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Effect of dopamine, arginine, tyrosine and tryptamine on the production of mescaline in callus culture of *Trichocereus pachanoi* Britton and Rose (San Pedro)

Master Thesis

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Statutory declaration:

I hereby certify that I have elaborated my thesis independently, only with the expert guidance of my thesis tutor Ing. Iva Viehmannová, Ph.D. and my official partner Ing. Jindrich Rejthar from Czech University of Lifes Sciencies Prague, Faculty of Tropical AgriSciences. I further declare that all data and information I have used in my thesis are stated in the reference.

In Prague April 2014

……..……...…………………………………

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Abstract

The main aim of this thesis was evaluation the effect of mescaline precursors and elicitors on the production of mescaline in callus culture of *Trichocereus pachanoi*. Callus used for the experiment was obtained by regular subcultivations on MS cultivation medium containing 1 mg l^{-1} of 2,4-D and 1 mg l^{-1} of thidiazuron. This medium was also used for the experiment. Effect of dopamine, tryptamine, arginine and tyrosine at concentrations 100, 200 and 500 mg l^{-1} on mescaline production was studied. Calli were kept on media with precursors/elicitors for 2 and 4 weeks. Mescaline analysis was performed using HPLC. Dopamine at the highest concentration (500 mg l^{-1}) and applied for 4 weeks was the only treatment able to produce mescaline. The contents of mescaline were 0.413 x 10^{-8} mg kg^{-1} in fresh callus and 9.43 x 10^{-8} mg kg^{-1} in dry matter. The growth of calli was also observed. It was found that dopamine accelerated callus growth compared to control treatment. Media enriched with tryptamine and arginine shown even higher rate of callus growth as compared to dopamine, but they did not produce mescaline. Tyrosine had toxic effect on callus and no mescaline was in this callus detected. Rate of callus growth did not differ significantly among various concentrations within each substance treatment. This research provides the first results about mescaline production under *in vitro* conditions in *T. pachanoi* and clearly indicates that dopamine, as the closest chemical substance in metabolic pathway of mescaline, is the most effective in production of this alkaloid. The result also suggests that dopamine should be tested in further research in suspension cultures and organ cultures of *T. pachanoi*.

Keywords: biosynthesis of alkaloids, elicitors, *in vitro*, high performance liquid chromatography, mescaline, precursor, *Trichocereus pachanoi*

Abstrakt

Cílem diplomové práce bylo stanovení vlivu prekurzorů meskalinu a vybraných elicitorů na produkci meskalinu v kalusové kultuře kaktusu *Trichocereus pachanoi*. Kalusové kultury byly pro účely pokusu namnoženy na MS médiu s přídavkem 1 mg l^{-1} 2,4-D a 1 mg l^{-1} TDZ. Toto médium bylo rovněž využito pro založení pokusu. Byl testován vliv dopaminu, tryptaminu, argininu a tyrosinu o koncentracích 100, 200 and 500 mg l^{-1} na produkci meskalinu v kalusových kulturách pěstovaných na uvedených médiích 2 či 4 týdny. Stanovení meskalinu v rostlinném pletivu bylo provedeno pomocí vysokoúčinné kapalinové chromatografie (HPLC - high-performance liquid chromatography). Studie ukázala, že pouze aplikace dopaminu v nejvyší testované koncentraci (500 mg l^{-1}) po dobu 4 týdnů vede k produkci meskalinu. Obsah meskalinu byl v čerstvém hmotě stanoven na 0.413 x 10^{-8} mg kg^{-1} a na 9.43 x 10^{-8} mg kg^{-1} v sušině. Rovněž byl sledován vliv prekurzorů/elicitorů na růst kalusu. Bylo zjistěno, že dopamine urychluje růst ve srovnání s kontrolní variantou. Média s obsahem tryptaminu a argininu měla na tvorbu kalusu ještě výraznější pozitivní vliv než dopamin, avšak v těchto kalusech meskalin nebyl zjištěň. Tyrosin vykazoval toxický vliv na kalus. Meskalin v tomto kalusu rovněž nebyl detekován. Dále bylo zjištěno, že růst kalusu není výrazně ovlivněn koncentrací prekurzoru/elicitoru. Tato studie poskytuje prvotní výsledky o produkci meskalinu v *in vitro* kulturách druhu *T. pachanoi*. Rověž dokládá, že dopamin, jakožto chemicky nejbližší látka v metabolické dráze meskalinu, je nejefektivnější při tvorbě meskalinu *in vitro*. Tyto výsledky ukazují, dopamine je slibnou látkou při produkci meskalinu, a že by měl být aplikován v rámci dalšího výzkumu v suspenzních a orgánových kulturách *T. pachanoi*.

Klíčová slova: biosyntéza alkaloidů, elicitory, *in vitro*, meskalin, prekurzor, *Trichocereus pachanoi*, vysokoúčinná kapalinová chromatografie,

List of the abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
CULS Prague	Czech University of Life Sciences Prague
KIN	kinetin
MS	Murashige & Skoog (1962) medium
NAA	α -naphthaleneacetic acid
PGR	plant growth regulator
HPLC	high performance liquid chromatography
GLC-MS	combined gas-liquid chromatography with mass spectrometry
L-DOPA	L-dihydroxyphenylalanine
TYDC	tyrosine decarboxylase
4-HPA	4-hydroxyphenylacetaldehyde
BIAs	benzylisoquinoline alkaloids
ODC	ornithine decarboxilase
ADC	arginine decarboxilase
NCS	norcoclaurine synthase
AIH	agmatine iminohydrolase.
CPA	N-carmaboylputrescine amidohydrolase.
PMT	putrescine N-methyltransferase
PDA-LC	photodiode array detec
UV-Vis	ultraviolet/visible

