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Application of plant tissue culture technique for production of tropane alkaloids from *Datura innoxia* Mill

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By

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أَفَرَأَيْتُم مَّا تَحْرُثُونَ ﴿٦٣﴾

[63] Then tell Me about the seed that you sow in the ground.

أَأَنتُمْ تَزْرَعُونَهُ أَمْ نَحْنُ الزَّارِعُونَ ﴿٦٤﴾

[64] Is it you that make it grow, or are We the Grower?

((سورة الواقعة))

Declaration

I certify that this dissertation was prepared under my supervision at the Department of Crop Science, College of Agriculture/ University of Sulaimani and hereby recommended to be accepted in partial fulfillment of the requirement for the degree of Ph.D. in Agriculture, Crop Science (Plant Tissue Culture).

Supervisor
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College of Agriculture
University of Duhok
06 / 05 / 2010

In view of the above recommendation, I forward this dissertation for debate by the examining committee.

Assist. Prof. Dr. Abdulsalam A. Rasool
Head of Crop Science Department
College of Agriculture
University of Sulaimani
06 / 05 / 2010

DEDICATION

Dedicated to …..

- *My father's virtuous spirit who had wished to see me a Ph.D. holder;*
- *My mother, brother and sisters;*
- *My wife Zana;*
- *My son Rebaz;*
- *My daughters Roza and Shanaz;*
- *All my beloved friends.*

With Great Love and Gratitude

Ahmad

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Table of Contents

Summary

Several experiments were carried out in Plant Tissue Culture Laboratories at both Duhok and Sulaimani to investigate the tropane alkaloids in medicinal plant species, ***Datura innoxia* Mill** which belongs to the (***Solanaceae***) family.

The conducted experiments included: leaf explants sterilization, studying the effect of different combinations and concentrations of cytokinin (BA) and auxins (NAA and 2,4-D). The cultures were grown on **MS** medium with normal $\mathbf{S_0}$ (30 gl^{-1}), $\mathbf{S_1}$ (15 gl^{-1}), and $\mathbf{S_2}$ (45 gl^{-1}) sucrose. The MS media also contained 3 levels of salts, normal, half, and double salt strength i.e., $\mathbf{X_0}$, $\mathbf{X_1}$, and $\mathbf{X_2}$ respectively and all the cultures were placed in three local Aluminum made Growth Chambers maintained in an air conditioned room at a temperature of 25±1 C°, the source of illumination in each chamber was with irradiance of 60–100 µmol $m^{-2}s^{-1}$ provided by 1.25 feet white cool fluorescent tubes (20 watt). In addition to the above mentioned, treatments were placed under different illumination intensities which were 1000, 2000, and 3000 lux (about 19, 37 and 56 $\mu molm^{-2}s^{-1}$ respectively) at the level of cultures in 3 chambers and a 16 hour light regime was followed by 8 hour darkness. Histological examination was carried out to find out the mode of cell differentiation in ***Datura*** during callus formation at fourth subculture, while we continued up to 12^{th} subcultures. Organogenesis and shoot formation ***in vitro*** culture depend on the application of exogenous plant growth regulators, specially auxins and cytokinin, and also organogenesis depends on the ability of the tissue to respond to these plant growth regulators during culture period, as shown by ***Datura*** callus which responded very well to these plant growth regulators.

Of the various pretreatment trials for ***Datura innoxia* Mill,** ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.) gave the maximum survival percentage (97%).

From the results of these experiments, it can be concluded that the best characters of ***Datura*** callus growth studied {frequency of callus formation (%), dry matter (DM) %, dry weight (g), and fresh weight (g)} was obtained from the combination of cytokinin (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) at 2^{nd} subculture. The highest frequency % of callus formation (99.42) was obtained, which was highly significantly different from all

other combinations even from the value of 93.35 for (BA 0.4 mgl^{-1}) / auxin (2,4-D 1.0 mgl^{-1}) combination. The maximum values of calli (DM %), fresh weight (g), and dry weight (g), for (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) combinations were 11.97%, 11.39g, and 1.57g respectively, while for the correspondent values for cytokinin (BA) / auxin (2,4-D) combinations were 11.08%, 8.39g, and 0.844 g at (0.4 mgl^{-1} BA) / (1.0 mgl^{-1} 2,4-D) combinations respectively.

The highest number of shoots (46.167) was obtained at (BA, 2 mgl^{-1}) / auxin (NAA, 0.6 mgl^{-1}) combination at sixth subculture, which was highly significantly different from other plant growth regulator combinations. Maximum growth of the callus as expressed on the basis of dry weight (1.357g) was observed at the end of the 2^{nd} subculture (8^{th} week) for the treatment (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) combination, calli growth was declined thereafter.

It appeared that the effects of growing ***Datura*** leaf explants at different sucrose concentrations on alkaloid concentrations and their totals were found to be increased especially the first three alkaloids (atropine, hyoscyamine and scopolamine) at $\mathbf{S_1}$ (15 gl^{-1}) sucrose treatment when the means of $\mathbf{S_1}$ and $\mathbf{S_2}$ were compared to those of **So** treatments.

In general the effect of growing ***Datura*** leaf explants at different salt strength on alkaloid concentrations and their totals especially at $\mathbf{X_1}$ treatment followed the similar trend of sucrose concentrations. While reducing the salt strength into half ($\mathbf{X_1}$) caused an increase in alkaloid concentrations compared to the effects of double strength ($\mathbf{X_2}$) and normal salt strength (**Xo**).

It was shown that the effect of growing ***Datura*** calli under different light intensities on four alkaloids, atropine, hyoscyamine, scopolamine and tropine concentrations and their totals were found to be increased when the means of **LI1** (1000 lux) were compared to those of $\mathbf{LI_2}$ (2000 lux) and $\mathbf{LI_3}$ (3000 lux) treatments. However, treatment of $\mathbf{LI_2}$ significantly decreased the concentrations (28.250 and 22.155 µg/g DW) of both alkaloids, 7-hydroxyhyoscyamine and tiglohyoscyamine respectively as compared to mean concentrations of the same alkaloids under $\mathbf{LI_1}$ (61.04 and 56.452 µg/g DW) and $\mathbf{LI_3}$ (50.472 and 31.816 µg/g DW) respectively.

In general, when the concentrations of all alkaloid types (except scopolamine) of ***Datura innoxia*** calli grown at different sucrose and salt concentrations under different light intensities were compared to their correspondent concentrations of leaves from greenhouse grown plants which showed greater concentrations differences under **LI_1**(1000lux) condition, treated with **S_1** (15 gl^{-1} sucrose) and **X_1** (half strength salt). However, in case of scopolamine it was clear that the treatment **LI_2** (2000 lux) significantly decreased the concentration of scopolamine by 25.051 (µg/g DW) compared with calli from leaves of greenhouse plants. Consequently, the present results indicated that growing leaf explants of ***Datura innoxia*** in MS media at different sucrose, salt concentrations under light intensity of (1000 lux) might be regarded as the best growing MS media to give higher concentrations of all alkaloids apart from scopolamine.

CHAPTER ONE

Introduction

Plants are valuable source of a vast array of chemical compounds; they synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful as raw materials for various commercial applications. Industrialization coupled with urbanization is constantly putting pressure on natural resources. Due to depletion of habitat and ruthless collection, medicinal plants are on the verge of extinction (Naika and Krishna, 2008).

A part from primary metabolites (i.e. carbohydrates, lipids & amino acids), plants synthesize a wide variety of secondary metabolites like alkaloids, glycosides, steroids, flavonoids, volatile oils, tannins, resins…etc. (Bhalsingh and Maheshwari. 1998 and Evans, 2001). However these compounds are sometimes considered as waste or secondary products of plant metabolism (Hedge, 1984 and Aissa, 1991).

Medicinal plants have been the subject of man's curiosity and purpose since the time immemorial. The importance of medicinal plants in the treatment of chronic diseases needs no elaborations (Srivastava *et al*., 2004). Medicinal plants are also unique sources of life saving drugs for the majority of the world population. Many of these compounds are also acknowledged as having pharmaceutical values, for example, in developing countries, 25% of all prescription dispensed from community pharmacies from 1959 through 1980 contained plant extracts or active principles prepared from higher plants, e.g. codeine (Fransworth *et al*., 1985). In fact, even with more recently tremendous advancement in the field of synthetic chemistry, almost 50% of the commercial drugs available in the market remain of plant origin (Srivastava *et al*., 2004). For developing countries , the World Health Organization (WHO) recently reported that more than 3.5 billion people rely on plants as components of their primary health care (WHO, 2002).

Moreover, secondary plant metabolisms are also generally recognized as being critical to plant protection because they serve as a chemical defense against herbivores or invasion by pathogens while the physiological function of these metabolites regarding plant protection is well recognized (Ziska *et al*., 2005).

Secondary metabolites often accumulate in the plant in small quantities, sometimes in specialized cells, hence their extraction is often difficult (Heble and Chadha, 1985b). There are many compounds which are commercially important as medicinal substances, fragrances, food additives (pigments, flavoring and aromatic compounds) and herbicides (Heble and Chadha, 1985b).

In spite of the progress made in organic synthesis or semi-synthesis of a wide range of compounds similar to those produced by the plants, extraction of secondary metabolites from plants is still of considerable commercial importance. A large number of those metabolites are difficult or virtually impossible to synthesize at economic values. In several cases, the natural product is more easily accepted by consumers than an artificially produced one (Sasson, 1991).

Both reasons as an objective and subobjective explain that natural extraction still applies to a large number of aromas or fragrances which are the result of a mixture of hundreds of different compounds as the case of jasmine and strawberry, or to biochemical that have complex molecular structures, e.g. some alkaloids and glycosides (Dicosmo and Misawa, 1995).

In recent years, there has been an increased interest in ***in vitro*** culture techniques, which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants (Li *et al*., 2004 and Emma *et al*., 2005).

Plant tissue culture technology has become a powerful tool in plant biotechnology. The potential of plant tissue culture for plant propagation and production of secondary metabolites has itself provided substantial impetus for research (Dicosmo and Misawa, 1995). Many biotechnological strategies have been hypothesized and experimented for enhanced production of secondary metabolites from medicinal plants (Namdeo *et al*., 2007).

Tissue culture technology is widely applied for the improvement of Field Crops, Forests, Horticulture and Plantation Crops for increased Agricultural and Forestry production. This technology has been commercialized globally and contributed significantly towards the enhanced production of high quality planting material (Kumar, 2005).

In the present study the genus ***Datura*** belonging to the ***Solanaceae*** family and are a group of a pharmaceutically important medicinal plants was selected. Plants of ***Datura innoxia*** **Mill**. were used because of the following criteria favoured its choice:-

a) Plants are of easy cultivation and seeds are readily available.

b) A scrutiny of literature revealed that the plant has not been previously worked out for tissue culture studies in Kurdistan region.

c) These plant species are most available in the city gardens compared to other ***Datura*** species.

d) The question arose as to whether an effect of salt strength on alkaloid accumulation is in dependant of the light regimes. This can be of practical importance as ***Datura innoxia* Mill** is grown in different regions, under different light conditions.

Moreover, they are known to be rich sources of alkaloids which are synthesized in several ***Datura*** species, these are scopolamine and hyoscyamine (Tailang *et al*., 1997) all of which can cause anticholinergic poisoning if taken in large concentrations (Shervette *et al*., 1979; Sakuta *et al*., 1987; Kurzbaum *et al*., 2001;Salen *et al*., 2003 and Ertekin *et al*., 2005).

Secondary metabolism in higher plants is strongly influenced by environmental factors. In plant tissue culture, plant growth regulators, nutritional factors and light intensity affect the production of secondary metabolites as well as growth under control conditions. Different types and concentrations of growth regulators i.e. auxins and cytokinins are known to show different effects on plant growth and production of secondary metabolites (Sakuta *et al*., 1987).

The aim of the study

The goal of this study was to apply plant tissue culture technique in Kurdistan region as a new technique for the region, through this technique we might be able to increase the tropane alkaloids contents in callus culture of (***Datura innoxia* Mill**) compared with those naturally grown plants and producing them all year around without depending on the season. Moreover, determining the relationship between the physiological age of the plant and its chemical contents, through applying the following treatments:

1. Using different concentrations of auxins and cytokinins to follow up their effect on both callus production and their contents of some alkaloids, as well as their effect on shoot formation.
2. Study the effect of different concentrations of MS salts (strength of MS medium salts) on some alkaloids' production.
3. Study the effect of carbon concentration on the production of these materials.
4. Testing the effect of light intensity on the production of some alkaloids.

More recently (Dechaux and Boitel-Conti, 2005) **f**ound that transformed roots in axenic culture present a good model system for studying the regulation aspects of plant secondary metabolism in ***Datura innoxia.*** However, for industrial use, scopolamine is the more valuable than hyoscyamine. Thus they confirmed that there is increasing interest in obtaining cultures with enhanced content of scopolamine. Accordingly based on the literature that was the reason why we have chosen ***Datura*** genus and in particular ***Datura innoxia.***

CHAPTER TWO

Literature Review

2.1. The Genus Datura:

The genus ***Datura*** comprises more than fifteen species of vespertine (in botany, a vespertine flower is one which opens or blooms in the evening) flowering plants belonging to the family Solanaceae. Its precise and natural distribution is uncertain, owing to its extensive cultivation and naturalization throughout the temperate and tropical regions of the globe. ***Datura stramonium*** was introduced by the first American settlers in a settlement called (James town) and hence the name James weed came as a result because it became a noxious weed, its distribution then started from the United States south through Mexico, where the highest species diversity occurs. The english names are Thorn apple; Trumpet; Devil's Trumpet flower; Stink weed; Jimson weed; Mad apple (Chakravatry, 1976).

2.1.1. Description

Datura are woody-stalked, leafy annuals and short-lived perennials which can reach up to 2 meters in height. The leaves are alternate, 10–20 cm long and 5–18 cm broad, with a lobed or toothed margin. The flowers are erect or spreading (not pendulous like those of the closely allied Brugmansiae), trumpet-shaped, 5–20 cm long and 4–12 cm broad at the mouth; colours vary from white to yellow, pink, and pale purple. The fruit is a spiny capsule 4–10 cm long and 2–6 cm broad, splitting open when ripe to release the numerous seeds. The seeds disperse freely over pastures, fields and even wasteland locations (Al-abdalli, 1975 and Chakravatry, 1976).

There are three common species of ***Datura*** in Iraq (Al-Khalidi, 2005) as follows:

- ***Datura stramonium*** **Linn.**
- ***Datura inoxia*** **Mill .**
- ***Dtura metel*** **Linn.**

In the present study only one species (***Datura innoxia*** Mill **.**) was selected**.** Angel's trumpet **(*Datura inoxia* Mill**) is an annual shrubby plant that typically reaches a height of 0.6 to 1.5 metres. Its stems and leaves are covered with short and soft grayish hairs, giving the whole plant a grayish appearance. It has elliptic entire-edged leaves with pinnate venation(Preissel and Hans-Georg, 2002). All parts of the plant emit a foul odor similar to rancid peanut butter when crushed or bruised, although most people find the fragrance of the flowers to be quite pleasant

when they bloom at night (Hawkes *et al.*, 1991 and Preissel and Hans-Georg, 2002). The flowers are white, trumpet-shaped, 12–19 cm long (Fig. 1).

Description

Scientific classification	
Kingdom:	*Plantae*
(unranked):	*Angiosperms*
(unranked):	*Eudicots*
(unranked):	*Asterids*
Order:	*Solanales*
Family:	*Solanaceae*
Genus:	*Datura*
Species:	***D. innoxia***
Binomial name	
***Datura innoxia* Mill**.	

Figure 1. ***D. inoxia*** with ripe, white-open flower.

They first grow upright, and later incline downward. It flowers from early summer until late fall. The fruit is an egg-shaped spiny capsule, about 5 cm in diameter (Fig. 2 and 3). It splits open when ripe, dispersing the seeds. Another means of dispersal is by the fruit spines getting caught in the fur of animals, which then carry the fruit far from the mother plant. The seeds have hibernation capabilities, and can last for years in the soil. The seeds, as well as the entirety of this plant, are also hallucinogenic, but have a high probability of overdose (Taha, 1984 and Djibo and Bouzou, 2000).

Figure 2. ***D. inoxia*** with fruit.

Figure 3. ***D. inoxia*** with ripe, split-open fruit.

2.1.2. Geographical Distribution of *Datura innoxia* Mill.

Mexico and USA are considered the habitat of this species, it grows near the Himalayan mountains and widespread in equator, and in house gardens of many Iraqi provinces including Abi-ghreb, Zaafaranniah,etc. (Al-abdalli, 1975 and Muhamed, 1996) and in Sulaimani province as well, especially widely distributed along the banks of Tanjaro river.

Date of Planting

The seeds are sown between March 1-15; germinate when the soil temperature ranges between 8-15°C (Al-Abdalli, 1975).

Soil and fertilization

This plant species favours clay loam soil and the fertilizer is applied at budding stage at rate of 35-40 kg ammonium nitrate and 40-45 kg super phosphate / Donum **(**Al-Abdalli, 1975).

2.1.3. Medicinal Uses

All parts of the plant are anodyne, antispasmodic, hallucinogenic, hypnotic and narcotic (Emboden, 1979 and Chopra *et al.*, 1986). It has been used in the past as a pain killer and also in the treatment of insanity, fevers with catarrh, diarrhoea and skin diseases (Chopra *et al.*, 1986). The plant contains several alkaloids, the most active of which is scopolamine (Chopra *et al*., 1986). This is a potent cholinergic-blocking hallucinogen, which has been used to calm schizoid patients (Weiner, 1980). The leaves contain 0.52% scopolamine, the calices 1.08%, the stems 0.3%, the roots 0.39%, the fruits 0.77%, the capsules 0.33%, the seeds 0.44% and the whole plant 0.52 - 0.62% (Chopra *et al*., 1986).

2.1.3.1. General Poisoning Notes

Angel's trumpet (***Datura innoxia***) contains toxic alkaloids that have caused poisoning and death in humans and other animals. Most of the literature concerns poisoning by jimsonweed (***Datura stramonium***), but angel's trumpet should be considered poisonous as well. This plant is occasionally grown as an outdoor ornamental herb because of its spectacular tubular flowers (Cheeke and Shull, 1985).

2.1.3.2. Toxic parts

They include leaves, stems and seeds (Al-Abdalli, 1975).

2.1.4. Plant Chemistry (Phytochemistry)

Every plant is a chemical factory for array of complex substances which exceeds human capability to synthesize. In their poisons, antibiotic agents, prickles and foul tastes, they developed defenses against attack long before human stockades and pesticides.

Plants provide us with an enormous array of chemicals essential to industry and to our daily lives, but why are the chemicals there and why does the plant produce them? Many of the chemicals are linked to the ingenious strategies that plants have developed to help them flourish and survive (Price, 1998 and Raven and Freeman, 1999).

Plants can't run away from their enemies, be the animals or bacteria. Some of their defenses include the thick, insulating bark of many trees, the vicious thorns on roses, etc. (Price, 1998 and Raven *et al*., 1999), as well as chemical components to deter the attackers.

Most plants can be regarded as complex chemical factories, since an astonishing array of compounds has evolved within them over millions of years. Scientists divide these compounds into two categories:

2.1.4.1. Primary Metabolites

Primary Metabolites are found in all plant cells. These include sugars (carbohydrates), lipids and proteins which are involved in the fundamental biochemical reactions common to all life (Kurz, 1989). However, we're not interested in primary metabolites here; we're more interested in the second category.

2.1.4.2. Secondary Metabolites

Secondary Metabolites tend to be more specialized, and some are usually peculiar to only one plant or species. Their biological function is not always obvious, but they are not formed without a reason. They are important for the survival and propagation of the plant. While some secondary metabolites are designed to attract creatures that can pollinate their flowers or distribute their seeds, others protect the plant from the sun's radiation, or serve as 'chemical signals' that enable the plant to respond to 'environmental clues'. Others are defensive compounds, designed to deter or kill disease-causing organisms, potential predators or competitors. Oddly enough, it's among the plant chemicals that are generally poisonous to mammals, that so many of our medicines are found. Examples of such drugs in common use today include morphine and digitoxin. These are both secondary metabolites, and still isolated from plant sources (Price, 1998 and Raven and Freeman, 1999).

There are three major categories of secondary metabolites: alkaloids, phenols and terpenoids (Price, 1998 and Raven and Freeman, 1999).

2.1.4.2.1.Alkaloids

Alkaloids are important groups of plant chemicals, more than 10000 of which have now been isolated. Many are extremely poisonous to humans but a number, like morphine, atropine and cocaine, are widely used in medicine. Alkaloids represent a highly diverse group of compounds that are related only by the occurrence of a nitrogen atom in a heterocyclic ring. Alkaloids can be organized into groups according to their carbon skeletal structures. Their biosynthesis in plants involves many catalytic steps, catalyzed by enzymes and their starting materials are a wide range of amino acids (Jorg and Facchini, 2008).

Alkaloids are a class of "secondary" plant metabolites that traditionally have been classified as basic compounds derived from amino acids that contain one or more heterocyclic nitrogen atoms. Although this definition holds for most known alkaloids, recently any N containing secondary compound is considered an alkaloid if it cannot readily be classified otherwise i.e. not an amine, cyanogenic glycoside, glucosinolate….etc. Oksman-Caldentey and Saito (2005) also showed that the best characterized groups of alkaloids are tyrosine-derived isoquinoline alkaloids, the tryptophane-derived indole alkaloids, and ornithine-derived nicotine and tropane alkaloids. The word alkaloid is derived from the Arabic *al-qali*, a plant from which soda was 1st isolated. The original definition for alkaloids is pharmacologically active, N-containing basic compounds of plant origin. Humans have been using alkaloids in the form of plant extracts for poisons, narcotics, stimulants and medicines for at least the past several thousand years. Morphine was isolated from poppy seeds in 1806 although its structure wasn't known until 1952. The antimalarial properties of quinine, an alkaloid extracted from the bark of *Cinchona* spp. trees indigenous to the high eastern slopes of the Andes Mountains have long been known. More than 12000 different alkaloids (Jorg and Facchini, 2008) of widely differing structures are now known from the small fraction of the planet's plants that have so far been examined. Currently more than 15000 different alkaloids are known and they are classified into several subclasses based on the amino acids from which they are derived and according to their chemical structures (Verpoorte, 2000). He also showed that contrary to other secondary metabolites, e.g. plant phenolics, which are abundant throughout the whole plant kingdom, alkaloids are often restricted to certain plant families or even certain plant species. Most medicinal compounds have traditionally been extracted from some plant tissues although modern synthetic chemistry has also attempted to synthesize all important medicinal compounds. Still 25% of compounds used in western medicine are plant-derived and most are still derived from plants in traditional medicine. Recent advances in plant genetic engineering are likely to make plants the preferred source of many medicinal compounds again in the future with "Pharming" developments (University of Kentucky / Research Annual Report 2002).

Alkaloids are physiologically active, nitrogen containing low molecular-weight compounds produced predominantly, although not exclusively, in higher plants (Kutchan, 1995). Mankind’s use of alkaloid-containing plants as medicinal agents can be traced back thousands of years, and indeed the modern pharmaceutical industry finds its roots in physiologically active

secondary metabolites. A role in the chemical defense of the plant also emerges for alkaloids, causing this class of metabolites to be of both eco-chemical and pharmaceutical interest (Pauli and Kutchan, 1998). Like many secondary metabolites, plants apparently synthesize alkaloids for defensive purposes. Nicotine and derivatives are among the earliest known and most potent insecticides. Some plants had already evolved the ability to synthesize alkaloids at the beginning of angiosperm evolution 200 million years ago. Like most natural product chemistry, the accumulation of alkaloids tends to run in families. Griffin and Lin, (2000) illustrated in their review the distribution of tropane alkaloids also within other families including, ***Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae*** and ***Cruciferae.***

2.1.4.2.2. Classification of Alkaloids

The biosynthetic precursors of most alkaloids are amino acids. At a very basic level it is possible to distinguish between aromatic and aliphatic amino acid precursors (Figure 4).

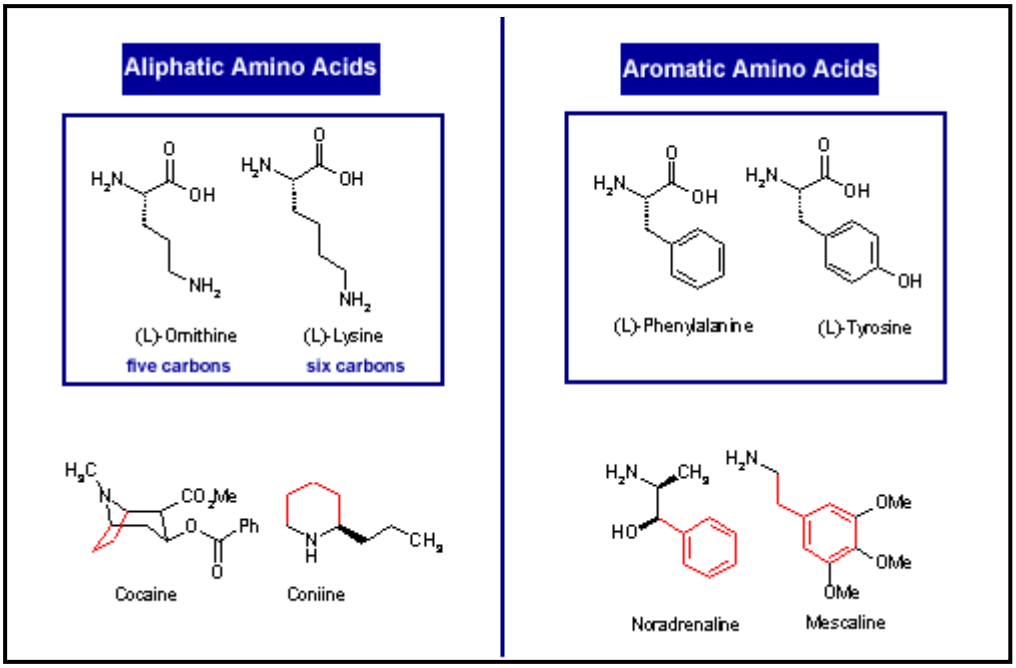


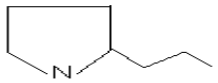
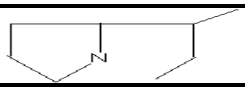
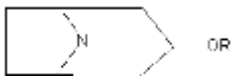
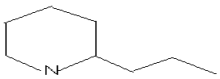
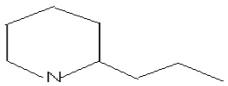
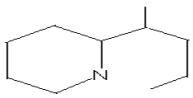
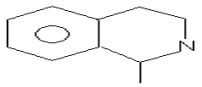
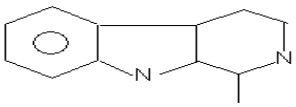
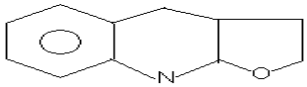
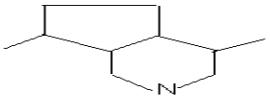
Figure 4. Precursors of aromatic and aliphatic amino acids (Griffin and Lin, 2000). Even in complex alkaloids the aromatic amino acid portion can easily be identified.

Most alkaloid skeletons are derived from amino acids with many different amino acids being alkaloid biosynthetic precursors. Some alkaloid carbon skeletons are derived from other groups of molecules such as the steroid alkaloids with the nitrogen from glutamine or another N

donor being added in later biosynthetic steps. Alkaloids are classified based on the structure of the N-heterocycle. Some major alkaloids and their structures are given in Kutchan (1995).

In this section we will cover a few of the alkaloid groups, some representative structures and some examples of biosyntheses are shown in table 1 below (Jakubke and Jeschkeit, 1975).

Table 1. General structural classification of alkaloids (Jakubke and Jeschkeit, 1975).

Group name	Base structure
Pyrrolidine	
Pyrrolizidine	
Tropane	
Piperidine	
Punica, Sedum and Lobelia alkaloids	
Quinolizidine	
Isoquinolizidine	
Indole	
Rutaceae alkaloids	
Terpene alkaloids	

The following figures 5 and 6 show the main pathway leading to secondary metabolites:

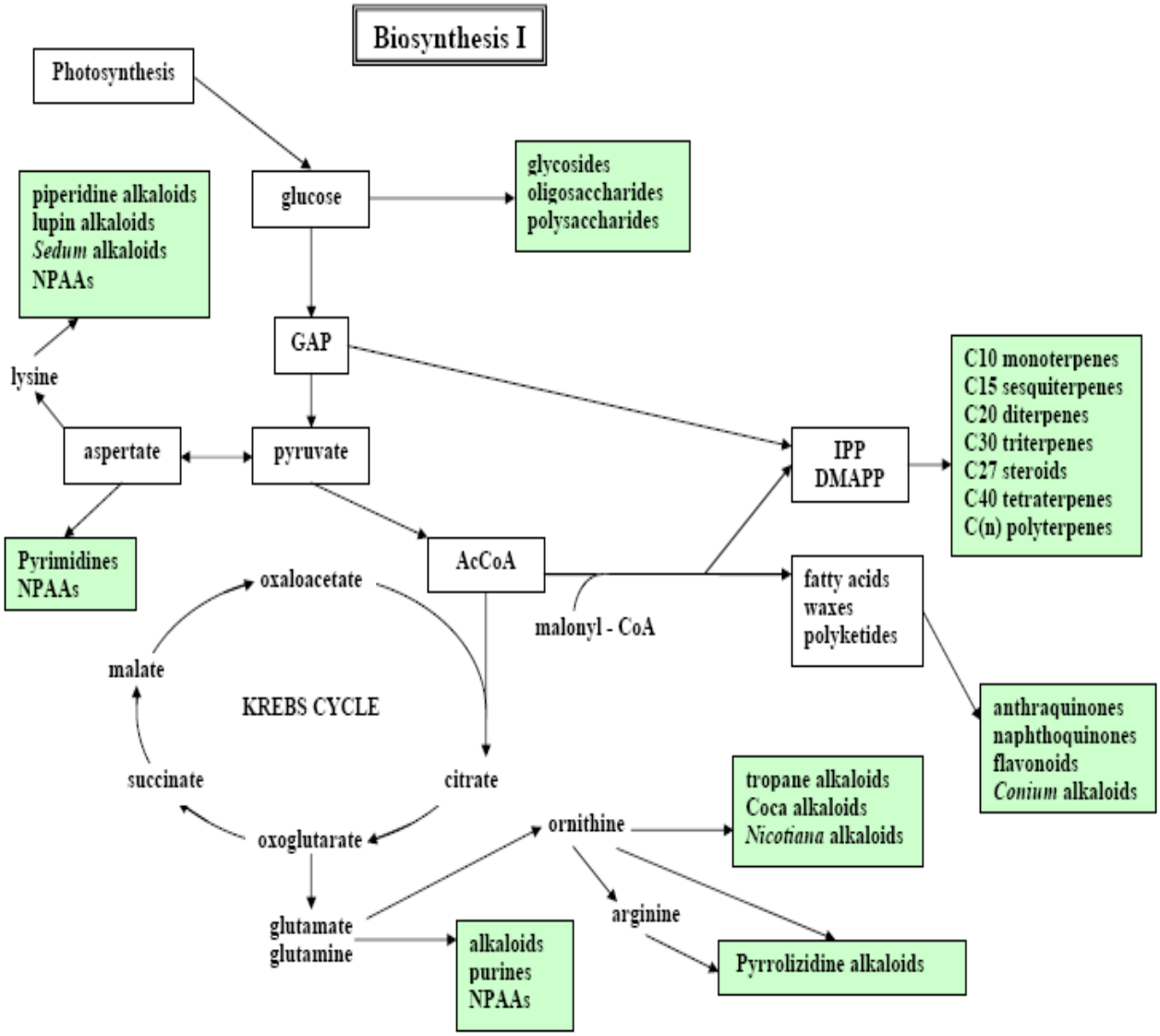


Figure 5. Main pathways leading to secondary metabolites.
Abbreviations: IPP, isopentenyl diphosphate; DMP, dimetyl allyl diphosphate; GAP, glyceraldehydes-3-phosphate; NPAAs, nonprotein amino acids; AcCoA, acetyl coenzyme A (Michael, 1999).

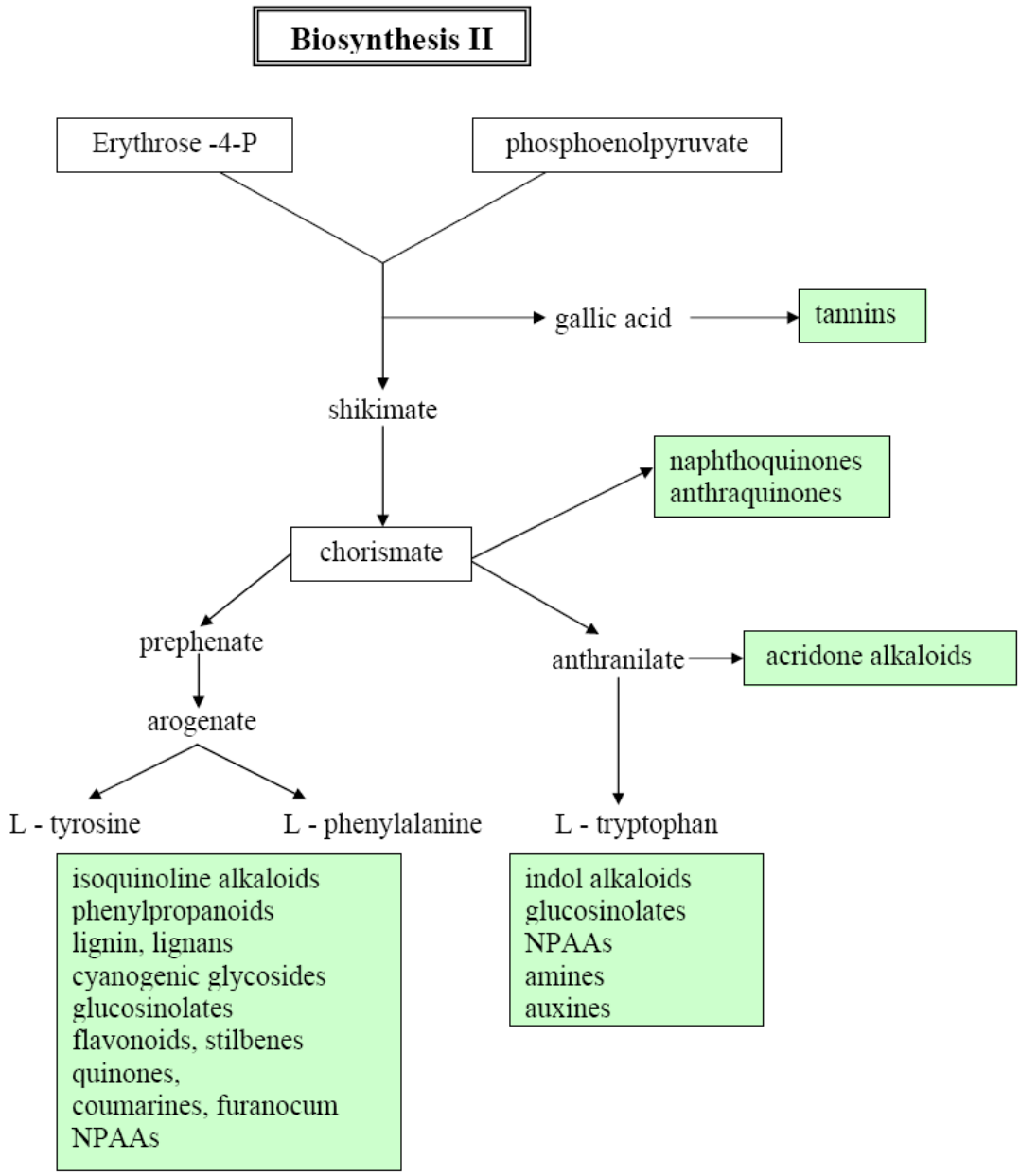


Figure 6. Several pathways of secondary metabolites derive from precursors in the shikimate pathway. Abbreviation: NPAAs, nonprotein amino acids (Michael, 1999).

2.1.5. Notes on Toxic angel's trumpet (*D. innoxia)* Plant Chemicals

Many plants belonging to the ***Solanaceae*** family have been used as a source of pharmaceuticals for centuries because of their active principles, tropane and nicotine alkaloids. Tropane alkaloids, atropine, hyoscyamine and scopolamine, are among the oldest drugs in medicine. These alkaloids are used in the chemo taxonomy of *Solanaceae* family (Griffin and Lin, 2000). On the other hand nicotine, the addictive agent in tobacco, has only recently gained attention as a backbone for novel potential alkaloids to be used for certain neurological diseases (Oksman-Caldentey, 2007). It has been found from the literature that ***Datura*** contains the tropane alkaloids atropine, hyoscine, and hyoscyamine (Cheeke and Shull, 1985).

2.1.5.1. Tropane alkaloids

Since the discovery of the opium alkaloid morphine almost two centuries ago, alkaloids are still one of the most studied groups of plant secondary metabolites often possessing strong physiological properties leading to their use as pharmaceuticals (Oksman-Caldentey, 2007). Tropane alkaloids, such as atropine, hyoscyamine and scopolamine have an important role as medicinal compounds acting as parasympatolytes. They have been isolated from many different plant families, *e.g.* ***Solanaceae, Erythroxulaceae, Convolvulaceae, Proteaceae, Rhizophoraceae*** and ***Euphorbiaceae***. In the ***Solanaceae*** family, e,g of common tropane alkaloid producers are ***Duboisia, Atropa, Hyoscyamus, Scopolia*** and ***Datura*** **sp**. Other Solanaceous plants such as ***Scopolia carniolica, Mandragora officinarum, Brugmansia candida*** and some food crops (e.g.potato, eggplant) also contain tropane or tropane-like alkaloids. The total alkaloid content varies from 0.01 – 3 % of dry weight depending on the species and plant organs. Plant breeding has resulted in contents up to 5% (Oksman-Caldentey, 2007). Cocaine is also tropane alkaloid but it is found outside *Solanaceae* plants, namely in ***Erythroxylon coca*** (Latorre and Klimek, 1999). have been reported to cause degenerative neurological disorders in cattle (Todd *et al*., 1995).

L-Hyoscyamine is a levorotatory component of the racemic atropine. Scopolamine demand is estimated to be tenfold compared to that of hyoscyamine and atropine combined (Hashimoto *et al.*, 1993). Due to the difficult synthesis of these rather complex molecules, both alkaloids are still extracted from plants.

2.1.5.1.1. General Medical Uses of Tropane Alkaloids

1. Atropine, hyoscyamine and scopolamine affect the parasympathetic nervous system.
2. They competitively inhibit muscarinic receptors for acetylcholine and act as nonselective muscarinic antagonists, producing peripheral antimuscarinic and central sedative, antiemetic, and amnestic effects.
3. The semi-synthetic quaternary muscarinic antagonists ipratropium and, more recently, tiotropium, are two effective bronchodilators that have been developed and are used extensively in the treatment of chronically obstructed pulmonary disorder (Rodrigo and Nannini, 2006).

2.1.5.1.2. Biosynthetic Pathway of Tropane Alkaloid

Roots are the main sites of nicotine and tropane alkaloid biosynthesis, and generally have higher alkaloid content and a wider variety of alkaloids than the other parts of the plant. However, secondary modifications, e.g. epoxidation of hyoscyamine to scopolamine, and the storage of alkaloids occur in aerial parts (Kanage *et al*., 1994). As a consequence, leaves are often the preferred source of isolation. Tropane alkaloids have a pyrrolidine and a piperidine ring, which share the nitrogen atom and two carbon atoms. In tropane alkaloid producing plants, the tropane ring system requires the condensation of an appropriate acetate-derived intermediate with *N*-methylpyrrolinium to form tropinone (Humphrey and Hagan, 2001). Tropane alkaloid is considered the key intermediate in the biosynthesis of tropane esters in Solanaceous plants (Portsteffen *et al*., 1991).

Biosynthesis of tropane alkaloids (Figure 7) starts from the amino acids arginine and ornithine, as is the case with tobacco alkaloids. Tobacco and tropane alkaloid pathways share common steps until *N*-methylpyrrolinium, which is directed towards tropinone in tropane alkaloid producing systems (Sévon *et al.*, 2001 and Humphrey and Hagan, 2001). The role of tropinone in tropane alkaloid biosynthesis was controversial for a long time, until in 1990 in which some researchers showed it to be an intermediate in the biosynthesis of tropine (Landgrebe and Leete, 1990). Tropinone is further converted into tropine and pseudotropine by the reactions catalysed by two distinct enzymes, tropinone reductase I :TRI (Nakajima *et al*., 1993) and tropinone reductase II; TRII (Nakajima *et al*., 1993 and Keiner *et al*., 2002), respectively.

Littorine, a positional isomer of hyoscyamine, is formed from tropine and phenyllacetic acid, the latter deriving from phenylalanine.

Recently, Li and co-workers exploited virus-induced gene silencing (VIGS) to discover the rearrangement of littorine to hyoscyamine (Li *et al*., 2004). It was suggested that the (*R*)-littorine is converted to hyoscyamine in a two-step process, first by formation of hyoscyamine aldehyde catalyzed by a cytochrome P450 enzyme, proceeding to the formation of hyoscyamine by an alcohol dehydrogenase-catalysed reaction. Hyoscyamine-6β-hydroxylase (H6H; EC 1.14.11.11) catalyzes both the hydroxylation of hyoscyamine leading to 6β-hydroxyhyoscyamine and the epoxidation of the latter leading to scopolamine (Hashimoto and Yamada, 1986 and Matsuda *et al*., 1991). The hydroxylase activity of *H6H* has commonly been observed to be much higher than the epoxidase activity leading to the formation of scopolamine. Scopolamine production has been increased in hairy roots of hyoscyamine-producing plants by overexpressing *H6H* alone (Yun *et al*., 1992; Hashimoto *et al*., 1993 and Jouhikainen *et al*., 1999), and simultaneously with *PMT* (Zhang *et al*., 2004). Calystegines, which are synthesized in the other branch of the tropane pathway, are polyhydroxy nortropane alkaloids, possessing strong glycosidase inhibitory activity (Asano *et al.,* 2000). The compounds were originally found in ***Calystegia sepium* (L.)** (Goldmann *et al.,* 1990), but they have also been detected in many ***Solanaceae*** and other families, even in some species which were not thought to possess the tropane alkaloid pathway, such as ***Solanum tuberosum* (L.)** (Dräger *et al*., 1995). Calystegines are formed from tropinone in a reaction catalysed by TRII (Dräger *et al*., 1994).

