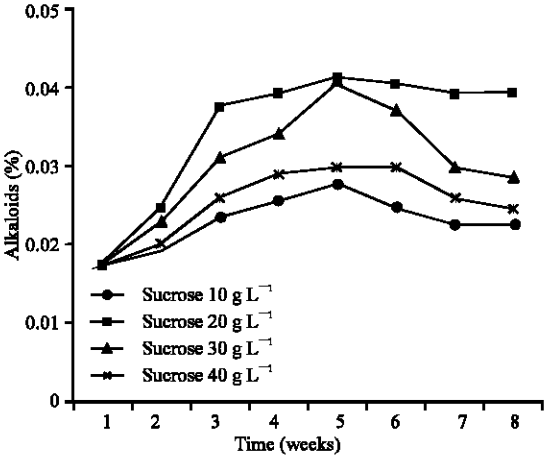

Fig. 2: The role of different quantities of sucrose on the cell number of *D. strananium* planted in MS medium with hormone treatment of Kin = 0.5 and NAA = 2

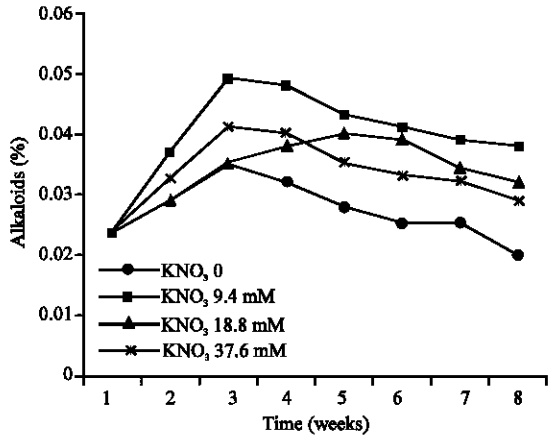

Fig. 3: The role of different quantities of KNO_3 on the fresh weight of biomass of *D. strananium* planted in MS medium with hormone treatment of Kin = 0.5 and NAA = 2

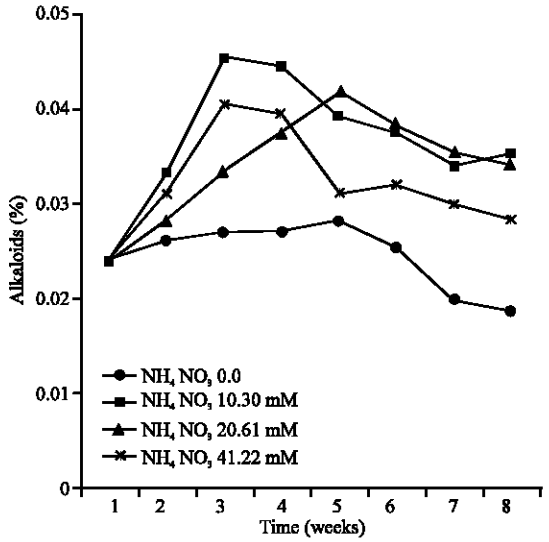

Fig. 4: The role of different quantities of NH_4NO_3 on the dry weight of biomass of *D. strananium* planted in MS medium with hormone treatment of Kin = 0.5 and NAA = 2

Comparison between concentrations of 0, 1.98, 3.96 and 7.92 mM $CaCl_2$ showed that maximum alkaloid production took place in the medium with 7.92 mM $CaCl_2$ in the fourth week (Fig. 5). Also maximum fresh or dry weights crop up with 7.92 mM $CaCl_2$ in the eighth week. However, maximum number of cells happened in the medium with 3.96 mM $CaCl_2$ in the eighth week.

The maximum alkaloids production and maximum number of cells was occurred in the medium hold in 20°C

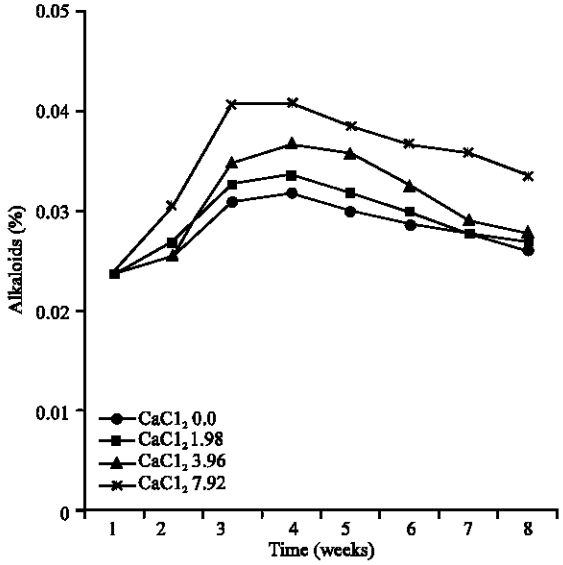

Fig. 5: The role of different quantities of $CaCl_2$ on the dry weight of biomass of *D. strananium* planted in MS medium with hormone treatment of Kin = 0.5 and NAA = 2