TABLE OF CONTENT

Page

1. Introduction ... 1

2. Literature review ... 2

2.1 Taxonomy classification ... 2

2.2. Origin and general distribution ... 4

2.3. Botanical description and reproductive biology ... 5

2.4. Propagation ... 7

2.4.1. Generative propagation ... 8

2.4.2 Vegetative propagation ... 8

2.4.2.1. Propagation by cuttings ... 9

2.4.2.2. *In vitro* propagation of Cactaceae memebers ... 9

2.5. Chemical composition ... 11

2.5.1. Content of mescaline and other compounds in *Trichocereus pachanoi* ... 11

2.5.2. Biosynthesis and importance of mescaline ... 12

2.6. Tradicional uses ... 14

2.7. *In vitro* production of secondary metabolites ... 15

2.7.1. Importance and classification of secondary metabolites ... 15

2.7.2. Types of *in vitro culture* techniques for the production of secondary metabolites ... 17

2.7.2.1. Cell culture ... 17

2.7.2.2. Callus culture ... 18

2.7.2.3. Organ culture ... 18

2.8. Developed strategies to increase the content of secondary metabolites ... 19

2.8.1. Selection of cell lines ... 19

2.8.2. Use of elicitors and precursors ... 19

2.9. Dopamine, tyrosine, arginine and tryptamine in the biosynthesis of alkaloids 22
3. Objetives 26
3.1. General objectives 26
3.2. Specific objectives 26
4. Materials and methods 27
4.1. Plant material 27
4.2. *In vitro* propagation of plant material 27
4.3. Preparation of media for the experiment 27
4.4. Preparation and application of precursors/elicitors. 27
4.5. Establishment of the experiment 28
4.6. Mescaline analysis 29
4.6.1. Preparation of sample solutions 29
4.6.2. High performance liquid chromatography (HPCL) 30
5. Results 31
5.1. Effect of precursors/elicitors on callus growth and color 31
5.2. Analysis of mescaline in calli cultivated on media containing precursors and elicitors 33
6. Discussion 38
7. Conclusion 42
8. References 43

List of figures

page

Figure 1. Distribution of the genus *Trichocereus* 5

Figure 2. Some morphological characteristics of *Trichocereus pachanoi* 7

Figure 3. Chemical structure of mescaline in relation with the drugs amphetamine, epinephrine and norepinephrine.. 12

Figure 4. Biosynthesis of mescaline 14

Figure 5. The native pathway for the production of the BIA precursor (S)-norcoclaurine synthesized from two molecules of tyrosine... 23

Figure 6. Primary metabolism precursors... 24

Figure 7. Biosynthesis of N-methylputrescine from arginine and ornithine 24

Figure 8. Tryptamine as precursor of indole alkaloids 25

Figure 9. Different effects of precursors on callus growth in *Trichocereus pachanoi*............ 32

Figure 10. Mescaline content in fresh callus and dry matter under the effect of dopamine.... 34

Figure 11. Comparison between studied parameters in callus induced on 500....................... 35

Figure 12. Chromatograms of mescaline in callus cultures.. 36

Figure 13. Comparison of the HPLC chromatograms of the treatments 37

List of tables

Table 1. Solubility of the precursors/elicitors…………………………………………………...28

Table 2. General diagram of the experiment……………………………………………….29

Table 3. Rate of callus growing under the effect of different precursors after 4 weeks……...33

Table 4. Mescaline content in mg x 10-8 mg kg^{-1} of fresh callus after 2 and 4 weeks……….33

Appendix

1. Plant material, *Trichocereus pachanoi* (San Pedro)……………………………………….56

2. HPLC parameters…………………………………………………………………………57

1. Introduction

In vitro techniques are considered as an alternative source to the whole plant for the production of valuable compounds. Different *in vitro* strategies have been developed to increase the content of plant secondary metabolites. These strategies can be cell and callus culture, organ culture, cell immobilization, addition of precursors and elicitors, etc. (Naivy, 2011). Secondary plant compounds of commercial interest have been included in three main categories according to their biosynthetic pathways: terpenes, phenolic compounds and alkaloids (Costa, 2012). Alkaloids are a diverse group of compounds with about 4,000 known structures. These are physiologically active in humans (cocaine, nicotine, morphine) and of course of great interest in the pharmaceutical industry (Sajc *et al*., 2000). There are studies that show the successful production of alkaloids in callus culture and states that this method is suitable for enhancement of the production of alkaloids easier than other methods (Vanisree, 2004).

The large columnar cactus *Trichocereus pachanoi* Britton and Rose, also called San Pedro has been used for centuries in South America as the basis of a hallucinogenic drink (Schultes, 1970). San Pedro has an essential function in Andean folk medicine to cure diseases related to headache and many others related to the spiritual part (De Feo, 2002). The main alkaloid present in San Pedro is mescaline which is responsible for hallucinations caused when the drink is ingested. The alkaloid mescaline has been studied for years because of its strong effect on the psychic states of human subjects.

Recently there are not many reports available confirming cultivation of San Pedro for the commercial purposes. Biotechnological techniques may help to conserve this plant material and exploit its benefits. The addition of mescaline precursors or elicitors in cultivation medium may represent an attractive strategy to increase the content of mescaline in tissue cultivated under *in vitro* conditions.

Thus, the aim of this thesis is evaluation of four different precursor/elicitors, namely arginine, tyrosine, tryptamine and dopamine at three concentrations on mescaline production in callus culture of *Trichocereus pachanoi*.

2. Literature review

2.1 Taxonomy classification

The genus *Trichocereus* belongs to the subtribe Trichocereinae Buxbaum, which in turn belongs to the tribe Trichocereeae Buxbaum, subfamily Cactoideae (Buxbaum, 1958). Spegazzini (1896) was the first that described the genus *Trichocereus* but it was established by Riccobono (1909) who relied on the subgenus of the same name created by Berger (1905) that recognized *Trichocereus* as a genus based on the columnar stems and pilosity flowers by the description of two species which were *Trichocereus macrogonus* and *Trichocereus spachianus*. Above different species were almost included all in the giant genus *Cereus*, covering all of columnar stems cacti, others in *Echinopsis* genus due to the shape and hairiness of flowers and a few in other genus, such as *Echinocereus*, *Pilocereus*, etc. The first key to differentiate the species was established by Britton and Rose (1920) who recognized 19 species, and designating *Cereus macrogonus* as the type species. This genus was not modified, except for some new species added, until Backeberg (1949) created the type *Helianthocereus* for *Trichocereus* species with short flowers (about 13 cm), usually colorful and with day-opening flowers. Kiesling (1975) indicated that there are 50 species and 20 of them are endemic from Argentina but actually Torres (2008) showed that *Trichocereus* has 40 species. Kiesling (1975) also mentions that *Helianthocereus* corresponds to a subgenus of *Trichocereus*, because the characteristics that were used to distinguish these species presents wide variation and species are also so related, this makes it difficult to have only one reference about them, especially with *H. pseudocandicans* and *T. candicans.* Finally Friedrich (1974) subsumed *Trichocereus* under *Echinopsis*. It is based on the similarity of the flowers (those of *Echinopsis* are much narrower and looser hairiness) and existence of *Trichocereus* species whose stems are globose and *Echinopsis* with columnar stems (only two species, one of each is genus, with the exception characters). However, this opinion was not shared by a number of other authors such as Kiesling (1978), Ritter (1980), Lambert (2010), Schick (2011), and Albesiano and Terrazas (2012). Geographically *Echinopsis* and *Trichocereus* both overlap only marginally, because *Echinopsis* extends from the Atlantic to the Andes and *Trichocereus*