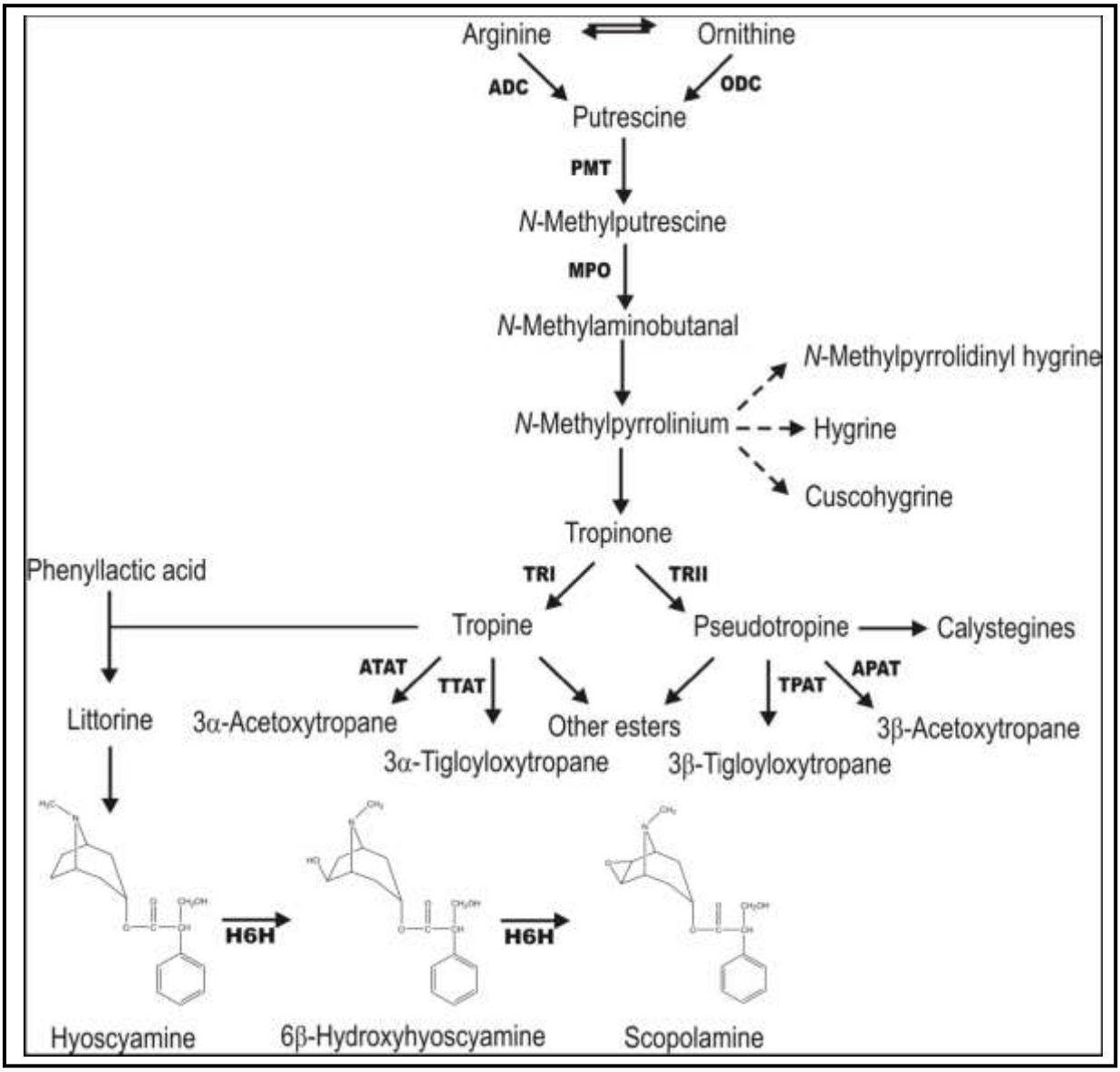


Figure 7. Tropane alkaloid pathway. One arrow may represent more than one step. Hypothetical steps are indicated with dashed lines. Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PMT, putrescine N methyltransferase; MPO, N-methylputrescine oxidase; TRI, tropinone reductase I; TRII, tropinone reductase II; ATAT, acetyl-CoA:tropine acyl transferase; TTAT, tigloyl-CoA:tropine acyl transferase; TPAT, tigloyl-CoA:pseudotropine acyl transferase; APAT, acetyl- CoA:pseudotropine acyl transferase; H6H, hyoscyamine-6β-hydroxylase (Portsteffen *et al*., 1992).

2.1.6. Atropine

Atropine is the best-known member of a group of drugs known as muscarinic antagonists, which are competitive antagonists of acetylcholine at muscarinic receptors. This naturally occurring tertiary amine was first isolated from the ***Atropa belladonna*** plant by Mein in 1831 (Weiner, 1980).

Name and chemical properties of Atropine and L-Hyoscyamine which is a levorotatory component of the racemic atropine:

Atropine is DL mixture (Racemis) of Hyoscyamine when the position of OH-group (alcoholic) is either L (above) or D (bellow) the α carbon next to COO group.

Both alkaloids have the same empirical formula $C_{17}H_{23}O_3N$ while are having different chemical structures.

2.1.6.1. Medical Uses of Atropine

Although atropine earlier enjoyed widespread use in the treatment of peptic ulcer, today it is mostly used in resuscitation, anaesthesia, and ophthalmology, usually as the more soluble sulphate salt. By competitively blocking the action of acetylcholine at muscarinic receptors, atropine may act as a specific antidote. As such, it may also be used to counteract adverse parasympathomimetic effects of pilocarpine, or neostigmine administered in myasthenia gravis. It is a specific antidote for the treatment of poisoning with organophosphorus and carbamate insecticides and organophosphorus nerve agents (Heath, 2002).

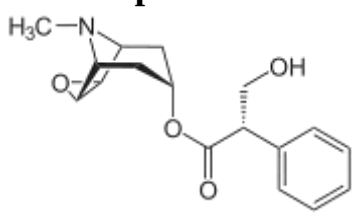
2.1.6.2. Name and Chemical Properties of Scopolamine

(Karch, 2003; Spinks *et al*., 2004 and White *et al*., 2007)

Scopolamine is a thick, syrupy, colourless alkaloid, $C_{17}H_{21}NO_4$, extracted from plants such as alkaloid drug obtained from plants of the nightshade family (Solanaceae), chiefly from henbane, ***Hyoscyamus niger***. Scopolamine is more valuable and is preferred for its higher physiological activity and fewer side-effects (Häkkinen *et al*., 2005).

Scopolamine: Chemical formula: $C_{17}H_{21}O_4N$

Scopolamine



Etymology

Scopolamine is named after the plant genus ***Scopolia.*** The name "hyoscine" is from the scientific name for henbane, ***Hyoscyamus niger*.**

Physiology

Scopolamine acts as a competitive antagonist at muscarinic acetylcholine receptors, specifically M1 receptors; it is thus classified as an anticholinergic,anti-muscarinic drug.

2.1.6.3. Medical Uses of Scopolamine

In medicine, scopolamine has these uses (Joubert *et al*., 1984; Wedin, 1988 and White *et al.* 2007) :

1. For treatment of nausea It is used as an antiemetic in the form of a transdermal patch and motion sickness.
2. For treatment of intestinal cramping.
3. For ophthalmic purposes used in eye drops to induce mydriasis (papillary dilation).
4. Used as a general depressant and adjunct to narcotic painkillers. As a preanesthetic agent.
5. Used as a drying agent for sinuses, lungs, and related areas.
6. It can be used as a depressant of the central nervous system.
7. Due to its effectiveness against sea-sickness it has become commonly used by scuba divers.
8. Used to enhance the pain-killing ability of various opioids.
9. Because of its anticholinergic effects, scopolamine has been shown to prevent the activation of medial temporal lobe structures for novel stimuli during spatial memory tasks.

2.1.7. Plant Tissue Culture

2.1.7.1. Callus Culture

Plant tissue culture forms the backbone of plant biotechnology, i.e. micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant Tissue Culture is an essential component of plant biotechnology. Plant cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future (Kumar and Kumar, 1996). Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations. Tissue culture techniques are now being widely applied for improvement of field crop, forest, horticulture and plantation crop for increased agricultural and forestry production. Today, tissue culture technology is being exploited mainly for large-scale production or micropropagation of elite planting material with desirable characteristics. This technology has now been commercialized globally and has contributed significantly towards the enhanced production of high quality planting material (Baran Jha and Ghosha, 2005).

The growth and maintenance of unorganized cell masses initiated from disorganized growth of pieces of plant tissues or the explants are known as callus. Callus cultures are initiated from a small part of an organ or tissue segment called the explants on growth supporting solidified nutrient medium { One of the most commonly used media for plant tissue culture is that developed by Murashige and Skoog (MS) for tobacco tissue culture in (1962), table 4 – chapter 3} under sterile conditions. Any part of the plant organ or tissues may be used as the explants (Baran Jha and Ghosha, 2005).

Furthermore, the callus, derived from surface sterilized plant material on a medium promoting rapid growth (inorganic nutrients, vitamins, growth regulators, or a carbon/energy source) consists of undifferentiated plant cells. The callus usually contains a mixture of cell types and/or cells in an early phase of differentiation. The part of the explants providing the source of callus initiation (e.g., root, leaf, or stem) can result in differences in the biosynthetic characteristics of the callus (Dörnenburg and Knorr, 1996). Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to a fast proliferation of cell mass and to a condensed biosynthetic cycle. This is the most important advantage of plant cell cultures as model systems for the study of biosynthetic

pathways, because secondary metabolite formation may take place within a short cultivation time of about 2 to 4 weeks (Dörnenburg and Knorr, 1996). Also, plant cell cultures are an excellent source for the isolation of enzymes, much better ones than differentiated plant itself. "A pot of gold " as it was described by (Zenk, 1991).

Plant cell cultures are generally considered totipotent. This means that they retain the genetic information and capacity to regenerate the whole plant when the callus or suspension is given the environmental signals. The ability of the plant cells to differentiate also seems to be important for the formation of secondary metabolites (Dörnenburg and Knorr, 1996).

Plant cell culture have been pursued for more than thirty years as a potential source of secondary metabolites to rival extraction processes based on whole plant material. However, progress has been somewhat disappointing, with only a very limited number of successes in the market. Many questions still remain, with a particular need for enhanced productivities in cell cultures and simplified and improved process technologies (Häkkinen *et al*., 2005 and Oksman-Caldentey, 2007).

The large-scale production of secondary metabolites such as pigments and alkaloids by field grown plants had been limited in the last century due to the dependency of the metabolism on rate of propagation, cultivation of plants, and climate during the cultivation. Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavuor and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson et al., 1990). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Kamada *et al*., 1986 and Ramachandra and Ravishankar, 2002). In spite of these efforts, the large-scale production of only few substances such as shikonin has succeeded (Fujita *et al.*, 1981). Even though alkaloids have been widely used and studied for decades, surprisingly little is known about their synthesis in the plant cells. The lack of understanding of the functions of various enzymes and their respective genes in directing and controlling the long multistep biosynthetic pathways has hampered the development of biotechnological production systems in the cell and tissue cultures. Characterization of many alkaloid pathways is currently under way in

several laboratories using classical biochemical or new functional genomics approaches (Verpoorte, 2000; Hirai *et al*., 2004 and Oksman-Caldentey and Saito, 2005). As cross point of these technologies, hydroponics called Plant Milking Technology (PMT) has been used to grow entire plant in non sterile conditions and to force root secondary metabolite exudation in order to collect these compounds from the nutrient solution in which the plant has grown (Thi Le *et al*., 2007). They studied the efficiency of detergent (Tween 20) and demonstrated that hyoscyamine and scopolamine can be obtained from living ***Datura innoxia*** **Mill** plants.

2.1.7.2. Tissue Cultures Producing Pharmaceutical / Products of Interest

2.1.7.2.1. Tropane Alkaloids

Oksman-Caldentey (2007) reviewed the tropane and nicotine alkaloids and their pharmacological properties. He highlighted recent achievements in the biosynthesis of these alkaloids and had given special focus on the new possibilities of metabolic engineering towards efficient biotechnological production of plant secondary metabolites using plant cell and tissue culture systems.

Although tropane alkaloid-based drugs have been known for a long time, they are still recognized as effective and readily available drugs and in most cases have not been superseded by better or cheaper alternatives (Arroo *et al*., 2007). Novel derivatives of naturally occurring tropane bases have been developed and are likely to be used in the foreseeable future. As an example, pervilleine A, a novel tropane alkaloid isolated from ***Erythoxylum pervillei*** has shown it's potential to act as a multi-drug resistance (MRD)-reversing agent (Mi *et al*., 2001).

Tropane alkaloids that include scopolamine and hyoscyamine are being used commercially as anesthetic and antispasmodic drugs. The production of tropane alkaloids by callus, cell, shoot, root and hair root cultures has been reported before for several genera of *Solanaceae* including *Atropa, Datura, Duboisia, Hyoscyamus and Scopola* and occur in leaves of (*Solanaceae*) plants including generally Datura species (Evans, 1989 and Kinsara and Seif El-Naser, 1994). They found that the ***in vitro*** plant culture resulted in obtaining high percentage of alkaloids more than in the intact plant. Studies on production of tropane alkaloids by plant tissue cultures have been actively carried out by many researches (Tabata *et al*., 1971 and Yamada and Hashimoto, 1982). It has been shown repeatedly that undifferentiated cultures such as callus and cell suspension cultures produce only very low contents of secondary metabolites. High

production, dependent on primary differentiation of cells, is often lost in undifferentiated cell suspension cultures especially during long periods of subculturing. Many cultures have shown fluctuation in productivity or even dramatic decrease with time. Several attempts have been carried out to solve these problems. However, selection of high producing cell lines, optimization of the growth medium and the use of elecitors had only a marginal influence on enhancement or control of tropane alkaloid production (Oksman-Caldentey and Inzé, 2004).

The connection between the expression of alkaloids and the differentiation programme has been often pointed out. In higher plants, the occurrence of certain pathways can depend on the general development of the organism and/or on the development of single organs, tissue and particular specialized cells. Synthesis and accumulation can be endogenously controlled by development-dependent differentiation processes, and/or can be regulated by exogenous factors (Kanage *et al*., 1994). Therefore production of alkaloids in differentiated tissue cultures, first using conventional root cultures and later hairy roots, has gained much interest in recent years since tropane alkaloid production is associated with the organogenesis of roots (Hashimoto and Yamada, 1986). Compared to conventional root cultures, hairy roots possess higher growth rates, which are comparable to those of cell suspension cultures, and they are genetically stable. Furthermore, they are able to produce tropane alkaloids at levels which are often comparable to or greater than that of the intact plants (Sevón and Oksman-Caldentey, 2002). However, they are not as easily modified by changing the culture conditions as in the case of undifferentiated plant cells (Sevón, 1997). Careful optimization of the culture conditions in bioreactors is often needed to maximize the production of medicinal compounds from plant cell cultures. Several elicitors, for instance, sucrose as nutrients and abscisic acid and auxin as phytohormones used in root cultures of ***Atropa belladonna*** (Rothe and Dräger, 2002); and east extract also as elicitors used in root cultures of ***Brugmansia candida*** (Pitta-Alvarez *et al*.,2000), have been applied in an attempt to enhance tropane alkaloid production in hairy root cultures of ***Solanaceae.***

The concentration of scopolamine and hyoscyamine in cultured cells are generally very low in spite of many efforts to increase the yield using various approaches. Other investigators **(**Bhandary *et al*., 1969; Hiraoka and Tabata , 1974 and Hashimoto and Yamada 1983) also found that the production of tropane alkaloids by callus culture, was generally difficult. It seems that the biosynthesis of such alkaloids was correlated with the organization of cells as root (Kamada *et al*., 1986). Therefore, the plant cell cultures have not yet been employed to manufacture these

alkaloids. For example, Tabata *et al*. (1971) added tropic acid into ***Scopolia japonica*** suspension cultures as a precursor and could increase the level of alkaloids up to 15 times. Mitsuno *et al.* (1983) selected a high tropane alkaloids producing strain of ***Hyoscyamus niger*** which produced about 7 times more hyoscyamine ($13.9X10^{-3}$% fresh cells), than that of the parent strain. According to their results, there was no direct correlation between high producing ability and variation of chromosomal numbers. Endo *et al.*, (1985) and Kitamura (1988) and many other scientists reported that roots differentiated from cultured cells accumulate scopolamine, hyoscyamine and/or nicotine. However, Kitamura indicated that the alkaloids were not accumulated in leaves of the regenerated plantlets.

Since the alkaloids are synthesized in the root, Flores and Filner (1989) cultivated hairy roots transformed with ***Agrobacterium rhizogenes*** and showed the production of hyoscyamine and other alkaloids at the similar levels to the normal roots. Also, Kamada *et al*. (1986), showed the amount of the tropane alkaloids in the axenic cultures of hairy roots of ***Atropa belladonna*** was the same as or even higher than those of normal plants grown in the field.

In most cases, hyoscyamine is the major alkaloid (0.5-0.8 g/100g dry weight) **(** Huang *et al*., 1996; Lanoue *et al*., 2002 and Berkove *et al*., 2003)**.** For example ***Datura stramonium* L**., produce a range of biologically active alkaloids, including tropane alkaloids.The major alkaloids are hyoscyamine (generally the most abundant) and scopolamine; atropine may be formed from hyoscyamine by racemization during the extractive procedure. More recently Dechaux and Boitel-Conti (2005) found that transformed roots in axenic culture present a good model system for studying the regulation aspects of plant secondary metabolism in ***Datura innoxia.*** However, for industrial use, scopolamine is the more valuable of these two alkaloids. About 38 tropane alkaloids have been found in ***Datura innoxia*** (Berkove and Zayed, 2004). Hyoscyamine and scopolamine are present in low concentration in all organs whereas the ester derivatives of tropine (3a-tigloyloxytropine and 3-igloyloxy-6-hydroxytropine) are the major compounds. The results of Dechaux and Boitel-Conti (2005) pointed out that the alkaloid formation is nearly absent or very low in cultured callus and is induced upon differentiation of the callus to roots and shoots. Thus they confirmed that there is increasing interest in obtaining cultures with enhanced content of scopolamine.

In recent decades, approaches and research in the area of plant tissue culture technology (***in vitro***) aimed to production of natural compounds have employed callus induction and

differentiated organ culture (Parr *et al.,* 1990) and resulted in the production of many pharmaceutical substances for new therapeutics (Vanisree *et al*., 2004).They showed that advances in the area of plant tissue cultures for the production of medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids, and amino acids. Cultures showed stable production of the compounds, which typically resemble that of the parent plant, both qualitatively and quantitatively (Robins *et al.,* 1991). However, levels of alkaloid production have been observed to be different between cultured and naturally grown plants, and between different plant organs.

Several products were found to be accumulated in cultured cells at higher level than those in native plants through optimization of cultural conditions. For example, shikonin by ***Lithospermum erythrorhizon*** (Takashahi and Fujita, 1991) was accumulated in much higher levels in cultured cells than in the intact plants and Iranbakg *et al*. (2006) found idioblast cells in semi-hyaline calli derived from basal parts of ***Datura stramonium*** leaf cultured under a 16 h photoperiod; thus it is possible to obtain tropane alkaloids from cell cultures under appropriate conditions. However, many reports have described that yields of desired products were very low or sometimes not detectable in dedifferentiated cells such as callus tissues or suspension cultured cells. In order to obtain products in concentrations high enough for commercial manufacturing, therefore, many efforts have been made to stimulate or restore biosynthetic activities of cultured cells using various methods. The following are typical approaches that may increase production of cultured plant cells.

2.1.7.3. Optimization of Cultural Conditions

Various strategies have been followed to increase alkaloid production *in vitro*, including the optimization of medium composition, elicitation, genetic engineering, phytohormones (Whitmer *et al.,*1998) pH, temperature, aeration, light intensity, salt strength, different sucrose concentrations,…etc. This is the most fundamental approach in plant cell culture technology. Zenk *et al*. (1977) showed that the amount of serpentine depended on the composition of the basal medium used. Among them Murashige-Skoog's (MS) formulation was recognized to be the most suitable one for the production of serpentine alkaloid by ***Catharanthus roseus*** suspension.

2.1.8. Plant Growth Regulators

Phytohormones strongly influence secondary metabolism, affecting both culture growth and secondary metabolite production; efforts aimed at optimizing the chemical composition of the medium have thus largely dealt with the manipulation of the level of phytohormones. Among a number of other components in the medium, phytohormones such as auxins and kinetins have shown the most remarkable effects on growth and productivity of plant metabolites. There is extensive literature on the effects of plant growth regulators on secondary metabolism in cultures (Kurz, 1989, Staba, 1980 and Rhodes et al., 1994). The importance of using the appropriate concentration of an auxin and a cytokinin to induce proliferative growth and callus formation from an explants is known to all tissue culturists, as are the standard recipes for inducing regeneration from callus. It is therefore not surprising that the concentration and balance between growth regulators in a culture medium would influence secondary metabolism, a facet of differentiation. Generally, treatments which encourage structural differentiation, e.g. shoots or roots from callus, also change the biochemical profile. For example, the regeneration of roots from callus of several species tends to be accompanied by a sharp rise in alkaloid content (Tabata *et al*., 1971; Hashimoto and Yamada, 1983 and Endo and Yamada, 1985). The best callus response was obtained from the stem explants derived from ***Datura insignis***, by using BA concentrations above 0,25 mg l^{-1} + 0.25 or 1 mg l^{-1} NAA. Combinations containing concentrations of BA and NAA below 0.06 and 2 mg l^{-1}, respectively, promoted root regeneration in leaf explants **(**Figueiredo and Esquibel, 1991).

In general, as an auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) or Naphthalenacetic acid is frequently used for example an increase of auxin levels, such as 2,4-D, in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites(Goddijn *et al*., 1992 and Arroo *et al*., 1995). The influence of various growth regulators, especially auxins, on alkaloid production by ***C. roseus*** cultures has been reviewed (Van der Heijden *et al.*, 1989). Auxins have repeatedly been shown to inhibit alkaloid accumulation in periwinkle cell suspensions (Sakuta and Komamine, 1987 and Arvy *et al*., 1994), Auxins negatively influence the TIA pathway primarily at the transcriptional level of gene expression. Pasquali *et al*. (1992) showed that the addition of 2,4-D and other auxins to cell cultures of ***C. roseus*** rapidly down-regulated the transcription of the genes 136 encoding STR and TDC. Transcriptional repression, triggered by addition of 2,4-D, may also be responsible for

the reduced abundance of a group of polypeptides that appeared to be related to TIA biosynthesis (Ouelhazi *et al*., 1993). This is why auxins are commonly added to the medium for callus induction, but they are added at a low concentration or omitted for production of metabolites. Constabel *et al.* (1982) reported that cytokinins stimulated alkaloid synthesis which was induced by removing auxin from the medium of a cell line of ***C. roseus*.** The concentration of auxins in the medium is generally between 0.1 to 50 µM. The kinetin or Benzyladenine as a cytokinin is occasionally required together with auxins for callus induction at concentrations of 0.1 to 10 µM. Other derivatives of auxins and kinetin are also used in some cases. Since each plant species requires different kinds and levels of phytohormones for callus induction, growth and metabolites production, it is important to select the most appropriate growth regulators and to determine their optional concentrations (Masanaru, 1994).

Tropane alkaloids were detected in non-rooted shoots regenerated from calluses induced with benzyladenine (BA) 10 µM + naphthaleneacetic acid (NAA) 1 µM and BA 10 µM + indole-3-butyric acid(IBA) 0.1 µM. The peak of hyoscyamine and scopolamine contents were $38.5 * 10^{-4}$ and $6.5 * 10^{-4}$ dry weight, respectively (Khanam *et al*., 2001). Also, hyoscyamine and scopolamine were detected in the 11-week-old callus induced with 10 µM BA + 1 µM NAA. No tropane alkaloids were detected in the 4-week-old shoot cultured in liquid medium which were induced with 10 µM BA + 0.1 µM NAA (Khanam *et al*., 2000).

Moreover, Dessouky et *al.,* (2001), induced cell suspension cultures from different explants cultures of ***D. stramonium* L.** and ***D. metel* L..** Influence of different concentrations of various growth regulators i.e., 2,4-D, Kin, NAA and BA on callus production was investigated. They reported that ***D. stramonium*** showed better results as compared with ***D. metel*** . The best supplementation to the liquid MS medium was 1 mgl^{-1} of each of NAA and BA. The establishment of calli cultures from different explants of ***Datura metel (*L*.)*** had been carried out by Miskat *et al*. (2003). They reported that internodal segment showed best and prompt calli response in MS medium supplemented with 2 mgl^{-1} 2,4-D. Furthermore Zayed *et al*. (2006) established undifferentiated callus from the stem explants of ***Datura innoxia*** using MS medium supplied with BA at 1 mgl^{-1} and IAA at 0.5 mgl^{-1} in combination for 6 weeks.

Concerning the *in vitro* propagation of Datura, Dos Santos *et al*. (1990) reported that ***Datura insignis*** Bard Rod nodal explants were cultured on MS medium supplemented with either BA alone or in combination with 2,4-D or IAA. Shoots multiplication and elongation were

obtained in various growth regulator different concentrations. However the best results were obtained in a medium with 1 mgl^{-1} of BA. Furthermore, Sethi *et al.* (1990) investigated the effect of various inhibitors on differentiation (shoot morphogenesis) in calli cultures of ***Brassica, Datura*** and ***Nicotiana*** . Hormone medium without any inhibitor (control) resulted in 6% shoot formation. Addition of inhibitors such as actinomycin D,cordyceptin , abscisic acid, trigonelline and theophylline greatly enhanced shoot formation. The results suggested that inhibitors played a regulatory role in the control of differentiation sequences. Moreover, Muthukumar *et al*. (2000), studied plants regeneration from hypocotyl explants of ***Datura metel* (L.)** on MS basal medium with different concentrations of BA and 2,4-D. They reported that, maximum percentage of shoot proliferation was observed at 1.5 mgl^{-1} BA followed by 1 mgl^{-1} BA. The regenerated shoots were elongated on MS basal medium without any growth regulator and rooted with different concentrations of IBA. The rooted plantlets were hardened properly and transferred to soil. Furthermore, De, (2003) described the regeneration of adventitious shoots from young leaves of ***Datura metel.*** He reported that shoots buds were developed on MS medium with 2 mgl^{-1} BA and elongated on hormone-free solid basal medium.

The production and evaluation of some active ingredients from ***Solanaceae*** family using plant cell cultures had been carried out by Kinsara and Seif El- Nasr, (1990). They investigated cell suspension of ***Hyoscyamus albus L.*** for growth and production of tropane alkaloids in three different media. The effects of sucrose concentration and initial pH were also studied. 2,4-D and 1% sucrose enhanced the production of tropane alkaloids. The percent of alkaloids (0.75%) was higher in cell cultures than in intact plant (0.352%). Furthermore, Missaleva *et al.* (1993) tested five nutrient media for expression of morphological pathway in ***Datura innoxia*** callus cultures. Regenerants – rooted plantlets and non-rooted shoots obtained from three subsequent subcultures of callus cultures were assayed for tropane alkaloids content and composition. Although no wide variation of biochemical traits of regenerants was observed. For a given number of plantlets, decrease of hyoscyamine in their roots was detected. Alkaloids content and composition of upper parts of plantlets having roots and shoots and / or lacking roots was studied-receiving an idea about biosynthesis of these organs.

Also, Raoufa *et al*. (2008) showed that MS-medium supplemented with 1 mg/l of NAA and BA gave the best results of calli production in ***Datura metel* (L.).** However, MS medium

containing 1 mgl^{-1} of 2,4-D and Kin resulted in maximum number of shoots formation from leaf explants as compared with other types of explants.

2.1.9. Sucrose Concentration of MS Medium

Sucrose is the most commonly used carbon source in heterotrophic and/or mixotrophic tissue culture. However, contradictory effects of supplied sugar on plant metabolism have been reported (Sima and Desjardins, 2001). Exogenous sucrose negatively affected growth and photosynthesis of various ***in vitro*** plantlets (Schäfer *et al*., 1992; Kozai *et al*., 1995 and Serret *et al*., 1997). On the other hand, stimulating effects of sugars on growth and photosynthesis have also been reported for ***in vitro*** plantlets of tobacco (Paul and Stitt, 1993); for nicotine accumulation in tobacco **(**Mantell and Smith, 1983); for polyphenol production by cells of *Rosa* (Davies, 1972) and also for potato (Tichá *et al*., 1998). In addition, Hdide and Desjardins, (1994) showed that sucrose induced non-photosynthetic carbon fixation by providing PEP necessary for PEPC activity. It has been suggested that sucrose can enhance the PEPC activity of C3-plants (Hdide and Desjardins, 1994). Under high N assimilation, degradation of starch and sucrose, which in turn, provide PEP, phosphorylated sugars (Glc-6-P, triose-P) and DHAP (dihydroxyacetone phosphate), can drive PEPC activity. The regulation of light-induced PEPC phosphorylation by PEPC protein Kinases is common in plants (Jiao and Chollet, 1991; Rajagopalan *et al*., 1993 and Lepiniec *et al*., 1994). Light greatly activates C4-PEPC and has a reduced effect on C3-PEPC (Rajagopalan *et al*., 1993). The presence of Glc 6- P, a PEPC activator, lowered the L-malate inhibiting effect in the three treatments as earlier mentioned by Wedding *et al*. (1989). A low inhibition of PEPC observed in sucrose fed plants suggested that sucrose led to enhanced phosphorylation level, thus protecting the enzyme desensitizing it to malate (Krömer *et al*., 1996). ***In vivo***, PEPC might also be protected against malate effect by the rapid conversion of this organic acid into isocitrate when the demand of α-ketoglutarate is increased by N assimilation. Sima and Desjardins (2001) concluded from their study that Plants grown with 3% Sucrose + N had the highest phosphorylation level compared to those cultured with 3% Sucrose and the control (1% Sucrose + N). These investigations demonstrated clearly that sucrose enhanced phosphorylation. This induction probably modulates the N effect.

Since 3-PGA is thought to influence enzyme phosphorylation, exogenous sucrose could be involved in such a mechanism by way of its glycolytic degradation. Cytosolic sucrose degradation may provide phosphoglycerate (PGA) that increases pH by capturing H^+ ions and

liberating Ca^{2+} from the vacuole, thus inducing PEPC and PEPC-kinase synthesis and activation of the former. Sucrose or its metabolism may cause PEPC to become less sensitive to L-malate inhibition (Sima and Desjardins, 2001).

During their study, Pruski *et al*. (2000) demonstrated that all cultures **{**of potato (***Solanum tuberosum*****) cv.** Atlantic, chokecherry (***Prunus virginiana* L**.) cv. Garrington and Saskatoon berry (***Amelancher alnifolia* Nutt.) cv.** Northline grown *in vitro* for 3 weeks at 24/22 Ċ, 16-h photoperiod, 150 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) mixed fluorescent/incandescent light were stored for 6, 9 and 12 weeks at 4 C° under 0 (darkness) and 3 µmol m^2 s^1 PPFD (690 nm red light continuous illumination). Growth regulators free MSMO medium either with or without 30 g l^{-1} sucrose was used to store the cultures, retained capacity to re-grow after storage. Tested factors, sucrose, light and the length of the storage period had an impact on shoot quality and re-growth capacity of the cultures. For either light treatment, sucrose was essential for the low temperature maintenance of vigorous stock plants of potato, if stored for over 6 weeks. Chokecherry and saskatoon cultures stored well without sucrose; although chokecherry benefited from sucrose in the storage medium when the stock cultures were kept at the low temperature for 12 weeks. Low light significantly improved quality of the stored potato cultures, but had very little effect on both chokecherry and saskatoon berry cultures. All RGR values calculated for potato cultures grown from the cultures stored on media without sucrose are negative. The positive RGR are only for cultures stored on media with sucrose, irrespective of the length of the storage period. Cultures also benefited from the light during storage producing significantly higher RGR than those stored in dark (Pruski *et al*., 2000).

In general, raising the initial level of sucrose leads to an increase in the secondary-metabolite yield of cultures. There are many other examples, including the production of the steroidal alkaloids, solasodine, in callus cultures of ***Solanum nigrum*** (Bhatt *et al*., 1983) and in suspension cultures of ***Solanum elagnifolium*** (Nigra *et al*., 1990), alkaloids and polyphenol production in dark-grown suspension cultures of ***Catharanthus*** *roseus* (Knobloch and Berlin, 1980), and anthocyanin accumulation in *Vitis* suspension cultures (Yamakawa *et al.*, 1983, Cormier *et al*., 1990 and Do and Cormier, 1991). The increased accumulation of anthocyanin in ***Vitis vinifers*** suspension cultures when the sucrose levels were increased has been shown to be the result of osmotic stress (Do and Cormier, 1990). However, apart from these findings and those of Boyd (1991) with cultures of ***Bixa orellana*** accumulating carotenoids, most of the

reports gave little indication as to how the metabolism of the cells is affected by increased sucrose levels. Interaction between sucrose and nitrogen have also been reported. Yamakawa *et al.* (1983) have reported that increased anthocyanin levels occurred in ***Vitis*** cell cultures when sucrose levels were increased. However, they also found that at lower sucrose concentrations reduced nitrogen levels also resulted in higher amounts of anthocyanin and suggested that there was an optimal C:N ratio for pigments production. These observations from a variety of laboratories working with several different species further support the concept of an inverse relationship between major aspects of primary metabolism, example of protein synthesis and the synthesis and accumultion of secondary metabolites, based on the differential and ontagonistic utilization of common precursors. Attention will be now focused on the possible role of plant growth regulators on this partitioning (Yeoman and Yeoman, 1996).

Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. Increased sugar concentration favoured synthesis of shikonin in cell cultures of ***Lithospermum erythrohizon***, diosgenin production in ***Dioscorea***, and anthraquinone in cell cultures of ***Gallium mollugo.*** On the contrary, lesser amounts of sucrose favoured the production of ubiquinone 10 in ***Coleus blumei*** (Roja and Rao, 1998). The maximum yield of rosmarinic acid produced by cell suspension cultures of ***Salvia officinalis*** was 3.5 gl^{-1} when 5% of sucrose was used but it was 0.7 gl^{-1} in the medium containing 3% sucrose **(**Whitaker *et al***.,** 1984).

2.1.10. Salt Strength

There is no universal medium for *in vitro* culture, since plant species and cultivars are genetically specific with regard to different components of the medium, which include not only organic substances, but also mineral elements (Sari´c *et al*., 1995). According to Williams (1995), the *in vitro* chemical micro environment can be regarded as having three main phases, the medium, the atmosphere or head space and the plant material. Under *in vitro* conditions, plant growth depends on the mineral elements and organic components of the medium, due to a very low level of photosynthesis and small leaf area of the plants. Hence, the choice of mineral and organic components is very important (Lumsden *et al*., 1990).

Regarding other plant species and cultivars, Ruži´c *et al*. (2000) stated that mineral composition of many media is deficient in or in excess of some macroelements, thus inducing abnormal growth, vitrification and other undesirable effects. Hence, the widely used salts

proposed by Murashige and Skoog (1962) are deficient in some macroelements for pear and crab apple (Singha *et al*., 1988). After varying the concentrations of potassium (K) and carbon (C) in MS medium, Pasqualetto *et al*., (1988) concluded that the percentage of vitrified apple shoots **(*Malus domestica* Borkh.)** was highest on medium with lowest potassium (K) and carbon (C) concentrations. Severe signs of deficiency in elements in sweet cherry rootstock culture occurred after individual exclusion of nitrogen (N), phosphorus (P) and calcium (Ca) from the media (Ruži´c *et al*., 2000). Hence, a clear determination of both the effect of the essential elements in mineral nutrition on *in vitro* plant growth and optimal concentrations for specific stages or culturing systems is of prime importance (Ruži´c *et al*., 2000**)**.

The mineral nutrition can exert an effect by the relative ionic composition, by the pH of the culture medium, and by the total mineral dose. All three parameters can simultaneously exert their own specific influence (Demeyer and Dejaegere, 1989; 1993; 1995; 1996).

There are numerous reports in the literature of how the application of nutrient stress to plant-cell and tissue cultures can reduce growth and primary metabolism and promote differentiation of secondary metabolism (Dougall, 1980; Mantell and Smith, 1983 and Collin, 1987). It has already been shown in the literature that the pathways of primary and secondary metabolism compete for common precursors and this competition depend on whether the culture condition support rapid or slow growth (Phillips and Henshaw, 1977; Lyndsey and Yeoman, 1985; and Yeoman, 1987b). As the cells cease to synthesize the polymers necessary of the formation of new cells and larger cells, the precursors will be directed towards secondary metabolism. Therefore, any manipulation of the culture medium which inhibits cell division and cell expansion might be expected to enhance cell differentiation and the capacity to synthesize and accumulate secondary metabolites (Yeoman, 1987b).

There are two obvious targets for this empirical approach, the sources of phosphate and nitrogen (N) in the culture medium. Nitrogen is essential for the synthesis of protein and nucleic acids; reducing or removing the source of nitrogen (N) will reduce/stop growth. There are many references in the literature which demonstrate unequivocally that a depletion or deficiency of N and/or phosphate is associated with growth limitation and a concomitant increase in the level of secondary metabolism (Knobloch and Berlin, 1981; Yamakawa *et al*., 1983; Lindsey, 1985; Collin, 1987 and Oksman-Caldentey *et al*., 1994). Over 30 years ago, Nettleship and Slaytor, (1974) showed that of a variety of nutrient-limited media, phosphate-free was especially

conducive to the production of alkaloids and other secondary metabolites by a callus culture of *Peganum harmala*. Knobloch and Berlin (1981) showed that the accumulation of cinnamoyl putrescines in cultures of tobacco was enhanced greatly by phosphate limitation, whereas growth of the cultures was reduced under such conditions, and concluded that the levels of inorganic phosphate used routinely for cell culture are prohibitively high for certain types of secondary metabolism to proceed. Mantell and Smith, (1983) concluded that lack of phosphate more than any other nutrient stimulates secondary-metabolite biosynthesis.

In addition to those reports on the effects of phosphate, there are also numerous references to the effects of a reduction in nitrogen level on the production of secondary metabolites (Yamakawa *et al*., 1983; Lindsey, 1985 and Do and Cormier, 1991**).** In the majority of cases a reduction in nitrogen was found to reduce the growth of the cultures and bring about a premature stationary phase in which the product accumulated. In total contrast there is a report of stimulated betacyanin accumulation accompanying an increase in the nitrogen supply in suspension cultures of ***Phytolacca americana*** (Sakuta *et al*., 1987), but in this case there is a close correlation between growth and betacyanin accumulation. Moreover (Ruži´c *et al*., 2000) found that as fresh and dry weight of the explants of Sweet cherry rootstock Gisela 5 where { micropropagated on Murashige and Skoog (MS**)** medium, on MS medium containing double-strength macro salts (MS 2X), 1/2 strength (MS 1/2) and 1/4 strength (MS 1/4) with 4.4 μM BA, 0.5 μM NAA, and 0.3 μM GA_3} increased during subculturing, the fresh and dry weight of the media decreased. The pH of the media declined during subculturing following by slow increase on media MS 1/2 and MS1/4. Gisela 5 showed the best growth and development on MS 2X and MS media with the highest N and P uptake. Growth and multiplication depend on the uptake of these elements from the medium.

According to Ingestad and Ågren, (1992), one of the models for the study of ***in vitro*** mineral nutrition and plant growth is based on the observation that the relative plant growth is linearly related to the external concentration of the elements in the medium. However, in their experiment (Ruži´c *et al*., 2000), investigated the effect of the concentration of mineral elements in the medium on plant growth and found that it is closely related to the uptake of mineral elements from the medium and to the culture growth. Gisela 5 on MS 2x and MS media, on which it had the best growth and development, absorbed the highest levels of nitrogen (N) and phosphorus (P) as a result of apart from (N) and phosphorus (P), which remained in small

quantities in MS 2x and MS medium after day 40, residues of other elements were considerably higher in these media. Thus, it can be assumed that the growth and multiplication depend on the uptake of these elements**.** Williams (1995) also reported that a limited addition of an essential ion is one of the possible factors leading to a limited plant growth. Sakano *et al*. (1995) also reported the correlation between the uptake of P from MS medium and ***Catharanthus roseus* (L.)** G. Don cell growth. Phosphorous is not only the element which plants take up in the highest amount and most rapidly, but its concentration in the medium is of great importance as well. Pryce *et al*. (1993) found out that the growth rate of ***Delphinium*** plants increases with the increase in the levels of mineral elements or only in the phosphate concentration in the medium. Schmidt *et al*., (1992) investigated the mechanism of the uptake of non-organic P in ***Catharanthus roseus*** cell suspension, and reported that cell growth on MS medium is linearly related to phosphate level in the medium. This clearly showed that phosphate had become growth limiting factor (Ruži´c *et al*., 2000).

Miraldi *et al*. (2001) reported that the levels of hyoscyamine and scopolamine within datura plant parts depend on several factors, the nutritional state is one of them, and atropine is often formed from hyoscyamine racemization and the hyoscyamine content is approximately two fold that of scopolamine.

Microelements such as iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), Crome (Cr), Nickel (Ni), lead (Pb), cadmium (Cd) and cobalt (Co) have an important physiological role in human organism while metals like nickel, lead and cadmium are very toxic substances. Microelements enter human body mainly by consumption of food of plant and animal origin. Moreover, the micro-elements found in a reasonably balanced level and chelated form gave the fertilizer a good opportunity to play an important role in the physiological activities of a plant. In this concern, the majority of the micro elements were found to enhance the activity of many enzymes within plant cells (Liptay and Arevalo, 2000). In addition, Fe, Mn, and Zn somehow participate in chlorophyll formation (Salisbury and Ross, 1992). Molybdenum is essential for N fixation in the soil and for nitrate reductase enzyme in the plant (Mengel and Kirkby, 1987). Copper and B are essential factors for phytohormone synthesis, carbohydrate translocation, and nucleic acid formation (Bernier, 1988).

Demeyer and Dejaegere, (1989) have stated that when aiming to increase the yield of the medicinally used alkaloids in ***Datura stramonium* L.,** mineral nutrition is one of the most

obvious environmental parameters to manipulate. Data on the influence of the ion-balance on yield and alkaloid content in ***Datura stramonium*** have shown differences in alkaloid accumulation in the upper vegetative plant parts when potassium or calcium were the dominant cations within the interionic balance of the six major elements.

Moreover, Demeyer and Dejaegere (1989), also found that contents of both main alkaloids present in this plant were affected differently. Hyoscyamine content in leaves and stems was positively influenced when the balance between potassium, calcium and magnesium was shifted to calcium, which also promoted growth, while scopolamine accumulation increased with the relative proportion in which potassium was present in the culture medium. The latter response coincided with a retarded development of the plants. As it is well known that the epoxydation of hyoscyamine to scopolamine is strongly dependant on the developmental stage of the plant (Cosson, 1969; Cosson *et a.,* 1978 and Van de Velde *et al.,* 1988), the observed effect of the Ca^{2+}/K^{+} balance on the hyoscyamine: scopolamine ratio could have resulted from a positive action on hyoscyamine epoxydation and from an indirect influence exerted by the developmental stage of the plants.