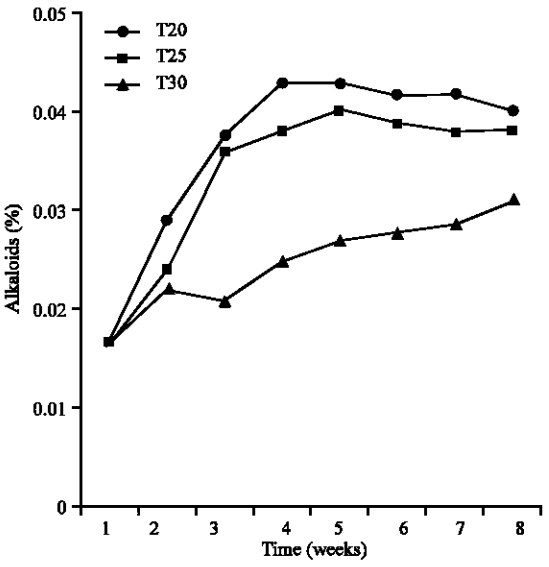

Fig. 6: The role of different temperature on the cell number of *D. strananium* planted in MS medium with hormone treatment of Kin = 0.5 and NAA = 2

in the seventh week (Fig. 6). The most amounts of fresh and dry weights suggested itself in the medium kept in 25°C in fifth week.

DISCUSSION

This study showed that the maximum tropane alkaloid production happens with 30 g L^{-1} glucose in sixth week

since culture while 10 g L^{-1} glucose in fifth week causes the maximum cells division. Also it was proven that the maximum dosage of tropane alkaloid production is related to the concentration of 20 g L^{-1} sucrose in the fifth week. This is in line with findings of Majd and AmirJani (1996). Schripsema and Verpoorte (1992) showed that biomass production of root culture in sucrose medium was more than monosaccharide medium while alkaloid production was in the most rank in monosaccharide medium. This research also showed that the maximum number of cells, dry or fresh weight was related to 40 g L^{-1} sucrose. This is in accordance with the fact that the highest concentration of monosaccharide causes the maximum production of biomass (Woo *et al*., 1998). Studies on plants of *Hyoscyamus niger* showed that increasing of sucrose causes the increase in biomass as well as stimulation of production of scopolamine alkaloid (Hilton and Rhodes, 1994). Nhat Hanh (1992) have mentioned that sucrose in medium increases photosynthesis. They reported that sugar nutrition prevents photo inhibitor and the photosynthesis capacity excessively increases, especially with the presence of sucrose. Maldonado and Loyola (1995) reported that photosynthesis reaction makes tropic acid and co-factor and prevents the alkaloids damage. Hilton and Rhodes (1990) considered the growth and production of Hyocyamine in the culture of *D. stramonium* hairy root inside of bio-reactor. They reported that growth with 3% concentration of sucrose is the best.