is almost exclusively from Andean extending outside in the area of Sierras Pampeanas, both in the southern half of South America (Kiesling, 1978). *Trichocereus* is also close to *Lobivia*, so some its species (the shorter flowers) were also included in that genus. A group of *Lobivia* species has stems extraordinarily big for that genus. The systematic position of these taxa is questionable, but it is closer to *Lobivia* than *Trichocereus* (Kiesling, 1978). But Friedrich (1974) considered them as "Series" within the subgenus *Trichocereu.* According to mentioned above the taxonomy and systematics of *Trichocereus* have been problematic, and acceptance of the genus has been questioned, as well as the number of its related species and groups.

Britton and Rose (1920), and Friedrich (1974) mention that *Trichoereus pachanoi* San Pedro belongs to the following taxonomic classification:

Kingdom: Plantae

Division: Angiosperms

Order: Caryophyllales

Family: Cactaceae

Sub family: Cactoideae

Tribe: Trichocereeae

Genus: *Trichocereus*

Species: *T. pachanoi*

Synonym: *Echinopsis pachanoi*

Common names:

According to Kvist and Moraes (2006):

San Pedro (General name) Achuma, Giganton, Huachuma (Ecuador, Peru, Bolivia), Wachuma, Agua-collo and San pedrillo (Ecuador and Peru), Cimarron and Huando (Peru), Olala and Curi (Bolivia).

2.2. Origin and general distribution

Cactaceae family is native to the Americas only. It was unknown prior to the discovery of America by Columbus represented by 100-200 genera and over 2000 species, typical of arid regions, but also habit in rainforests and cold-temperate areas (Kiesling, 2003; Cabral, 2010; Kiesling *et al*., 2011).

The genus *Trichocereus* expanded from the Andes of Ecuador, Peru, Bolivia, Chile and Argentina, reaching in this last country the extra-Andean provinces of Cordoba, La Pampa and Buenos Aires, as shown in the figure 1 (Britton and Rose, 1920; Shultes, 1976; Kiesling, 1975; Kiesling, 1978; Torres, 2008; Lambert 2010; Kiesling *et al*., 2011).

Britton and Rose (1920) and Backeberg (1944) were the first who reported the origin of *Trichocereus pachanoi*. They indicated that this species is widely cultivated in Ecuador and it is apparently native to Andes from regions of South America and occurs at altitudes of 2,000-3,000 m.a.s.l. This information was shared later by Schultes (1972) and Katarzyna *et al*. (2013) who stated specifically that the San Pedro is from Peru and Ecuador.

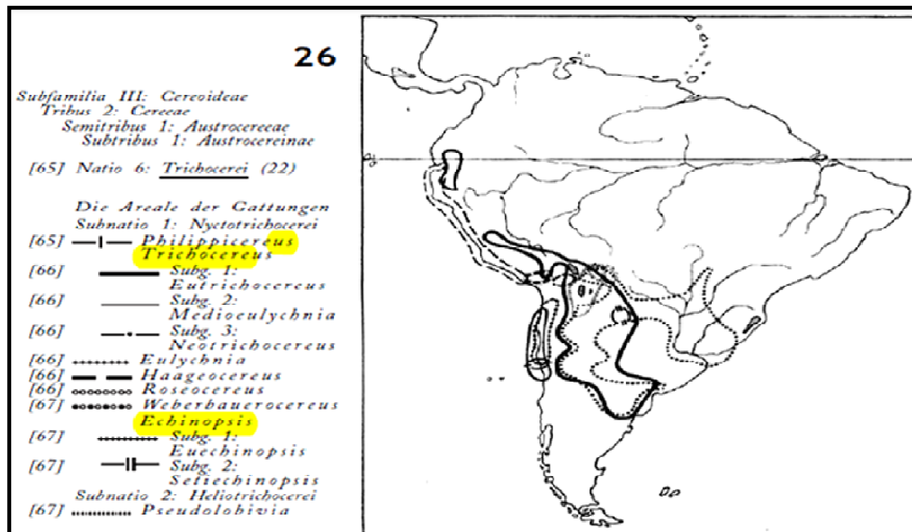


Figure 1. Distribution of the genus *Trichocereus* (Source: Backeberg, 1944)

2.3. Botanical description and reproductive biology

The genus *Trichocereus* are columnar plants up to15 m hight, with cylindrical stems, more or less branched; the branches can be from 6-26 cm light green, dark or gray, narrow, straight or slightly curved (Fig. 2C), the shallow ribs are short from 9-40 cm long and wide up to 5cm, they can be few to numerous, either low or prominent, usually very spiny. Areoles could be round, oblong or obovate, short (up to 10 mm high) and pannose with white, yellow, gray, brown or black hairs. They are also strongly woolly when young (Albesiano, 2012; Albesiano and Kiesling, 2012; Buxbaum, 1958; Lambert, 2010). Spines are terete flexible or rigide, they have brown tips and a light green base (Fig. 2A). Flowers are nocturnal, zygomorphic, ranging in shape from campanulate to infundibuli form, with large tepals from 8-30 cm long, funnel form usually white or whitish (Fig. 2B), or brightly colored in some taxa (Shick, 2011).They also are apical or subapical the perianth are persistent or separating from the fruit by abscission; perianth-segments are elongated; it has numerous stamens, filiform, arranged in two groups. Stigma-lobes are numerous; ovary (2-3 cm in diameter) and flower-tube (4-7 cm long) are bearing with numerous scales, from axils bearing with gray, or white long hairs, nectaries 3–4 mm in diameter, tubular; stamens white, 13–23 mm long, arranged in

two series; the first series distributed along the floral tube (Albesiano, 2012; Albesiano and Kiesling, 2012). Fruit is an umbilicate berry (globose to ovate –fleshy capsule) from 3–4 cm long and 3–5 cm in diameter, deshiscent for one longitudinal scars, yellow to orange and greenish when are ripen, covered with subulate scales, axils have abundant brown, gray or white hairs of 4–6 mm long; exocarp is dark and green, mesocarp and endocarp are white. Seeds are black to brown color with double testa (Kiesling, 1978). The cells of tegument are hexagonal or subhexagonal, with its sides more or less marked and perforations in their angles. Roots are fibrous (Buxbaum, 1958; Lambert, 2010).