Although high levels of nitrate (NO_3) decreased the alkaloid concentration in relatively young plants of ***Datura stramonium L.***, high levels of nitrate (NO_3) increased nitrogen uptake and alkaloid yield as plants matured. In younger plants, increased alkaloid yield was associated with vegetative plant parts while in older plants increased alkaloid yield was associated with the generative plant parts. Nitrate (NO_3) dose effects on the hyoscyamine to scopolamine ratio were influenced by plant development (Demeyer and Dejaegere, 1997). Sikuli and Demeuer (1997**)** showed that the highest biomass yield of "Hairy roots " of ***Datura stramonium L.*** was found with NO_3^{-} and K^{+}- dominance, whereas hyoscyamine yield was highest with the cultures medium in which SO_4^{2-} and K^{+} were dominant. Shifting the inter-cationic balance to strongly towards Ca^{2+} caused and overall reduced metabolism, since as well biomass yield as hyoscyamine was lost with the NO_3^{-} - Ca^{2+} - medium. Also tropine yield was affected by the ion-balance, indicating that this culture parameter also influences alkaloid synthesis.

Micronutrients are often neglected in using the single-fertilizer form, although vigorous plant growth and crop production require an adequate supply and balanced amounts of all nutrients (Mengel and Kirkby, 1987) in order to maximize plant health and vigor by optimizing the plant nutrient uptake. This can be only achieved if the nutrient content of the fertilizer is

appropriate to the needs of the plants. Compound-fertilizers, containing both macro and micro-elements, may possess this characteristic and in several studies have been found to escape leaching losses of nutrients when applied in chelating form (Brown *et al.*, 1982; Snyder *et al.*, 1984 and Mancino, 1991).

Sodium chloride, potassium chloride and sorbitol were found to stimulate alkaloid accumulation in ***C. roseus*** as well as abscisic acid (Smith *et al*., 1987a**)**. Higher concentrations of phosphate results in an increase in the production of indole alkaloids in ***Catharanthus roseus***, whereas in callus cultures of ***Peganum***, low phosphate levels stimulate the secondary metabolism (Nettleship and Slayter, 1994). Transfer of suspension cultures of ***Thuja occidentalis*** from MS to B5 medium induced the synthesis of terpenoids. Both, different NH_4^+ content and the stress due to transfer to specific media, seemed responsible for accelerated shikonin production in suspension cultures of ***Lithospermum erythrorhizon*** (Fujita *et al*., 1981). The type and the amount of N source seems to affect the yield of secondary products. The ratio of nitrate and ammonia in the culture media also influences growth and secondary metabolite production. Fujita *et al*. (1981**)** reported increase in the yield of shikonin with increase in the concentration of sole nitrogen source, nitrate till 6.7 mM, but the production decreased with above 10 mM nitrate level. Decreased levels of N are reported to stimulate the production of secondary metabolites such as, certain polyphenols, anthocyanins (Datta and Srivastava, 1997).

Manipulation of concentrations of microelements in the nutrient media offers a strategy to increase the production of secondary metabolites in plant cell cultures (Demeyer and Dejaegere, 1993). Trace elements have indeed been considered as abiotic elictors or as inducing factors (Pinol *et al*., 1999) that trigger the biosynthesis of secondary metabolites. There are results showing the effect of divalent ions; Co^{2+} and Cu^{2+} seem to have received more attention because of their positive effects on the production of secondary metabolites (Fujita,*et al.*, 1981; Furze, 1991; Sri Andrijany *et al*., 1999 and Trejo-Tapia, 2001**).** Increase in Co^{2+} from 1 to 5 µM resulted in the enhanced production of betalains in ***Beta vulgaris*** (Trejo-Tapia, 2001). Enhanced shikonin production in the cultures of ***Lithospermum erythrorhizon*** have been attributed to the increased concentration of both copper and sulphate (Fujita *et al*., 1981).

Cupper (Cu^{2+}) enhanced both, the growth and the alkaloid yield in ***Hyoscyamus albus*** hairy roots. Similar results have been obtained in the production of shikonin derivative by cell suspension cultures of ***Lithospermum erythrorhizon*** (Christen *et al*., 1992). Copper

concentration up to 11 µM stimulated hyoscyamine production but had no influence on growth of hairy root cultures of ***Hyoscyamus.*** Hairy root cultures of ***Brugmansia candida*** produce the tropane alkaloids scopolamine and hyoscyamine. $CaCl_2$ had little effect on accumulation or release of either alkaloid. $CdCl_2$ acted positively on the release of both alkaloids (3- to 24-fold), but was highly detrimental to growth (Sandra *et al*., 2000).

2.1.11. Light Intensity

Several aspects such as wavelength (quality), intensity (quantity) and duration of light are important factors affecting plant growth (Arditti and Ernst, 1992). High light intensity substantially increased the total number of expanded leaves, dry matter, sugar content and nitrogen absorbed in *Phalenopsis* (Kubota and Yoneda, 1993). But, excessive light intensity causes stunting of the stem and leaf of alpine plants (Datta, 1994). High light intensity stimulated growth, tillering and yield per tiller and increased the stem proportion of ***Brachiria bizantha and*** Panicum ***maximum***. It greatly increased the number of sclerenchyma cells and their wall thickened in all organs (Deinum et al., 1996). Higher light intensity has more violet and ultra-violet radiation that causes the production of excess phenolic compounds in ***Zosteria marina*** (Vergeer et al., 1995). Most lower plants like mosses and ferns, as well as several woodland wild flowers are retarded in their growth or killed by high-sunlight intensity (Datta, 1994).

In moderate light intensity, plants generally bear longer internodes, and are less tough and more succulent with larger leaves than those grown in intense light (Barber and Anderson, 1992). Plant growth is related to the function of growth hormones like auxin, which is sensitive to high light intensity. Cytokinins act in concert with auxin to cause cell division in plant tissue culture (Soontornchainaksaeng, et al., 2001). They showed that light intensity plays a significant role not only on dry weight accumulation but also on plant height, leaf number, leaf shape and leaf area. The best results were found at 74 µmol m^{-2} s^{-1}. On the other hand, plantlets of ***V. coerulea***, as well as ***P. tankervilliae***, grown under the light intensity of 56 µmol m^{-2} s^{-1} grew less than those grown under 37 and 74 µmol m^{-2} s^{-1}. Generally, plant growth and development are affected by both internal factors including genotype and plant hormones and external factors such as light, temperature and moisture supply. This result may be due to the interaction between light intensity and internal factors which directly affect plant growth. The suitable light intensity and duration will give the best result of product. Stronger light intensity costs more in terms of energy input.

Consequently, the results suggest that light intensity of 37 µmol m^{-2} s^{-1} was sufficient to culture both ***V. coerulea*** and ***P. tankervilliae*** economically.

Light intensity in the range of 25-500 µmol m^{-2} s^{-1} altered the growth of ***Brassica*** seedlings. Plant height and gibberellin concentration increased progressively when light intensity decreased. In contrast, plant dry weight decreased with decreasing light intensity (Potter et al, 1999). Light is the ultimate substrate for photosynthetic energy conversion, it can also harm the plants. Higher light intensity causes photooxidation which involves the destruction of chlorophyll, resulting in less biomass production. High light intensity is damaging to the water-splitting photosystem II (PSII), leading to degradation of the reaction center. The frequency of this damage is relatively high when light intensity is increased, especially when combined with other environmental factors. There is an exception for lily plants in which no photoinhibition or damage to PSII was observed in the critical condition of strong light and high temperature in the culturing season (Sorrentino et al., 1997**).** Moreover, ***Scindapsus*** yielded plants that were more vigorous when the light intensity of the pretransplant stage was either 3,000 or 10,000 lux, whereas ***Cordyline*** and ***Dracaena*** showed progressive increases in the vigor of plants with increasing light intensity up to 10,000 lux (Miller and Murashige, 1976).

Light is the physical condition that can be manipulated to increase product yield. Light is known to affect the production of several secondary metabolites (Mantell and Smith, 1983 and Towers and Yamamoto, 1985). Contacting effects of light on the production of secondary metabolites have been reported; for example, the inhibition of nicotine accumulation in tobacco cell cultures (Ohta and Yatazawa, 1978**)** and the stimulation of anthocyanine production by ***Haplopappus*** cultures (Stickland and Sunderland, 1972) and of betalains by cell cultures of ***Chenopodium rubrum*** (Berlin *et al*., 1986).

Callus production from leaves of quince clone BA 29 was increased with increasing 2,4-D concentration from 2.5 mg/l to 5mg/l and after 2, 4, or 5 days of induction period, but it was not influenced by light quality where the leaf explants cultured under red, blue, red+blue, far-red +blue, white, far-red light or darkness conditions, the only exception was far-red +blue light, which reduced callusing response (Morini *et al*., 2000). These results suggested the involvement of the blue-absorbing photoreceptor system in the callus formation processes. For root regeneration, phytochrome seemed to be the only photoreceptor involved.

Alkaloid content and production of both ***Datura metel*** and ***Datura stramonium*** are also influenced by the radiation the plants receive (Cosson, 1969 and Trease and Evans, 1978) respectively.

Demeyer and Dejaegere (1997) found that when plants of ***Datura stramonium*** **var.** ***tatula*** **L.** Torr were grown on two different mineral media, varying only in the relative proportions between these two cations (Ca^{2+}/K^{+} balance) under different light conditions, those grown under higher light energy regime, had more alkaloids accumulated in the plants during a certain stage of development, but also a higher proportion was found in fruits and seeds. This was associated with lower alkaloid content in leaves and stems. The observed effect of the Ca^{2+}/K^{+} balance on alkaloid content in the leaves was affected by the different light regimes. They also concluded that the Ca^{2+}/K^{+} balance exerts an effect on alkaloid concentration. This influence is, however, only noticeable during a certain stage of the development of the plant, while the intensity of it is affected by the amounts of light energy received by the plants. As both ecophysiological parameters are synergistic, a different influence of a changing Ca^{2+}/K^{+} balance may be expected when plants are grown at different locations**.** In addition to the mineral composition, Alkaloid content and production are also influenced by the radiation the plants receive (Cosson, 1969 and Trease and Evans, 1978).

Kleiber and Mohr (1967) observed that light stimulated TE (tracheary elements are useful experimental systems to study the biosynthesis of secondary cell walls *in vitro* **)** differentiation in hypocotyls of ***Sinapis alba*** **L**. (mustard) seedlings**.** Mizuno *et al*. (1971) found that light was necessary to induce TE differentiation in ***Daucus carota*** L.(carrot) root explants and Fosket (1968) reported that light increased TE differentiation in cultured stem segments of ***Coleus blumei Benth*** (coleus). Light has been found to increase the proportion of tracheary elements differentiating in callus cultures derived from xylem-parenchyma of ***Pinus radiata D***. grown on an induction medium containing activated charcoal but no phytohormones. The differentiation rate increased from 20% when callus was grown in darkness to 45% when callus was grown with a 16 h or 24 h photoperiod (Möller, 2006).

CHAPTER THREE

Materials and Methods

3.1. Equipments and Instruments

The following equipments and instruments were used throughout the present study:

Table 2. The equipments and instruments used throughout this study**.**

No.	Equipment / Instrument	Company	Origin
1	Laminar Air Flow Cabinet	Envirco	U.S.A
2	Incubator	Binder	Germany
3	Autoclave	Certoclar	Austria
4	pH-meter	Fisher	Germany
5	Oven	Memmert	Germany
6	Analytical Balance	Meltter	England
7	Compound Light Microscope	Olympus	Japan
8	Growth Chamber	Labconco	U.S.A
9	Hotplate Magnetic Stirrer	Bibby	England
10	Refrigerator	Vestel	Turkey
11	Shaking Water Bath	Eppendorf	Germany
12	Rotary Evaporator	Buchi	Germany
13	Centrifuge	Beckman	Germany
14	High Performance Liquid Chromatography (HPLC)	Shimadzu/ Kyoto	Japan
15	Air Dryer	Philips	Holland
16	Pasteur Pipettes	Analar	England
17	Millipore Filters	Halzfeld	Germany
18	Filter Papers (Watman)	Analar	England
19	Glass and Plastic(Petridishes)	Sterilin	England
20	Glass Flasks	Terumo	Japan
21	Glass Culture Tube	Halzfeld	Germany
22	Glass Pipette	Halzfeld	Germany
23	Micropipette	Eppendorff	Germany
24	Loop and Needle	Analar	England
25	Forceps	Analar	England
26	Gas Burner	Analar	England

3.2. Chemicals

The following chemicals were used throughout these experiments:

Table 3. The chemicals used throughout this study.

Chemicals	Company
2,4-Diclorophenoxyacetic acid (2,4-D)	BDH
Ammonium nitrate (KNO_3)	BDH
Benzyladenine (BA)	BDH
Boric Acid	BDH
Calcium Chloride Anhydrate	BDH
Cobalt Chloride.$6H_2O$	BDH
Ethanol	BDH
Ferric Chloride	BDH
Ferrous Sulfate.$7H_2O$	BDH
Magnesium Sulphate Anhydrate	BDH
Magnesium Sulphate.$4H_2O$	BDH
NaCl	BioMerieux sa
Petroleum ether	BDH
Potassium iodide	BDH
Potassium nitrate	BDH
Potassium Phosphate Monobasic	BDH
Pyrodoxine.HCl	BDH
Sulphuric Acid	BDH
Thiamine.HCl	BDH
Zinc Sulphate.$7H_2O$	BDH
Methanol	BDH
Chloroform	BDH
Acetic acid	BDH
Ammonia Solution	Fluka
Ferrie Chloride	Fluka
Sodium Hydroxide	BDH
Dhydrogen Potassium Phosphate	Fluka
Thiamine – HCl	Sigma
Pyridoxine – HCl	Sigma
Myo – inositol	BDH
Formaldehyde	Merck
Glacial Acetic Acid	BDH
Sucrose	Fluka
Tween 20	BDH
Hyoscine Standard	Renaudin - France

Atropine Standard	Renaudin – France
Atropine Sulphate Standard	Renaudin – France
Naphthalene Acetic Acid (NAA)	Sigma
Glycine	BDH
Commercial Bleach (NaOCl 5%)	Turkish-Chlorox and Dax and Iranian – Buzhna)
Mercuric Chloride ($HgCl_2$)	BDH
Ascorbic Acid	BDH
Citric Acid	BDH
Activated Charcoal	BDH
Chloramphenicol	BDH
Diethylether	BDH
Ethylacetate	BDH
Methanol Special Grade for HPLC	BDH
Agar	Sigma
Hydrochloric Acid	BDH
Ammonium hydroxide 25%	BDH
Sodium Hydroxide	BDH
Glycine	Sigma
Nicotine	Sigma
Pyroledoxine	Sigma
Thiamine	Sigma

3.3. Experimental locations:

This study was carried out at the following laboratories:

a) Plant Tissue Culture Laboratory, College of Agriculture / University of Duhok during the period from Dec.25, 2007 to Dec.1, 2008,

b) Plant and Cell Culture Laboratory, Kurdistan Institute For Strategic Studies and Scientific Research / Sulaimani, during the period from Dec.15, 2008 to Sep.20, 2009,

c) Phytochemistry Laboratory, Department of Crop Science and Laboratory of Food Technology, Department of Food Industry, College of Agriculture / University of Sulaimani where the extraction of alkaloids was carried out after final collection of samples.

d) Research Laboratory, College of Pharmacy/University of Sulaimani where the alkaloid samples were analyzed with High Performance Liquid Chromatography (HPLC) until end of Dec., 2009.

3.4. Plant Materials and Explants Preparation

The medicinal plant ***Datura*** **(*Datura innoxia* Mill)** which belongs to the family ***Solanaceae*** was used in this study.

3.4.1. Greenhouse Grown Plants

3.4.1.1. The seeds of *Datura innoxia* Mill. were collected from local public gardens in Sulaimani city. Plant leaves were used as explants and as a source of alkaloids. Seeds of both plant species, 5 seeds each were planted separately in plastic pots (30 cm in diameter) in the greenhouses / Colleges of Agriculture / Universities of Duhok and Sulaimani.

Leaves and single nodes were removed from their main axis and used for explants preparation and they were washed with tap water for 60 minutes to remove soil and other superficial contaminants followed by tap water and liquid soap for 20 minutes, followed by 3-5 minutes rinses in sterile distilled water, and then surface sterilized as follows:

All steps of sterilization were carried out under septic conditions (Laminar air flow cabinet) by using sterilized instruments, different concentrations of different origins of NaOCL (Sodium Hypochlorite) were used. These concentrations were chosen according to active chlorine available in the detergents. Moreover, the use of different concentrations of $HgCl_2$ as sterilant was also investigated. The trails were also pertained at the use of mixture of NaOCl, $HgCl_2$ and ethanol in the sterilization procedure.

The following treatments were used for surface sterilization of ***Datura innoxia*** **Mill** leaf explants:

1. Erasing the leaves with ethanol %70 + NaOCl 1.0% (10 min.)
2. Erasing the leaves with Ethanol %70 + NaOl 1.0% (5 min.).
3. Erasing the leaves with Ethanol %70 + NaOCl 0.5% (10 min.)
4. Erasing the leaves with Ethanol %70 + NaOCl 0.5% (5 min.)
5. Ethanol 70% (2 min.) + NaOCl %1.0 (5 min.) + $HgCl_2$ 0.1% (1 min.)
6. Ethanol 70% (1 min.) + NaOCl %1.0 (5 min.)
7. Ethanol 70% (2 min.) + NaOCl %1.0 (5 min.)
8. Ethanol 70% (3 min.) + NaOCl %1.0 (5 min.)
9. Ethanol 70% (4 min.) + NaOCl %1.0 (5 min.)

10. Ethanol 70% (5 sec.) + NaOCl %1.0 (10 min.)
11. Ethanol %70 (2 min.) + $HgCl_2$ % 0.05 (5 min.)
12. Ethanol %70 (2 min.) + $HgCl_2$ % 0.01 (5 min.)
13. Ethanol %70 (2 min.) + $HgCl_2$ % 2.0 (5 min.)
14. Ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.)
15. Ethanol %70 (2 min.) + $HgCl_2$ % 0.01 (2 min.)
16. Ethanol %70 (2 min.) + $HgCl_2$ % 0.01 (4 min.)
17. Ethanol %70 (2 min.) + $HgCl_2$ % 0.1 (2 min.)
18. Ethanol %70 (2 min.) + $HgCl_2$ % 0.1 (4 min.)
19. Ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.)+ Activated charcoal 1.5 gl^{-1} to (MS) medium.
20. Ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.)+ Ampiciline 125 mgl^{-1} to (MS) medium.
21. Ethanol %70 (2 min.) + NaOCl% 2.0 (2 min.)+ Chloramphenicol 125 mgl^{-1} to (MS) medium.
22. Ethanol %70 (2 min.) + NaOCl% 2.0 (2 min.)+ Ascorbic acid & Citric acid 100 mgl^{-1} for (5 min.).
23. Ethanol %70 (2 min.) + NaOCl% 2.0 (4 min.) + Ascorbic acid & Citric acid 100 $mgl^{-1}$for (5 min.).

Moreover, all sterilization treatments contained one drop of Tween-20 and after each treatment, the single nodes were rinsed 3 to 5 times using sterilized distilled water. All the sterilization steps were carried out under vacuum pump and all the tissues visibly damaged by the sterilant were removed.

The leaves were cut into suitable explants as follows:

1. Leaf blades were cut into pieces of 1.0 cm^2.
2. Single nodes were cut into 1.0 cm long.

One explant was placed horizontally in 25×150 mm test tube or 500 ml conical flask or 250 ml bottle. Besides, before culturing, all excised leaves were dried-off on sterilized filter papers. The cultures were maintained at 25 ± 1 °C and 60 ± 5% relative humidity in a culture room under 16 hour photoperiod provided by white fluorescent tubes (Karim, 2008). The records on percent survival of explants were made after 2 weeks of incubation, from these experiments; a sterilization regime was adopted and used for future experiments.

3.5. Sterilization of the Media and Instruments

The cultures were grown on (Murashige and Skoog, 1962) medium (Table 4) with normal $\mathbf{S_0}$ (30 gl^{-1}), $\mathbf{S_1}$ (15 gl^{-1}), and $\mathbf{S_2}$ (45 gl^{-1}) sucrose. The MS also contained 3 levels of salts, normal, half, and double salt strength i.e., $\mathbf{X_0}$, $\mathbf{X_1}$, and $\mathbf{X_2}$ respectively. All the media contained agar (Agar-agar, Gum agar, A-9915, "Sigma") at 8 gl^{-1}, and 100 mgl^{-1} Myoinositol. Medium

components were placed on a hotplate magnetic stirrer and its pH was adjusted to 5.6 - 5.7 using 0.1 N of NaOH or HCl. Various containers of tissue culture medium, tools and instruments were wrapped with aluminum foil, autoclaved at 121 C° under a pressure of 1.04 $kg.cm^{-2}$ for 15 minutes (Cappuecino and Sherman, 1987 and Karim, 2008).

Table 4. Murashige and Skoog's medium components (Murashige and Skoog, 1962).

Murashige-Skoog Medium Composition		
Components	**Chemical formula**	**Weight (mgl^{-1})**
Macronutrients		
Ammonuim Nitrate	NH_4NO_3	1650
Potassuim Nitrate	KNO_3	1900
Calcium Chloride Anhydrate	$CaCl_2.2H_2O$	440
Magnesium Sulphate Anhydrate	$MgSO_4.7H_2O$	370
Potassium Phosphate Monobase	KH_2PO_4	170
Micronutrients		
Boric Acid	H_3BO_3	6.20
Potassium Iodide	KI	0.83
Manganese Sulphate.$4H_2O$	$MnSO_4.4H_2O$	22.30
Zinc Sulphate.$7H_2O$	$ZnSO_4.7H_2O$	8.60
Molybdic Acid (Sodium Salt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric Sulphate.$5H_2O$	$CuSO_4.5H_2O$	0.025
Cobalt Chloride.$6H_2O$	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium Ethylene Diamine Tetraacetate	Na_2-EDTA	37.35
Ferrous Sulphate.$7H_2O$	$FeSO_4.7H_2O$	27.8
Vitamins		
Thiamine. HCl	$Cl2Hl_7ClN_4OS.HCl$	0.1
Nicotinic Acid(free acid)	$C_8H_{11}NO_3.HCl$	0.5
Pyrodoxine HCl	$C_6H_5NO_2$	0.5
Glycine (free base)	$C_2H_5NO_2$	2.0
Common organic sources		
Myo-inositol		100
Sucrose		30000
Agar		8000

3.6. Plant growth regulators

Different PGRs at different concentrations were prepared and added to the culture media as required. The PGRs used were as follows:

Benzyl adenine (BA) at 0.0, 0.3, 0.6 and 0.9 mgl^{-1} were used in combination with auxins including naphthalene acetic acid (NAA) at 0.0, 1.0, 2.0 and 3.0 mgl^{-1} for callus induction and also BA at 0.0, 1.0, 2.0 and 3.0 mgl^{-1} in combination with NAA at 0.0, 0.2, 0.4 and 0.6 mgl^{-1} were used for shoot formation. Benzyl adenine (BA) at 0.0, 0.2, 0.4 and 0.6 mgl^{-1} were used in combination with 2,4-diclorophenoxyacetic acid (2,4-D) at 0.0, 0.5, 1.0 and 1.5 mgl^{-1} for callus induction and also BA at 0.0, 1.0, 2.0 and 3.0 mgl^{-1} were used in combination with 2,4-D at the same concentrations for shoot formation. Each combination was used with 10 replications.

3.7. Preparation of Laminar Air Flow Cabinet and Cultural conditions:

3.7.1. Preparation of Laminar Air Flow Cabinet

All experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench fitted with a U.V. tube (15 W, peak emission 2637 A). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The chamber was then sterilized with U.V. rays continuously for one hour before starting cultures. The surface of all the vessels and other accessories such as (spatula, forceps, scalpels, blade..etc.,) gas burner, lighter, tube containing, absolute alcohol..etc., were also cleaned with alcohol. Alcohol was then sprayed in the chamber with the help of an atomizer.

Hands and arms which were to be used inside the Laminar were also scrubbed with alcohol before culturing. The rims of the test tubes and the sides of the plugs were flame sterilized. Structures (like forceps, scalpels, spatula.etc.) were all sterilized by dipping them in the alcohol and flaming them several times. Care was taken to cool the instruments before using them under Laminar Air Flow Cabinet (Figure 8).

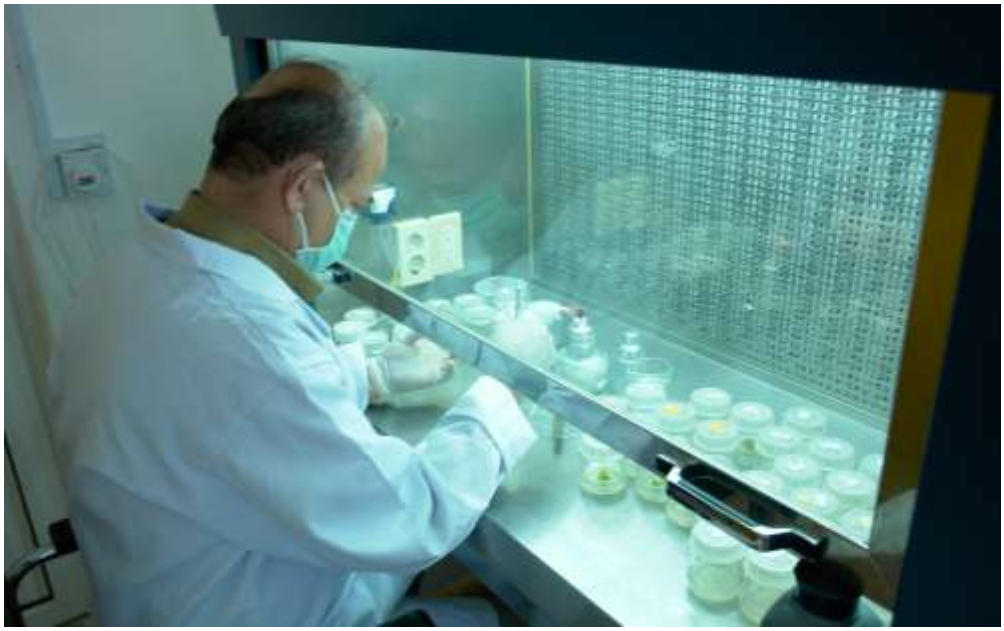


Figure 8. Laminar Air Flow Cabinet.

3.7.2. Cultural conditions

All the cultures were placed in three locally made Aluminum Growth Chambers maintained in an air conditioned room at a temperature of 25±1 C°, the source of illumination in each chamber was with irradiance of 60–100 µmol $m^{-2}s^{-1}$ provided by 1.25 feet white cool fluorescent tubes (20 watt).The intensities of illumination were 1000, 2000, and 3000 lux (about 19, 37 and 56 µmol$m^{-2}s^{-1}$ respectively) at the level of cultures in 3 chambers and a 16 hour light regime was followed by 8 hour darkness. From the calli present at the age of eight weeks, i.e. after 2nd subcultures, the 2nd experiment was carried out via another ten subcultures maintaining enough number of jars containing calli by four weekly transfers for 48 weeks (i.e. total of 12 subcultures) to the same freshly prepared medium in the same conditions. The young matured leaves from greenhouse grown plants (control or intact two months age plants) were separately dried at 60C° in air drying oven until having constant weight and powdered. Moreover, the alkaloid content of callus of the 2nd subculture was extracted and determined and compared to that of the control leaves.

3.8. Histological Examination

This examination was carried out to find out the mode of cell differentiation of ***Datura*** during callus formation at fourth subculture**.**

3.8.1. Killing and Fixation

Formalin-acetic acid-alcohol (FAA) solution was used for this purpose. FAA solution was prepared through mixing 90 ml of 70% alcohol, 5 ml of glacial acetic acid and 5 ml of formalin. Calli of ***Datura*** were placed in FAA solution for 24 hrs. Vials containing the fixed tissue in FAA solution were placed inside a desecrator connected to a mild vacuum pump for 5 min. to remove air bubbles from the tissue (Al-Mukhtar *et al.*, 1982).

3.8.2. Dehydration Series

The samples were dehydrated through passing them in a series of increasing ethanol (EtOH) concentrations (50, 70, 80, 90, 96 and 99%) for 45 min. at each step. Dehydrated tissues were then transferred to absolute alcohol and xylene combinations as follows:

1. Mixture absolute alcohol+ xylene 3: 1 (45 min)
2. Mixture absolute alcohol+ xylene 1: 1 (45 min)
3. Mixture absolute alcohol+ xylene 1: 3 (45 min)
4. Xylene 2-3 min.
5. Micrografted tissues were infiltrated in a mixture of 1/3 xylene + 2/3 paraffin in room temperature (20- 22°C) for 30 min., then transferred to 40°C for 24 hrs. Tissues were embedded in fresh melted paraffin and left in wax at 60°C for 24- 72 hrs (Al-Mukhtar, *et al*., 1982).

3.8.3. Preparation of Sample Blocks

Tissues embedded in melted paraffin were transferred to a rectangular cavity framed by two previously L-paper boat smeared with glycerol. The tissues were quickly oriented in a proper direction in the melted paraffin with the help of warm tweezers and needle. When the tissue was oriented in the proper position, the boat were quickly cooled over cold water.

3.8.4. Trimming and Sectioning the Blocks

Trimming was the first procedure towards sectioning the blocks which was performed with a razor blade, placing them on flat surfaces. All the surfaces of the block were plane and the edges were parallel to each other. Samples were cooled, a small piece of wax was mounted over the sample and melted with a hot scalpel holder. The block was placed on the melted wax and the base was strengthened with extra wax using the same scalpel holder. After ascertaining that the razor had satisfactorily been sharpened, the section cutting may be started by setting the microtome at the desired thickness and inserting the block holder in the microtome. The long axis of the material was oriented towards the blade of knife. Sections were cut at 12 μ thick and obtained as a ribbon of successive sections.

3.8.5. Affixing the Ribbon on the Slide

A suitable length (2-3 cm) of the ribbon was cut with a razor blade, and placed on clean slides smeared with a Haupt's adhesive (Al-Mukhtar *et al.*, 1982) which consists of the following: Pure gelatin 1 g, distilled water 100 ml, glycerol 15 ml and phenol crystals 2 mg. For Haupt's adhesive preparation, gelatin was suspended in water for 12- 24 hrs, and next all other ingredients were mixed.

One drop of the adhesive was added at nearly one end of slide and rubbed with finger in one direction to make a fine smear over the slide, a drop of distilled water was placed on the slide and then 1 or 2 ribbons were put on the slide. The slide was placed on a hot plate (50 C^o) or passed over spirit lamp. When sections were stretched, the slides were carefully drained by a piece of filter paper. The slides were placed in an incubator (35 C^o) for 24h.

3.8.6. Deparaffin and Hydration

Slides with sections were passed in a series of xylene and an alcohol to remove the wax then hydrated through decreasing alcohol concentrations at 10 min. intervals as follows:

Xylene 10 min.
Xylene 10 min.
Xylene: absolute alcohol 3: 1 10 min.
Xylene: absolute alcohol 1: 1 10 min.
Xylene: absolute alcohol 1: 3 10 min.

Absolute alcohol 10 min.
Alcohol 96% 10 min.
Alcohol 92% 10 min.
Alcohol 70% 10 min.
Alcohol 50% 10 min.

3.8.7. Staining

The sections were stained with safranin and fast green. Safranin is a micro stain and water and alcohol soluble. The safranin stain was prepared by dissolved in 100 ml of 50% ethanol. Fast green (Fast green, micro stain, pure) was prepared by dissolving 0.1 g of fast green in 100 ml of 92% ethanol.

The sections were dipped in safranin solution for 24- 72 hrs. The slides were rinsed in distilled water (two changes) to remove excess stain and passed through increasing concentrations of alcohol to dehydrate the slides for 10 min. intervals as follows:

Alcohol 50% 10 min.
Alcohol 70% 10 min.
Alcohol 80% 10 min.
Alcohol 92% 10 min.

The slides were then stained with fast green for 10 min. and rinsed in isopropanol (two changes) (Al-Mukhtar *et al.*, 1982).

The slides were then mounted with Canada balsam and covered with a cover slip and allowed to dry on a hot plate (40°C) for 24 hrs, and permanent preparation were attained following the sealing of cover slip with melted wax.

The slides were systematically examined under magnifying compound microscope (Axiostar, Japan) to find out the mode of cell differentiation of ***Datura*** during callus formation at fourth subculture, the slides were carefully studied, photographed and documented using digital camera (Canon 8.0 mega pixels, Japan).

3.9. Callus induction and *in vitro* studies

3.9.1. Callus Induction

A set of experiments were conducted to find out the most appropriate plant growth regulator supplements used for callus formation of ***Datura innoxia*** **Mill**. The explants were transferred to similar fresh medium every 4 weeks for a total of 48 weeks i.e. total of 12 subcultures. All cultures were incubated as mentioned in (3.5.2). The experiments were arranged in completely randomized design with 10 replications each consisted of 10 culture jars. After 8

weeks i.e. after the 2nd subculture the frequency % of callus initiation was calculated according to Liskova *et al.* (1994). The dry matter content (%) and fresh weight of the calli were measured as described by Ferreira and Janick (1996) as follows:

Dry matter content (%) = Dry weight x 100 / Fresh weight.

The numbers of shoot formation was measured at the stage of shoot induction and proliferation which occurred at 6th subculture in case of treatment of 2 mgl^{-1} BA + 0.6 mgl^{-1} NAA and at 8th subculture in case of the treatment 1 mgl^{-1} BA + 0.5 mgl^{-1} 2,4-D.

3.10. Extraction and Analysis of Alkaloids

3.10.1. Alkaloids Extraction

The following steps were followed for alkaloid's extraction procedure:

1- For each of the 10 replicates vials per experiment, the harvested samples were combined, cut into tiny pieces and weighed as fresh samples for extraction and analysis. Alkaloids were extracted from the calli of different treatments according to a method reported by Djilani *et al.* (2006).
2- Sample of fresh callus of ***Datura*** plant leaf explants (various weights g.) was placed in the beaker and treated with 15 ml of NH_4OH (25%, m/m).
3- At room temperature the solvent extraction was performed with 300 ml of ethyl acetate for 72 h.
4- The extract was filtered.
5- The solvent was evaporated in a rotary evaporator under reduced pressure at 40 °C.
6- The residue, dissolved in H_2O and acidified with H_2SO_4 to pH 3-4.
7- It was later extracted with petroleum ether and diethyl ether to remove lipophilic, acidic and neutral material.
8- The aqueous solution was basified to pH 9-10 with NH_4OH (25%, m/m).
9- It was extracted with chloroform, the extract was washed with distilled water to neutral pH.
10- It was dried with Na_2SO_4 and concentrated to dryness in a rotary evaporator under reduced pressure at 40 °C to obtain crude alkaloids.
11- Finally the crude alkaloids was collected using a mixture of equal volumes of Chloroform: Methanol for alkaloids analysis by HPLC.

3.10.2. Alkaloids Analysis

Due to the availability of the main alkaloid constituents (atropine, hyoscyamine, scopolamine, tropine, 7-hydroxyhyoscyamine, and tiglohyoscyamine) as a standard reference compound, Rts of plant sample extracts were compared with that of the reference using 50 µl. The extracts were qualitatively and quantitatively analyzed by High Performance Liquid Chromatography (HPLC) {Research Laboratory of the College of Pharmacy, Sulaimani University} using a model, Shimadzu Corporation, Kyoto Japan. With a pump 6000 A waters was used: column Phenomenex Nuc / eosi /HSC-18 (250 * 4.6 mm 5µ ID); mobile phase: Methanol MeOH and KH_2PO_4, 0.01M, (95:5) absorbance. The flow rate was 0.7 ml/min throughout the analyses; UV detector: at 280 nm; at temperature 30C°. The Rt (Retention time) values obtained from the extracts were compared with those obtained for authentic standards. The samples were previously prepared in equal volumes of Chloroform: Methanol. Fifty µl were injected with micro jets. The instrument was firstly run with deionized water followed by 50 ml 20% Ethanol, it was also ran daily with 50 ml of buffer solution, composed of Methanol MeOH and KH_2PO_4, 0.01M, (95:5) before operating and running the samples. After every day finishing it was washed with 50 ml Ethanol 20%.

3.10.3. Alkaloid Measurements

Extraction and analysis of alkaloids were conducted as mentioned in section **(3.10)**. The concentration of each type of alkaloid was measured in fresh weight (g) and thereafter converted to dry weight bases in μg^{-1} according to the following calculations.

$$X = R * 10^{-6} * V \qquad \text{1}$$

Where, X = Amount of alkaloid in (g) in the fresh sample (callus),

R= Reading from HPLC μgml^{-1} measured as follows:

$$\text{Concentration of alkaloid } (\mu gml^{-1}) = \frac{\text{Area of Sample}}{\text{Area of Standard}} \text{ X Concentration of Standard } (\mu gml^{-1})$$

(Illiana and Alfermann, 1989)

10^{-6} = µg/g

V = Final volume (ml) of the prepared alkaloid sample.

$$Y = \frac{X}{FW} \times 100 \quad \text{——— 2}$$

Y = Percentage of alkaloid in Fresh sample (callus).

FW = Fresh weight of sample (callus).

$$Z = Y (1 + W), \quad \text{——— 3}$$

Where, Z = Percentage of alkaloid on dry bases.

W = Water content of sample (callus) expressed as decimal (Hanks and Ashcroft, 1980).

3.11. Statistical Methods

3.11.1. Calli Growth Characters

The characters of frequency of callus initiation %, dry matter content (DM) %, dry weight (g), fresh weight (g), and number of shoots induced/leaf explant were analyzed by analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test, to assess the statistical significance ($P \leq 0.05$) which was considered to be statistically significant, using statistical software SPSS Version 18 (SPSS Inc., Chicago, USA).

3.11.2. Alkaloid Statistical Analysis

The factors (Sucrose, Salt strength and Light intensity) were implemented separately in Completely Randomized Block Design (CRBD) with nine replications. Means comparisons were carried out by using Least Significant Difference (LSD) Test **(**Sucrose; **S_0, S_1,** and **S_2,** Salt strength; **X_0, X_1,** and **X_2,** and Light intensity; **LI_1, LI_2,** and **LI_3)** at a significant level of ($P \leq 0.05$) in all analysis.

CHAPTER FOUR

Results and Discussion

4.1. Sterilization

A prerequisite for successful culture is the establishment of an aseptic technique. Thus, the first experiment in this investigation involved the establishment of suitable sterilization regimes for the explants.

Concerning the leaf explants of ***Datura innoxia*** **Mill,** figure (9) shows the effect of different sterilants concentration and duration on the survival percentage of leaf explants. Of the various pretreatment tried, **(N)** treatment which is consisted of ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.) gave the maximum survival percentage (97%). This sterilization regime was therefore selected for subsequent experimentations. As sterilants ethanol and $HgCl_2$ concerned, it was found that ethanol became deleterious when the duration of treatment exceeded 2 minutes and the chemical $HgCl_2$ also damaged the explants tissues irrespective of the concentration and duration of the treatment. Also sodium hypochlorite (Iranian Buzhna) proved to be the best of sterilants as it was effective not only in decontamination but is easy to remove resulting in minimal damage to the explants.

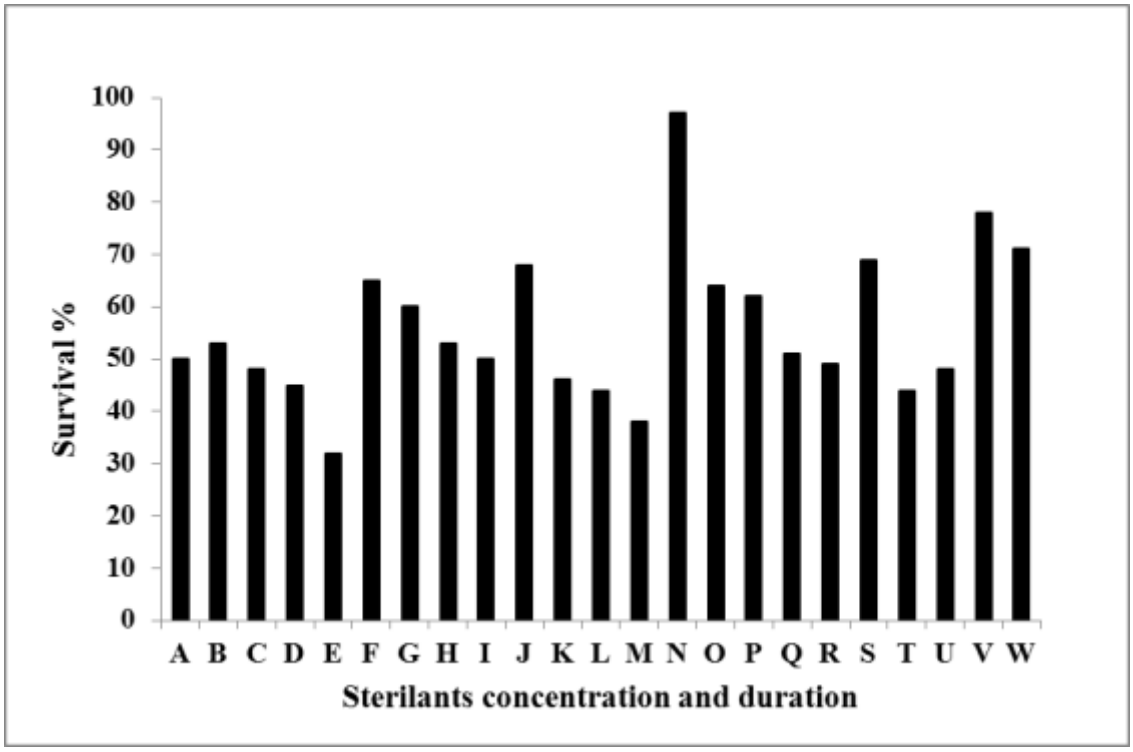


Figure 9. Effect of different sterilants concentration and duration on the percentage survival of leaf explants of ***Datura innoxia*** **Mill** after two weeks of culture.

A: Erasing the leaves with Ethanol %70 + Naocl 1.0% (10 min.)

B: Erasing the leaves with Ethanol %70 + Naocl 1.0% (5 min.)

C: Erasing the leaves with Ethanol %70 + Naocl 0.5% (10 min.)

D: Erasing the leaves with Ethanol %70 + Naocl 0.5% (5 min.)

E: Ethanol 70% (2 min.) + NaOCl %1.0 (5 min.) + $HgCl_2$ 0.1% (1 min.)