Plants contain on average 0.4-1.6%, of potassium, with more of this present in young, actively growing parts. Potassium is basically present as chlorides, hydrocarbonates and-phosphates and also in salts of pyruvic, oxalic and citric acids. The physiological and biochemical role of potassium in plants is very diverse (Chen *et al*., 1997). The results obtained from considering the effect of different concentrations of potassium nitrate on alkaloid tropane bio-synthesis showed a negative trend in which an increase in nitrate concentration causes a decrease in alkaloid biosynthesis. This might be explained by the antagonist effect of potassium on calcium and magnesium. The optimum applied concentration was 9.4 mM and low dosage of nitrate has stimulating effect on tropane alkaloid production. The maximum number of cells, the maximum fresh and dry weight, in medium has reached with 37.6 mM concentration of KNO_3. An increase in the KNO_3 concentration was followed by an increase in biomass. These findings are not in line with Sauerwein and Shimomura (1991) who reported that increase in KNO_3 did not have effect on alkaloid production. Since nitrogen is a necessary element in cell culture and plant tissue and also is vital in DNA, RNA, amino acids and protein structure, it seems that the increase of nitrogen concentration could cause an increase in root growth and other plant organs. In other hand, our results are in agreement with studies of Payne *et al*. (1987) who worked on *D. stramonium*. Demeyer and Dejaegere *et al*. (1988) reported that the increase of nitrate concentration in culture of root transformed of *Datura* caused the increase of biomass production, but control the alkaloid tropane bio-synthesis. They reported that alkaloids contain nitrogen and they are basically used as nitrate in plant nutrition. In plants, nitrate reductase is an important enzyme in absorption. These researchers explained the causes of biomass increase along with the increase of nitrate concentration in the way that substrate amino acids are used for the metabolism. The common substrates in the first and second metabolism are used as the production of biomass and the growth process. The stimulant factors increase the biomass production, such as nitrate increase and decrease the alkaloid production. These authors stated that when the growth reached the stationary phase then the Hyocyamine concentration increased. The results of present study, in this case, are just the same as the researcher's results.

The results obtained from considering the effect of different concentration of amino nitrate shows that the highest production of tropane alkaloid is happened with 10.3 mM amino nitrate at third week. The increase of amino nitrate dosage causes the decrease of tropane alkaloid production. The maximum number of cells, the heaviest fresh and dry weight of biomass, was observed in medium with 41.22 mM amino nitrate. The decrease of amino nitrate causes a decrease of the biomass production. Hilton and Rhodes (1995) examined different species culture of *Datura* and reported that NH_4^+ is totally absorbed from medium. It may be possible that NH_4^+ is a substrate for alkaloid bio-synthesis.

Demeyer and Dejaegere (1993) reported that the dosage of Hyocyamine in leaves and stems of *Datura stramonium* (4 to 12 weeks after culture) was at highest rank in treatment with NO_3^- and then it decreased. On the other hand, treatments of NH_4^+ and NO_3^- up to 16 weeks after culture caused a high dosage of Hyocyamine. Also, these researchers' results showed a high Hyocyamine along with the highest biomass.

From the point of view of uptake calcium is an antagonist for potassium, sodium, ammonium, magnesium, iron, manganese, several heavy metals and especially for hydrogen. Due to this fact the excess of calcium in the earth somewhat decreases the harmful effect of toxic concentrations of some of the previously mentioned ions on the development of the plants. The effects of calcium on the production of tropane alkaloid hyoscyamine have also been studied using hairy root cultures of

D. stramonium and has been shown that the ability of hairy root clones to produce hyoscyamine decreased with the decreased content of the calcium in the medium (Pinol *et al*., 1999). The results of this study showed that the highest dosage of calcium chloride used in this study (7.92 mM) is along with the maximum dosage of alkaloid production in the fourth week. The highest biomass production reached in medium with 7.92 mM $CaCl_2$. These considerations showed that calcium concentration has a significant effect on tropane alkaloid synthesis. It seems that this is because of alkaloid congregation in vacuoles and it means neutral tropane alkaloid, which can penetrate tonoplast, are ionized and saved as ionic trap. This result is the same as Chalapian and Majd's (2003) reports. Lee *et al*. (1999) reported that tropane alkaloid is saved in tonoplast. Gontier *et al*. (1994) considered the effects of calcium on the growth and dosage of alkaloid tropane in *D. innoxia* cell suspension lines. They reported that 10 mM calcium effect increasing the production efficiency 10 times as mush to control cells in standard medium. It is known that calcium effects on tropane alkaloid biosynthesis since it is effective in regulating amino acid metabolism (substrate alkaloid). It can also be effective in other enzyme functions in order to change the form of alkaloids (Pinol *et al*., 1999; Pudersell *et al*., 2003).

The results obtained from considering the effect of temperature on alkaloid production shows that the maximum dosage of alkaloid production was in 20°C with the highest fresh and dry weight of biomass in 25°C. Rhoton and Bouteraouy (1994) reported that hyocyamin in tissue culture with 20 or 25°C increases as compared with tissue culture with 30°C in *Datura stramonium* which is in accordance with our findings.

Collectively these results suggest that in addition to temperature media composition such glucose, sucrose, potassium nitrate, ammonium and $CaCl_2$ also play a significant role in the production of tropane alkaloids. The results obtained could be recommended for production of tropane alkaloids at the semi- industrial and industrial scales.

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