On the other hand *Trichocereus* is a nocturnal genus whose period of anthesis (Fig. 2B) extends into the morning hours of the next day (Schick, 2011). Pollination has been found to be bimodal in *Trichocereus* genus. It first occurs from about dusk to midnight carried out by hawkmoths, then resumes during the following morning by insects, mainly bees. This has been shown by Schlumpberger and Badano (2005) in *Trichocereus atacamensis* subsp. *Pasacana*; also by Walter (2009) in *Trichocereus chiloensis* subsp. *chiloensis*; and Ortega-Baes *et al*. (2010) in *Trichocereus terscheckii*.

Badano and Schlumberger (2005) observed that flowers *of Echinopsis atacamensis* subsp. *pasacana* are xenogamous, therefore depending on animals to promote outcrossing (*Echinopsis* genus is a columnar cactus similar to *Trichocereus* genus). The white-pinkish flowers open in the evening and remain open during the next day (De Viana *et al*., 2001). These studies also showed that nectar production in the genus *Trichocereus* was highest at night but only diurnal bees were reported to visit the flowers (Badano and Schumberger, 2005).

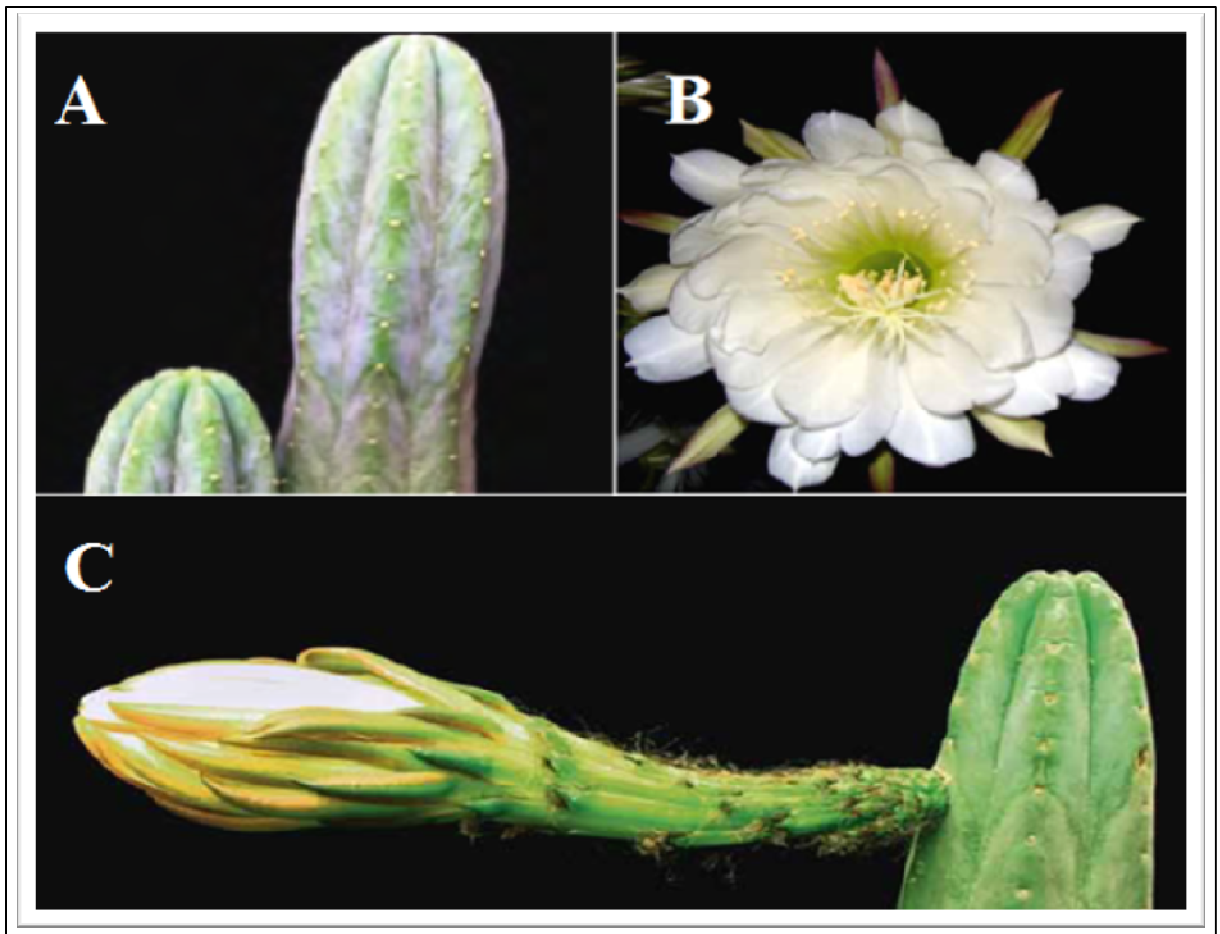


Figure 2. Some morphological characteristics of *Trichocereus pachanoi*, Britton and Rose: A) Flower with nocturnal habit, B) Stem covering with spines, C) Branches with flower buds. (Source: Ostalaza, 2011)

2.4. Propagation

Reyes (1994) mentioned that succulent plants are propageted by two main ways, sexual reproduction through seeds and vegetative propagation through buds, cuttings, stems, leaves and grafting.

2.4.1. Generative propagation

Reyes (1994) and Álvarez and Montaña (1997) stated that the method of propagation is the most important because most cacti and succulents produces lots of seeds and allows the production of thousands of plants with genetic variation, and it is a very important factor for a program of ecological restoration. Reyes (1994) also indicates one way how to propagate cacti by seeds which is immersing the seeds in sterile distilled water at 50 °C for 5 minutes and let cool to room temperature for 24 hours. Many seeds germinate without this treatment, but at a lower percentage. In the case of other succulents the pregerminative treatment (soaking) is not necessary because the seeds are too small.

Most seeds need three factors. Water, temperature and light for germination (Vasquez *et al.,* 1995). Maiti *et al.* (1994) developed a simple technique to induce germination of several species of cacti. The technique is to germinate seeds in Petri dishes with fine sand and organic material within a growth chamber with a photoperiod of 14 hours. This treatment is supporting by Gómez (2002), which is for to break the dormancy of seeds.