F: Ethanol 70% (1 min.) + NaOCl %1.0 (5 min.)

G: Ethanol 70% (2 min.) + NaOCl %1.0 (5 min.)

H: Ethanol 70% (3 min.) + NaOCl %1.0 (5 min.)

I: Ethanol 70% (4 min.) + NaOCl %1.0 (5 min.)

J: Ethanol 70% (5 sec.) + NaOCl %1.0 (10 min.)

K: Ethanol %70 (2 min.) + $HgCl_2$ % 0.05 (5 min.)

L: Ethanol %70 (2 min.) + $HgCl_2$ % 0.01 (5 min.)

M: Ethanol %70 (2 min.) + $HgCl_2$ % 2.0 (5 min.)

N: Ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.)

O: Ethanol %70 (2 min.) + $HgCl_2$ % 0.01 (2 min.)

P: Ethanol %70 (2 min.) + $HgCl_2$ % 0.01 (4 min.)

Q: Ethanol %70 (2 min.) + $HgCl_2$ % 0.1 (2 min.)

R: Ethanol %70 (2 min.) + $HgCl_2$ % 0.1 (4 min.)

S: Ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.)+ Activated charcoal 1.5 gl^{-1} to (MS)

T: Ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.)+ Ampiciline 125 mgl^{-1} to (MS)

U: Ethanol %70 (2 min.) + NaOCl% 2.0 (2 min.)+ Chloramphenicol 125 mgl^{-1} to (MS)

V: Ethanol %70 (2 min.) + NaOCl% 2.0 (2 min.)+ Ascorbic acid and Citric acid 100 mgl^{-1} for (5 min.).

W: Ethanol %70 (2 min.) + NaOCl% 2.0 (4 min.)+ Ascorbic acid and Citric acid 100 mgl^{-1} for (5 min.).

4.2. Experiments of plant growth regulators:

The first set of experiments carried out aimed at finding out the best concentrations of cytokinin and auxin combination enhancing callus growth of ***Datura innoxia*** plant leaf explants, the plant growth regulators were {NAA, 2,4-D, and cytokinin (BA)} according to the basic proposal of the present study. From the results of these experiments, it can be concluded that the best characters of callus growth studied (frequency of callus formation (%), dry matter (DM) %, dry weight (g) and fresh weight (g), were obtained from the combination of cytokinin (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) at 2^{nd} subculture, and number of shoots induced / leaf explants was obtained from the combination of cytokinin (BA, 2.0 mgl^{-1}) / auxin (NAA, 0.6 mgl^{-1}) at 6^{th} subculture (Figure 19 a and b).

Figures (10 and 11) show the effects of cytokinin (BA) / auxin (NAA) and cytokinin (BA) / auxin (2,4-D) combination**s** on frequency % of callus formation of ***Datura innoxia*****.** The highest frequency % of callus formation 99.42 was obtained at **(**BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) combination, which was highly significantly different from the value of 93.35 for (BA, 0.4 mgl^{-1}) / auxin (2,4-D, 1.0 mgl^{-1}) combination.

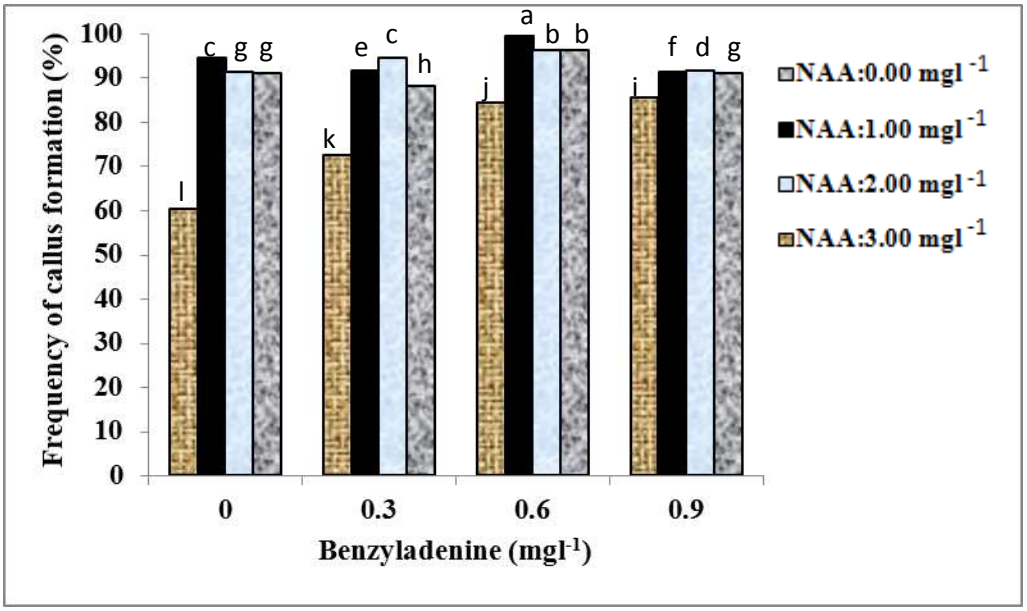


Figure 10. Effects of cytokinin (BA) / auxin (NAA) combinations on frequency (%) of callus formation of ***Datura innoxia*** **Mill** callus.

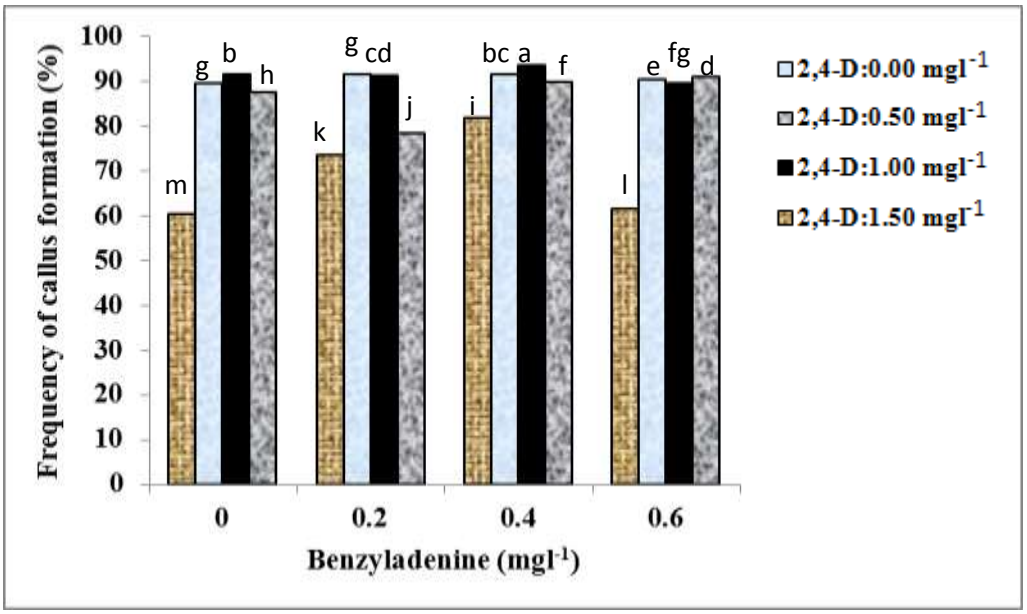


Figure 11. Effects of cytokinin (BA) / auxin (2,4-D) combinations on frequency of callus formation (%) of ***Datura innoxia*** **Mill** callus.

Moreover, Figures (12 and 13, 14 and 15, 16 and 17, and 18 and 20) show the effects of cytokinin (BA) / auxin (NAA) and cytokinin (BA)/auxin (2,4-D) combinations on dry matter (DM) %, fresh weight (g), dry weight (g), and number of shoots induced/leaf explants of the callus of ***Datura innoxia*** respectively. In general, among the different treatments, the highest

values of callus production were obtained from the addition of (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) to the medium which were highly significantly different from the correspondent values for cytokinin (BA) / auxin (2,4-D) combinations at all concentrations .The maximum values of calli dry matter (DM %), fresh weight (g), and dry weight (g), for (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) treatment were 11.97%, 11.39g, and 1.57g respectively, while the correspondent values for cytokinin (BA) / auxin (2,4-D) combinations were 11.08%, 8.39g, and 0.844 g at (0.4 mgl^{-1} BA) / (1.0 mgl^{-1} 2,4-D) combinations, respectively.

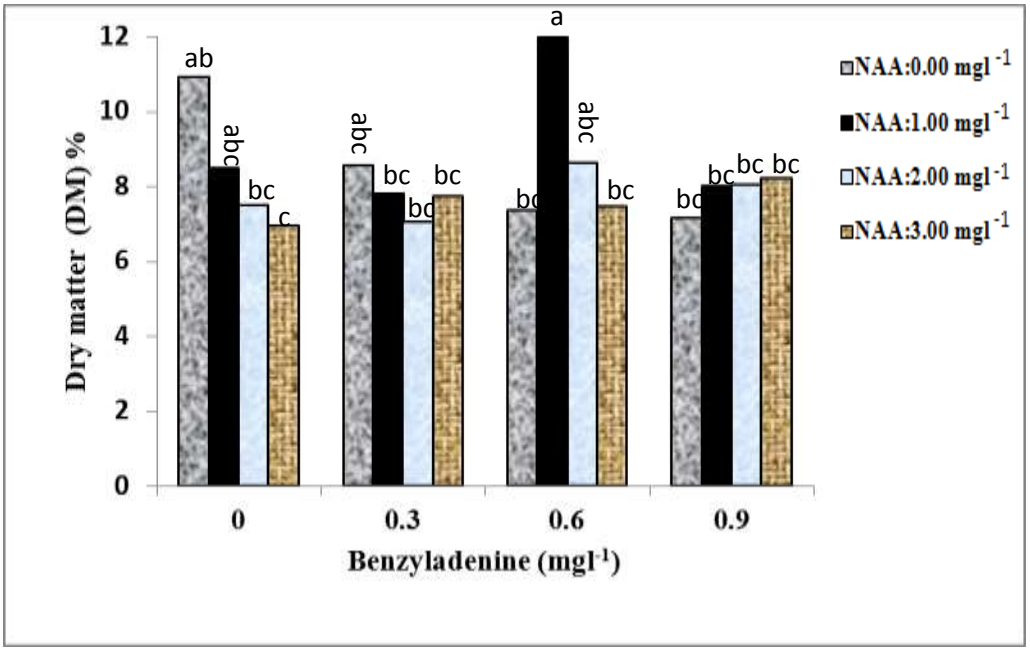


Figure 12. Effects of cytokinin (BA) / auxin (NAA) combinations on dry matter (DM) % of ***Datura innoxia*** **Mill** callus.

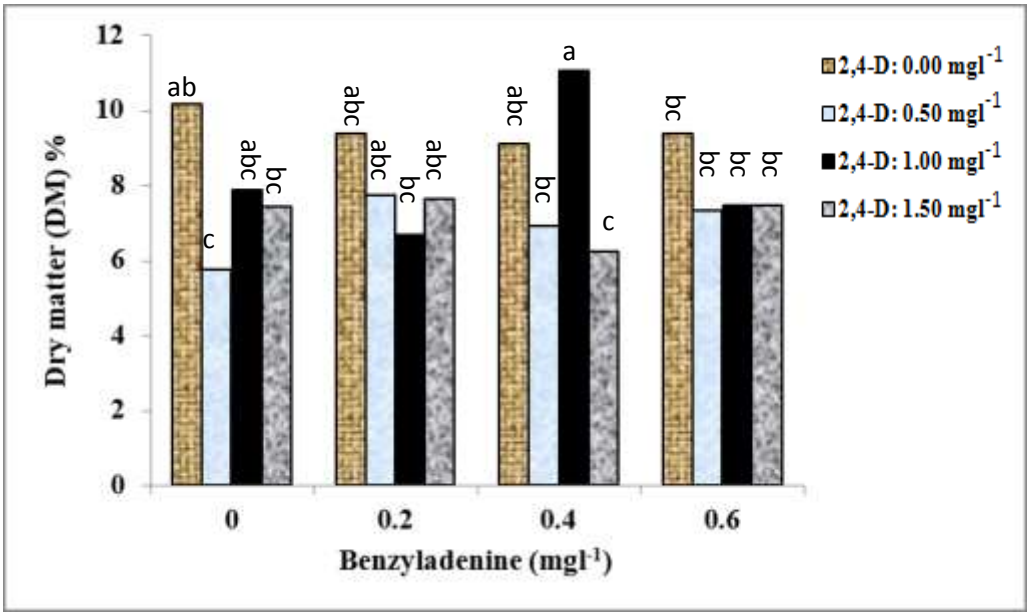


Figure 13. Effects of cytokinin (BA) / auxin (2,4-D) combinations on dry matter (DM) % of ***Datura innoxia* Mill** callus.

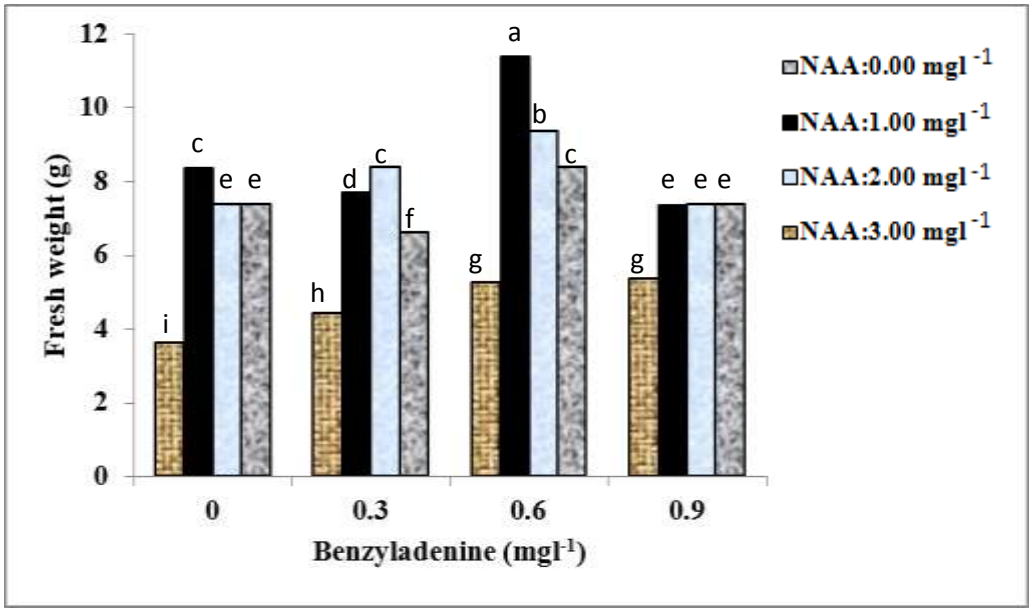


Figure 14. Effects of cytokinin (BA) / auxin (NAA) combinations on the fresh weight (g) of ***Datura innoxia* Mill** callus.

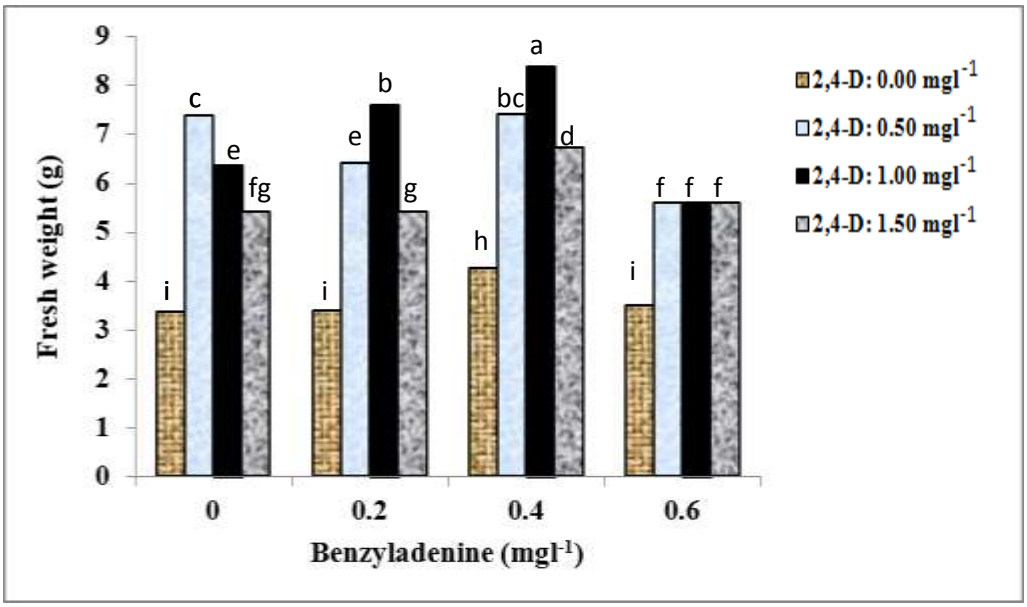


Figure 15. Effects of cytokinin (BA) / auxin (2,4-D) combinations on the fresh weight (g) of ***Datura innoxia* Mill** callus.

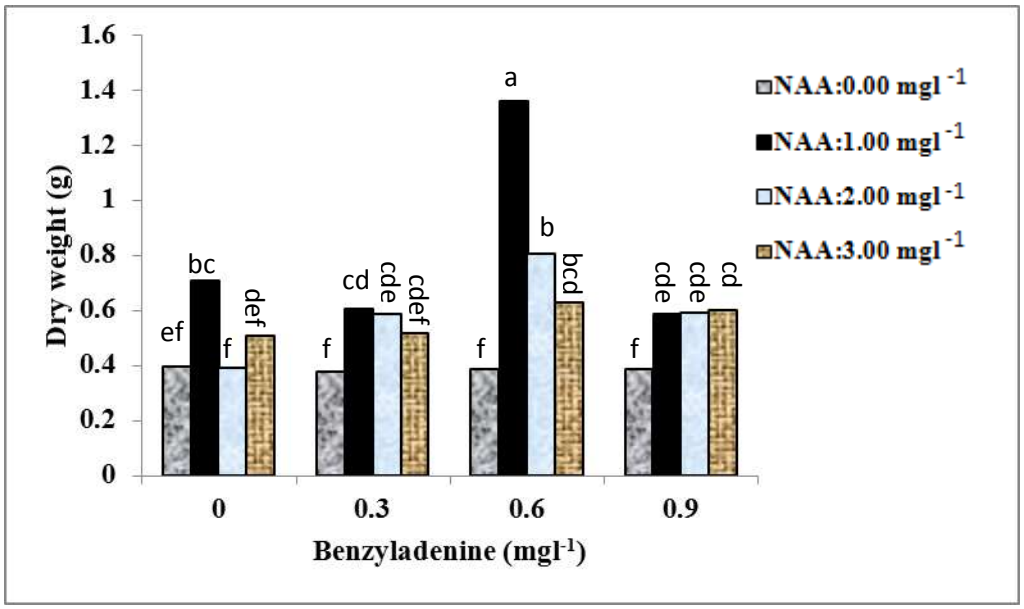


Figure 16. Effects of cytokinin (BA) / auxin (NAA) combinations on dry weight (g) of ***Datura innoxia* Mill** callus.

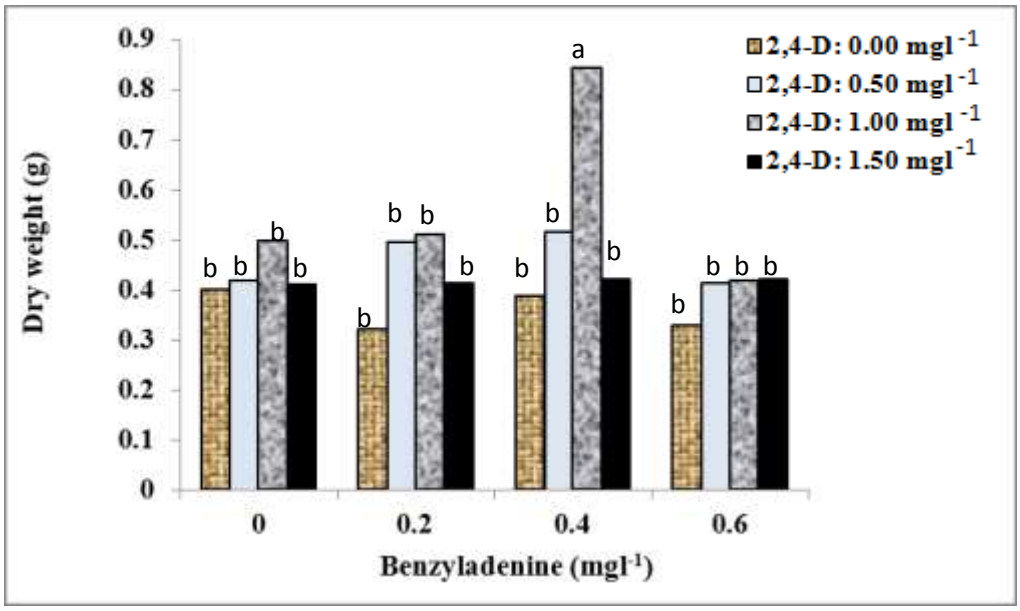


Figure 17. Effects of cytokinin (BA) / auxin (2,4-D) combinations on dry weight (g) of ***Datura innoxia* Mill** callus.

From the above results, one may conclude that supplementation of MS medium with 0.6 mgl^{-1} BA in combination with 1.0 mgl^{-1} of NAA is more suitable for callus production from leaf explants of ***Datura innoxia*** than BA and 2,4-D combination. In this respect, such results are in agreement with those of Nussbaumer *et al.* (1998). They reported that supplementation of B5 medium with 1.0 mgl^{-1} from each of NAA and BAP gave the best results of growth value for ***Datura candida*** and ***Datura aurea*** calli. Also, our results are close to those of Dessouky *et al.* (2001) who reported that the addition of 1.0 mgl^{-1} of each of NAA and BAP to the liquid MS medium was more suitable for callus production from ***Datura stramonium* L**. and ***Datura metel* L.** than the addition of NAA and Kinetin. In this respect, they concluded that callus formation and production are due to the presence of auxins and cytokinins that stimulated cell division and cell enlargement. Furthermore, our results with ***Datura innoxia*** calli induced from leaf explants, showed higher values for calli fresh, dry weights (g), and dry matter (%), 11.393, 1.574 and 11.97% respectively, compared to the results of El-Rahman *et al*. (2008) who reported that MS-medium supplemented with1.0 mgl^{-1} of both NAA and BA gave the best results of calli production, the maximum values of calli fresh weight (g), 6.76, dry weight (g), 0.673, and dry matter, % 9.05, were recorded with leaf explants of *Datura metel* L. The obtained results are in agreement with those of (Torres, 1988, El-Bahr, *et al*., 1989 and Dos Santos, *et al.*, 1990) who

reported that the addition of 1.0 mgl^{-1} of each BA and NAA was more suitable for callus production from ***D. metel*** and ***D. stramonium*.**

Figures 18 and 20 show the effects of cytokinin (BA) / auxin (NAA) and cytokinin (BA)/auxin (2,4-D) combinations on the number of shoots induced / leaf explants of the callus of ***Datura innoxia*** **Mill** at six and eight subcultures respectively. The highest number of shoots (46.167) (Figure 19) was obtained at cytokinin (BA, 2.0 mgl^{-1}) / auxin (NAA, 0.6 mgl^{-1}) at six subculture, which was highly significantly different from that value of 39.0 shoots for cytokinin (BA 1.0 mgl^{-1}) / auxin (2,4-D 0.5 mgl^{-1}) at 8^{th} subculture. However, concerning the number of shoots induced / leaf explants, El-Rahman *et al*. (2008) reported that MS medium containing 1.0 mgl^{-1} of 2,4-D and Kinetin gave the maximum number of shoots formation from leaf explants as compared with other types of explants.

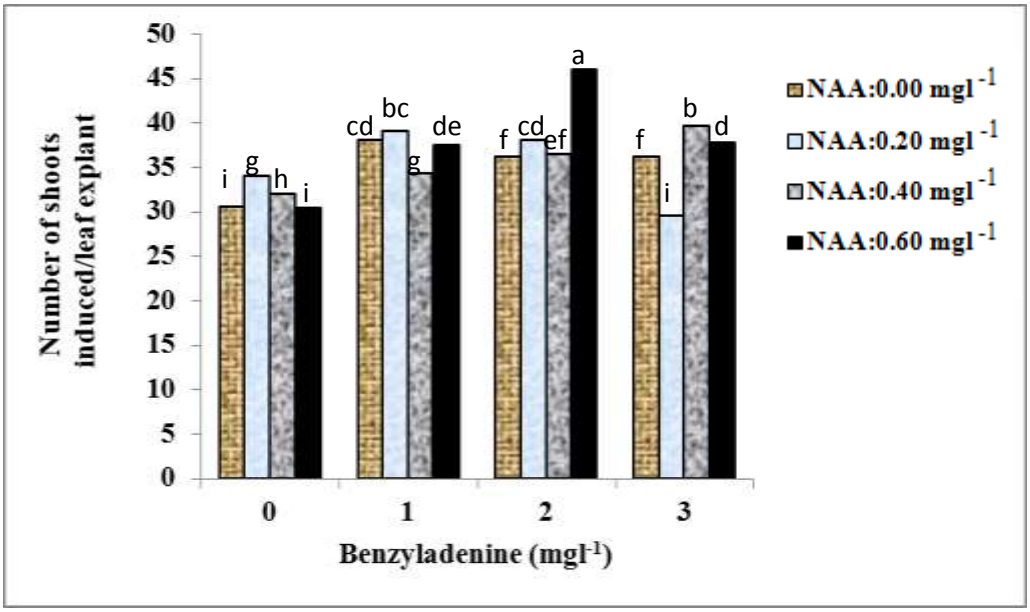


Figure 18. Effects of cytokinin (BA) / auxin (NAA) combinations on the number of shoots induced/leaf explant of ***Datura innoxia*** **Mill** callus at sixth subculture.

Figure 19a. Shoot formation on ***Datura innoxia*** **Mill** treated with (2.0 mgl^{-1} BA + 0.6 mgl^{-1} NAA).

Figure 19b. Further development of shoots on the same treatment at 8th subculture into complete regenerated plants.

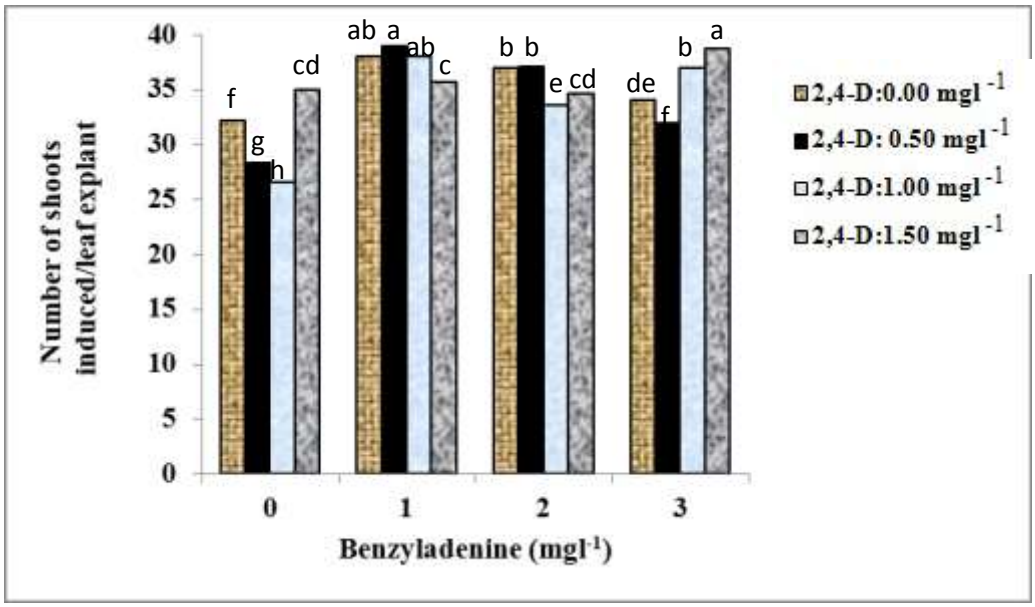


Figure 20. Effects of cytokinin (BA) / auxin (2,4-D) combinations on number of shoots induced/leaf explants of ***Datura innoxia*** **Mill** callus at 8th subculture.

(Figures from 10 to 17 represent the average means of 2nd subcultures only).

However, other researchers found the establishment of calli cultures but from different explants of ***Datura metel*** **L**. (Miskat *et al.* 2003). They reported that internodal segment showed best and prompt calli response in MS medium supplemented with 2.0 mgl^{-1} 2,4-D. Furthermore, Zayed *et al*. (2006) established undifferentiated callus from the stem explants of ***Datura innoxia*** using MS medium supplied with BA at 1.0 mgl^{-1} in combination with IAA at 0.5 mgl^{-1} for 6 weeks. Concerning the shoot multiplication in ***in vitro*** from nodal explants of ***Datura*** Dos Santos *et al*. (1990) reported that ***Datura insignis*** **Barb. Rod.** nodal explants were cultured on MS medium supplemented with either BA alone or in combination with 2,4-D or IAA. Shoots multiplication and elongation were obtained in various growth regulators at different concentrations. However, the best results were obtained in a medium with 1.0 mg/l^{-1} of BA, whereas in our study the highest number of shoots 46.167 was obtained at (BA, 2.0 mgl^{-1}) in combination with auxin (NAA, 0.6 mgl^{-1}) at 6th subculture, and the highest value for (BA, 1.0 mgl^{-1}) in combination with auxin (2,4-D, 0.5 mgl^{-1}) was 39 shoots at 8th subculture.

Similarly, Khanam *et al.* (2001) have reported that shoot regeneration from organogenic and non-organogenic calli of ***Duboisia myoporoides*** (an Australian medicinal plant containing

Atropine) can be induced with cytokinin (BA) more than kinetin in combination with different auxins (10µM IAA or NAA or 1µ M IBA).

As suggested previously for different ***Duboisia*** as well as other plant species, BA alone can cause shoot-bud initiation on the callus induced with various cytokinin and auxin combinations (Kitamura, 1988; Nin *et al.,* 1996 and Panizza *et al*., 1997). Kaminek *et al.,* (1997) who reported that in plant cells, cytokinin levels depend on cytokinin biosynthesis and/or uptake from extracellular sources and in addition, auxins may influence cytokinin levels. Patel and Thorpe (1984) stated that cell division mediated by BA leads to shoot formation in radiata pine which support the results of previous researchers and also our results. Therefore, from the results of the present study, NAA appear to be suitable auxin for quicker callus induction and shoot regeneration in ***Datura innoxia*** **Mill** in culture. To our knowledge the regeneration of shoots from about 25% only of the available calli in our study, can be well explained that by varying cytokinin/auxin combinations at the calli induction stage shoot buds or even root induction can be enhanced. Our conclusion can be supported by the findings of Khanam *et al.* (2001) in ***Duboisia myoporoides.***

Figure (21) shows the growth of callus in (g) of ***Datura innoxia*** **Mill** at different subcultures or passages (4 weeks / subculture, total of twelve subcultures). Maximum growth of the callus as expressed on the basis of dry weight, 1.357g was observed at the end of the 2nd subculture (8th week). Despite our results were in disagreement with Kinsara and Seif El-Nasr (1994) but generally both results had similar trend, they reported that maximum rate of growth rate of the cell suspension culture was 2.476 g dry weight / 100 ml medium, reached at the end of the 3rd week but both results followed similar trend in the rest of their growth passages where declined thereafter (Figure 21)**.**

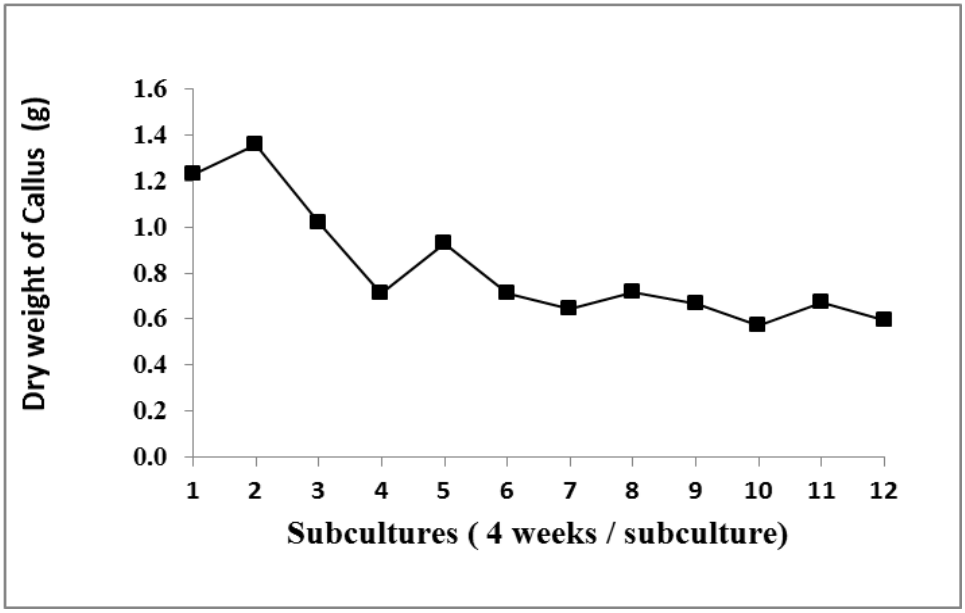


Figure 21. Growth of ***Datura innoxia*** **Mill** callus at different subcultures. (This figure represents the average means of twelve subcultures of the treatment: 0.6 mgl^{-1} BA + 1.0 mgl^{-1} NAA).

The obtained results are similar with those of Smith (2000) and El-Rahman *et al*. (2008) who reported that the rate of growth of calli tissue parallels in many ways the sigmoid curve seen in population of single celled organisms. There are usually five stages for callus growth rate; a lag phase in which cells prepare to divide, a period of exponential growth in which cell division is maximal, a period of linear growth in which division slows down and cells enlarge, a period of decelerating growth and stationary or no-growth period in which the number of cells is constant. The behavior of cells of callus tissue is different during each stage of growth. These differences in the growth stages may be attributed to the various auxins (IBA) and continuous illumination of 3000 lux used compared to normal light condition and auxin (NAA) used in our experiment. The media composition can also influence how long the callus remains at a particular stage. The calli grew as pale green cultures with isolated cell and small cell aggregates of cells (Figure 29).

This stock calli was used in subsequent subcultures in the present study. The calli were pale green, fine and friable till the end of the 3rd week of 1st subculture. After that, it became light green, fine and friable till the end of the seventh week of 2nd subculture.

Furthermore, the calli turned yellow- green with small aggregate cells and clumps till the end of the experiment (twelve subcultures). The incubation of the growth of calli subcultures for

total of 24 weeks may be well attributed to the previous researches of Khanam *et al.* (2001) who reported that histochemical study showed the presence of only meristemoid regions in the 11-week-old non-organogenic calli of ***Duboisia myoporoides*** and Thorpe (1980**),** the presence of meristemoid regions in the calli tissues of various plants, that may be used as a good indicator of active cell division in the calli.

Histological examination of the calli which are formed in fourth subculture (Figure 22) and showed the formation of different structures like root primordial, leaf primordial, globular embryo-like structure and epidermal cells (Figures 23). Organogenesis ***in vitro*** culture depends on the application of exogenous phytohormones, specially auxins and cytokinin, and also organogenesis depends on the ability of the tissue to respond to these plant growth regulators during culture period, where ***Datura*** callus responded very well to these plant growth regulators (Figure 23)**.** The manipulatable nature of tissue culture can be exploited for the physiological dissection of organogenesis *in vitro* culture. In general, three phases of organogenesis are recognizable, on the basis of temporal requirement for a specific balance of plant growth regulators in the control of organogenesis. In the first phase, cells in the explants acquire "competence" which is defined as the ability (not capacity) to respond to hormonal signals of organ induction. The process of acquisition of organogenic competence is referred to as "dedifferentiation" hereafter. The competent cells in cultured explants are canalized and determined for specific organ formation under the influence of the phytohormone balance through the second phase. Then after that the morphogenesis proceeds independently of the exogenously supplied plant growth regulators during the third phase (Christianson and Warnick,1983; Attfield and Evans, 1991 and Lo *et al*., 1997). Furthermore, figure 23 (C, D and E) clearly shows the distribution of some idioblast cells within ***Datura*** tissues which are responsible for alkaloid biosynthesis and accumulation (Iranbakhsh *et al*., 2006).

Figure 22. ***Datura innoxia*** **Mill** callus formed as a result of treatment with (BA, 0.6 mg/l^{-1}) + (NAA, 1.0 mg/l^{-1}) at the 4th subculture.

Furthermore, our results are also in agreement with results of Missaleva *et al.* (1993) who reported that in the 4th passage of callus culture of ***Datura innoxia*** **Mill** spontaneous formation of meristematic structures was observed. The calli of present study were also characterized by structures, resembling bulbs in appearance, were easy to disingwish by their more compact texture and dark green colour, their number increased in the next passage. However, in later passages the ability for self-initiaion of such structures was reduced as shown by Missaleva *et al.* (1993).

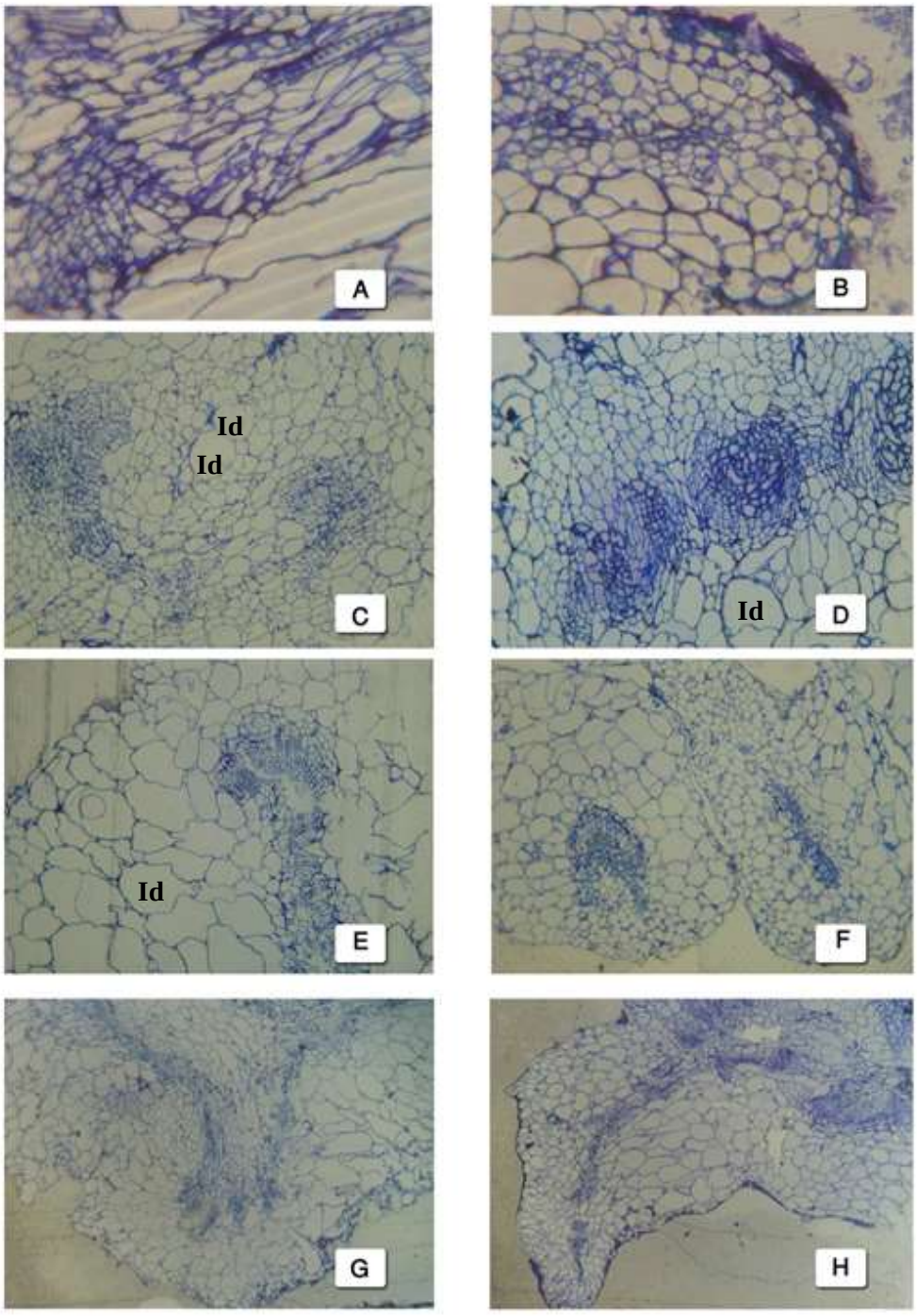
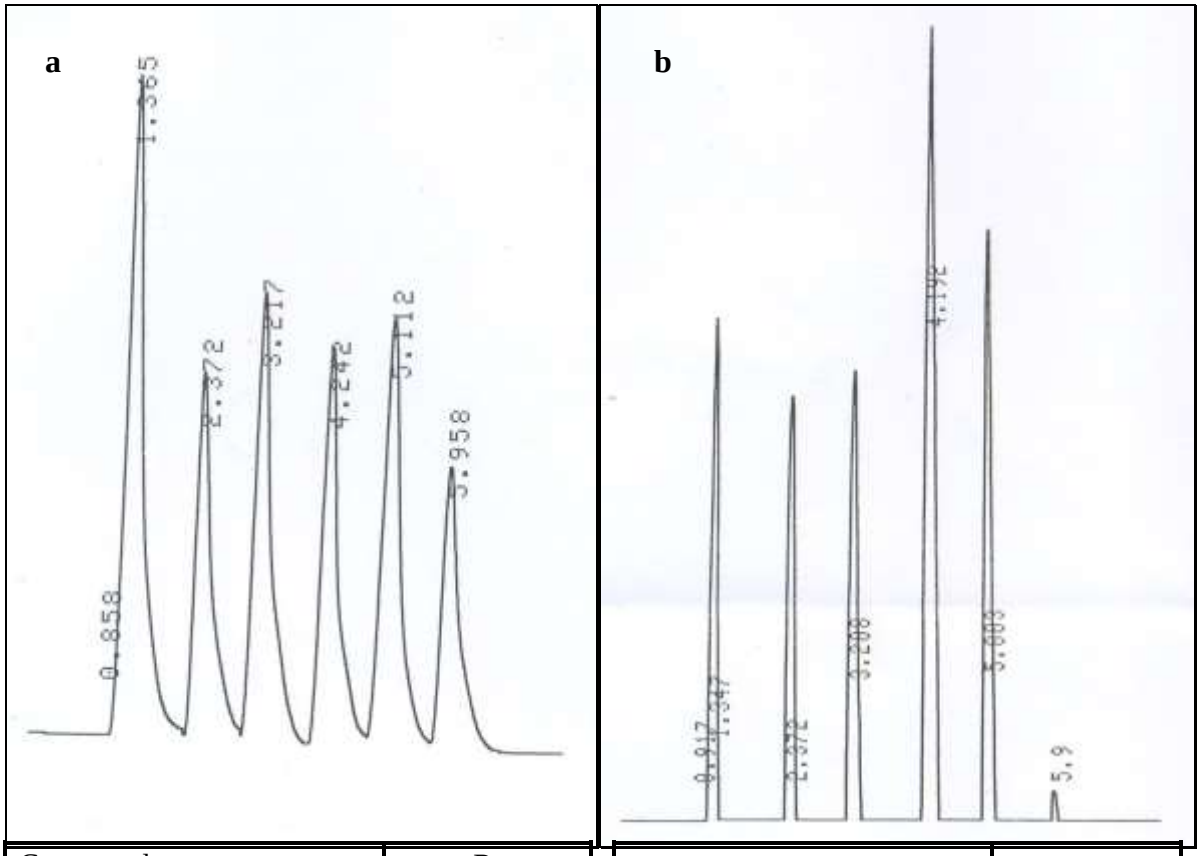


Figure 23. The development of cells during callus formation of ***Datura innoxia*** **Mill**.(A and B show the beginning of vascular tissue formation, C, D and E show the advance stage of vascular tissue formation and the presence of **idioblast** cells, F shows the formation of root primordia and embryo like structure, and G and H show the formation of leaf primordia).