Other similar method was proposed by Kelly (2009) who mentions the seed should be sown in shallow wide containers in a soil mix that assures good drainage, provides air and some organic matter to hold nutrients and moisture. Mixes should contain 50% inorganic material such as perlite or pumice. The other half of the mix should be either sphagnum peat or well composted organic material. Animal manures should be avoided because it may inhibit seedling establishment. The appropriated night time temperatures is 18–21 °C this rank is conducive to good germination and growth.

2.4.2 Vegetative propagation

Kelly (2009) mentions that vegetative propagation relies on the plant ability to produce new roots and shoots from an existing part of the plant such as a stem or leaf.

2.4.2.1. Propagation by cuttings

As many other plants, *Trichocereus pachanoi* can be propagated by cuttings. The result is a genetic clone of the parent plant cuttings (Verne, 2004). Reyes (1994), Kelly (2009) and López (2006) stated that cuttings are the easiest method of asexual propagation. The stems of cacti are cut into pieces and putted in a dry place to wait them to heal and planting them in the substrate.

Verne (2004) describes one example of propagation by cuttings and he recommended that the cutting must be 15 cm long and it is preferable the end part of a cactus column. This can be cut off with a knife, then the cutting can be left to dry for about two weeks in the shade, or in a dry place. This is so that the surface of the cut end dries to keep the cuttings out microbes such as fungi and bacteria. The cutting can be dipped in rooting hormone (optional, but effective) and planted on the surface of or buried to a maximum deep of 2.5 cm in good propagation media mixed with some sand and perlite. The sand and perlite will ensure that the media will drain and not stay too wet. The cutting is kept in the shade or indirect sunlight, so that the root system can develop and the cactus does not grow too thinly.

2.4.2.2. *In vitro* propagation of Cactaceae memebers

Plant tissue culture represents a viable alternative for the propagation and *ex situ* conservation of cacti and acquires greater value if they are in a category of threat. The micropropagation techniques have been applied to various members of the Cactaceae family with significant progress. Many of these studies have been conducted in various countries in Europe, United States of America , Japan and others, with Mexican species several years ago (Smith *et al*., 1991; Stuppy and Nagl, 1992; Machado and Prioli, 1996; Papafotiou *et al*., 2001; Giusti *et al*., 2002). *In vitro* techniques have the potential to produce many plants in a short time in minimal space and their success has already been demonstrated for several cacti species. Micropropagation by axillary shoot proliferation is primarily applied for the conservation of rare and endangered species (Ramirez-Malagon *et al*., 2007). In addition, the aim can be the mass propagation of plants for commercial purposes (Pérez-Molphe-Balch *et*

al., 2002; Sánchez-Martinez and Hernandez-Martinez, 2002). Somatic embryogenesis-based plant regeneration has also been reported in several cactus species (Santacruz-Ruvalcaba *et al*., 1998). Callus cultures were used as target tissues to produce transgenic plants in economically important cacti (Silos-Espino *et al*., 2006). This technique can overcome certain limitations associated with conventional propagation of rare cacti and can be excellent tool for cacti micropropagation and has been used on species especially in the genera *Opuntia* (Garcia *et al*., 2005). It has been described that auxins affect development, cell growth, embryo formation and callus induction in different species. Kinetin (KIN) and benzylaminopurine (BAP) are synthetic cytokinin commonly used in plant cell culture to generate somatic embryogenesis, organogenesis and callus formation in cacti (Shedbalkar *et al*., 2010). Seeman *et al*. (2007) realized one study on micropropagation of *Trichocereus* genus. It had been proved that the use of 0.1 mg $l^{-1}$of α-naphthaleneacetic acid (NAA) and 1.0 mg l^{-1} of BAP is excellent for the formation of buds. No callus was obtained.

Addition of amino acids (proline and glutamine) and organic supplements (carbohydrates and peptone) also have a promoting effect on *in vitro* culture development stimulating cell proliferation. Vitamins, like biotin improves the physiological response of *in vitro* cultured recalcitrant species (Al-Khayri, 2001). An additional advantage of micropropagation is that *in vitro* development of cactus plantlets can be extremely rapid in comparison with *ex vitro* cultured seedlings (Ault and Blackmon, 1987; George, 1996). Malda *et al*. (1999) showed that *in vitro* cultured plants of *Coryphantha minima* grew more than plants cultured under similar *ex vitro* conditions. This may be due to high humidity, plant growth regulators, and high sugar concentration in the culture media. *In vitro* propagation of Mexican species of cacti has been demonstrated for *Mammillaria san-angelensis* (Martínez-Vázquez and Rubluo, 1989), *M. candida* (Elias-Rocha *et al*., 1998), *Obregonia denegrii* and *Coryphantha minima* (Malda *et al*., 1999). In a previous study, Pérez-Molphe-Balch *et al.* (1998) developed systems for micropropagation of 21 species of Mexican cacti belonging to the genera *Astrophytum, Cephalocereus, Coryphantha, Echinocactus, Echinocereus, Ferocactus, Mammillaria, Nyctocereus,* and *Stenocactus*. Many different media and hormones have been tested for cacti propagation, but only in few cases the concrete protocol have been useful for more than one species (Mauseth, 1979; Clayton *et al*., 1990). Several research

papers on the micropropagation of Cactaceae can be found in the literature, but *in vivo* acclimatization of the plantlets obtained is not always reported, and in some cases this is shown to be critical in phase of micropropagation (Rubluo *et al*., 1993; Rodriguez-Garay and Rubluo, 1992; Smith *et al*., 1991). Nevertheless, for conservation purposes it is crucial that micropropagated plants are re-established in the wild and complete their life cycle (Rubluo *et al*., 1993).

2.5. Chemical composition

2.5.1. Content of mescaline and other compounds in *Trichocereus pachanoi*

Schultes (1976) mentions that San Pedro has the same main alkaloid found in Peyote (*Lophophora williamsii*), which is mescaline (3,4,5-trimethoxy-phenethylamine) responsible for the visual hallucinations caused by both species of catci. Mescaline has been isolated not only from San Pedro but also from another species of *Trichocereus* as was desmonstrated in the realized experiment by Retti (1947) who found one enzime called tyrosinase which is responsible for the process of mescalinization in animal and plants. He also found mescaline in plants of *Trichocereus candidans* and *Trichocereus terchecki*. Other report was carried out by Agurel (1969) who identified mescaline from 12 species of *Trichocereus: T. bridgessi*, *T. comarguensis, T. candidnas, T. chilensis, T. lanprochlorus, T. macrogonus, T. pachanoi, T. peruvianus, T. schickendantzii, T. spachianus*, *T. terscheckii* and *T. werdermannionus*.