4.3. Authentication of the Isolated Alkaloids of *Datura innoxia* Mill. Under Tissue Culture Conditions Using HPLC

Figures (24 to 38) represent the chromatograms of callus extract of ***Datura innoxia*** at different sucrose and salt concentrations under different light intensities with their retention times (Rts). Alkaloid analysis was carried out under the same conditions as mentioned in chapter three (Materials and Methods).



Compound	Rt
Atropine	1.365
Hyoscyamine	2.372
Scopolamine	3.217
Tropine	4.242
7-Hydroxyhyoscyamine	5.112
Tiglohyoscyamine	5.958

Others unknown compounds.

Compound	Rt
Atropine	1.365
Hyoscyamine	2.372
Scopolamine	3.217
Tropine	4.242
7-Hydroxyhyoscyamine	5.112
Tiglohyoscyamine	5.958

Others unknown compounds.

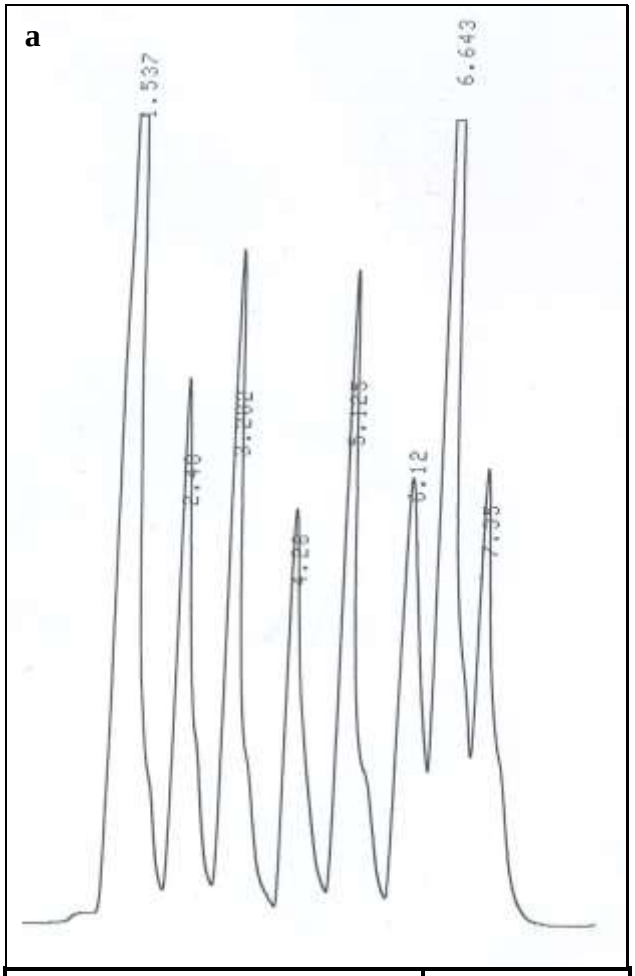
Figure 24 a and b. Detection of Rt (min.) of different alkaloids in of ***Datura innoxia*** **Mill** calli, from:

a. Reference

b. Leaves from greenhouse plants (***in vivo;*** Control), 8 weeks old, identified by (HPLC).

Compound	Rt
Atropine	1.365
Hyoscyamine	2.372
Scopolamine	3.217
Tropine	4.242
7-Hydroxyhyoscyamine	5.112
Tiglohyoscyamine	5.958

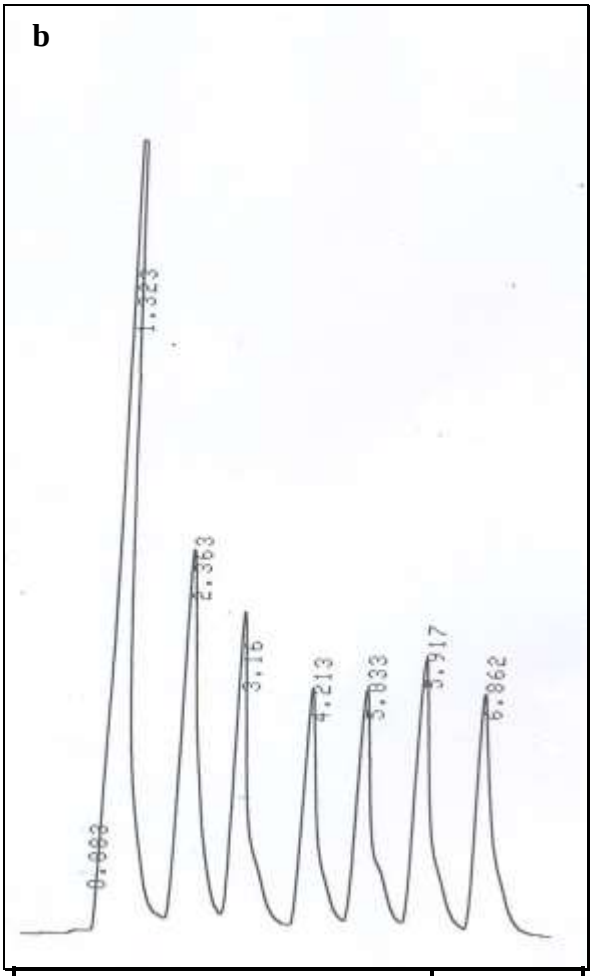
a



Compound	Rt
Atropine	1.537
Hyoscyamine	2.48
Scopolamine	3.282
Tropine	4.28
7-Hydroxyhyoscyamine	5.125
Tiglohyoscyamine	6.12

Others unknown compounds.

b



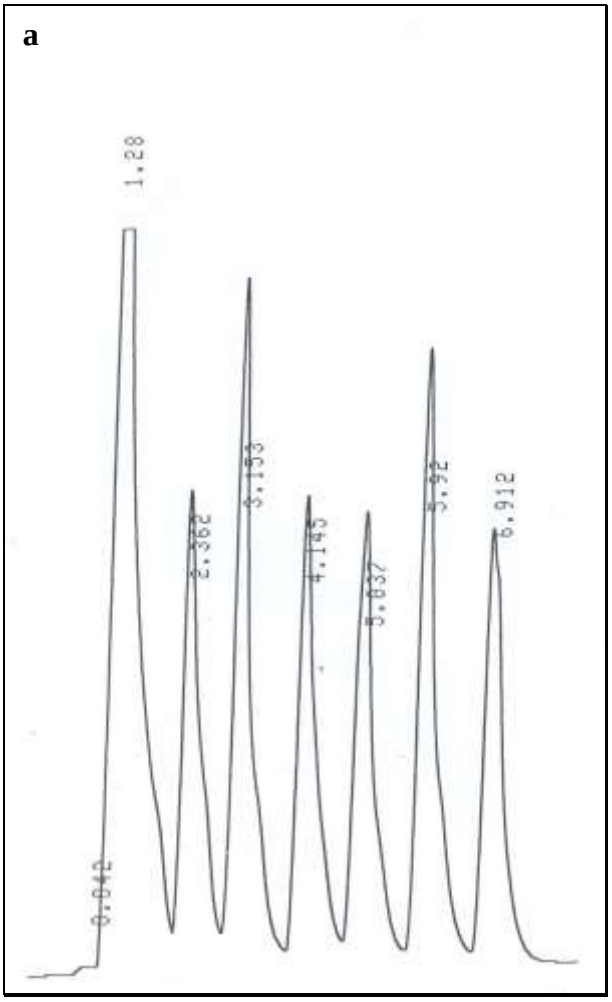
Compound	Rt
Atropine	1.323
Hyoscyamine	2.363
Scopolamine	3.16
Tropine	4.213
7-Hydroxyhyoscyamine	5.033
Tiglohyoscyamine	5.917

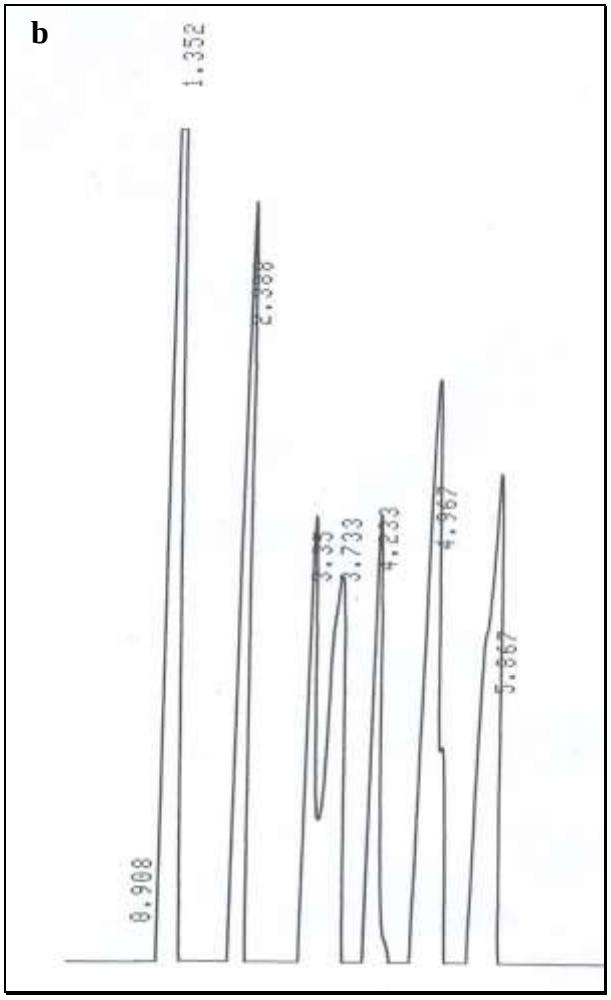
Others unknown compounds.

Figure 25 a and b.

a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($SoXoLI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($SoXoLI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).





Compound	Rt
Atropine	1.28
Hyoscyamine	2.362
Scopolamine	3.153
Tropine	4.145
7-Hydroxyhyoscyamine	5.037
Tiglohyoscyamine	5.92

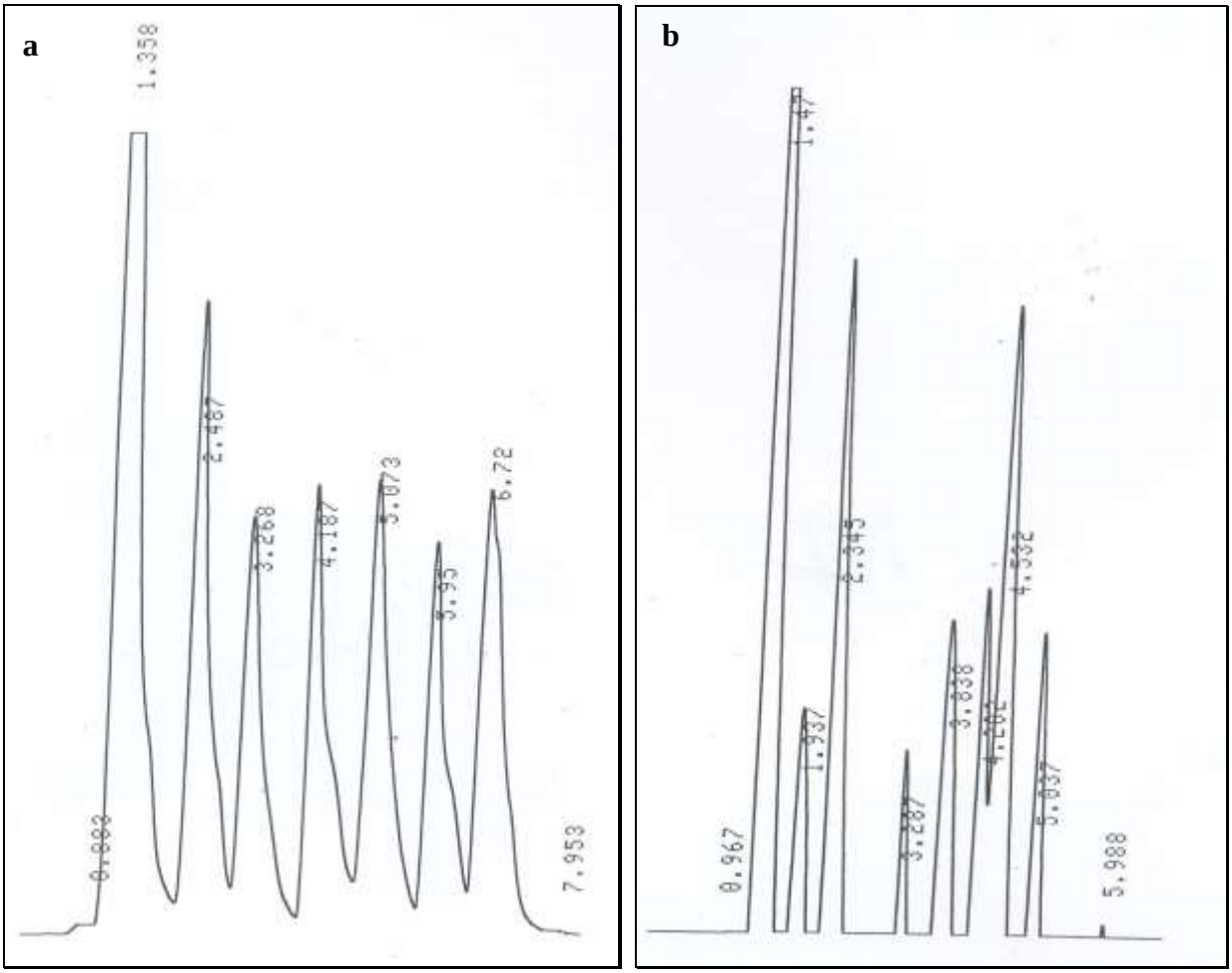
Others unknown compounds.

Compound	Rt
Atropine	1.352
Hyoscyamine	2.388
Scopolamine	3.35
Tropine	4.233
7-Hydroxyhyoscyamine	4.967
Tiglohyoscyamine	5.867

Others unknown compounds.

Figure 26 a and b.

- **a.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($SoXoLI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).
- **b.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, (SoX_1LI_1 treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC):



Compound	Rt
Atropine	1.358
Hyoscyamine	2.487
Scopolamine	3.268
Tropine	4.187
7-Hydroxyhyoscyamine	5.073
Tiglohyoscyamine	5.95

Others unknown compounds.

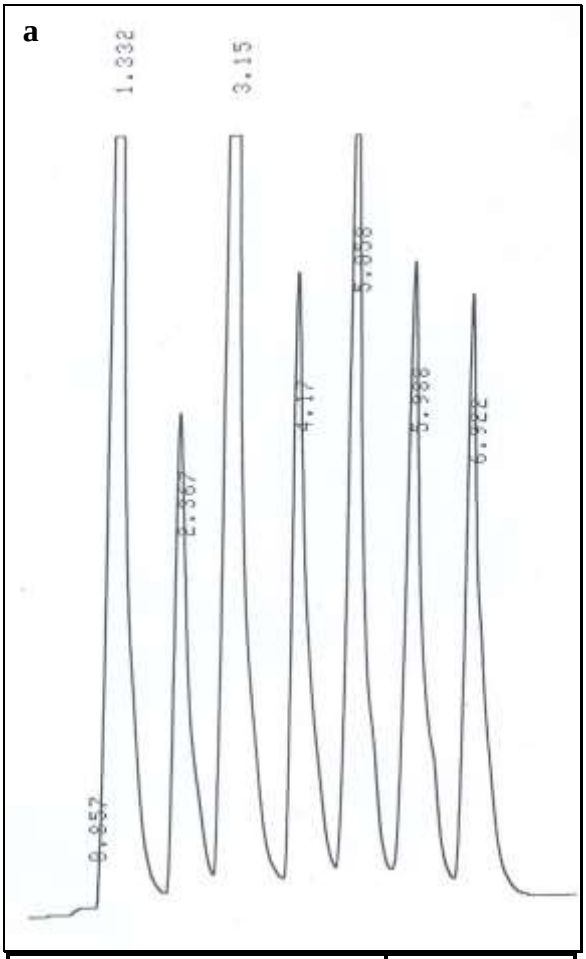
Compound	Rt
Atropine	1.47
Hyoscyamine	2.345
Scopolamine	3.287
Tropine	4.282
7-Hydroxyhyoscyamine	5.037
Tiglohyoscyamine	5.988

Others unknown compounds.

Figure 27 a and b.

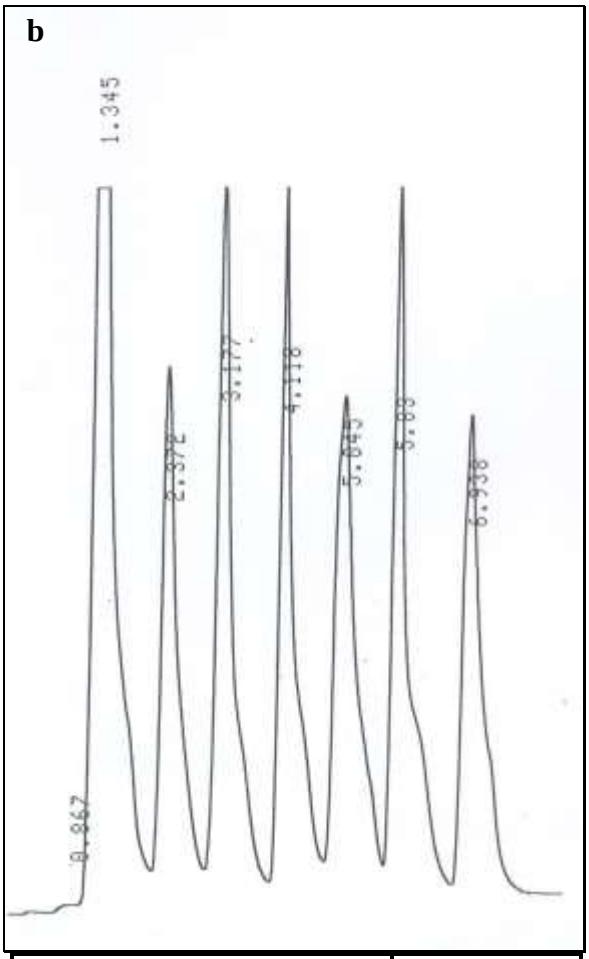
a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** , (SoX_1LI_2 treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** , (SoX_1LI_3 treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.332
Hyoscyamine	2.367
Scopolamine	3.15
Tropine	4.17
7-Hydroxyhyoscyamine	5.058
Tiglohyoscyamine	5.988

Others unknown compounds.



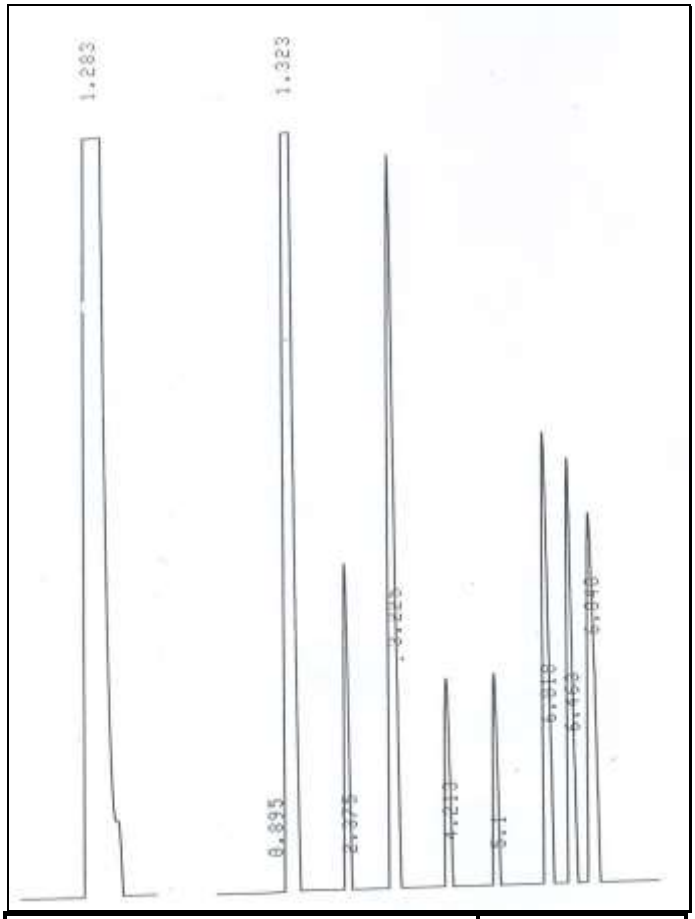
Compound	Rt
Atropine	1.345
Hyoscyamine	2.372
Scopolamine	3.177
Tropine	4.118
7-Hydroxyhyoscyamine	5.045
Tiglohyoscyamine	5.83

Others unknown compounds.

Figure 28 a and b.

a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, (SoX_2LI_3 treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

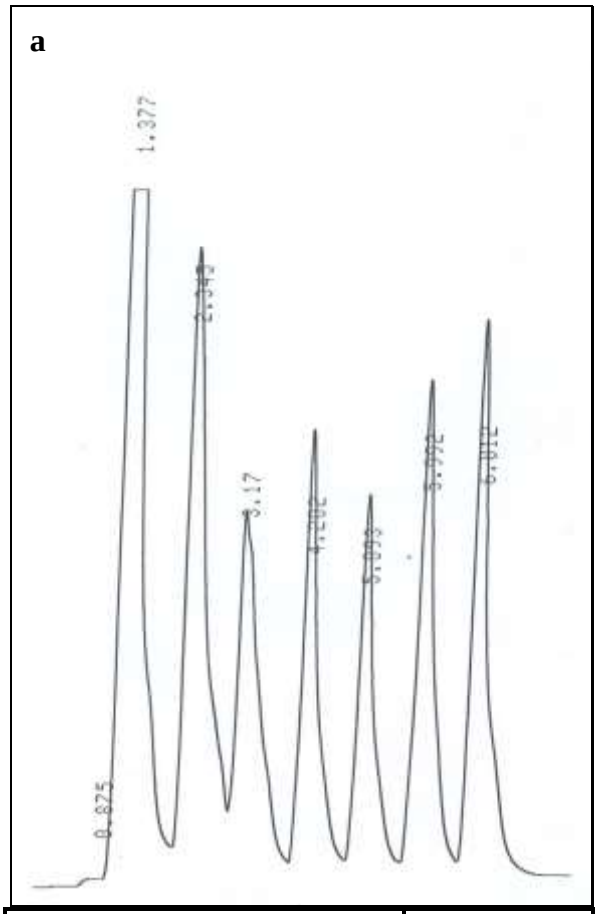
b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, (SoX_2LI_1 treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.323
Hyoscyamine	2.375
Scopolamine	3.225
Tropine	4.213
7-Hydroxyhyoscyamine	5.1
Tiglohyoscyamine	6.018

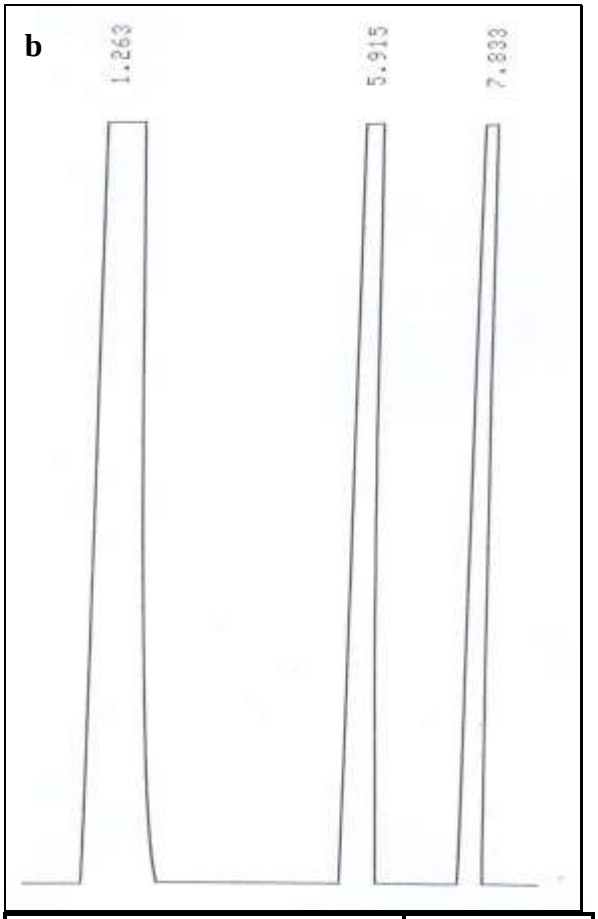
Others unknown compounds.

Figure 29. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, (SoX_2LI_2 treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.377
Hyoscyamine	2.345
Scopolamine	3.175
Tropine	4.202
7-Hydroxyhyoscyamine	5.992
Tiglohyoscyamine	6.018

Others unknown compounds.

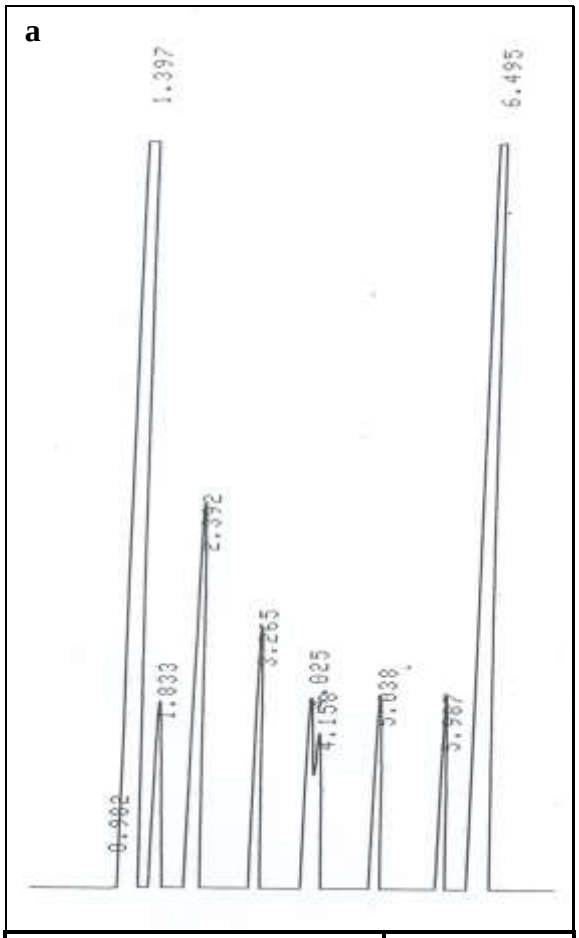


Compound	Rt
Atropine	1.263
Tiglohyoscyamine	5.915

Figure 30 a and b.

- **a.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** , ($S_1X_0LI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).
- **b.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** , ($S_1X_0LI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

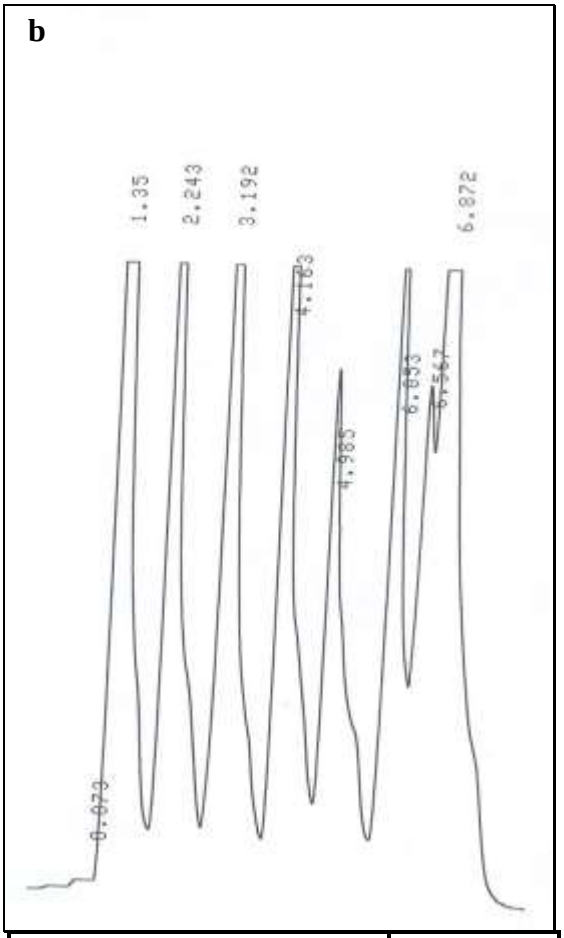
a



Compound	Rt
Atropine	1.397
Hyoscyamine	2.392
Scopolamine	3.265
Tropine	4.025
7-Hydroxyhyoscyamine	5.038
Tiglohyoscyamine	5.987

Others unknown compounds.

b



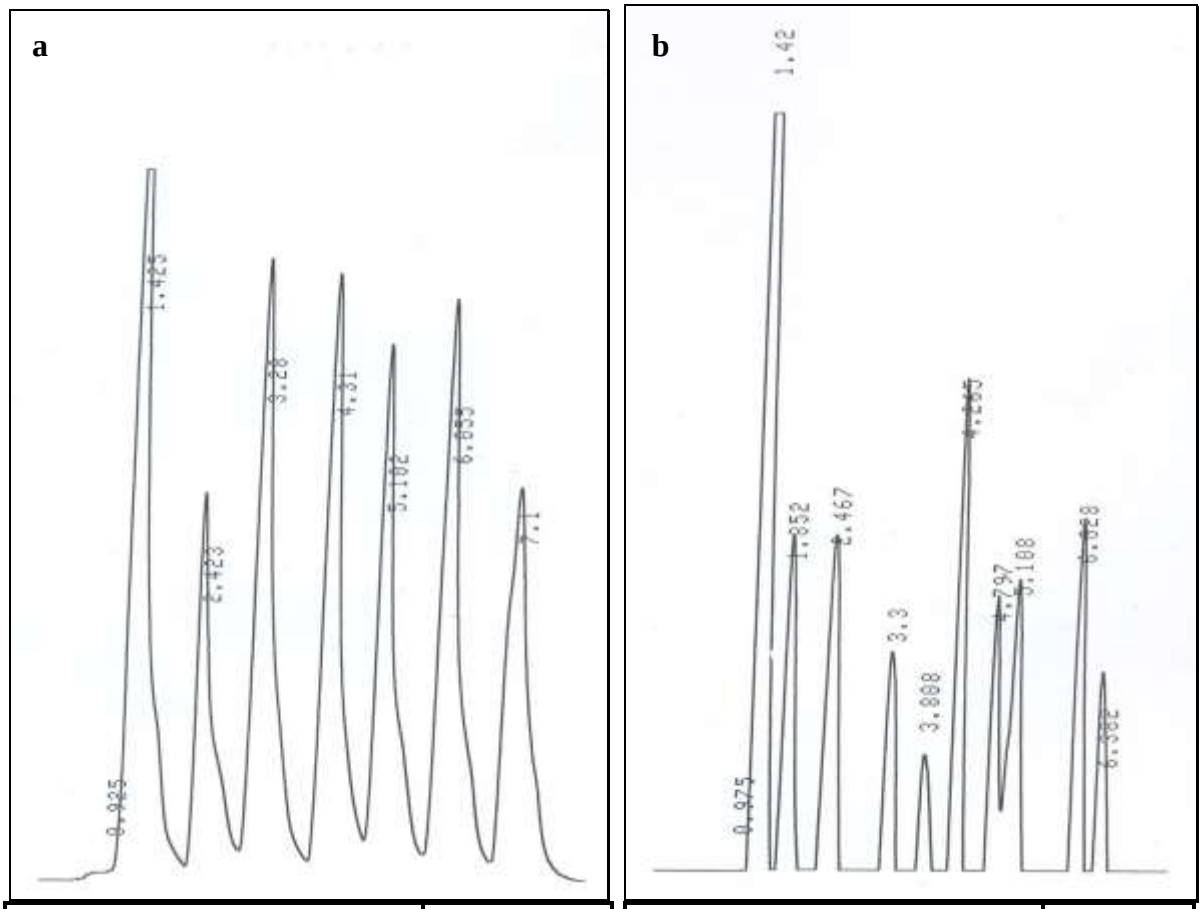
Compound	Rt
Atropine	1.35
Hyoscyamine	2.243
Scopolamine	3.192
Tropine	4.163
7-Hydroxyhyoscyamine	4.985
Tiglohyoscyamine	6.053

Others unknown compounds.

Figure 31 a and b.

a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_0LI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_1LI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.425
Hyoscyamine	2.423
Scopolamine	3.28
Tropine	4.31
7-Hydroxyhyoscyamine	5.102
Tiglohyoscyamine	6.055

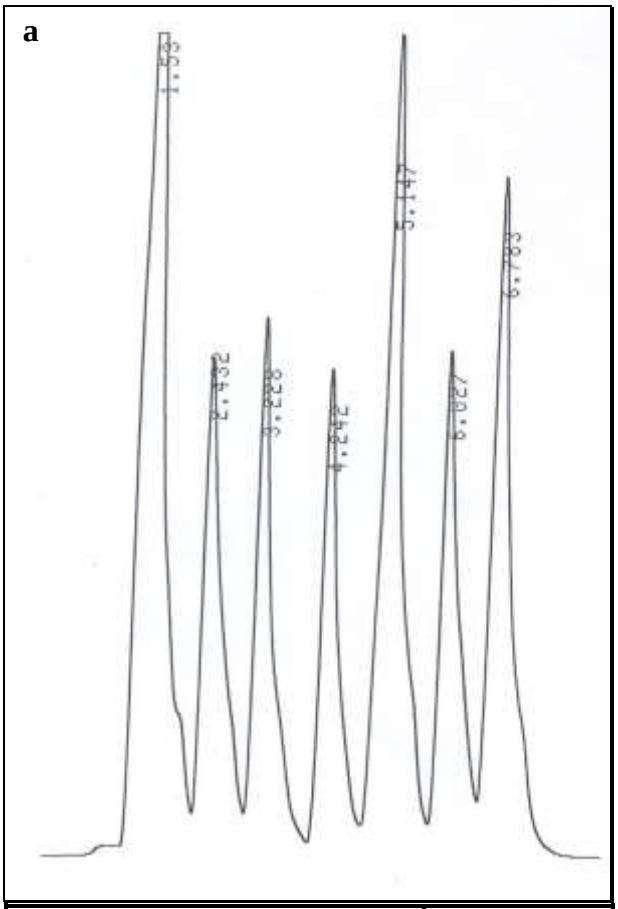
Others unknown compounds.

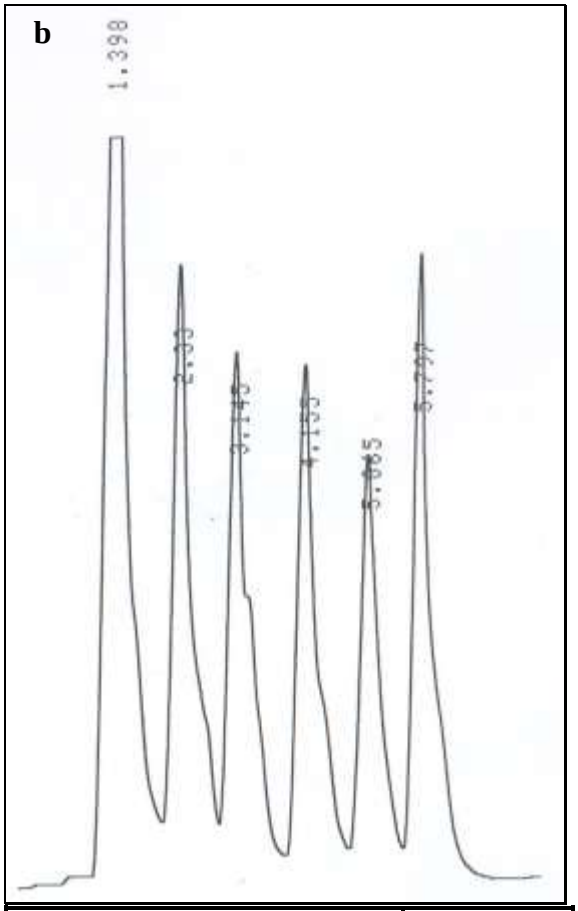
Compound	Rt
Atropine	1.42
Hyoscyamine	2.467
Scopolamine	3.3
Tropine	4.265
7-Hydroxyhyoscyamine	5.108
Tiglohyoscyamine	6.028

Others unknown compounds.

Figure 32 a and b.

- **a.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_1LI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).
- **b.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_1LI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).





Compound	Rt
Atropine	1.53
Hyoscyamine	2.432
Scopolamine	3.228
Tropine	4.242
7-Hydroxyhyoscyamine	5.147
Tiglohyoscyamine	6.027

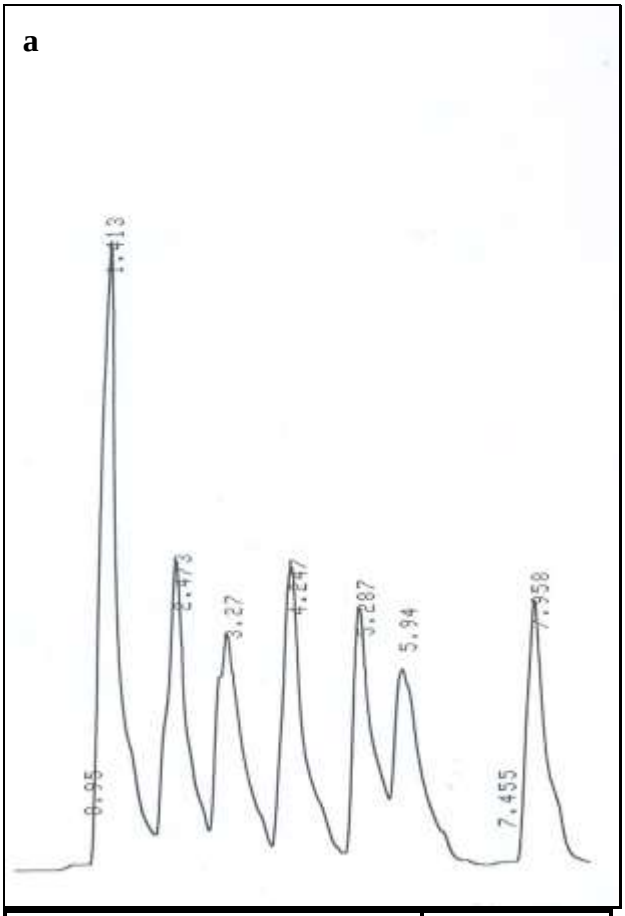
Compound	Rt
Atropine	1.398
Hyoscyamine	2.33
Scopolamine	3.145
Tropine	4.155
7-Hydroxyhyoscyamine	5.065
Tiglohyoscyamine	5.797

Others unknown compounds.

Figure 33 a and b.

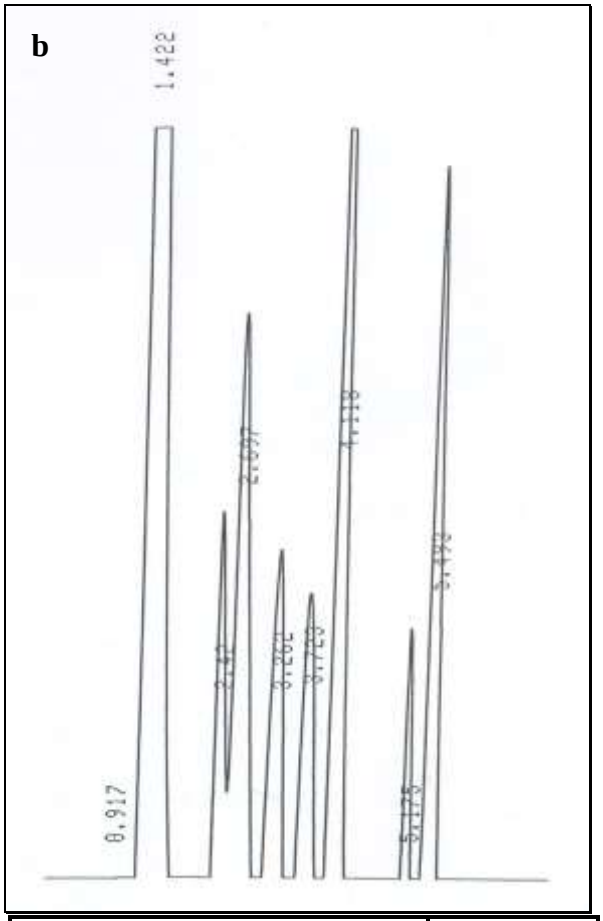
a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_2LI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_2LI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.413
Hyoscyamine	2.473
Scopolamine	3.27
Tropine	4.247
7-Hydroxyhyoscyamine	5.287
Tiglohyoscyamine	5.94

Others unknown compounds.



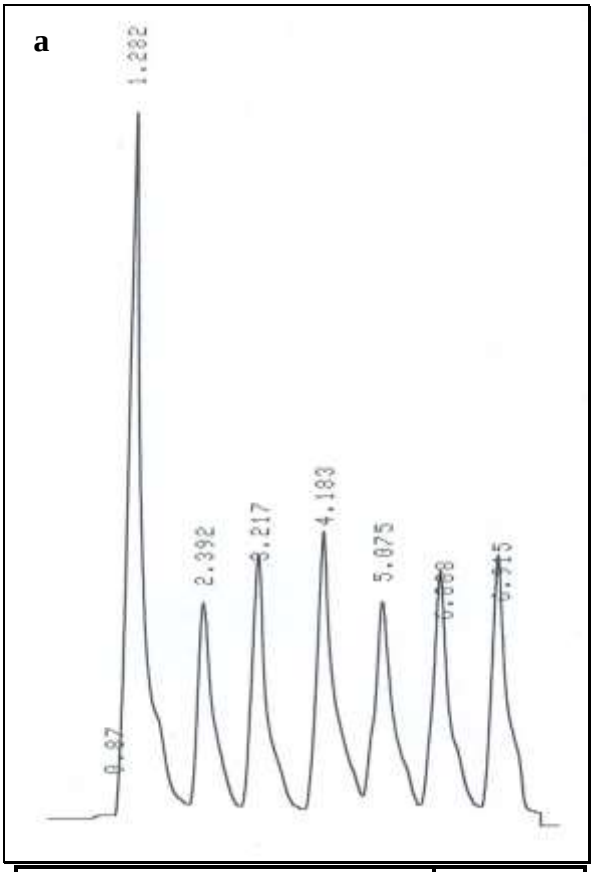
Compound	Rt
Atropine	1.422
Hyoscyamine	2.42
Scopolamine	3.262
Tropine	4.118
7-Hydroxyhyoscyamine	5.175
Tiglohyoscyamine	5.493

Others unknown compounds.

Figure 34 a and b.

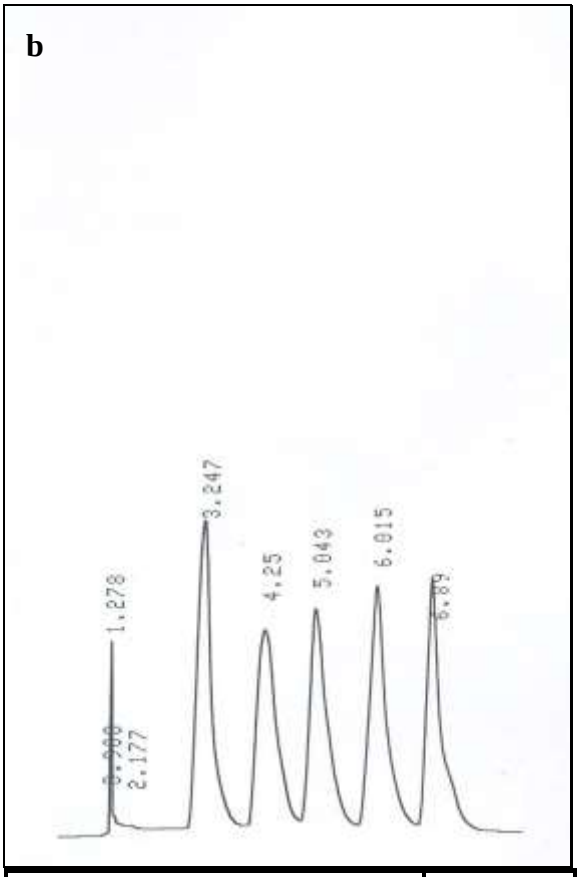
a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_1X_2LI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_2X_0LI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.282
Hyoscyamine	2.392
Scopolamine	3.217
Tropine	4.183
7-Hydroxyhyoscyamine	5.075
Tiglohyoscyamine	6.008

Others unknown compounds.

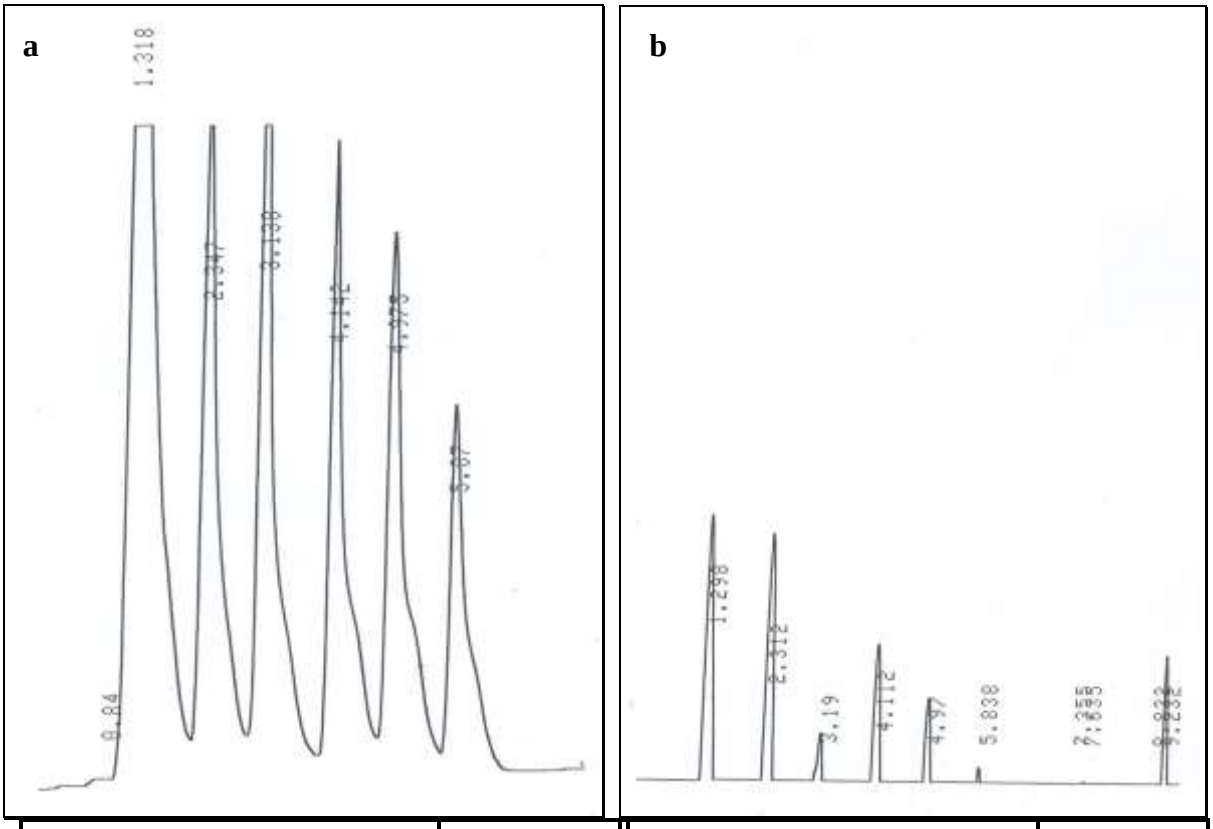


Compound	Rt
Atropine	1.278
Scopolamine	3.247
Tropine	4.25
7-Hydroxyhyoscyamine	5.043
Tiglohyoscyamine	6.015

Others unknown compounds.

Figure 35 a and b.

- **a.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_2X_0LI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).
- **b.** Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_2X_0LI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



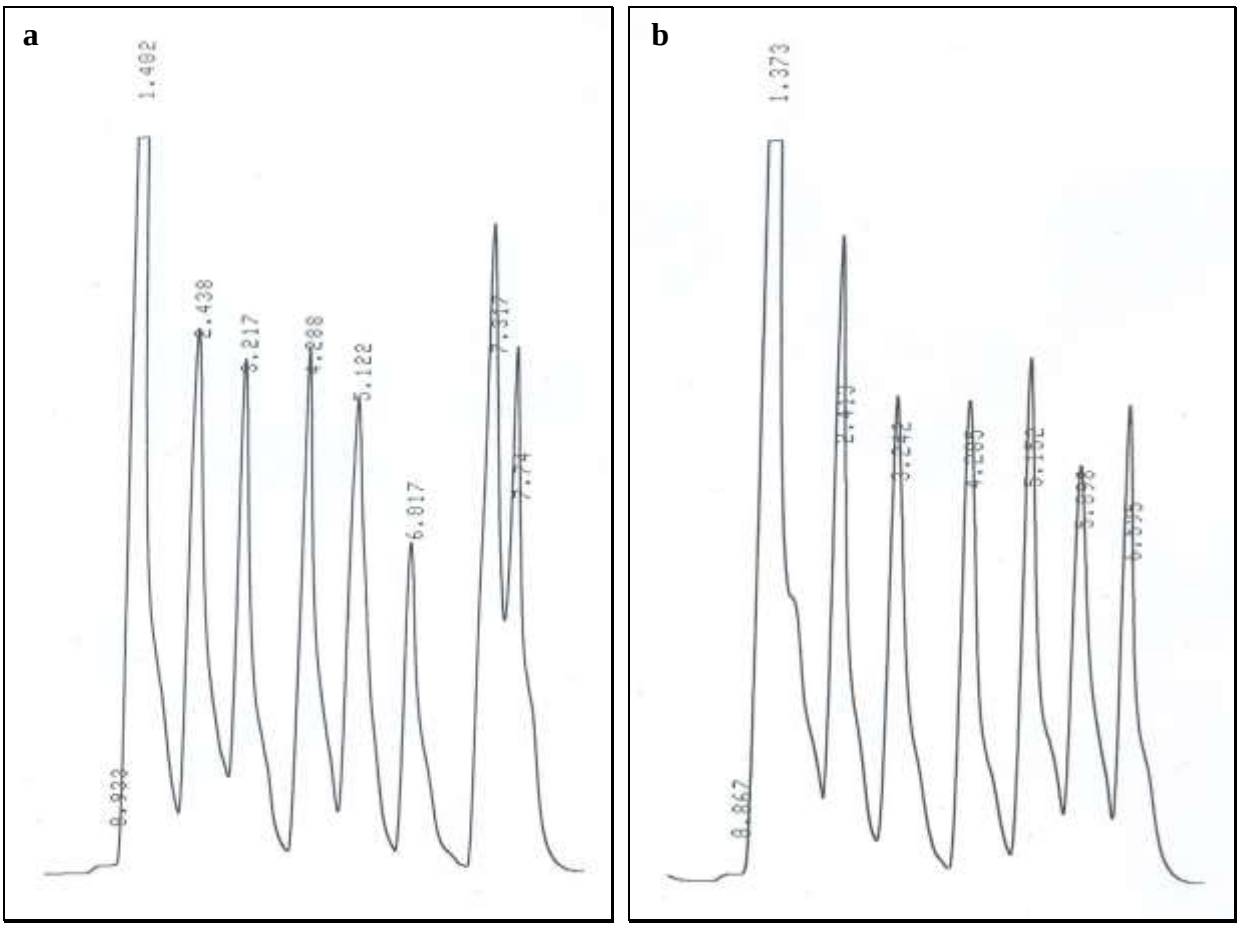
Compound	Rt
Atropine	1.318
Hyoscyamine	2.347
Scopolamine	3.138
Tropine	4.142
7-Hydroxyhyoscyamine	4.975
Tiglohyoscyamine	5.87
Others unknown compounds.	

Compound	Rt
Atropine	1.298
Hyoscyamine	2.312
Scopolamine	3.19
Tropine	4.112
7-Hydroxyhyoscyamine	4.97
Tiglohyoscyamine	5.838
Others unknown compounds.	

Figure 36 a and b.

a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia* Mill** calli, ($S_2X_1LI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia* Mill** calli, ($S_2X_1LI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.402
Hyoscyamine	2.438
Scopolamine	3.217
Tropine	4.288
7-Hydroxyhyoscyamine	5.122
Tiglohyoscyamine	6.017

Others unknown compounds.

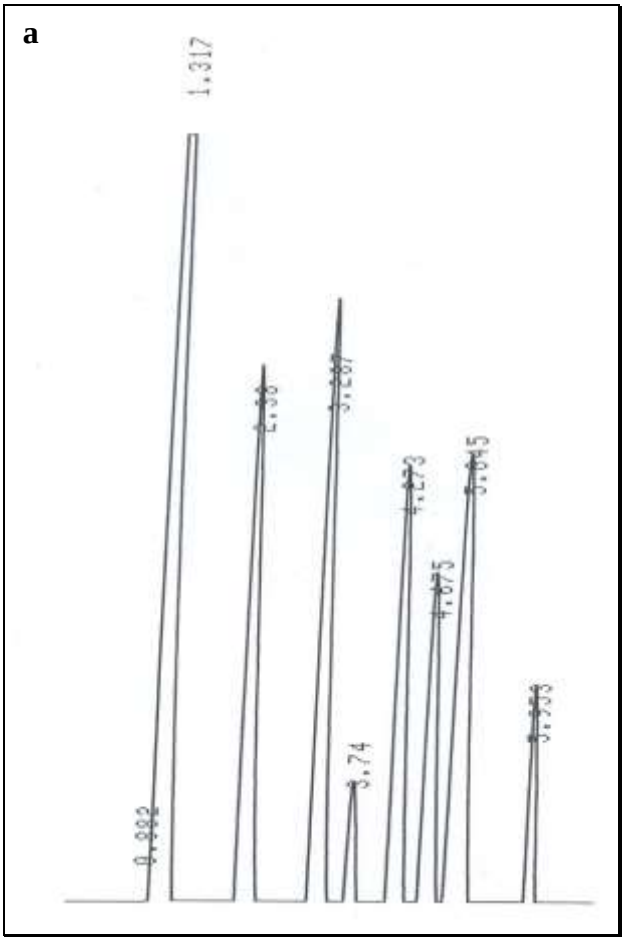
Compound	Rt
Atropine	1.373
Hyoscyamine	2.413
Scopolamine	3.242
Tropine	4.285
7-Hydroxyhyoscyamine	5.152
Tiglohyoscyamine	5.898

Others unknown compounds.

Figure 37 a and b.

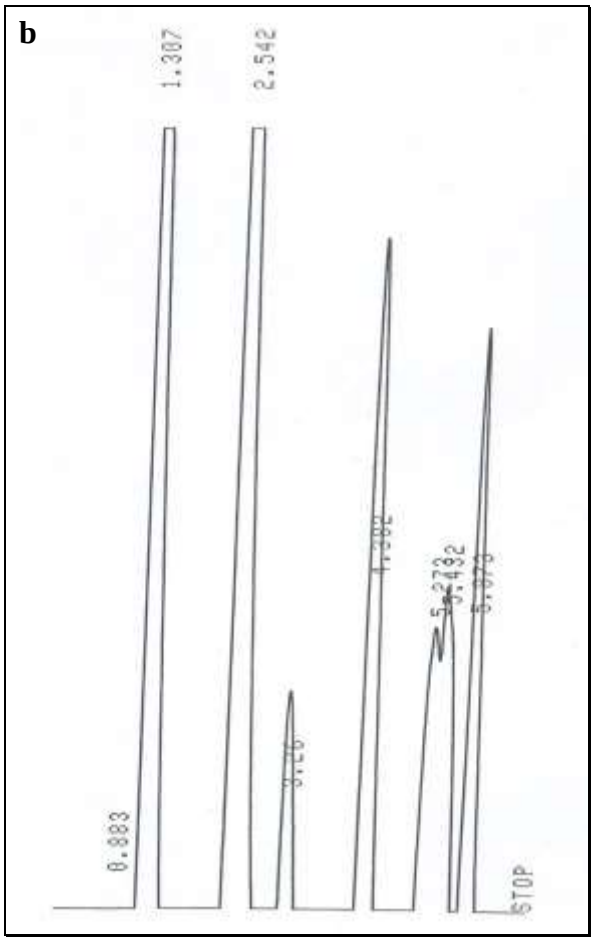
a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_2X_2LI_1$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia*** **Mill** calli, ($S_2X_1LI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).



Compound	Rt
Atropine	1.317
Hyoscyamine	2.38
Scopolamine	3.287
Tropine	4.273
7-Hydroxyhyoscyamine	5.045
Tiglohyoscyamine	5.953

Others unknown compounds.



Compound	Rt
Atropine	1.307
Hyoscyamine	2.542
Scopolamine	3.26
Tropine	4.382
7-Hydroxyhyoscyamine	5.273
Tiglohyoscyamine	5.873

Others unknown compounds.

Figure 38 a and b.

a. Detection of Rt (min.) of different alkaloids of ***Datura innoxia* Mill** calli, ($S_2X_2LI_2$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

b. Detection of Rt (min.) of different alkaloids of ***Datura innoxia* Mill** calli, ($S_2X_2LI_3$ treatment), cultured on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}), identified by (HPLC).

The chromatograms in the previous mentioned figures showed different number of peaks in each of them (mainly six peaks), representing atropine, hyoscyamine, scopolamine, Tropine, 7-hydroxyhyoscyamine and tiglohyoscyamine according to their retention times (Rts) when compared to the references of those alkaloids. Besides, some other minor peaks were also presented in most of the chromatograms, which was not identified because of the lack of all alkaloid references.

Concerning the quantitative evaluations of these major alkaloid components in the calli of ***Datura innoxia,*** tables (5, 6 and 7) show the means of the main alkaloids of ***Datura innoxia* Mill** calli at different sucrose **(S)**, salt **(X)** concentrations and under different light intensities **(LI)**of MS medium supplied with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}). Many studies were carried out concerning the biosynthesis of tropane alkaloids dealing with the callus tissues of this plant (Hiraoka and Tabata 1974). These studies indicated that total alkaloid content of the callus tissue was less than that of the intact plant. The total alkaloid in cell suspension of ***Datura innoxia*** was far below than that detected in the intact leaves. However there was also contradicting studies indicating the ***in vitro*** plant culture resulted in obtaining high percent of alkaloids more than in the intact plant (Kinsara and Seif El-Nasr 1994). Sucrose is the most commonly used carbon source in heterotrophic and/or mixotrophic tissue culture. However, contradictory effects of supplied sugar on plant metabolism have been reported (Sima and Desjardins, 2001). Exogenous sucrose negatively affected growth and photosynthesis of various ***in vitro*** plantlets (Schäfer *et al*., 1992; Kozai *et al*., 1995 and Serret *et al*., 1997). On the other hand, stimulating effects of sugars on growth and photosynthesis have also been reported for ***in vitro*** plantlets of tobacco (Paul and Stitt, 1993); for nicotine accumulation in tobacco (Mantell & Smith, 1983); for polyphenol production by cells of ***Rosa*** (Davies, 1972) and also for potato (Tichá *et al*., 1998**).** In addition, Hdide and Desjardins, (1994) showed that sucrose induced non-photosynthetic carbon fixation by providing PEP necessary for PEPC activity.

Table (5) represents the mean concentrations of the six alkaloids in ***Datura innoxia*** calli, cultured in MS medium at different sucrose concentrations (**So, S_1** and **S_2**). It appeared that the effects of growing **Datura** callus at different sucrose concentrations on alkaloid concentrations and their totals were found to be increased but not significantly especially the first four alkaloids at **S_1** (15 gl^{-1}) sucrose treatment when the means of **S_1** and **S_2** were compared to those of **So** treatments.

Our results are in agreement with those of Kinsara and El-Naser (1994) who studied the effects of sucrose concentration and reported that auxin, 2,4-D and 1% sucrose (10 gl^{-1}) enhanced the production of tropane alkaloids in ***Datura innoxia*** calli. In addition the total concentrations at sucrose treatment (**S_1**) 356.688 µg/g DW for all alkaloids was higher than those of **So** and **S_2** treatments (206.423 and 268.051 µg/g DW) respectively. However, in general, raising the initial levels of sucrose leads to an increase in the secondary-metabolite yield of cultures. There are many other examples, including the production of the steroidal alkaloids, solasodine, in callus cultures of ***Solanum nigrum*** (Bhatt *et al*., 1983) and in suspension cultures of ***Solanum elagnifolium*** (Nigra *et al*., 1990), alkaloids and polyphenol production in dark-grown suspension cultures of ***Catharanthus roseus*** (Knobloch and Berlin, 1980), and anthocyanin accumulation in ***Vitis*** suspension cultures (Yamakawa *et al.*, 1983, Cormier *et al*., 1990 and Do and Cormier, 1991**)**. The increased accumulation of anthocyanin in ***Vitis vinifers*** suspension cultures when the sucrose levels were increased has been shown to be the result of osmotic stress (Do and Cormier, 1990). Yamakawa *et al.* (1983) have reported that increased anthocyanin levels occurred in ***Vitis*** cell cultures when sucrose levels were increased. However, they also found that at lower sucrose concentrations, reduced nitrogen levels also resulted in higher amounts of anthocyanin and suggested that there was an optimal C:N ratio for pigments production. Thus, decreasing sucrose concentration in MS medium to (15 gl^{-1}) in our experiment can be explained by the presence of an optimal C:N ratio for alkaloid production. Moreover, Huimei *et al.*, (2007) proved that adventitious root formation is strongly dependent on sucrose supply. They showed that on the medium lacking sucrose, the plantlets remained green in the culture period and no adventitious roots produced. Sucrose concentration was positively correlated with the percentage of rooting and root number per rooted shoot. The rooting percentage and root number increased with the increasing concentrations of sucrose from 10 gl^{-1} to 30 $gl^{-1.}$ Higher concentrations of sucrose inhibited root formation which may explain the reflection of a genuine regulatory role of sucrose in adventitious regeneration.

These observations from a variety of laboratories working with several different species further support the concept of an inverse relationship between major aspects of primary metabolism, example of protein synthesis and the synthesis and accumultion of secondary metabolites, based on the differential and antagonistic utilization of common precursors.

Attention will be now focused on the possible role of plant growth regulators on this partitioning (Yeoman and Yeoman, 1996). Sucrose and glucose are the preferred carbon source

for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. Increased sugar concentration favoured synthesis of shikonin in cell cultures of ***Lithospermum erythrohizon***, diosgenin production in ***Dioscorea***, and anthraquinone in cell cultures of ***Gallium mollugo.*** On the contrary, lesser amounts of sucrose favored the production of ubiquinone 10 in ***Coleus blumei*** (Roja and Rao, 1998). The maximum yield of rosmarinic acid produced by cell suspension cultures of ***Salvia officinalis*** was 3.5 gl^{-1} when 5% of sucrose was used but it was 0.7 gl^{-1} in the medium containing 3% sucrose (Whitaker *et al.*, 1984).

Table 5. Means of the main alkaloids concentrations (µg/g DW) identified by (HPLC) of ***Datura innoxia*** **Mill** calli at different sucrose concentrations of MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}).

Concentrations of Alkaloids (µg/g DW)							
Treatments	Atropine	Hyoscyamine	Scopolamine	Tropine	7-Hydroxy-hyoscyamine	Tiglohyosc-yamine	Total
So	38.646	35.063	30.958	29.507	30.97	41.279	206.423
S1	69.695	79.961	51.47	58.718	43.428	53.416	356.688
S2	53.3	54.517	36.434	42.436	36.025	45.339	268.051
LSD (P≤0.05)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
$\bar{X}$±SE	53.88 ± 12.98	56.51 ± 14.09	39.62 ± 11.76	43.55 ± 11.08	36.81 ± 9.23	46.68 ± 7.44	277.05 ± 43.61
CI 95%	26.36 – 86.39	26.63 – 86.38	14.68 – 64.55	20.06 – 67.04	17.24 – 56.38	30.91 – 62.45	184.59 – 369.50

n.s: not significant. (**So**: normal sucrose concentration - 30 gl^{-1} in MS medium; **S_1**: sucrose concentration - 15 gl^{-1} in MS medium; **S_2**: sucrose concentration - 45 gl^{-1} in MS medium).
CI : Confidence Interval

Table (6) represents the mean concentrations of the six alkaloids in ***Datura innoxia*** calli, cultured in MS medium at different salt concentrations (**Xo, X_1** and **X_2**). In general the effect of growing ***Datura*** leafexplants at different salt strength on alkaloid concentrations and their totals especially at **X_1** treatment followed the similar trend of sucrose concentrations. While reducing the salt strength into half (**X_1**) caused non-significant increased in alkaloid concentrations as compared to the effects of double strength (**X_2**) and normal salt strength (**Xo**).

It seems possible that despite reducing the salt strength into half (**X_1**) it might be explained that at this salt concentration, the majority of the microelements were found to enhance the activity of many enzymes within explant cells (Liptay and Arevalo 2000). In this regard, several studies have indicated that the treatment (**X_1**) could have been considered by the callus cells as the suitable amounts of mineral nutrition which enhanced the hyoscyamine, scopolamine, and other alkaloids synthesis in ***Datura*** as a result of influencing the formation of amino acids that are converting to the drug components (Pinol *et al.*, 1999, Demeyer and Dejaegere 1993).

These findings are similar to those researches conducted previously by (Knobloch and Berlin, 1981; Yamakawa *et al*., 1983; Lindsey, 1985; Collin, 1987 and Oksman-Caldentey *et al*., 1994), which demonstrated unequivocally that a depletion or deficiency of N and/or phosphate is associated with growth limitation and a concomitant increase in the level of secondary metabolism. Mantell and Smith (1983) concluded that lack of phosphate more than any other nutrient stimulates secondary-metabolite biosynthesis. Ruži´c *et al*. (2000) found that fresh and dry weight of the explants of Sweet cherry rootstock Gisela 5 when micropropagated on Murashige and Skoog **(MS)** medium, on MS medium containing double-strength macro salts (MS 2X), 1/2 strength (MS 1/2) and 1/4 strength (MS 1/4) with 4.4 μM BA, 0.5 μM NAA, and 0.3 μMGA3 increased during subculturing. However, in their experiment (Ruži´c *et al*., 2000), investigated the effect of the concentration of mineral elements in the medium on plant growth and found that it is closely related to the uptake of mineral elements from the medium and to the culture growth. These findings were in harmony with those reported by Mazrou (1985), on ***Atropa belladonna*** and Mazrou and Al-Humaid (2000) on gladioli plants. On the other hand, Al-Humaid (2004) found that total alkaloid and drug (hyoscyamine and scopolamine) contents also increased with increasing the fertilization level to a peak value at 600 kg / ha, but he found the harmful effect of the extreme amounts of fertilization (800 kg ha^{-1}) on root growth and branching, which then, decreased at 800 kg / ha level., which lowered their ability to adsorb the nutrients

sufficiently. This may help us to explain the deleterious effects of double strength salt treatment on decreasing the alkaloid content of ***Datura innoxia*** calli in the present study. However, (**X_2**) treatment gave mixing results. It has been reported that high ionic media strength has growth inhibitory effects on several woody species (McCown and Sellmer, 1987).

Regarding other plant species and cultivars, Ruži´c *et al*. (2000) stated that mineral composition of many media which is deficient in or in excess of some macroelements, resulted in abnormal growth, vitrification and other undesirable effects.

Table 6. Means of the main alkaloids concentrations (µg/g DW) identified by (HPLC) of ***Datura innoxia*** **Mill** calli at different salt concentrations of MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}).

Concentrations of Alkaloids (µg/g DW)							
Treatments	Atropine	Hyoscyamine	Scopolamine	Tropine	7-Hydroxy-hyoscyamine	Tiglohyosc-yamine	Total
Xo	47.401	46.299	22.255	22.434	26.44	43.014	207.843
X_1	71.669	78.671	62.123	60.726	59.013	61.577	393.779
X_2	42.571	44.348	36.137	38.501	24.97	46.554	233.081
LSD ($P \leq 0.05$)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s	n.s.
$\bar{X} \pm SE$	53.88 ± 14.74	56.44 ± 17.79	40.17 ± 13.78	40.55 ± 12.98	36.81 ± 10.28	50.38 ± 11.16	278.23 ± 58.23
CI 95%	22.63 – 85.13	18.73 – 94.15	10.95 – 69.38	13.03 – 68.07	15.02 – 58.60	26.72 – 74.04	154.78 – 401.67

n.s: not significant, (**Xo**: normal salt concentration in MS medium; **X_1**: half - salt strength in MS medium, **X_2**: double - salt strength in MS medium).
CI : Confidence Interval.

Table (7) represents the mean concentrations of the six alkaloids in ***Datura innoxia*** calli, cultured in MS medium under different light intensities (**LI_1, LI_2** and **LI_3**). It generally showed that the effect of growing ***Datura*** leafexplants under different light intensity conditions on the first four alkaloid concentrations and their totals were found to be increased but not significantly when the means of **LI_1**(1000 lux) were compared to those of **LI_2** (2000 lux) and **LI_3** (3000 lux)

treatments. However, **LI_2** significantly decreased the concentrations (28.250 and 22.155 µg/g DW) of **both** alkaloids, 7-hydroxyhyoscyamine and tiglohyoscyamine respectively when

compared to mean concentrations of the same alkaloids under **LI_1** (61.04 and 56.452 µg/g DW) and **LI_3** (50.472 and 31.816 µg/g DW) respectively (table 7). Concerning the totals of the six alkaloids, generally they followed the same trend of the previous treatments of sucrose and salt strength. It is clear from the table (**7**) that the total alkaloids concentrations under **LI_1** was higher (387.307) µg/g DW compared to **LI_2** (185.450) µg/g DW and **LI_3** (257.221) µg/g DW (Figure 39 a and b). In general the effects of different light intensities on plants growth characters either ***in vivo*** or ***in vitro*** have been studied in the literature with contradicting results. Light is known to affect the production of several secondary metabolites (Mantell and Smith, 1983 and Towers and Yamamoto, 1985). Contradicting effects of light on the production of secondary metabolites have been reported; for example, the inhibition of nicotine accumulation in tobacco cell cultures (Ohta and Yatazawa, 1978 and Hobbs and Yeoman, 1991), and the stimulation of anthocyanine production by ***Haplopappus*** cultures (Stick-land and Sunderland, 1972*)* and of betalains by cell cultures of ***Chenopodium rubrum*** (Berlin *et al*., 1986).

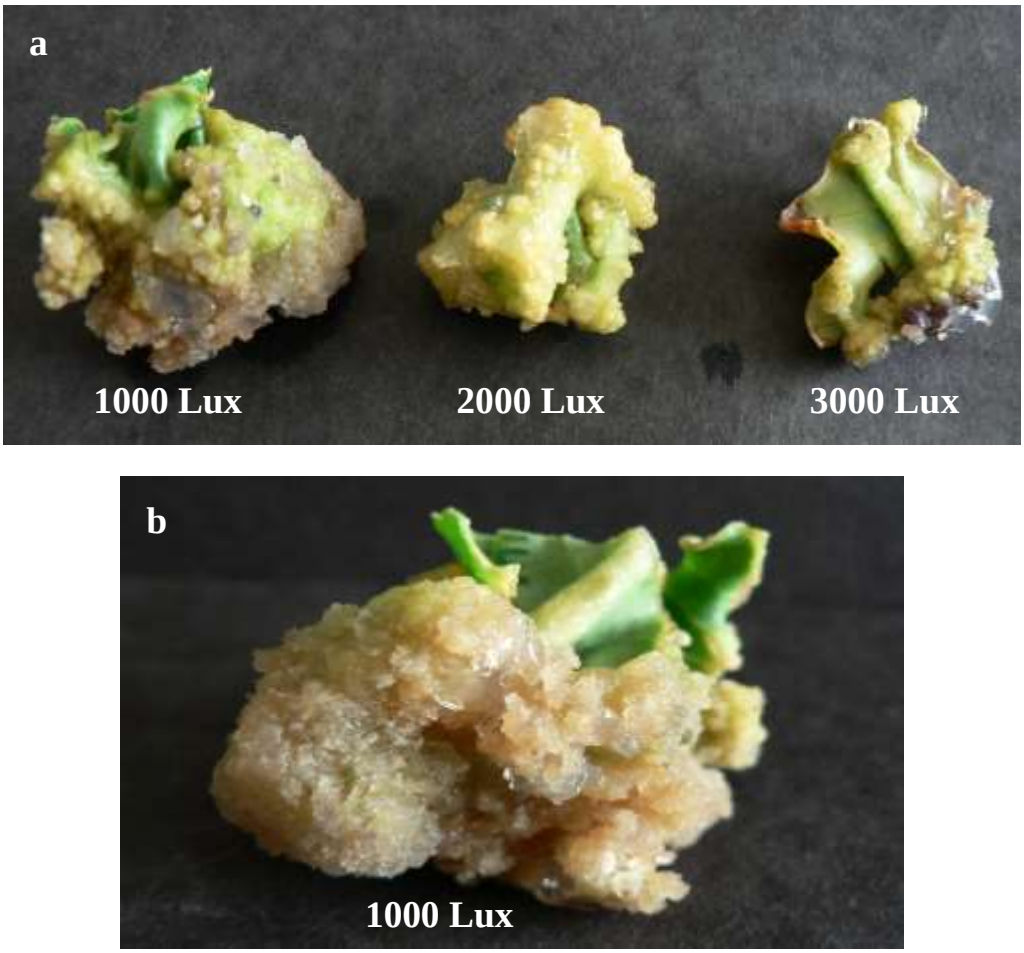


Figure 39 a and b. a) The effect of different light intensity treatments (**LI_1, LI_2 and LI_3**) on growth and development of ***Datura innoxia*** **Mill** calli. **b)** The effect of **LI_1** treatment on growth and development of ***Datura innoxia*** **Mill** calli.

Table 7. Means of the main alkaloids concentrations (µg/g DW) identified by (HPLC) of ***Datura innoxia* Mill** calli under different light intensities of MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}).

Concentrations of Alkaloids (µg/g DW)							
Treatments	Atropine	Hyoscyamine	Scopolamine	Tropine	7-Hydroxy-hyoscyamine	Tiglohyosc-yamine	Total
LI_1	75.733	71.295	59.117	63.669	61.041 a	56.452 a	387.307
LI_2	42.214	48.658	20.57	23.605	28.25 b	22.155 b	185.45
LI_3	43.694	47.143	40.828	43.387	50.472 a	31.816 a	257.221
	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
LSD (P≤0.05)					24.697	23.383	
$\bar{X}$±SE	53.88 ± 14.76	55.69 ± 17.74	40.17 ± 11.66	43.55 ± 11.18	46.58 ± 8.24	36.81 ± 7.80	276.66 ± 59.07
CI 95%	22.59 – 85.17	18.08 – 93.30	15.45 – 64.89	19.85 – 67.25	29.11 – 64.05	26.27 – 53.34	151.43 – 401.88

n.s: not significant; (**LI**: Light intensity;**LI_1**: 1000 lux; **LI_2**:2000 lux; **LI_3**: 3000 lux).

***** Significant at (P≤0.05) level

CI : Confidence Interval.

Moreover, Pretto and Santarem (2000) reported that tissues of ***Hypericum perforatum*** cultured in the presence of light have increased the activity of IAA-Oxidase which altered the endogenous balance between auxin/cytokinin and consequently decreasing the callus growth and affecting the content of secondary metabolites. However, Dong *et al.* (2006) tested different light intensities to determine the optimal intensity for shoot organogenesis from guayule leaf strips. The rate of organogenesis significantly improved when the light intensity dropped to 12µmol m^{-2} s^{-1}**;** both the number of explants producing shoots and the total shoots produced were doubled. Lowering of the light intensity appeared to enhance shoot production, down to 1.5 µmol m^{-2} s^{-1}, although the differences were not statistically significant. Dark grown leaf strips had less brown sectors than those grown under low light, but they produced fewer shoots.

Our results are in agreement with those of Soontornchainaksaeng, *et al*. (2001) who found that plantlets of ***V. coerulea***, as well as ***P. tankervilliae***, grown under light intensity of 56 µmol

m^{-2} s^{-1} grew less than those grown under 37 and 74 µmol m^{-2} s^{-1}. On the other hand, they showed in their study that light intensity plays a significant role not only on dry weight accumulation but also on plant height, leaf number, leaf shape and leaf area. The best results were found at 74 µmol m^{-2} s^{-1}. In moderate light intensity, plants generally bear longer internodes, and are less tough and more succulent with larger leaves than those grown in intense light (Barber and Anderson, 1992). Likewise, in papaya, the root formation of shoots or embryoids derived from callus or shoot tips occurred at light intensities of 3,000 to 4,000 lux (Yie and Liaw, 1997). Plant growth is related to the function of growth hormones like auxin, which is sensitive to high light intensity. Cytokinins act in concert with auxin to cause cell division in plant tissue culture (Soontornchainaksaeng *et al.*, 2001).

Generally, plant growth and development are affected by both internal factors including genotype and plant hormones and external factors such as light, temperature and moisture supply. This result may be due to the interaction between light intensity and internal factors which directly affect plant growth. The suitable light intensity and duration will give the best result of product. Stronger light intensity costs more in terms of energy input. Consequently, the results suggest that light intensity of 37 µmol m^{-2} s^{-1} was sufficient to culture both ***V. coerulea*** and ***P. tankervilliae*** economically. Similarly our results also showed that the treatment of **LI_2** had deleterious effects in decreasing the concentration of different alkaloids particularly 7-hydroxyhyoscyamine and tiglohyoscyamine in calli of ***Datura innoxia*** **Mill** cultured in MS media treated with different sucrose and salt concentrations, supplemented with 0.6 mgl^{-1} BA + 1.0 mgl^{-1} NAA. In other words it is apparent from table (8) that the normal light intensity, 1000 lux gave the best results although were not significant in first four alkaloids but were significant in the last two alkaloids. Consequently, the results suggest that light intensity of 1000 lux was sufficient to culture Datura leafexplants economically. Aoshima and Takemoto (2006) reported that light is the ultimate substrate for photosynthetic energy conversion; it can also harm the plants. Higher light intensity causes photo oxidation which involves the destruction of chlorophyll, resulting in less biomass production. High light intensity is causing the damage of the water-splitting photosystem II (PSII), leading to degradation of the reaction center. The frequency of this damage is relatively high when light intensity is increased, especially when combined with other environmental factors. There is an exception for lily plants in which no photoinhibition or damage to PSII was observed in the critical condition of strong light and high temperature in the culturing season (Sorrentino et al., 1997). Moreover, ***Scindapsus*** yielded

plants that were more vigorous when the light intensity of the pretransplant stage was either 3,000 or 10,000 lux, whereas ***Cordyline*** and ***Dracaena*** showed progressive increases in the vigor of plants with increasing light intensity up to 10,000 lux (Miller and Murashige, 1976).

Table (8) shows the comparison differences in alkaloid concentrations (µg/g DW) between leaves from greenhouse plants and callus of ***Datura innoxia*** grown at different sucrose and salt concentrations under different light intensities.

The results of the table (8) showed that in general the alkaloids production *in vitro* compared to that *in vivo* which was insignificantly higher indicated clearly the success of alkaloids production process from *Datura innoxia* Mill calli grown on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}) at different sucrose (S) and salt (X) concentrations under different light intensities (LI). The results showed that concentrations of all alkaloid types (except scopolamine) of ***Datura innoxia*** calli grown under **LI_1** (1000lux) condition, treated with **S_1** (15 gl^{-1} sucrose) and **X_1** (half strength salt) were greater than those obtained from leaves of ***in vivo*** plants.

Table 8. The comparison differences in alkaloid concentrations (µg/g DW) between leaves from greenhouse plants (***in vivo***) and callus of ***Datura innoxia*** **Mill** (***in vitro***) grown on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}) at different sucrose and salt concentrations under different light intensities.

Atropine									
Difference	S_0	S_1	S_2	X_0	X_1	X_2	L_1	L_2	L_3
in vivo _ in vitro	-0.087	-31.135	-14.740	-8.841	-33.109	-4.011	-37.173	-3.655	-5.133
$t_{Cal.}$	-0.008	-0.835	-0.386	-0.306	-0.766	-0.249	-0.879	-0.136	-0.286
$t_{0.05}(10)=$	2.228								
Hyoscyamine									
Difference	S_0	S_1	S_2	X_0	X_1	X_2	L_1	L_2	L_3
in vivo _ in vitro	5.047	-39.851	26.072	-6.189	-38.561	-2.915	-31.185	-7.002	-9.478
$t_{Cal.}$	0.684	-0.820	0.489	-0.181	-0.869	-0.104	-0.911	-0.206	-0.337
$t_{0.05}(10)=$	2.228								

Table (8) continued.

Scopolamine									
Difference	S_0	S_1	S_2	X_0	X_1	X_2	L_1	L_2	L_3
in vivo _ in vitro	14.664	-5.866	7.516	23.369	-16.519	9.465	-13.514	25.051	4.778
$t_{Cal.}$	1.036	-0.132	0.209	1.994	-0.370	0.568	-0.297	2.508	0.281
$t_{0.05}(10)=$	2.228								
Tropine									
Difference	S_0	S_1	S_2	X_0	X_1	X_2	L_1	L_2	L_3
in vivo _ in vitro	9.881	-19.331	-3.049	16.953	-30.339	0.887	-24.281	15.782	-3.999
$t_{Cal.}$	1.050	-0.423	-0.124	1.490	-0.696	0.049	-0.541	1.162	-0.210
$t_{0.05}(10)=$	2.228								

Table (8) continued.

7-Hydroxyhyoscyamine									
Difference	S_0	S_1	S_2	X_0	X_1	X_2	L_1	L_2	L_3
in vivo _ in vitro	-2.466	-14.924	-7.522	2.064	-30.509	3.533	-27.948	6.348	-3.312
$t_{Cal.}$	-0.251	-0.450	-0.305	0.103	-1.045	0.265	- 0.505	0.501	-0.240
$t_{0.05}(10)=$	2.228								
Tiglohyoscyamine									
Difference	S_0	S_1	S_2	X_0	X_1	X_2	L_1	L_2	L_3
in vivo _ in vitro	-17.906	-29.977	-21.966	-8.462	-38.204	-23.184	-37.601	-4.879	-27.369
$t_{Cal.}$	-1.279	-1.110	-0.999	- 0.484	- 0.460	- 0.479	-1.770	-0.366	-1.216
$t_{0.05}(10)=$	2.228								

The (-) sign indicates that the mean difference is in favor of increased alkaloid concentration of ***in vitro*** over ***in vivo*** treatment.
The (+) sign indicates that the mean difference is in favor of dominated alkaloid concentration of ***in vivo*** over ***in vitro*** treatment.

However, in case of scopolamine it was clear that the treatment **LI_2** (2000 lux) significantly decreased the concentration of scopolamine by 25.051 (µg/g DW) as compared with those of calli from leaves of greenhouse plants . Consequently, the present results indicate that growing leafexplants of ***Datura innoxia*** in MS media at different sucrose, salt concentrations under light intensity of (1000 lux) might be regarded as the best growing MS media to give higher concentrations of all alkaloids apart from scopolamine. Morini *et al*. (2000) suggested the involvement of the blue-absorbing photoreceptor system in the callus production from leaves of quince clone BA 29. For root regeneration, phytochrome seemed to be the only photoreceptor involved. Alkaloid content and production of both ***Datura metel*** and ***Datura stramonium*** are also influenced by the amount of radiation the plants received (Cosson, 1969 and Trease and Evans, 1978). Our results are in disagreement with those of Demeyer and Dejaegere (1997) who found that when plants of ***Datura stramonium*** **var. tatula L.** Torr were grown on two different mineral media, varying only in the relative proportions between two cations (Ca^{2+}/K^{+} balance) under different light conditions, those grown under higher light energy regime, had more alkaloids accumulated in the plants during a certain stage of development. The observed effect of Ca^{2+}/K^{+} balance on alkaloid content in the leaves was affected by the different light regimes. They also concluded that Ca^{2+}/K^{+} balance exerts an effect on alkaloid concentration. This influence is, however, only noticeable during a certain stage of the plant development, while the intensity of it is affected by the amounts of light energy received by the plants. Cosson (1969) and Trease and Evans (1978) also stated that in addition to the mineral composition, alkaloid content and production are also influenced by the radiation the plants received.