In addition the same author Agurel (1969) combined gas-liquid chromatography with mass spectrometry (GLC-MS) and identified in the plant of *Trichocereus pachanoi*, apart of mescaline, traces of alkaloids as tyramine (substance found in the brain to communication of neurons), hordenine (cardiovascular stimulant), 3-methoxy tyramine, 3,4-dimethoxy-S-phenethylamine, 3,4-dimethoxy-4-hydroxy-/3-phenethylamine, 3,5-dimethoxy-4-hydroxy-/3-phenethylamine, and anhalonidine (central nervous system depressants). According to Kowalczuk (2012) in *T. pachanoi* can be found 5% of mescaline in parenchyma calculated on dry material basis and 0.8% in the whole plant (Ogunbodede *et al*., 2010). On the other hand

the content o mescaline in *T. pachanoi* can be variable, depending of edafoclimatic conditions (origin), age, and the efficiency of isolation and identification process of alkaloids. This analysis was verified in the studies realized by Ogunbodede *et al*. (2010) who determined the mescaline content of *T. pachanoi* plants in Peru, the values varied between 0.54 and 4.7% (Matucana-Peru), and surely could be contrasted for other authors.

2.5.2. Biosynthesis and importance of mescaline

Mescaline is the found in key hallucinogenic substance peyote (*Lophophora williamsii*) and several other psychedelic plants, including the *Trichocereus* genus from *Cactaceae* family. Mescaline belongs to a class of chemicals called phenethylamines (Olive, 2007). Mescaline was discovered in 1960 by Turner and Heyman as the plant's major alkaloid, in the cactus *Opuntia cylindrica* (Turney and Heyman, 1960). The chemical name of mescaline is 3,4,5-trimethoxy-phenethylamine, and its chemical structure resembles that of other illegal drugs such as amphetamine as well as the brain's own neurotransmitters epinephrine (adrenaline) and norepinephrine as shown in the figure 3 (Olive, 2007).

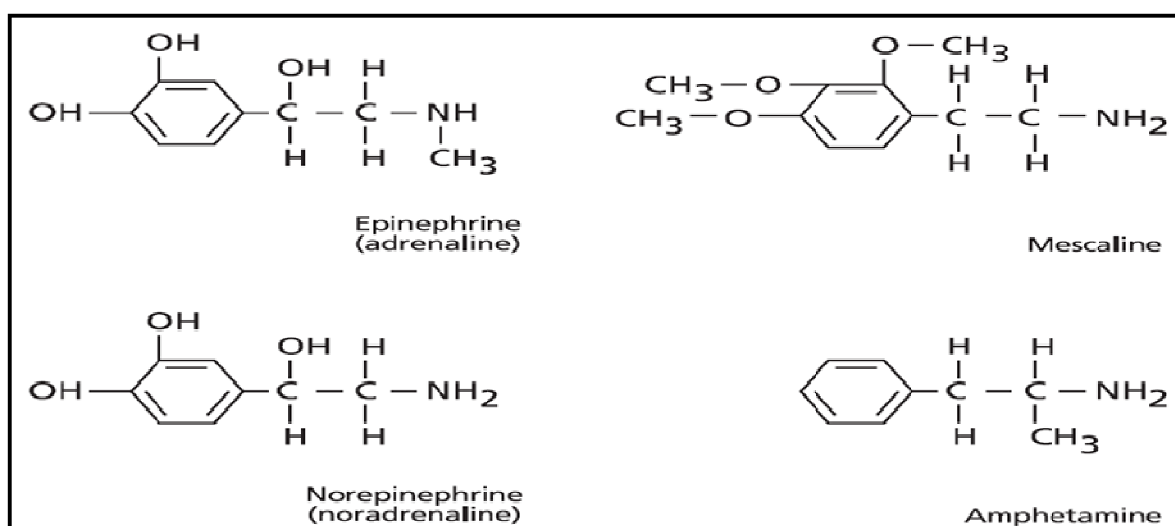


Figure 3. Chemical structure of mescaline in relation with the drugs amphetamine, epinephrine and norepinephrine (Source: Olive, 2007)

Olive (2007) describes the effect of mescaline in the human body apart of the psychedelic effects, these are: increased heart rate and blood pressure, increased patellar reflex of the knee, dilation of the pupils, increased perspiration, increased motor activity and “fidgeting”, changes in posture or difficulty walking, changes in body temperature, changes in levels of sugar, potassium, and white blood cells in the blood. One example of the use of mescaline was found during the Second World War, many prisoners of war were given hallucinogens in attempt by their Nazi captors to gain mind control and extract information. In this case the mescaline acted as a hypnotizer (Olive, 2007).

On the other hand, the mechanism of action of mescaline has not yet been fully clarified, but there are some known mechanism such as alteration of normal metabolism such a way as to cause hallucinations and mescaline-protein complex that is responsible for the characteristic central effects (Kovacic and Somanathan, 2009). Recently it has been observed that mescaline (similarly to other hallucinogens) acts as a partial agonist 5-HT2A receptors for serotonin, the stimulation of which leads to an increased release of glutamate in the cortex brain which, in turn, appears to be responsible for the cognitive distortions, caused by the perceptual and affective compound (Aghajanian and Marek, 1999).

Malits (1966) mentions the effects of hallucinogens such as mescaline and others compounds and he states that they have a unique property of evoking a number of powerful perceptual distortions such as visual hallucinations, illusions, and disturbances of body image and synesthesia with minimal disturbances in orientation or consciousness. The same author describes the possible therapeutic benefits of hallucinogens from the psychiatric point of view. There are three major areas:

- Abreaction: is a psychoanalytical method for reliving an experience in order to purge it of its emotional excesses; a type of catharsis. This method with sodium was used during the World War II.
- Intensification of the transference: is the most important factor in facilitating psychotherapy with hallucinogens.
- The recovery of repressed material, particularly childhood experiences.