Kleiber and Mohr (1967) observed that light stimulated TE **(** tracheary elements which are useful experimental systems to study the biosynthesis of secondary cell walls ***in vitro*** **)** differentiation in hypocotyls of ***Sinapis alba*** **L**. (mustard) seedlings**.** Mizuno *et al*. (1971) found that light was necessary to induce TE differentiation in ***Daucus carota*** L.(carrot) root explants and Fosket (1968) reported that light increased TE differentiation in cultured stem segments of ***Coleus blumei Benth*** (coleus). Light has been found to increase the proportion of tracheary elements differentiating in callus cultures derived from xylem-parenchyma of ***Pinus radiata D***.

grown on an induction medium containing activated charcoal without phytohormones. The differentiation rate increased from 20% when callus was grown in darkness to 45% when callus was grown under 16 h or 24 h photoperiod (Möller, 2006).

In case of general viewing the data shown in table (8), one may conclude that when checking the total concentrations of each alkaloid type within all treatments applied in the study, the alkaloid types could be arranged in the following order starting from greater concentration (µg/g DW): Tiglohyoscyamine – Atropine – Hyoscyamine – 7-hydroxyhyoscyamine – Scopolamine – Tropine. Consequently, it appears from the above data that the Tiglohyoscyamine was the first major alkaloid in the callus of ***Datura innoxia* Mill.** However, Berkov and Zayed (2004) found that scopine was main alkaloid in the leaves of the Egyptian ***Datura*** plants whereas hyoscyamine was main compound in the leaves of Bulgarian ***Datura*** plants. Moreover, scopolamine was main compound in the fruit alkaloid fractions of both Egyptian and Bulgarian ***Datura*** plants. As a comparison, Witte *et al*. (1987) reported 3, 6-ditigloyloxytropane and scopolamine as main alkaloids in the roots and leaves, respectively, of ***D. innoxia*** plants grown in Germany.

Our results as well as the results of Witte *et al*. (1987**)** and Berkov and Zayed (2004) clearly showed that the alkaloid spectra and accumulation in ***Datura innoxia*** are strongly influenced by the environmental factors.

Conclusions

1. Of the various pretreatments used for sterilization of leafexplants of ***Datura innoxia* Mill,** the treatment ethanol %70 (2 min.) + NaOCl % 2.0 (2 min.) gave the maximum survival percentage (97%) (Figure 15). Sodium hypochlorite (Iranian Buzhna) proved to be the most effective sterilants.
2. The best characters of callus growth studied (frequency of callus formation (%), dry matter (DM) %, dry weight (g), and fresh weight (g), were obtained from the combination of cytokinin (BA, 0.6 mgl^{-1}) / auxin (NAA, 1.0 mgl^{-1}) at 2^{nd} subculture, and the highest number of shoots induced/leaf explant(46.167) was obtained from the combination of cytokinin (BA at 2.0 mgl^{-1}) in combination with auxin (NAA at 0.6 mgl^{-1}) at sixth subculture.
3. Organogenesis *in vitro* culture of *Datura* callus responded very well to the plant growth regulators (auxins and cytokinin).
4. Supplementation of MS medium with cytokinin (BA at 0.6 mgl^{-1}) in combination with auxin (NAA at 1.0 mgl^{-1}) was the best treatment for callus production from leaf explants of ***Datura innoxia* Mill** as compared with that of 2,4-D.
5. Maximum growth of the callus as expressed on the basis of dry weight, 1.357g was observed at the end of the 2^{nd} subculture (8^{th} week). However, in the rest of their growth passages i.e. from 3^{rd} to the 12^{th} subculture it declined thereafter and gave almost stable line with little or no change from 6^{th} to the last subculture.
6. In general the alkaloids production *in vitro* compared to that *in vivo* which was insignificantly higher indicated clearly the success of alkaloids production process from *Datura innoxia* Mill calli grown on MS medium supplemented with BA (0.6 mgl^{-1}) + NAA (1.0 mgl^{-1}) at different sucrose (S) and salt (X) concentrations under different light intensities (LI).
7. The effects of growing ***Datura*** leafexplants under different light intensities on the alkaloids (atropine, hyoscyamine, scopolamine and tropine) concentrations and their totals were found to be increased under $\mathbf{LI_1}$ (1000 lux) treatment. However, $\mathbf{LI_2}$ significantly decreased the concentrations of the alkaloids, 7-hydroxyhyoscyamine and tiglohyoscyamine respectively as compared to mean concentrations of the same alkaloids under $\mathbf{LI_1}$ and $\mathbf{LI_3}$.

8. In general when the concentrations of all alkaloid types except that of scopolamine of ***Datura innoxia*** **Mill** calli grown at different sucrose and salt concentrations under different light intensities were compared to their correspondent concentrations of greenhouse plant leaves showed greater concentrations differences under $\mathbf{LI_1}$(1000lux) condition, treated with $\mathbf{S_1}$ (15 gl^{-1} sucrose) and $\mathbf{X_1}$ (half strength salt).

9. Among the six alkaloids measured in the present study, it appeared from the data that tiglohyoscyamine was the first major alkaloid in the callus of ***Datura innoxia*** **Mill** leaf explants.

10. It is clearly showed that the alkaloid spectra and accumulation in ***Datura innoxia*** are strongly influenced by the environmental factors such as light factor in the present study.

Recommendations

1. Production of plants regenerated from ***Datura innoxia*** **Mill** callus and analyzing it's alkaloid contents and comparing them with those of ***in vivo*** plants.
2. Culturing ***Datura*** roots to manipulate the alkaloids production.
3. Analyzing the alkaloids contents by using GC/MS instrument and replicating the samples for ***Datura*** for more identifications**.**
4. Micro propagation of different species of ***Datura*** cultivated in the area or wild grown *Datura* to compare their alkaloids contents.
5. Possibility of selecting particular type of alkaloid content of ***Datura*** via micro propagation technique for future drug manufacturing companies in Iraqi Kurdistan Region.
6. Micropropation of some rare or an endangered plant species in Iraqi Kurdistan Region.

Aissa, F. B. 1991. Les Plantes Mèdicinales en Algère, Coèdition bouchène et Ad. Diwan, Alger, p.159.

AL-Abdalli, S. A. 1975. ***Datura*** plant. Ministry of Agriculture and Agrarian Reform,Plant Department. Publication No. **232**.(In Arabic).

Al-Humaid, A. L. 2004. Effects of Compound Fertilization on Growth and Alkaloids of Datura Plants. J. Plant Nut., **27**(12): 2203–2219.

AL-Khalidi, M. S. S. 2005. Production and purification of some alkaloids from ***Datura*** plant using tissue culture technique. A PhD Thesis in Biotechnology, submitted to the College of Sci. – University of Baghdad (In Arabic).

Al-Mukhtar, K. A., S. M. Al-llaf and A. A. Al-Attar. 1982. Microscopic preparations. Iraq: Ministry of Higher Education and Scientific Research and fulminant hepatitius. *J. Emerg. Med.* **28**: 227-228.

Arditti, J. and R. Ernst. 1992. Micropropagation of orchids. John Wiley & Sons Inc, USA.

Arroo, R. R. J.,A. Develi,H. Meijers, E. Vandewesterlo, A. K. Kemp, A. F. Croes, and G. J. Wullems. 1995. Effect of exogenous auxin on root morphology and secondary metabolism in ***Tagetes patula*** hairy root cultures, *Physio.l Plant* **93**: 233-240.

Arroo, R., Woolley J., and Oksman-Caldentey .M., (2007). Henbane, Belladonna, ***Datura*** and ***Duboisia***. *Biot. in Agric. and For.*. **61**: 189-204.

Arvy, M. P., N. Imbault, F. Naudascher,M. Thiersault and P. Doireau. 1994. 2,4-D and alkaloid accumulation in periwinkle cell suspensions. *Biochimie.* **76**: 410–416.

Asano, N.,R. J. Nash, Molyneux and G. W. Fleet. 2000. Sugar-mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. Tetrahedron: *Asymmetry*. (**11**): 1645-1680.

Attfield, E.M. and P.K. Evans. 1991. Stages in the inhibition of root and shoot organogenesis in cultured leaf explants of ***Nicotiana tabacum*** cv. **Xant**h, *J. Exp. Bot.* **42**: 59-63.

Balandrin, M. J. and J.A. Klocke. 1988. Medicinal, aromatic and industrial materials from plants. In: Bajaj YPJ, editor. Biotechnology in agriculture and forestry. *Medicinal and aromatic plant***, 4**: 1–36.

Barber, J. and B. Andersson. 1992. Too much of a good thing: light can be bad for photosynthesis. *Trends Biochem Sci.* **17**: 61–66.

Baran J. T. and B. Ghosha. 2005. Plant Tissue Culture: Basic and Applied University Press (India) Private Limited, Hyderabad, 206 pages.

Berkov, S. and R. Zayed. 2004. Comparison of tropane alkaloid spectra between ***Datura innoxia*** plants grown in Egypt and Bulgaria. *Z. Naturforsch*. **59c**: 184-186.

Berkov, S., A. Pavlov, P. Kovatcheva, P. Stanimirova and S. Philipov. 2003. Alkaloid spectrum in diploid and tetraploid hairy root culyures of ***Datura stramonium***. *Z. Naturforsch*. **58c**: 42-46.

Berlin, J., S. Slag, D. Strack, M. Bokem and H. Harms. 1986. Production of betalains by suspension cultures of ***Chenopodium rubrum* L**. *Plant Cel., Tis. and Org. Cult.*, **5**: 163-174.

Bernier, G. 1988. The control of floral evocation and morphogenesis. *Annu. Rev. Plant Physiol. Plant. Mol. Biol*. **39**:175–219.

Bhalsingh S. B. and V.L. Maheshwari. 1998. Plant Tissue Culture-A Potential Source of Medicinal Compounds. *J. Sci. Indus.t Res.* **57**:703-708.

Bhandary, S. B. R, H. A. Collin, Thomas E, H. E. Street. 1969. Root, callus, and cell suspension cultures, from ***Atropa belladonna,* L.** and ***Atropa belladonna***, cultivar **lutea D ll.** *Ann. Bot*. **33**: 647-656.

Bhatt P. N., D. P. Bhatt and I. Susswx. 1983. Studies on some factors affecting solasodine contents in tissue cultures of ***Solanum nigrum***. *Physiol. Plant.* 57: 159-162.

Boyd, M. G. 1991. Studies on carotenoid production in cultures of ***Bixa orellana***. Ph.D. Thesis, University of Edinburgh, UK.

Brown, K.W., J. C. Thomas and R. L. Duble. 1982. Nitrogen source effect on nitrate and ammonium leaching and runoff losses from greens. *Agron. J*. **74**: 947–950.

Cappuecino, J. and N. Sherman. 1987. Microbiology. A Laboratory Manual. 2nd Edition. Benjamin/Cummings Publishing, *Inc. California.*: 153-171.

Chakravarty, H. L.1976. Plant Wealth of Iraq. A Dictionary of Economic Plants Vol.**1**, *Botany Directorate*. Ministry of Agricultural and Agarian Reform. Iraq.

Cheeke, P. R., and R. L. Shull. 1985. Natural toxicants in feeds and poisonous plants. AVI Publishing Company, *Inc., Westport, Conn., USA*. 492 pp.

Chopra, R. N., S. L. Nayar and I. C. Chopra. 1986. *Glossary of Indian Medicinal Plants (Including the Supplement).* Council of Scientific and Industrial Research, New Delhi.

Christen, P., T. Aoki, and K. Shimomura. 1992. Characteristics of growth and tropane alkaloid production in ***Hyoscyamus albus*** hairy roots transformed with ***Agrobacterium rhizogenes* A4**. *Plant Cell Rep*. **11**: 597–600.

Christianson, M.L. and D. A. Warnich. 1983. Competence and determination in the process of ***in vitro*** shoot organogenesis, *Dev Biol*. **95**: 288-293.

Collin, H. A. 1987. Determinants of yield of secondary products in plant tissue cultures. *Adv. in Bot. Res.* **113**: 146-187.

Cormier, P., H. A. Crevier and C. B. Do. 1990. Effects of sucrose concentration on the accumulation of anthocyanins in grape ***(Vitis vinifera)*** cell suspension. *Can. J. of Bot*. **68**:1822-1825.

Constabel, F., Gaudet-LaPrairie, P., Kurz, W.G.W., and Kutney, J.P. 1982. Alkaloid production in *Catharanthus roseus* cell cultures. XII. Biosynthetic capacity of callus from original explants and regenerated shoots. Plant Cell Rep. **1**:139-142.

Cosson, L. 1969. Influence de l'éclairement sur les variations ontogéniques des teneurs en scopolamine et en hyoscyamine des feuilles de ***Datura metel***. *Phytochemistry*. **8**: 2227–2233.

Cosson, L., A. Escudero-Morales and N. Cougoul. 1978. La regulation écophysiologiques du métabolisme des alcaloides tropaniques (hyoscyamine et scopolamine). *Plantes Médicinales et Phytothérapie.* **XII**: 319–326.

Datta, A. and P. S. Srivastava. 1997. Variation in vinblastine production by ***Catharanthus roseus*** during ***in vivo*** and ***in vitro*** differentiation. *Phytochemistry*. **46**: 135–137.

Datta, S. C. 1994. Plant Physiology. An Introduction to Gymnosperms. *Wiley Eastern Inc, India.*

Davies, M. E. 1972. Polyphenol synthesis in cell suspension cultures of Paul's scarlet rose. *Planta.* **104**: 50-65.

De, B. 2003. Steroidal compounds from ***in vitro*** regeneration shoots of ***Datura metel.*** *Fitoterapia*. **74**: 14-17.

Deinum**,** B, R, D., M. I. Sulastri, H. J. Zeinab and A. Maassen. 1996. Effects of light intensity on growth, anatomy and forage quality of two tropical grasses (***Brachiaria brizantha*** and ***Panicum maximum*** var ***tricoglume*****)**. *Netherlands J Agri. Sci.* **44**(2): 111-24.

Demeye, K. and R. Dejaegere. 1997. Nitrogen and alkaloid accumulation and partitioning in ***Datura stramonium***. *J. Her., Spe. & Med. Pl. , Vol.* **5**, Issue 3:15-23.

Demeyer, K. and R. Dejaegere. 1989. Influence of the ion-balance in the growth medium on the yield and alkaloid content of ***Datura stramonium*** L. *Plant and Soil* **114**: 289–294.

Demeyer, K. and R. Dejaegere. 1993. Influence of nitrogen on the alkaloid content of ***Datura stramonium***. Acta Hort., **331**: 213–220.

Demeyer, K. and R. Dejaegere. 1995. Total mineral dose and pH of the culture medium affect alkaloid accumulation in ***Daruta stramonium*** L. J. Herbs, Spices, Medicinal Plants **3**(3): 35-44.

Demeyer, K. and R. Dejaegere. 1996. Alkaloid content and partitioning in ***Datura stramonium* L.** are influenced by the Ca^{2+}/K^{+}- balance and by the light energy received by the plants. Austr. J. Bot. **45**(1): 261-267.

Demeyer, K. and R. Dejaegere. 1997. The Influence of the Ca2+/K+ Balance and Light Energy on Alkaloid Content and Partitioning in ***Datura stramonium***. *Aust. J. Bot.*: 45:81–101.

Dessouky, M. M., H.S. Taha and M. K. El-Bahr. 2001. Enhancement of alkaloids production in suspension cultures of ***Datura stramonium* L**. and *Datura metel* L. Arab. J. Biotech., **4**(2): 271-2.

Dicosmo, F. and M. Misawa. 1995. Plant Cell and tissue culture: Alternatives for metabolite production. *Biotechnol. Adv.* **13**(3): 425-453.

Djibo, A, S. B. Bouzou. 2000. [Acute intoxication with "sobi-lobi" (***Datura***). Four cases in Niger]. *Bull Soc Pathol. Exot*. **93**: 294–297.

Djilani, A., B. Djilani, R. Soulimani, A. Dicko and C. Younos. 2006. New extraction technique for alkaloids. *J. Braz. Chem. Soc.* Print ISSN 0103-5053, Vol.,**17**, No.3, 16 pages.

Do, C. B. and F. Cormier. 1991. Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (***Vitis vinifera***) cell suspension. *Plant Cell Rep.* **9**: 500-504.

Do, C.B., and F. Cormier. 1990. Accumulation of anthocyanins enhanced by a high osmotic potential in grape (***Vitis vinifera*)** cell suspension. *Plant Cell Rep.* **9**: 143-146.

Dörnenburg, H. and D. Knorr. 1996. Generation of colors and flavors in plant cell and tissue cultures. Critical Reviews in Plant Sciences, **15**(2): 141-168.

Dong, W. K., J. H. Feng, and X. Q. Yang. 2006. Z. Kristallogr. *New Cryst. Struct*. **221**: 447–448.

Dos Santos, M. C. F., M. A. Esquibel and A. V. P. Dos Santos. 1990. In vitro propagation of the alkaloid producing plant ***Datura insignis*** *Barb. Rodr.* J. Plant Cell, Tiss. And Org. Cult., **21**(1): 75-8.

Dougall, D. K. 1980. Nutrition and metabolism. In : Staba EJ, ed. *Plant Tissue Cultures as a Source of Biochemicals.* Boca Raton, FL. USA : CRC Press,: 21-58.

Dräger, B., C. Funck, A. Hoehler, G. Mrachatz, A. Nahrstedt, A. Portsteffen, A. Schaal, and R. Schmidt. 1994. Calystegines as a new group of tropanealkaloids in ***Solanaceae***. *Plant Cell Tiss. Organ Cult. Vol. **38.***: 235.240.

Dräger, B., V.A. Almsick and G. Mrachatz. 1995. Distribution of calysteginesin several Solanaceae. *Planta Med. Vol. **61***: 577-579.

El-Bahr, M. K., M. S. Hussein and H. A. Moursy. 1989. Effect of some growth regulators on the growth and alkaloid production of ***Datura stramonium*** L. culture *in vitro*. *Egypt. J. Bot., **32***: 53-62.

El-Rahman, R. A., El-Wkil H. El-Din, Abou Gabal, A. El-Said and H. D. Khlifa. 2008. Production of Scopolamine and Hyoscyamine in calli and regenerate cultures of ***Datura metel (*** **L**.*)*. J. Appl. Sci. Res., **4**(12): 1858-1866.

Emboden. W. 1979. Narcotic Plants *Studio Vista . ISBN 0-289-70864-8* Engineering tropane biosynthetic pathway in ***Hyoscyamus niger*** hairy root cultures. Proc. *Natl. Acad. Sci. USA Vol. **101***: 6786-6791.

Emma, M. J., A. Sevdalina and A. C. J. Verkleij. 2005. Callus induction and plant regeneration in the metallophyte ***Silene vulgaris*** (***Caryophyllaceae***). *Plant Cell Tiss. Org. Cult., **80***: 25-31.

Endo, T. and Y. Yamada. 1985. Alkaloid production in cultured roots of three species of Duboisia. *Phytochem.*, **24**: 1233-1236.

Ertekin, V., M. A. Selimoglu and S. Altinkaynak. 2005. A combination of unusual presentations of ***Datura stramonium*** intoxication in a child: Rhabdomyolysis

Evans, W. C. 1989. Alkaloids In: Evans WC(ed). *Pharmac.* (13rd Edition): 544-636. Baillièr Tidal, London.

Evans, W. C. 2001. Plant cell and tissue culture; biochemical conversion; clonal propagation In *Pharmacognosy* Evans, W.C (ed.). 14th edition. W.B. Saunders Company Ltd. U.K.: 76-86.

Ferreira, J. F. S. and J. Janick. 1996. Roots as an enhancing factor for the production of artemisinin in shoot cultures of ***Artemisia annua***. Plant Cell Tiss. Org. Cult. **44**: 211–217.

Figueiredo, S. F., and M. A. Esquibel. 1991. Organogenesis and micropropagation of ***Datura insignis*** Barb. *Rodr. R. Bras. Fisiol. Veg.* **3**(2): 63-68.

Flores, H.E. and P. Filner. 1989. In "Primary and Secondary Metabolism of Plant Cell Cultures" Ed. Kurz, W.G.W. : 174-185. Springer-Verlag, Berlin, Heidelberg.

Fosket, D. E. 1968. Cell division and the differentiation of wound-vessel members in cultured stem segments of coleus. *PNAS **59***: 1089–1096.

Fransworth, N .R., O. Akerele and A. S. Bingel. 1985. Medicinal plants in therapy. *Bulletin of the WHO,* **63**: 965-981.

Fujita, Y., Y. Hara, C. Suga and T. Morimoto. 1981. Production of shikonin derivatives by cell suspension cultures of ***Lithospermum erythrorhizon*** II. A new medium for the production of shikonin derivatives, Plant Cell Rep., **1**: 61–63.

Furze, J. M, M. J. C. Rhodes, A. J. Parr, R. J. Robins, I. M. Whitehead and D. R. Threlfall. 1991. Abiotic factors elicit sesquiterpenoid phytoalexin production but not alkaloid production in transformed roots of ***Datura stramonium***, Plant Cell Rep. 10: 111–114.

Goddijn, O. J. M., R. J. Dekam, A. Zanetti, R. A. Schilperoort and H. C. Hoge. 1992. Auxin rapidly down regulates transcription of the tryptophan decarboxilase gene from ***Catharanthus roseus***. *Plant Mol Biol* ***18***: 1113-1120.

Griffin, W. and D. Lin. 2000. Chemo taxonomy and geographical distribution of tropane alkaloids. *Phytochem.*, **53**: 623-637.

Häkkinen, S. T., E. Moyano, M. R. Cusido, J. Palazon, M. T. Pinol and K. M. Oksman-Caldentey. 2005. Enhanced secretion of tropane alkaloids in ***Nicotiana tabacum*** hairy roots expressing heterologous hyoscyamine-6ß-hydroxylase.J. Exp. Bot., Vol. **56**, No.420: 2611-2618.

Hanks, R. J. and G. L. Ashcroft. 1980. Applies soil physics. Soil water and temperature a pplications. Springer-Verlag Berlinberg.

Hashimoto, T. and Y. Yamada. 1986. Hyoscyamine 6b-hydroxylase, a 2- oxoglutarate-dependent dioxygenase, in alkaloid producing root cultures. *Plant Physiol*., **32**: 713-718.

Hashimoto, T., D. J. Yun and Y. Yamada. 1993. Production of tropane alkaloidsin genetically engineered root cultures. Phytochemistry Vol. 32: 713.718.

Hawkes, J.G. (ed.), R. N. Lester, M. Nee and N. Estrada. 1991. ***Solanaceae*** *III – Taxonomy, Chemistry, Evolution (Proceedings of Third International Conference on* ***Solanaceae****). Royal Botanic Gardens, Kew*. : 197–210. ISBN 0947643311.

Hdide, C. and Y. Desjardins. 1994. Effects of sucrose on photosynthesis and phosph *o*enolpyruvate carboxylase activity of ***in vitro*** cultured strawberry plantlets. *Plant Cell Tiss. Org. Cult.,* **36**: 27–33.

Heath Andrew, J. 2002. Antidotes for poisoning by organophosphorous pesticides. Monograph on Atropine. International Programme on Chemical Safety Evaluation. National Poisons Centre, Penang, Malaysia.

Heble, M. R., M. S. Chadha. 1985b. Recent developments in plant biotechnology. In: Biot. in Heal. Car. : 55-64. New Delhi.

Hedge, C. 1984. Global Survey of the Biogeography of Labiatae. 1984. Harley R, Reynolds T (eds) Royal botanical gardens: *Kew*: **7**.

Hiraoka, N, and M. Tabata. 1974. Alkaloid production by plants regenerated from cultured cells of ***Datura innoxia***. *Phytochem*.,**13**: 1671-1675.

Hobbs, M. C. and M. M. Yeoman. 1991. Effect of Light on Alkaloid Accumulation in Cell Cultures of *Nicotiana* species. *Oxford Journals Journal of Experimental Botany,* **42 (11)**: 1371-1378

Huang, N., W. Abraham, H. Kim and E. Leete. 1996. 1-Methylpyrrolidine-2-acetic acid is not aprecursor of tropane alkalids. *Phytochem*. **41**: 767-773.

Humphrey, A. J. and O. D. Hagan. 2001. Tropane alkaloid biosynthesis. A century old problem unresolved. *Nat. Prod. Rep.* Vol. **18**: 494-502.

Illiana, I., L. Witte and A. W. Alfermann. 1989. Production of alkaloid by transformed root cultures of ***Datura innoxia***. *Planta Medica,* Vol. **55**: 229-230.

Ingestad, T. and G. Ågren. 1992. Theories and methods on plant nutrition and growth. *Physiol. Plant*. **84**: 177–184.

Iranbakhsh, A., M. A. Oshaghi and A. Majd. 2006. Distribution of atropine and scopolamine in different organs and stages of development in ***Datura stramonium* l.** (***Solanaceae***). Structure and ultra structure of biosynthesizing cells. *Acta Biologica Cracoviensia Series Botanica,* **48**, 1: 13–18.

Jakubke, H.D., H. Jeschkeit. 1975. Brockhaus Biochemie. Brockhaus-Verlag, Leipzig.

Jiao, J.A. and R. Chollet. 1991. Posttranslational regulation of phosphoenolpyruvate carboxylase in C4 and Crassulacean acid metabolism plants. *Plant Physiol.* **95**: 981–985.

Jörg, Z. and P. J. Facchini. 2008. Alkaloid Biosynthesis: Metabolism and Trafficking. Ann. Rev. Plant Biol.. Vol., **59**: 735-769.

Joubert, J., P. Joubert , M. Van der Spuy and E. Van Graan. 1984. Acute organophosphate poisoning presenting with choreoathetosis. *Clin Toxicol*, **22**: 187-191.

Jouhikainen, K., L. Lindgren,T. Jokelainen, R. Hiltunen, T. Teeri and K. M. Oksman-Caldentey. 1999. Enhancement of scopolamine production in ***Hyoscyamus muticus* L.** hairy root cultures by genetic engineering. *Planta*, Vol. **208**: 545.551.

Kamada, H., O. Nobuyuki, S. Motoyoshi, H. Hiroshi and S. Koichiro. 1986. Alkaloid production by hairy root cultures in ***Atropa belladonna***. *Plant Cell Rep.,: 239-242.*

Kaminek M, V. Motyka, and R. Vankova. 1997. Regulation of cytokinin content in plant cells. *Physiologia Plantarum* **101**: 689–700.

Kanage, T., H. Kajia, Y. Amano, T. Hashimoto and Y. Yamada. 1994. *Plant physiol*.,**105**: 483-490.

Karch, A. M. 2003. *Lippincott's Nursing Drug Guide*.Springhouse, PA: Lippincott Williams & Wilkins.

Karim, Z. J. 2008. ***In vivo*** and ***in vitro*** Studies on (***Rosmarinus officinalis* L.**) Secondary Metabolites. *M.Sc. Thesis, College of Science, Sulaimani University*.

Keiner, R., H. Kaiser, K. Nakajima, T. Hashimoto and B. Drager. 2002. Molecular cloning, expression and characterization of tropinone reductase II, an enzyme of the SDR family in ***Solanum tuberosum*** L. *Plant Mol, Biol.*,**48**: 299-308.

Khanam, N., C. Khoo and A. G. Khan. 2001. Effects of cytokinin-auxin combinations on cell arrangement in the basal stems and tropane alkaloid production in cultured non-rooted shoots of ***Duboisia myoporoides***. *Aust. J. Bot.,* **49** (4): 443-450.

Kinsara, A. M. and M. M. Seif El-Naser. 1990. Growth and production of tropane alkaloids by cell suspension cultures of ***Hyoscyamus albus* L**. *Qatar Univ. Sci. Bull,* **10**: 145-53.

Kinsara, A. M. and M. M.Seif El-Nasr. 1994. Organization and Alkaloid Production in Tissue Culture of ***Datura innoxia* Mill**. *JKAU: Sci*., **6**: 5-15.

Kitamura, Y. 1988. In "Biotechnology in Agriculture and Forestry 4" Ed. Bajaj, *Y.P.S.*: 419-436 .Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

Kleiber, H. and H. Mohr. 1967. Vom Einfluss des Phytochroms auf die Xylemdifferenzierung im Hypokotyl des Senfkeimlings (***Sinapis alba* L**.). *Planta* **76**: 85–92.

Knobloch, K. H. and J. Berlin. 1980. Influence of medium composition on the formation of secondary compounds in cell suspension cultures of ***Catharanthus roseus*** (**L**.) G. Don. ***Zeitschrift für Naturforschung****, C. Biosciences,* **35**: 551-556.

Knobloch, K. H. and J. Berlin. 1981. Phosphate mediated regulation of cinnamoyl putrescine biosynthesis in cell suspension cultures of ***Nicotiana tabacum***. *Planta Medica,* **42**: 167-172.

Kozai, T., K. Watanabe and B. R. Jeong. 1995. Stem elongation and growth of ***Solanum tuberosum*** L. ***in vitro*** in response to photosynthetic photon flux, photoperiod and difference in photoperiod and dark period temperatures. *Sci. Hortic*. **64**: 1–9.

Krömer, S., P. Gardestrom and G. Samuelson. 1996. Regulation of the supply of cytosolic oxaloacetate for mitochondrial metabolism via phosphoenolpyruvate carboxylase in barley leaf protoplasts. I. The effect of covalent modification on PEPC activity, pH response and kinetic-properties. *Biochim. Biophys. Acta,* **1239**: 343–350.

Kubota, S. and K. Yoneda. 1993. Effect of light intensity preceding day-night temperatures on the sensitivity of ***Phalaenopsi****s* to flower (Japanese). *J Jap Soc Hort. Sci.* **62**(3): 595-600.

Kumar, A. and V. A. Kumar. 1996. Plant Biotechnology and Tissue Culture Principles and Perpectives, International Book Distributing Co, Lucknow.(Cited by: tissue culture of a medicinal plant-***Aloe vera*** L. A Dissertation By Mr.Diwakar Aggarwal Roll No.3010108 *Masters of Science in Biotechnology Department of Biotechnology and Environmental Sciences Thapar Institute of Engineering and Technology (Deemed University) Patiala* –**147004** (2003).

Kumar, R. 2005. Clonal Propagation of Nasturtium –An Ornamental Plant Through Plant Tissue Culture. *M.Sc. Thesis in Biotechnology*, Thapar Institute of Engineering and Technology. (Deemed University).

Kurz, W. G. W. (ed.). 1989. Primary and secondary metabolism of plant cell cultures ll. *Biotechnology in agriculture and forestry*, 9. *Berlin, Springer-Veriage*, 450 pp.

Kurzbaum, A., C. Simsolo, L. Kvasha and A. Blum. 2001. Toxic delirium due to ***Datura stramonium***. *Israel Med. Assoc. J.* **3**: 538-539.

Kutchan, T. M. 1995. Alkaloid biosynthesis – the basis for metabolic engineering of medicinal plants. *Plant Cell*, 7: 1059–1070.

Landgrebe, M.E. and Leete, E. 1990. The metabolism of tropinone in intact ***Datura innoxia*** plants. *Phytochem.*, **29** : 2521.2524.

Lanoue, A., M. Boitel-Conti, J. C. Portais , J. C. Laberche, L. N. Barbotin , P. Christen and B. Sangwan-Norreel. 2002. Kinetic study of littorine re-arrangement in ***Datura innoxia*** hairy roots by 13C-NMR spectroscopy. *J. Nat. Prod*, **65**: 1131-1135.

Latorre, F. and L. Klimek. 1999. Does Cocaine Still Have a Role in Nasal Surgery? *Drug safe*.,**20** (1): 9-13.

Lepiniec, L., J. Vidal, R. Chollet, P. Gadal and C. Crétin. 1994. Phosphenolpyruvate carboxylase: structure, regulation and evolution. *Plant Sci*. **99**: 111–124.

Li , J. M., R. Vunsh *et al*., 2004. Callus induction and regeneration in ***Spirodela*** and ***Lemna.*** *Plant Cell Rep*., **22**: 457-464.

Lindsey, K. 1985. Manipulation by nutrient limitation, of the biosynthetic activity of immobilized cells of ***Capsicum frutescens*** **Mil** cv. **Annuum**. *Planta* **165**: 126-133.

Lindsey, K. and M. M. Yeoman. 1985. Dynamic aspects of plant cell cultures. *In : Vasil I, ed. Cell Cultures and Somatic Cell Genetics of Plants: A Comprehensive Treatise. New York, London : Academic Press,* 61-101.

Liptay, A., and A. E. Arevalo. 2000. Plant mineral accumulation, use and transport during the life cycle of plants: a review. *Can. J. Plant Sci.,* **80**: 29–38.

Liskova, D., R. J. Ordonez, A. Lux and A. P. Lopez. 1994. Tissue culture of ***Karwinskia humboldtiana*** – a plant producing toxis with antitumor effects. *Plant Cell Tiss. Org. Cult.* **36**: 339–343.

Lo, K. H; K. L. Giles and V. K. Sawhney. 1997. Acquisition of competence for shoot regeneration in leaf discs of ***Saintpaulia ionantha*** (**African violet**) cultured ***in vitro***. *Plant Cell Rep.,* **16**: 416-420.

Lumsden, P. J., S. Price and C. Leifert. 1990. Effect of mineral nutritionon the growth and multiplication of ***in vitro*** cultured plants. *In: Nijkamp HJ, Van Der Plas and Van Aartrijk J (eds) Progress in Plant Cell. and Mol. Biol.*: 108–113. Kluwer Academic Publishers, Dordrecht.

Mancino, C. F. 1991. Nitrate and ammonium concentrations in soil leachate and N leaching losses from fertilizers applied to turfgrass. *Golf Course Manager,1*: 66–72.

Mantell S. H., and H. Smith. 1983. Cultural factors that influence secondary metabolite accumulations in plant cell and tissue cultures. In : Mantell SH, Smith H, eds. *Plant Biotechnology. Society for Experimental Biology Seminar Series* **18**. Cambridge : Cambridge University Press, 75-108.

Masanaru, M. 1994. Plant Tissue Culture: an alternative for production of useful metabolites. *FAO Agricultural Service Bulletin* **108**, 90 pages.

Matsuda, J., S. Okabe, T. Hashimoto, and Y. Yamada. 1991. Molecular cloning of hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, from cultured roots of ***Hyoscyamus niger***. *J. Biol. Chem*. **266**: 9460-9464.

Mazrou, M. M. 1985. Okologische und Ontogenetische Einflusse auf Drogenertrag and Alkaloidgehalt von Atropa belladonna L (Tollkirsche). *Ph.D. dissertation, Giessen Univ., Fedral Republic of Germany*.

Mazrou, M. M. and A. I. Al-Humaid. 2000. Growth and flowering of somegladioli cultivars in relation to the chemical fertilization rates. *Zagazig J. Agric. Res*., 27: 635–644.

McCown, B. H. and J. C. Sellmer. 1987. General media and vessels suitable for woody plant cultures. In: Bonga, J.M., Durzan, D.J. (Eds.), *Tissue culture in forestry - General principles and biotechnology,* Vol. **2**. Martinus Nijhoff Publ., Dordrecht, Boston : 4-6.

Mengel, K. and E. A. Kirkby. 1987. *Principles of Plant Nutrition, 4th Ed.; International Potash Inst.: Bern, Switzerland.*

Mi, Q., Cui, B.,G. L. Silva, D. Lantvit, E. Lim, H. Chai, M. You, M. G. M. G. Hollingshead, J. G. Mayo, A. D. Kinghorn, and J. M. Pezzuto. 2001. Pervilleine Am a novel tropane alkaloid that reverses the multidrug-resistance phenotype. *Cancer Res.* **6**: 4030-4037.

Michael, W. 1999. Biochemistry of Plant Secondary Metabolism. *Sheffield Academic Press. Germany*: 5-26.

Miller, L. R. and T. Murashige. 1976. Tissue culture propagation of tropical foliage plants. *In vitro* **12** : 797-813.

Miraldi, E.,A. Masti, S. Ferri, I. B. Comparini. 2001. Distribution of hyoscyamine and scopolamine in ***Datura stramonium***. *Fitoterapia*, **72**: 644–648.

Miskat, A. J., S. Hadiuzzaman and M. A. Ghani. 2003. Plant regeneration through calli derived from explants of in vitro grown seedlings of three forms of ***Datura metel*** **L**. *Bangladesh J. Sci. Ind. Res*., **38**(1-2): 81-90.

Missaleva, N., G. Petri and E. Szoke,1993. Some morphological and biochemical peculiarities of ***Datura innoxia*** callus and regenerated cultures. *J. Plant Cell, Tiss. And Org. Cult.,* **35**(1): 87-92.

Mitsuno, M. V. B. Jipson, H. N. Lynt, and J. F. Graczyk. 1983. *8th Meeting of Plant Tissue Culture, Toyama, Japan, July*.

Mizuno K, A. Komamine, and M. Shimokoriyama. 1971. Vessel element formation in cultured carrot-root phloem. *Plant Cell Physiol.*, **12**: 823–830.

Möller R., D. B. Roderick, R. H. Anna, M. Günter, and F. Jens. 2006. Effect of light and activated charcoal on tracheary element differentiation in callus cultures of *Pinus radiata* D. Don. *Plant Cell Tiss. Org. Cult*., **85**:161–171.

Morini S., C. D'Onofrio, G. Bellocchi, and M. Fisichella. 2000. Effect of 2,4-D and light quality on callus production and differentiation from in vitro cultured quince leaves. *Plant Cell Tiss. Org. Cult*., **63**, (1): 47-55(9).

Muhammed S. I. 1996. Datura Plant. Treatment with medicinal plants and vitamins. *Forst Edition, Dar Al-Tabsheer.* (In Arabic).

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.* **15**,3: 473-497.

Muthukumar, B., D.I. Arockiasamy, and S.J. Britto. 2000. ***In vitro*** *propagation* of ***Datura metel*** L. from hypocotyl explants. *Plant Cell Tiss. Org. Cult..*, **10**,1: 39-44.

Naika, R. H. and V. Krishna. 2008. Plant regeneration from callus culture of ***Clematis gouriana*** **Roxb**. – A rare medicinal plant. *Turk J. Biol*.**32**: 99-103.

Nakajima, K., T. Hashimoto, and Y. Yamada. 1993. Two tropinone reductases with different stereo specific cities are short-chain dehydrogenases evolved from a common ancestor. *Proc. Natl. Acad. Sci. USA* , **20**: 9591-9595.

Namdeo A. G., T. S. Jadhav, P. K. Rai, S. Gavali, and K. R. Mahadik. 2007. Precursor feeding for enhanced production of Secondary metabolites: *Pharmacognosy Reviews*, **1**, Issue 2, Jul-Dec.

Nettleship, L. and M. Slaytor. 1974. Zellsuspensionskulturen von **Peganum harmala**. *J. Exp. Bot*., **25**: 1114.

Nigra H. M., M. A. Alvarez, and A. M. Giulietti. 1990. Effects of carbon and nitrogen sources on growth and solasodine production in batch suspension cultures of ***Solanum eleagnifolium*** *Cav. Plant Cell Tiss. Org. Cult.*, **21**: 55-60.

Nussbaumer P, I. Kapetanidis, and P. Christen. 1998. Hairy roots of *Datura candida* × *D. aurea*: effect of culture medium composition on growth and alkaloid biosynthesis. *Plant Cell Reports* **17**: 405–409.

Ohta, S., O. Matsui, and M. Yatazawa, 1978. Culture conditions for nicotime production in tobacco tissue culture. Agric. Biol. Chem. 42, 1245-1251.

Oksman-Caldentey K. M. 2007. Tropane and nicotine alkaloid biosynthesis-novel approaches towards biotechnological production of plant-derived pharmaceuticals. *Curr Pharm Biotechnol. Aug*; **8**, (4): 203-10.

Oksman-Caldentey, K.M., and Saito, K. (2005). Integrating genomics and metabolomics for engineering plant metabolic pathways, *Curr. Opin. Biotech.*, **16**: 174-179.

Oksman-Caldentey K. M., N. Sevon, L. Vanhala, and R. Hiltunen. 1994. Effects of nitrogen and sucrose on the primary and secondary metabolism of ***Hyoscyamus muticus.*** *Plant Cell Tiss. Org. Cult*., **38**: 263-272.

Oksman-Caldentey,K. M. and D. Inze. 2004. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends in Plant Sci.*, **9**: 433-40.

Ouelhazi, L., M. Filali, A. Décendit, J. C. Chénieux, and M. Rideau. 1993. Differential protein accumulation in zeatin- and 2,4-D-treated cells of ***Catharanthus roseus*** Correlation with indole alkaloid biosynthesis. *Plant Physiol*. *Biochem.* **31**: 421–431.

Panizza, S. 1997. Plantas que curam: cheiro de mato. 17ed. São Paulo: *IBRASA*: 44-45.

Parr, A. J, J. Payne, J. Eagles, B. T. Chapman, R. J. Robins, and M. J. C. Rhodes. 1990. Variation in tropane alkaloid accumulation within the solanaceas and strategies for its exploitation. *Phytoch.,* **29**: 2545–2550.

Pasqualetto P. L., W.P. Wergin, and R. H. Zimmerman. 1988. Changes in structure and elemental composition of vitrified leaves of `Gala' apple *in vitro*. *Acta Hortic*. **227**: 352-357.

Pasquali, G., O. J. M. Goddijn, A. De Waal, R. Verpoorte, R. A. Schilperoort, J. H. C. Hoge and J. Memelink. 1992. Coordinated regulation of two indole alkaloid biosynthetic genes from ***Catharanthus roseus***. *Plant Mol. Biol*. **18**:1121–1131.

Paul, M. and M. Stitt. 1993. Effects of nitrogen and phosphate deficiencies on levels of carbohydrates, respiratory enzymes and metabolites in seedlings of tobacco, and their response of exogenous sucrose. *Plant Cell Env*., **16**:1047–1057.

Pauli, H. H. and T. M. Kutchan. 1998. Molecular cloning and functional heterologous expression of two alleles encoding (*S*)-*N*-methylcoclaurine 39-hydroxylase (CYP80B1), a new methyl jasmonateinducible cytochrome P-450-dependent mono-oxygenase of benzylisoquinoline alkaloi biosynthesis. *The Plant J.,* **13**, 6: 793–801.

Phillips R. and G. G. Henshaw. 1977. The regulation of synthesis of phenolics in stationary phase cultures of ***Acer pseudoplatanus***. *J. Exptl. Bot.,* **28,** (4): 785-794.

Phillipson, J. D., B. V. Charlwood, and M. J. C. Rhodes. 1990. Plants as source of valuable products. *In* Secondary Products from Plant Tissue Culture. (eds.), *Oxford: Clarendon Press*: 1-21.