Dewick (2002) characterized the mescaline biosynthesis in the cactus *Lophophora williamsii* (Cactaceae) with the parallel biosynthesis of tyramine, horenine, dopamine, noradrenaline and adrenaline all of these alkaloids derivate from phenylalanine and tyrosine aminoacids. The first step of this process start when phenylethylamine derivatives possess 3,4-di- or 3,4,5-tri-hydroxylation patterns, are derived via dopamine, by decarboxylation product from L-DOPA (L-dihydroxyphenylalanine) (precursor of the mescaline) then the aromatic hydroxylation and O-methylation reactions convert dopamine into mescaline (Fig 4). This description is similar to the characterization of Spivey (2007) about mescaline biosynthesis.

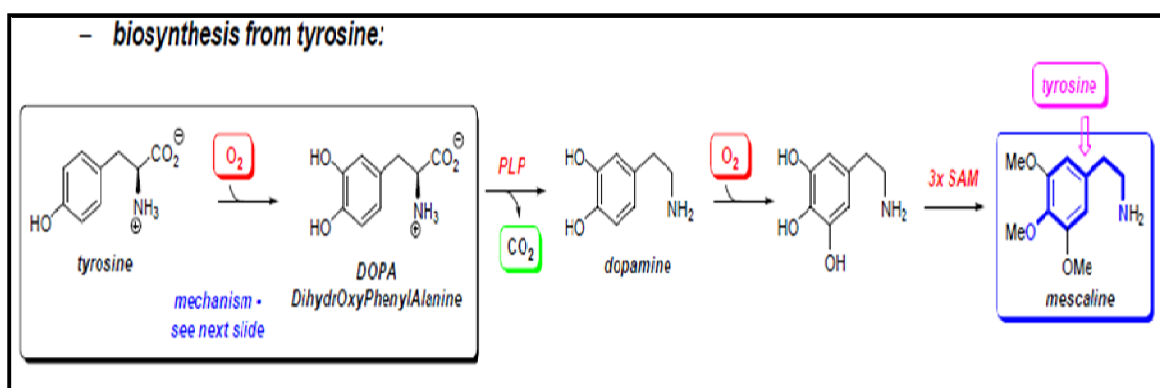


Figure 4. Biosynthesis of mescaline (Source: Dewick, 2002 and Spivey, 2007)

Acosta (2008) also described the same route mentioned by Dewick (2002) and Spivey (2007) of biosynthesis of mescaline and he also states that L-DOPA is the direct precursor of dopamine and other compounds such as mescaline, ephedrine and others.

2.6. Tradicional uses

Trichocereus pachanoi San Pedro has a long history of being used in South America for longer time. Archeological studies have found evidence of use going back two thousand years, the representations of the cacti have been found in Chavin engraved stones and textiles, Nazca, Moche and Chimú ceramics (Bussmann and Sharon, 2006). According to De Feo (2002), *T. pachanoi* has an essential function in the Andean traditional medicine. San Pedro importance is closely related to mescaline, because it is the main hallucinogen compound. The use of San Pedro, together with additives like Angel's-Trumpet (*Brugmansia spp*.), Jimson-weed (*Datura*

ferox), and tobacco, is still the central part of the curing ceremonies of healers in Northern Peru (Bussmann and Sharon, 2006). Schultes (1976) mentions that one of the ancient traditional uses of the native people is the Cimora which is an intoxicating drink made from the San Pedro cactus. The preparation consist in to cut the stems like loaves of bread and then boiled in water for several hours, sometimes with superstitious objects such as cemetery dust and powdered bones. The same author also mentions that Cimora is the basis of a folk healing ceremony that combines ancient indigenous ritual with imported Christian elements. The author also shows one observation about the plant indicating it as catalyst that activates all the complex forces at work in a folk healing session, especially the visionary and divinatory powers of the native medicine man. Feo (2002) realized a study in the Northern Peruvian Andes and he made a description of preparation of San Pedro drink which consists in the decoction of rounded pieces of stem or trunk in water and then they are boiled for several hours, until the volume of water is reduced to 1/4 of the initial volume. The dosage by mouth is 3 cups of cooled decoction during the ritual ("mesada") and may be given only by the "curandero." The same author also mentions that decoction of the plant is used with some ritual purposes: such as shamanistic diagnosis of the illnesses, divination in past and future times, location ("rastreo") of peoples and things lost in time and/or in the space and prescription of ritual therapies.

2.7. *In vitro* production of secondary metabolites

2.7.1. Importance and classification of secondary metabolites

Secondary metabolites are compounds derived from primary metabolism but of limited distribution in the plant kingdom, restricted to a particular taxonomic group (Shilpa *et al.*, 2010). Formerly it was accepted that secondary substances were produced with nonspecific relating functions, after it was found that many of these have high yields and have multiple roles in plants (Wink, 2007). The secondary compounds have no apparent function in primary metabolism but they have an ecological implication as a defense against herbivores, viruses, fungi and bacteria, as allelopathic substances, phytoalexin or dietary deterrent (Bourgaud *et*

al., 2001). Others have a physiological function, such as alkaloids, pectins that may serve to transport toxic nitrogen and storage compounds, while the phenolic compounds such as flavonoids realized the function of UV protectors (Wink, 2007). They are also an important source of active ingredients of drugs and valuable chemicals (Goossens *et al*., 2003). Although most of the drugs are obtained by chemical synthesis; most of the main structures are based on natural products. Globally there are 44% of new drugs based on natural products and in developed countries 25% of medicines are derived from plants (Haq, 2004). Such is its importance that approximately 60% of anticancer compounds and 75% of infectious diseases drugs are natural products or their derivatives (Cragg and Newman, 2005). There are many examples that demonstrate its use. Among them can be mentioned the isolated alkaloids of *Physostigma venenosum* used for constrict the pupil, unlike isolated alkaloids from *Atropa belladonna* that engorged them; cardiac glycosides of *Strophantus* species are effective as heart stimulants. Similarly, aspirinis used as an analgesic and to prevent heart attacks and blood clots is a derivative of salicylic acid and it is found naturally in the genus *Salix*. Secondary plant compounds of commercial interest have been included in three main categories according to their biosynthetic pathways: terpenes, compounds phenolic and nitrogen compounds (Chinou, 2008). Terpenes are formed by polymerization of isoprene units and steroids (Sarin, 2005) and divided into six groups: monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes sterols, within which carotenes, cardiac glycosides, taxol are among others (Shilpa *et al*., 2010). Phenolic compounds phenolic acids, coumarins, flavonoids and tannins are included. Pharmaceutical applications of these compounds are considerable concern about its effects as analgesic, antibacterial, antihepatotóxicos, antioxidant, antitumor, immunostimulant, among others (Gurib-Fakim, 2006). Nitrogen compounds are mainly alkaloids and cyanogenic glycosides. Moreover, cyanogenic glycosides are considered possibly secondary metabolites ratio greater defense functions (Bennett and Wallsgrove, 1994). A large group of natural products have also been ranked by application. These include pharmaceuticals that are grouped alkaloids (vincristine, vinblastine, ajmalicina atropine, berberine, codeine, reserpine, nicotine, camptothecin), cardenolides (digitoxin, digoxin), diterpenes (paclitaxel); flavor and flavonoids as stevioside, quinine, products used as pigments and perfumes such as anthocyanins, betalains, oil of roses and jasmine and end

products used for chemicals and agrochemicals purposes as proteases, vitamins, lipids, oils, latex , etc (Ramachandra and Ravishankar , 2002).