Pinol, M.T., J. Palazon, R. M. Cusido, and M. Ribo. 1999. Influence of calcium ion-concentration in the medium on tropane alkaloid accumulation in ***Datura stramonium*** hairy roots. *Plant Sci*., **141**: 41–49.

Pitta-Alvarez S.I., T. C. Spollansky, and A. M. Giulietti. 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of ***Brugmansia candida***. *Enzyme Microb Technol.,* **26**: 252-258.

Portsteffen, A., B. Drager, and A. Nahrstedt.1992. Two tropinone reducing enzymes from ***Datura stramonium*** transformed root cultures. *Phytochem.*, **31**: 1135-1138.

Potter, T. I., S. B. Rood, and K. P. Zanewich. 1999. Light intensity, gibberellin content and the resolution of shoot growth in ***Brassica***. *Planta.,* **207** : 505-11.

Preissel, U. and P. Hans-Georg. 2002. ***Brugmansia*** *and* ***Datura****: Angel's Trumpets and Thorn Apples*. Buffalo, New York: *Firefly Books*: 106–129. ISBN 1-55209-598-3.

Pretto, F. R. and E. R. Santarem. 2000. Callus formation and plant regeneration from *Hypericum perforatum* leaves. *Plant Cell Tiss.Org. Cult.,* **62**: 107-113.

Price, S. 1998. Shirley Price's Aromatherapy Workbook: Understanding Essential Oils - From Plant to Bottle *The Aromatherapy Workbook,* Thorsons, ISBN 0 7225 2645 8.

Pruski, K., T. Kozai, T. Lewis, T. Astatkie and J. Nowak. 2000. Sucrose and light effects on ***in vitro*** cultures of potato, chokecherry and saskatoon berry during low temperature storage. *Plant Cell Tiss. Org. Cult*., **63**: 215–221.

Pryce S, L. P. J., F. Berger, and C. Leifert. 1993. Effect of plant density and macronutrient on ***Delphinium*** shoot cultures. *J. Hort. Sci.,* **68**: 807–813.

Rajagopalan, A. V., M.T. Devi, and A.S. Raghavenra. 1993. Patterns of phosphoenolpyruvate carboxylase activity and cytosolic pH during light activation and dark deactivation in C3 and C4 plants. *Photosynth. Res*.,**38**: 51–60.

Ramachandra R, S. and G.A. Ravishankar. 2002. Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnol. Adv*., **20**: 101-153.

Raoufa, A. R., H. El. El-Wakil, A. El. Abou Gabal and H.D. Khlifa. 2008. Production of Scopolamine and Hyoscyamine in Calli and Regenerate Cultures of *Datura metel* (L.). *Journal of Applied Sciences Research*, **4**(12): 1858-1866.

Raven, P.H. and W. H. Freeman. 1999. *Biology of Plants*, 6th edn., ISBN 1 5725 9041 6.

Rhodes, M. J.C., Parr, A.J., Giuletti, A., Aird, E. L.H. 1994. Influence of exogenous hormones on the growth and secondary metabolite formation in transformed root cultures. *Plant Cell Tiss. Org. Cult.* **38**:143-151.

Robins. R. J., A. J. Parr, E. G. Bent, and M. J. C. Rhodes. 1991.Studies on the biosynthesis of tropane alkaloids in ***Datura stramonium*** **L**. transformed root cultures. *Planta,* **183**: 185–195.

Rodrigo, G.J. and L. J. Nannini. 2006. Role of tiotropium in the treatment of COPD. *Am. J. Med.,* **119**:S12–20.

Roja, G. and P.S. Rao. 1998. Biotechnological investigations in medicinal plants for the production of secondary metabolites, in: I.A. Khan, A. Khanum (Eds), Role of Biotechnology in Medicinal and Aromatic Plants, *Ukaaz Publication, Andhra Pradesh*: 95–115.

Rothe, G. and B. Dräger. 2002. Tropane alkaloids-metabolic response to carbohydrate signal in root cultures of ***Atropa belladonna***. *Plant Sci*., **163**: 979-985.

Ružic, D., M. Sari´c, R. Cerovi´c and L. Culafi´c. 2000. Relationship between the concentration of macroelements, their uptake and multiplication of cherry rootstock Gisela 5 ***in vitro***. *Plant Cell Tiss. Org. Cult*., **63**: 9–14.

Sakano, K., Y. Yazaki, K. Okihara, K. Mimura, and S. Kiyota. 1995. Lack of control in inorganic phosphate uptake by ***Catharanthus roseus*** **L**. G. Don Cells. *Plant Physiol.,* **108**: 295–302.

Sakuta, M., T. Takagi, and A. Komamine. 1987. Effects of nitrogen source on betacvanin accumulation and growth in suspension cultures of ***Phytolacca americana***. *Physiol. Planta*., **71**: 459-463.

Salisbury, F. B. and C. W. Ross. 1992. Lipids, essential oil, terpenoids, and other natural products. Plant Physiology, 4th Ed.; *Wadsworth Publishing Comp.: Belmont, CA,*: 116–135.

Sandra, I., A. Pitta, C. Tatiana, A. Spollansky and M. Giulietti. 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of ***Brugmansia candida***. Elsevier Science Inc. *Enzy. Microbiol Tech.,:* 252-258

Sari´c, M, S. Mezei, and D. Ruži´c. 1995. Genetic aspects of mineral nutrition of plants grown ***in vitro***. *Arch. Biol. Sci*., **47**: 1–12.

Sasson, A. 1991. Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects United Nations Educational, Scientific and Cultural Organization, *(UNESCO), 7 Place de Fontenoy, 75700 PARIS, FRANCE Options Mediterraneennes-Seri Seminaires-n°,* **14**: 59-74.

Schäfer, C., H. Simper, and B. Hofmann. 1992. Glucose feeding results in coordinated changes of chlorophyll content, ribulose-1,5-bisphosphate carboxylase-oxygenase activity and photosynthetic potential in photoautotrophic suspension cultured cells of ***Chenopodium rubrum***. *Plant Cell Env.,* **15**: 343–350.

Schmidt, M., E. Heim, S. Wylegalla, C. Helmbrecht. and K. G. Wagner. 1992. Characterization of phosphate uptake by suspension cultured ***Catharanhus roseus*** *Cells. J. Plant Physiol*., **140**:179–184.

Serret, M. D., M.I. Trillas, J. Matas and J. L. Araus. 1997. Development of photoautotrophy and photoinhibition of ***Gardenia jasmoides*** plantlets during micropropagation. *Plant Cell Tiss. Org. Cult*., **45**: 1–16.

Sethi, U., A. Basu and S.G. Mukherjee. 1990. Role of inhibitors in the induction of differentiation in callus cultures of ***Brassica***. ***Datura*** and ***Nicotiana***. *Plant Cell Rep.,* **8**,10: 598-600.

Sevon, N. 1997. Tropane alkaloids in hairy roots and rgenerated plants of ***Hyoscyamus muticus***, *PhD thesis, Univercity of Helsinki,Finland.*

Sevón, N. and K. M. Oksman-Caldentey. 2002. Agrobacterium rhizogenes-mediates transformation: Root cultures as a source of alkaloids. *Planta Med,,* **68**: 859-868.

Sevón, N., S. Biondi, N. Bagni, and . M. Oksman-Caldentey. 2001. Transgenic ***Hyoscyamus muticus*** (Egyptian henbane). In: Bajaj, Y.P.S. (Ed.). *Biotechnology in Agriculture and Forestry 48, Transgenic Crops III. Berlin-Heidelberg: Springer-Verlag.:* 171-200.

Sikuli, N. N. and K. Demeuer. 1997. Influence of ion-composition of the medium on alkaloid production by "hairy roots" of ***Datura stramonium*** **L**. *Plant Cell Tiss. Org. Cult.,* **47***: 261-267.*

Sima, B. D. and Y. Desjardins. 2001. Sucrose supply enhances phosphoenolpyruvate carboxylase phosphorylation level in ***in vitro Solanum tuberosum***. *Plant Cell Tiss. Org. Cult.,* **67**: 235–242.

Singha S. and S.K. Bhatia. 1988. Shoot proliferation of pear cultures on medium containing thidiazuron and benzylamino purine. *HortScience*, **23**: 803.

Smith, J. I., N. J. Smart, W. G. W. Kurz and M. Misawa. 1987a. Stimulation of indole alkaloid production in cell suspension cultures of **Catharanthus roseus** by abscisic acid. *Planta Med*., **53**: 470-474.

Smith, R. H. 2000. Callus induction: 98-103. In plant tissue culture techniques and experiments. *Second edition. Acad. press, San Diego, California.*

Soontornchainaksaenga P., Sarinee C., Matura S. and Maleeya K. (2001). In vitro Studies on the Effect of Light Intensity on Plant Growth of *Phaius tankervilliae* (Banks ex L' Herit.) Bl. and *Vanda coerulea* Griff. *ScienceAsia* **27**: 233-237

Sorrentino, G., L. Cerio and A. Alvino. 1997. Effect of shading and air temperature on leaf photosynthesis, fluorescence and growth in lily plants. *Scientia Horticulturae* **69**: 259-73.

Spinks, A. B., J. Wasiak, E. V. Villanueva, and V. Bernath. 2004. "Scopolamine for Preventing and Treating Motion Sickness." *Cochrane Database Systems Review* **3**, CD002851.

Sri Andrijany, V., G. Indrayanto, and L.A. Sochono. 1999. Simultaneous effect of calcium, magnesium, copper and cobalt ions on sapogenin steroids content in callus cultures of ***Agave amaniensis***, *Plant Cell Tiss. Org. Cult.,* **55**: 103–108.

Srivastava, P.S., N. Alka and S. Sheela. 2004. Biotechnological Approaches Towards Improvement of Medicinal Plants. *Plant Biotechnology and Molecular Markers. Anamaya Pulishers, New Delhi, India.*

Staba, J. 1980. Plant tissue culture as a source of Biochemical. C.R.C. Press, Bocaraton, Florida, (C.F. In vitro studies on (*Hypericum perferatum* L.) Ph.D. Thesis, by Taha, H.S. (1999), Fac. of Agric. Cairo Univ.

Stickland, R. G. and N. Sunderland. 1972. Photocontrol of growth, and anthocyanin and chlorogenic acid production in cultured callus tissues of ***Haplopappus gracilis***. *Ann. Bot.,* **36**: 671-685.

Tabata, M., H. Yamamoto and N. Hirakawa. 1971. *In "Les Cultures de Tissue de Plantes,*: 389. CNRS, Paris

Taha, S. A. 1984. ***Datura*** intoxication in Riyadh. *Trans R Soc Trop Med Hyg*., **78**,1: 134-5.

Tailang, M. K., M. D. Kharya, et al. 1997. Mutation induced bioproduction of glycyrrhetinic acid from callus culture of Glycyrrhiza glabra Linn. Indian *Journal of Pharmaceutical Sciences* **59(1):** 22-25. {a} Bhupal Nobles Coll. Pharm., Udaipur, Rajasthan 313 001, India

Takahashi, S. and Y. Fujita. 1991. In "Plant Cell Culture in Japan". Co. Ltd. Tokyo, ibid,: 72-78.

Thi Le, M. T., V. Thi Dao, B. Benoil, F. Flore, B. Jean-Paul, G. Michele, Bui C.T. Armand, B. Frederic and G. Eric. 2007. Hydroponics as a model technology for producing plant root secondary metabolites. P*lanta*., **225**: 1245-1253.

Thorpe, T.A. & Biondi, S. 1984. Conifers. - In Handbook of Plant Cell Culture vol. **2**. *Crop species* (W.R. Sharp, D.A. Evans, P.V. Ammirato, Y. Yamada, eds.): 435-470. Macmillian Publishing Company, New York. ISBN 0-02-949230-0.

Tichá, I., F. ˇCáp, D. Pacovská,P. Hofman,D. Haisel, V. ˇCapková and C. Schäfer. 1998. Culture on sugar medium enhances photosynthetic capacity and light resistance of plantlets grown *in vitro*. *Physiol. Plant*., **102**: 155–162.

Todd, F. G.,F. R. Stermitz, P. Schultheis, A. P. Knight and E. Traub-Dargatz. 1995. Tropane alkaloids and toxicity of ***Convolvulus arvensis***. Phytochem.,**39**: 301-303.

Torres, K. 1988. Isolation and culture of protoplast from carrot cell suspension culture. :. In *Tissue Culture Techniques for Horticultural Crops. Torres K., (ed.). Acad. Press, New York:* 187-200.

Towers, G. H. N. and E. Yamamoto. 1985. Interaction of cinnamic acid and its derivatives with light. In: Van Sumere CF, and Lea PJ, eds. *The Biochemistry of Phenolics. Oxford: Clarendon Press,*: 271-287.

Trease, G. E. and W. C. Evans. 1978. Factors involved in the production of drugs. In '*Pharmacognosy'.* (Eds G. E. Trease and W. C. Evans.): 169–182. (Baillière Tindall: London.).

Trejo-Tapia, G., A. Jimenez-Aparicio, M. Rodriguez-Monroy, A. De. Jesus-Sanchez and G. Gutierrez – Lopez. 2001. Influence of cobalt and other microelements on the

production of betalains and the growth of suspension cultures of ***Beta vulgaris***, *Plant Cell Tiss. Org. Cult*., **67**: 19–23.

University of Kentucky. 2002 Research Annual Report, Kentucky Agricultural Experiment Station. Pharming and Foam.

Van de Velde, H., Demeyer, K. and R. Dejaegere. 1988. Influence of IAA and DMAA on hyoscyamine and scopolamine production in ***Datura stramonium*** var. ***tatula***. *Acta Agronomica Hungaria,* **37**: 55–63.

Van der Heijden R, and R. Verpoorte and H. J. G. Ten Hoopen. 1989. Cell and tissue cultures of ***Catharanthus roseus*** (L.) G. Don: a literature survey. *Plant Cell Tiss. Org. Cult.* **80**: 231–280.

Vanisree, M. and C. Lee, S. Lo, S. M. Nalawade, C. Y. Lin and H. Tsay. 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot. Bull. Acad. Sinc.*., **45**: 1-22.

Vergeer, L. H. T., T. L. Aarts and J. D. Degroot. 1995. The wasting disease and the effect of abiotic factors (light intensity, temperature, salinity) and infection with ***Labyrinthula zosterae*** on phenolic content of ***Zostera marina*** shoots. *Aquatic Bot* **52**,(1-2): 35-44.

Verpoorte, R. 2000. In Metabolic Engineering of Plant Secondary Metabolism, (Verpoorte, R. and A. W. Alfemann, EDs), *Kluwer Academic Publisher, Dordrech*: 1-29.

Wedding, R. T., M. K. Black and C. R. Mayer. 1989. Activation of higher plant phosphoenolpyruvate carboxylase by glucose-6-phosphate. *Plant Physiol.* **90:** 648–652.

Wedin, G. P. 1988. Comment: anticholinesterase insecticide reactions. *Drug Intell Clin Pharm,* **22**: 919-920.

Weiner, M. A. 1980. Earth Medicine,Earth Food. *Ballantine Books. ISBN 0-449-90589-6.*

Whitaker, R. J.,T. Hashimoto and D. A. Evans. 1984. Production of the secondary metabolite, rosmarnic acid, by plant cell suspension cultures. *Ann. NY Acad. Sci.*, : 435-464.

White, P. F., J. Tang and D. Song. 2007. Transdermal scopolamine: an alternative to ondansetron and droperidol for the prevention of postoperative and postdischarge emetic symptoms". *Anesth. Analg.* **104** (1): 92–6.

Whitmer S, C Canel, D. Hallard, C. Goncalves and H. Verpoorte. 1998. Influence of precursor availability on alkaloid accumulation by transgenic cell line of *Catharanthus roseus*. *Plant Physiol*. **116**: 853-857.

WHO (World Health Organization). 2002. Traditional medicine: Growing needs and potential. *WHO Policy Perspectives on Medicines,* **2**: 1-6 .

Williams, R. R. 1995. The chemical microenvironment. In: Aitken-Christie J, Kozai T & Smith MAL (eds) Automation and Environment Control in Plant Tissue Culture: 405–439. *Kluwer Academic Publishers, Dordrecht.*

Witte, L., K. Muler, and H. A. Alfermann. 1987. Investigation of the alkaloid pattern of *Datura innoxia* plants by capillary gas-liquid-chromatography-mass spectrometry. *Planta Med*. **53**: 192-197.

Yamada, Y. and T. Hashimoto 1982. Production of tropane alkaloids in cultured cells of ***Hyoscyamus niger***. *Plant Cell Rep*., **1**: 101 – 103.

Yamakawa, T., S. Kato, N. D. K. Ishida, T. Kodama and Y. Minoda. 1983. Production of anthocyanins by ***Vitis*** cells in suspension culture. *Agric. and Biol.Chem.*, **47**: 2185-2191.

Yeoman, M. M. 1987*b*. Bypassing the plant. *Ann. Bot.*, **60** [Supplement]: 157-174.

Yeoman, M. M. and C. L. Yeoman. 1996. Manipulating Secondary Metabolism in Cultured Plant Cells. *New Phytologist*, **134**, (4): 553-569.

Yie, S. T. and S. I. Liaw. 1997. Plant regeneration from shoot tips and callus of papaya. *In vitro,* **9**: 564-8.

Yun, D. J., T. Hashimoto and Y. Yamada. 1992. Metabolic engineering of medicinal plants: transgenic ***Atropa belladonna*** with an improved alkaloid composition. *Proc. Natl. Acad. Sci. USA,* **89**: 11799-11803.

Zayed, R., M. Winka and H. El-Shamy. 2006. *In vitro* Organogenesis and Alkaloid Accumulation in ***Datura innoxia***. *Z. Naturforsch*, **61**: 560-564.

Zenk, M. H. 1991. Chasing the enzymes of secondary metabolism: Plant cell cultures as a pot of gold. *Photochem*., **30**: 3861-3863.

Zhang, L., R. Ding, Y. Chai, M. Bonfill, E. Moyano, K. M. Oksman-Caldentey, T. Xu, Y. Pi, Z. Wang, H. Zhang, G. Kai, Z. Liao, X. Sun and K. Tang. 2004. Engineering tropane biosynthetic pathway in ***Hyoscyamus niger*** hairy root cultures. *The Nat. Acad. Sci*.

Ziska, L. H., S . D. Emche, E . L . Johnson, K . George, D . R . Reed and R . C . Sicher. 2005. Alternations in the production and concentration of selected alkaloids as a function of rising atmospheric carbon dioxide and air temperature: implications for ethno-pharmacology. Alternate Crop and Systems Laboratory, USDA, Agricultural Research Service, Beltsville, MD 20705, USA ,*Global Change Biol.*, **11**: 1798-1807.

پوختەی کار و ئەنجامەکان

چەند تاقیکردنەوەیەك، لە تاقیگەکانی چاندنی شانەی ڕووەك لە شاری (دهۆك) و (سولەیمانیی)، لە سەر تفتەکانی (تروپەین) لە ڕووەکی پزیشکیی (ژووژیلە – چەز): *Datura innoxia* Mill کرا.

ئامانج لە ئەم لێکۆڵینەوەیە ئەوە بوو، لە ڕێی تەکنیکی شانە چاندنەوە لە هەرێمی (کوردستان)، وەك تەکنیکێکی نوێ لە ناوچەکەدا بتوانین، ئەگەری زیادکردنی بڕی بەرهەمهێنانی تفتی (تروپەین)ی نیو کاڵەسی ڕووەکی (داتورا)، لەگەڵ ئەوەی ڕووەکە ساغەکەدا بەراوردکرا، بە دریژایی سال، بێ ئەوەی پشت بە وەرزەکانی ساڵ ببەسرێ. هەروەها، پیوەندیی تەمەنی فیسۆلۆجیی ڕووەکەکە، لەگەڵ بڕی ئەو کەرەسە کیمیاییانەی تێی دایە، دیاریکرێ.

ئەم تاقیکردنەوانەی خوارەوەش جێبەجێکران:

1. بەشەکانی ڕووەکەکە (Explants) پاکژکرایەوە.
2. یەك جۆر (سیتوکاینین) و جۆرە جیاوازەکانی (ئۆکسینەکان) تێکەڵکرایەوە.

شانەی ڕووەکەکان، لە سەر ئاستی نیوەندی (MS)، بە چڕییەکانی (So 30گم/ لتر،S1 15گم/ لتر و S2 45گم/لتر) شەکری تێکرا. هەروەها، سێ ئاستی جیاوازی خوێ: (Xo چڕیی سروشتیی، X_1 نیوە چڕیی سروشتیی و X_2 دوو هێندەی چڕیی سروشتیی) کە لە نیو نیوەندی (MS)دا تێکرا. لە دواییدا، بەشە ڕووەکییە چاندراوەکان لە نیو کابینەی (ئەلەمنیوم) دانران و هەواکەشی کۆنترۆڵکرابوو، پلەی گەرمیی (1±25)ی سەدییدا بوو، سەرچاوەی ڕووناکییەکەش لە هەر کابینەیەکدا، ژمارەیەك گڵۆپی نیۆنی سپی کورت بوون، بە دریژیی (1.25) پێ دەبوو، کە چڕییەکەی لە نیوان (60– 100) مایکرۆمۆڵ /m^2 /چرکە، بڕەکەشی (20) وات بوو.

هەروەها، هەموو بەشە ڕووەکییە چاندراوەکان، لە نیو هەر سێ کابینەکەدا، لە ژێر مامەڵەی ڕووناکیی جیاوازدا دانران، لە چڕییەکەیدا لە (1000 لەکس، 2000 لەکس و 3000 لەکس) یەکەی ڕووناکیی پێکهاتبوو. واتە: لە ئاستی بەشە ڕووەکییە چاندراوەکاندا، بۆ ماوەی (16) دەمژمێر ڕووناکیی و (8) دەمژمێر تاریکیی لە شەو و ڕۆژێکدا، بەرامبەر بە (19، 37، 57) مایکرۆمۆڵ/ م^2 / چرکەیە.

تاقیکردنەوەی تێکاریی شانەکان کرا، بۆ ئەوەی چۆنێتی جیاکردنەوەی خانەکانی ڕووەکی (داتورا) بزانرێ. ئەمەش بە ئەوە دەبێ، شانەی کاڵەس لە قۆناغی چوارەم، لە چاندنەوەی کاڵەسەکەدا دروستبووە. سەرەتای دروستبوونی ئەندامەکان و لقەکان، لە

نیُوەوەی لەشی زیندوودا، پێوەندیی بە گەشەکەرە ڕێکخەرە ڕووەکییەکان، بە تایبەتیی (سایتۆکاینین) و (ئۆکسین)ەکانەوە هەیە. هەروەها، لە سەر توانای کاردانەوەی شانەکە بەندە، کە گەشەکەرە ڕێکخەرەکان، لە ماوەی گەشەکردندا هەیانە. ئەوەی شایەنی باسە، شانەی کالّەسی (داتۆرە)، کاردانەوەیەکی باشی هەبووە.

مامەلّەی گەلّای ڕووەکی (داتۆرا) بە گیراوەی (کهول) بە خەستیی (70٪) و بۆ ماوەی دوو خولەك مەمەلّەکرا، دوایی بە گیراوەی (NaOCl : 1٪)، بۆ ماوەی دوو خولەك، بەرزترین ڕێژەی پاکژکردنەوەی دا، کە (97٪) بوو.

لە ڕێی ئەم تاقیکردنەوانەی کران، بۆمان دەرکەوت: باشترین ڕەوشی گەشەکردنی کالّەس، بە ڕێژەی سەدیی، بڕی ڕێژەی سەدیی لە کەرەسەی وشك (DM٪)، کێشی ووشك/گم و کێشی تەڕ/گم، لە چاندنی بەشەکانی گەلّا لە سەر نیُوەندی(MS) بەدەسهات ، کە هەر دوو گەشەکەرە ڕێکخەرەکەی، بە چڕیی (0.6 ملگم/لتر BA+ 1.0 ملگم/ لتر ئۆکسین NAA)، لە قۆناغی دووەمی چاندنەوەی کالّەسەکان تێدا کرابێ. هەروەها، بەرزترین ژمارەی لقەکان، لە پێکهاتەی هەر دوو گەشەکەرە ڕێکخەرەکە، بە چڕیی (2.0 ملگم/لتر BA + 0.6 ملگم/لتر ئۆکسین NAA)، لە قۆناغی شەشەم لە چاندنەوەی کالّەسەکان بەدەسهات.

ئەنجامەکان دەریانخست: پێکهاتەی (0.6 ملگم/ لتر BA + 1.0 ملگم ئۆکسین NAA)، بەرزترین ڕێژەی هاندانی کالّەسی داوە: (99.42٪) کە بە سەر ڕێژەی (93.35٪) زالّبوون، کە هەر دوو گەشەکەری ڕێکخەری (0.4 ملگم/ لتر BA+ 1.5 ملگم/ لتر ئۆکسینی 2,4-D) بەدەسهاتووە. ڕێژەی سەدیی کەرەسەی ووشك (DM٪)، کێشی ووشك/گم و کێشی تەڕ/گم، لە (0.6 ملگم /لتر BA+ 1.5 ملگم/لتر اوکسین NAA) بەدەسمانهێنا. بەڵام بە بەراوردکردنی ئەم بەهایانە، لەگەڵ بەرانبەرەکانیاندا، کاتێ (0.4 ملگم/ لتر BA+ 1.5 ملگم/ لتر ئۆکسین 2,4-D)مان بەکارهێنا و بە دوای یەکدا، ئەمانە بوون: (11.05٪) و (8.39 گم و 0.844 گم)، بەرزترین ژمارەی لقەکان بۆ هەر کالّەسێ (46.167) لق بوو، لە مامەلّەی (2.0 لتر BA + 0.6 ملگم/لتر اوکسین NAA)، بە سەر هەمان خەسلەت لە ژمارەکەی (39.0) لق بوو، لە مامەلّەی (1.0 ملگم/ لتر BA+ 0.5 ملگم/ لتر ئۆکسین 2,4-D) زالّبوو.

هەروەها، بەرزتربن کێشی کالّەس لە ڕووی کێشی ووشك (1.350)گم مان لە کۆتایی قۆناغی دووەمی چاندنەوەی کالّەسەکەدا دەسکەوت. واتە: دوای (8) هەفتە. لە مامەلّەی (0.6 مگلم/لتر BA+ 1.0 ملگم/لتر ئۆکسین NAA) و دوای ئەو قۆناغە، ئیدی تێکڕای گەشەکردنی کالّەسەکان، بەرەو کەمیی دابەزیوە. بڕی پێکهاتە تفتەکان و جۆرەکانی، لە نیُو گەلّاکانی ڕووەکەکە و هەروەها، لە نیُو کالّەسە

بەرهەمهێنراوەکەشدا، بە ئامێری {HPLC} دیارییکرا. ئەنجامەکانیش دەریانخست: چاندنی بەشە گەڵاکانی ڕووەکی {داتورا}، لە نیۆەندی {MS}دا بە چڕیی جیاواز، شەکری تێ کرابوو، کاری لە زیادکردنی بڕی تفتەکانی پێکهاتەی گشتیی بە تایبەتیی سێ جۆری یەکەم {ئەترۆپین، هایوسیامین و سکۆپۆڵامین} کردووە، کاتێ چڕیی شەکرەکە: {S_1 15 ملگم /لتر} بوو، گەر لەگەڵ مامەڵەی {S_0 گم/لتر}دا بەراوردکرێ.

بە شێوەیەکی گشتیی، کارتێکردنی جیاوازی چڕیی خوێکان، لە گەشەکردنی بەشە گەڵاکانی ڕووەکی {داتۆرا} بە تایبەتیی لە بڕی تفتەکان و پێکهاتەی گشتیی جۆرەکانی دەرکەوت: مامەڵەی چڕیی خوێ{(X_1} نیوە سروشتییەکە، هەمان ئاراستەی کارتێکردنی چڕیی شەکرەکەی گرتووە، ئەمەش بە ئەوەی، کاتێ چڕیی خوێکە بۆ نیوە {X_1} دابەزیوە، بڕی تفتەکان زیادیکردووە، گەر لەگەڵ مامەڵەی چڕیی خوێی{X_2} بەراوردکرێ، کە دوو هێندەی سروشتییەکە بوو، یا {X_0} کە لە نیۆەندی {MS}ە سروشتییەکەدا بوو.

شایەنی باسە، کارتێکردنی گەشەکردنی کاڵەس لە بارودۆخی جیاوازی چڕییەکانی ڕووناکیدا، کارێکی جیاوازی لە چوار جۆری یەکەم لە تفتەکان: {ئەترۆپین، هایۆسیامین، سکۆپۆڵامین و ترۆپین} و پیکهاتەی گشتبی کردووە و دەرکەوت: زیادیان کردووە، کاتێ چڕییەکانی ڕووناکیی {LI_1 : 1000 لەکس} بوو، لەگەڵ هەر دوو مامەڵەی {LI_2: 2000 لەکس و LI_3: 3000 لەکس} بەراوردکرا. لەگەڵ ئەوەشدا، مامەڵەی {LI_2}، بڕی هەر دوو تفتەکەی کۆتایی: {7 هایدرۆکسی هایۆسیامین} و {تیگلۆهایۆ سیامین}ی، بۆ ئاستی {22.155 و 28.250} مایکرۆمۆڵ/گرام کەرەسەی ووشك بە دوای یەکدا دابەزاندووە، گەر لەگەڵ بڕی هەمان دوو تفتەکان: {61.04 و 56.452} مایکرۆمۆڵ/گم کەرەسەی ووشك، لە مامەڵەی {LI_1}دا و {50.472 و 31.816} مایکرۆگم/گم کەرەسەی ووشك لە مامەڵەی { LI_3}دا بە دوای یەکدا بەراوردکرێن.

بە شێوەیەکی گشتیی، کاتێ چڕیی هەر هەموو تفتەکان، جگە لە {سکۆپۆڵامین}، لە نیۆ کاڵەسی {داتـــۆرا}دا، لە نیۆەندی {MS}دا، کە شەکر و خوێکانی بە چڕیی جیاواز تێکرابێ، لە ژێر مامەڵەی جیاواز لە چڕییەکانی ڕووناکییدا دانرابن، لەگەڵ بڕی تفتەکان لە نیۆ گەڵای {داتورا}ی ساغدا بەراوردکران، دەرکەوت: جیاوازییەکی گرنگ و واتاداری گەورە، لە مامەڵەی { LI_1 } چڕیی شەکرەکە { S_1} بێ و چڕیی خوێکانیش {X_1} بێ، لە نیۆ نیۆەندی {MS}دا هەیە. بەڵام بە ڕوونبی بۆمان دەرکەت: لە بارەی تفتی {سکۆپۆڵامین}ەوە، مامەڵەی چڕییەکانی ڕووناکیی {LI_2}، بڕی تفتی ناوبراوی، بۆ {25.051 } مایکرۆگم/گم کەرەسەی ووشك دابەزاند، گەر لەگەڵ شانەکانی گەڵای ڕووەکە ساغەکەدا بەراوردکرێ.

لە کۆتاییشدا، ئەنجامەکانی ئەم توێژینەوەیە دەریانخستووە: گەشەکردنی گەڵی ڕووەکی (داتۆرا) لە نێوەندی (MS)دا، کە چڕییەکانی جیاوازیی لە خوێکان و شەکر تێکرابێ، لە مامەڵەی چڕییە ڕووناکیی (LI_1)دا دانرێ، باشترین جۆری نێوەندی (MS) دەبێ، بۆ ئەوەی شانەکانی گەڵەکە گەشەبکا، جگە لە (سکۆپۆلامین، تا ئاستێکی بەرزمان، لە هەموو جۆرە تفتەکان دەسکەوێ.

* * *

حکومەتی هەرێمی کوردستان

وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی

زانکۆی سلێمانی - کۆلێژی کشتوکاڵ

جێبەجێکردنی تەکنیکی چاندنی شانەی ڕووەك بۆ بەرهەمهێنانی تفتەکانی (ترۆپەین) لە ڕووەکی ژووژیلە (داتورا) دا

Datura innoxia Mill

تێزەکە

پێشکەش بە ئەنجومەنی کۆلێجی کشتوکاڵ / زانکۆی سلێمانی کراوە

وەك بەشێك لە پێداویستییەکانی بەدەستهێنانی پلەی دکتۆرای فەلسەفە

لە زانستە کشتوکاڵییەکان/ بەروبوومی کێڵگە

(چاندنی شانەی ڕووەك)

لە لایەن

ئەحمەد حەمە ئەمین حەمە ڕەشید

بەکالۆریوس لە زانستە کشتوکاڵییەکاندا / بەروبوومی کێڵگە / زانکۆی سلێمانی (1976)

ماستەر لە زانستە کشتوکاڵییەکاندا / فیسیۆلۆژیای ڕووەك / زانکۆی ملبۆرن / ئوسترالیا (*1983*)

بە سەرپەرشتیی

پ. د. موصلیح محەمەد سەعید دهۆکیی

نەورۆز 2710 کوردی　　ربیع الأول 1431 ه　　نیسان 2010 ز

حكومة اقليم كوردستان

وزارة التعليم العالي و البحث العلمي

جامعة السليمانية- كلية الزراعة

تطبيق تقنية الزراعة النسيجية لأنتاج قلويدات التروبين من نبات الداتورة *Datura innoxia* Mill

أطروحة مقدمة إلى

مجلس كلية الزراعة - جامعة السليمانية

كجزء من متطلبات نيل درجة دكتوراه فلسفة

في العلوم الزراعية/ المحاصيل الحقلية

(زراعة الأنسجة النباتية)

من قبل

أحمد حمه أمين حمه رشيد

بكالوريوس في العلوم الزراعية/ المحاصيل الحقلية / جامعة السليمانية (1976)

ماجستير في العلوم الزراعية/ فسلجة النبات / جامعة ملبورن / أستراليا (1983)

بإشراف

الأستاذ الدكتور مصلح محمد سعيد الدهوكي

ربيع الأول 1431 هـ **نةورؤز 2710 ك** **نيسان 2010 م**

الخلاصة

نفذت عدة تجارب في مختبرات زراعة الأنسجة النباتية في كلية زراعة دهوك و السليمانية لدراسة بعض مركبات الأيض الثانوية لنبات الداتوره :-

Datura innoxia Mill

من العائلة الباذنجانية ***Solanaceae***

استهدفت الدراسة تطبيق تقنيات زراعة الأنسجة النباتية في أقليم كردستان , بأعتبارها تقنية حديثة في المنطقة ..و مدى امكانية زيادة المحتويات الكيميائية في نسيج الكالس مقارنة مع تلك المنتجة من النبات الأم على مدار السنة دون الأعتماد على فصول السنة . علاوة على تحديد العلاقة بين العمر الفسلجي للنبات و بين محتوياته الكيميائية , و يمكن أيجاز التجارب بمايلي :-

دراسة تأثير أنواع مختلفة من السايتوكايتين **(BA)** و الأوكسينات ، تمت زراعة الأجزاء النباتية المعقمة في وسط **Murashige and Skoog (1962)** بتوليفة من تراكيز السكر **(S_0 , S_1 و S_2)** أي **30**, **15** و **45** غم/لتر على التوالي .كما إحتوى الوسط أيضاَ على ثلاثة مستويات من الأملاح **(X_0 , X_1 و X_2)** أي الطبيعي و نصف القوة و قوة الملح المضاعفة على االتوالي تم وضع الأجزاء النباتية المزروعة في ثلاثة حجرات محلية مصنوعة من الألمنيوم مكيف و بدرجات الحرارة **25±1** م و مصدر الأضاءة في كل حجرة **60-100** مايكرومول / م^2/ ثا ومزودة مصابيح ذات طول **1.25** قدم من نوع فلورسينت ذو سطوع 20 واط .

ومن ثم, تم وضع جميع المعاملات المذكورة أعلاه تحت ظروف الأنارة ذات كثافات ضوئية متفاوتة تراوحت بين 1000 ,2000و 3000 لكس (وحدة ضوئية) أي تقريباَ 19 , 37 ,57 مايكرومول/م^2 /ثا على التوالي و ذلك عند المستوى نفسه للأجزاء النباتية المزروعة و بتواقت 16 ساعة ضوء / 8 ساعة ظلام . بعدها تم تشريح الأنسجة النباتية للكشف عن كيفية تمايز خلايا الـ *Datura* من خلال تكوين نسيج الكالس في المرحلة الرابعة من اعادة زراعة الكالس و إعتمد نشوء الأعضاء و الأفرع داخل الجسم الحي على إضافة منظمات النمو النباتية و خصوصاَ السايتوكايتين و الأوكسينات و كذلك أعتمد على قدرة النيسج في الأستجابة الى تلك المنظمات خلال فترة النمو . فقد وجد استجابة نسيج كالس الـ*Datura*. و عند غمر أوراق نبات .*Datura innoxia* Mill بالكحول الأثيلي

(70%) لمدة دقيقتين و تليها دقيقتين بهايبوكلورايت الصوديوم (NaOCl) بتركيز 1% فقد اعطى أعلى نسبة مئوية للبقاء و هي (97%).

يستنتج مما سبق أن أفضل سلوك للنمو معبرا عنها كنسبة مئوية لنشوء الكالس او النسبة المئوية لمحتوى المادة الجافة و الوزن الجاف (غم) و الوزن الطازج (غم) تم الحصول عليها من التوليفة **0.6** ملغم/لتر من **BA** مع **1.0** ملغم/لتر من أوكسين **NAA** خلال المرحلة الثانية من اعادة زرع الكالس و كذلك عدد الأفرع المتكونة لكل نسيج ورقي مزروع فقد تم الحصول عليها من التوليفة 2.0 ملغم/لتر من **BA** مع **0.6** ملغم/لتر من **NAA** في المرحلة السادسة من اعادة زراعة الكالس . و كانت أعلى % لنشوء الكالس (99.42) وقد اختلفت معنويا عن قيمة **(93.35)** من التوليفة **0.4** ملغم/لتر من **BA** مع **1.0** ملغم/لتر أوكسين **2,4-D** . و كانت النسبة المئوية للمادة الجافة و الوزن الطري (غم) و الوزن الجاف (غم) من التوليفة **0.6** ملغم/لتر من **BA** مع **1.0** ملغم/لتر من أوكسين **NAA** هي **11.97%** و **11.3** (غم) و **1.57** (غم) على التوالي . في حين كانت القيم المماثلة من التوليفة **0.4** ملغم/لتر من **BA** مع **1.0**ملغم/لتر من أوكسين **2,4-D** هي **11.05%** , **8.39** (غم) و **0.844** (غم) على التوالي وتم الحصول على أعلى عدد من الأفرع **(46.167)** من التوليفة **2.0** ملغم/لتر من **BA** مع **0.6** ملغم/لتر من أوكسين **NAA** و التي أختلفت مئوية عن قيمة **(39)** من التوليفة **1.0** ملغم/لتر من **BA** مع **0.5** ملغم/لتر من أوكسين **2,4-D** .

و قد لوحظ بأن أعلى درجة لنشوء الكالس معبرا عنه بالوزن الجاف (**1.350** غم) و ذلك في نهاية المرحلة الثانية من إعادة زراعة الكالس بعد **ثمانية** اسابيع للتوليفة **0.6** ملغم/لتر من **BA** و **1.0** ملغم/لتر من أوكسين **NAA** وانخفض معدل نمو الكالس بعد ذلك، كما تم التقدير الكمي و النوعي للمركبات القلويدية في مستخلص أوراق النبات و أنسجة الكالس بأستعمال تقنية كروموتوغرافيا السائل ذات الأداء العالي **HPLC** .

اشارت النتائج الى تأثر نمو الاجزاء الورقية لنبات الـ ***Datura*** المزروعة في وسط **MS** ذات تراكيز مختلفة من السكر في تركيز القلويدات وفي محتواها الكلي حيث أزدادت و خصوصاَ (أتروبين و هايوسيامين و سكوبولامين عند معاملة السكر بتركيز **(S_1) 15** ملغم/لتر و كذلك مقارنتها بمعاملة المقارنة **(S_0)** .

و بصورة عامة , فأن تأثر نمو الأجزاء الورقية لنبات ***Datura*** عند وضعها في تراكيز ملحية مختلفة في تركيز القلويدات و محتواها الكلي و خصوصاَ في معاملة نصف قوة الملح **(X_1)** تلت الاتجاه نفسة بتراكيز السكر

بينما في حالة خفض قوة الملح الى النصف **(X_1)** فسببت زيادة في تركيز القلويدات مقارنة مع تأثير الملح المضاعف **(X_2)** و قوة الملح الطبيعية **(X_0)** .

و من الجدير بالذكر , أن تأثر نمو نسيج الكالس الـ ***Datura*** بظروف كثافات ضوئية متفاوتة على أربعة من القلويدات

(أتروبين , هايوسيامين , سكوبولامين و تروبين ، و محتواها الكلي , فقد وجد أن هنالك زيادة عند مقارنة **LI_1** (**1000** لكس وحدة ضوئية) مع معاملتي **LI_2** (**2000** لكس وحدة ضوئية) و **LI_3** (**3000**لكس وحدة ضوئية). على أية حال , فأن معاملة **(LI_2)** قد خفضت تركيز القلويدتين الأخيرتين (7هايدروكسى هايو سيامين و تيكلوهايوسيامين) (**22.155 و 28.250** مايكروغرام / غم مادة جافة) على التوالي, مقارنة مع تركيزات القلويدات نفسها تحت ظروف **(LI_1)** (**61.04 و 56.452** مايكروغرام / غم مادة جافة) و **(LI_3)** (**50.472 و 31.816** مايكروغرام / غم مادة جافة) على التوالي .

وبصورة عامة , عند مقارنة تراكيز أنواع القلويدات بأستثناء سكوبولامين في كالس ال ***Datura*** النامي في تراكيز مختلفة من السكر , والاملاح و تحت ظروف كثافات ضوئية مختلفة أيضاَ مع غيرها لأوراق النبات الأم النامية في البيت الزجاجي أعطت تركيزاَ مختلفاَ وكبيراَ لمعاملة الأضاءة **(LI_1)** و معاملتها مع السكر بتركيز **(S_1)** و تركيز ملحي نصف القوة **(X_1)** .

أما السكوبولامين فتبين بوضوح بأن المعاملة **(LI_2)** أدت الى انخفاض معنوي لتركيزها إلى **25.051** مايكروغرام / غم مادة جافة مقارنة بنسيج أوراق نبات الأم النامي في البيت الزجاجي. و بالنتيجة , فأن النتائج الحالية تشير الى نمو أوراق نبات الـ ***Datura*** في وسط **MS** عند تراكيز سكر و أملاح مختلفة تحت ظروف الكثافة الضوئية **(LI_1)** قد تعد أفضل أنواع وسط **MS** للنمو بغية الحصول على تراكيز عالية لكل أنواع القلويدات باستثناء سكوبولامين،