2.7.2. Types of *in vitro culture* techniques for the production of secondary metabolites

2.7.2.1. Cell culture

The advantages of cell culture, specifically cell suspensions, are to allow a similar operation to that performed with microorganisms, rapid cell division and can perform scaling by new techniques such as bioreactors (Vanisree *et al*., 2004). However, not all compounds are produced in undifferentiated cell in equal quantity and quality than those obtained from the mother plants. This is because many metabolites are integrated synthesized in differentiation events (Kreis, 2007). Various authors have reported the identification of cell lines that can produce metabolites in amount to equal or greater than that produced under natural conditions. Also new substances have been detected, which are not synthesized by plants in their natural habitat (Massot *et al*., 2000; Capote *et al*., 2008), so it is stated that the cultivation of cell lines is a technology of great importance for the development of new secondary metabolites. The production of secondary metabolites in cell cultures, mainly cell suspensions has materially and comprehensively in the production of bioactive substances (Vanisree and Tsay, 2007). The development of cell culture systems aims to obtain a high yield of the compound, high productivity and high product performance through novel techniques of biosynthesis. The main problems associated with commercial production are low productivity, instability of cell lines and the difficulty for scaling production (Zhong, 2001). A relevant example of the use of cell suspension culture is taxol production. Associated with low productivity and commercial value importance has stimulated the development of multiple researches. Authors as Ketchum *et al*. (1999) and Wang *et al.* (2001) have reported that paclitaxel and other toxoids obtained from different tissue culture systems such as calluses, cell suspensions, buds, roots and embryogenic cultures in different species of *Taxus*. The suspension culture is considered the most viable for obtaining taxol method but the values obtained are still low for commercial production (Cusidó *et al*., 2002).

2.7.2.2. Callus culture

Plant tissue cultures have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2002). Actually, callus tissues and suspension cultures are used widely for production of plant secondary metabolites (Acemi, 2012). Salma *et al*. (2008) mentions that callus culture facilitated the optimization of alkaloid production, whereas media composition was effective for the callus induction so as to enhance the alkaloid production and conservation of threatened genotype. There are several studies where it is described the use of callus culture for the production of secondary metabolites.

Capote *et al*. (2008) found in the callus culture of *Morinda royoc* the greatest number of identified compounds, which were not detected in the leaves of plants in the field or *in vitro* shoot. Among the compounds identified in callus culture is the morindin which is disaccharide derivative of anthracenedione (aromatic organic compound), a specific compound of the genus *Morinda*.

Teramoto and Komamine (1988) induced callus tissues of *Mucuna hassjoo, M. pruriense, and M. deeringiana* and optimized the culture conditions. The highest concentration of L-DOPA was obtained when *M. hassjoo* cells were cultivated in MS medium with 0.025 mg l^{-1} of 2.4 -D and 10 mg $l^{-1}$of kinetin.

2.7.2.3. Organ culture

Kreis (2007) mentions the aspects which may be associated with the accumulation of secondary metabolites. These are: the presence of certain cell types, the presence of certain organelles and the expression and regulation of catabolic or biosynthetic genes. Therefore, organ culture represents an interesting alternative for the production of plant secondary metabolites. Two types of organs are considered most important: the shoots and roots (Bourgaud *et al*., 2001.). These organs can be grown on a large scale. The shoot culture has been investigated as sources of essential oils, alkaloids and flavonoids. For example, according Wilken *et al*. (2005) cultivation of shoots of *Lavandula officinalis* in semisolid

culture media showed rosmarinic acid content higher than that obtained in plants under natural conditions. However, other species such as *Hypericum perforatum, Fabian imbricata* and *Cymbopogon citratus* the desired concentrations of compounds were lower than those obtained in plants under natural conditions. The most used of root culture is based on the infection of the tissue with *Agrobacterium rhizogenes* method which is developing in aerial roots at the site of infection (Kreis, 2007). These aerial roots have the advantage of rapid multiplication and require exogenous application of growth regulators (Giri and Narasu, 2000). Several investigators have compared the cultivation of transformed and untransformed roots regarding productivity and growth and showed clear evidence that confirms the assumption that the roots transformed generally have rapid growth and faster production of compound desired.

2.8. Developed strategies to increase the content of secondary metabolites

2.8.1. Selection of cell lines

Once established cell culture, a continuous process is observed epigenetic or genetic changes, which causes the population is heterogeneous. That is why it is necessary to select clones with high growth and high production of metabolites of interest (Shilpa *et al*., 2010). The cell lines were obtained by selection using following strategies that include the microscopic examinations, macroscopic and chemical (Kreis, 2007). Selection of cell lines has been a favorable strategy for obtaining pigments in cultures of *Lithospermum erythrorhizon* where clones were found with a high increase of shikonin (Fujita *et al*., 1984). However, this approach has not been successful in investigations such as cell culture of *Papaver somniferum* to produce significant quantities of codeine (Park *et al*., 1992).

2.8.2. Use of elicitors and precursors

Elicitors are compounds stimulating any type of plant defense (Radman, 2003). This broader definition of elicitors includes both substances of pathogen origin (exogenous

elicitors) and compounds released from plants by the action of the pathogen (endogenous elicitros) there are aboitic and biotic or physical and chemical elicitors. Elicitors could be used to enhance plant secondary-metabolite synthesis and could play an important role in biosynthetic pathways to enhanced production of commercially important compounds (Radman *et al*., 2003). Elicitation is one of the most effective methods established processes to large scale to induce the expression of genes associated with enzymes responsible of synthesis of secondary metabolites as a defense mechanism against attack of pathogens or damaged in plants (Namdeo, 2007; Baldi *et al*., 2007). Among the physical elicitors belong water deficit, salinity, extreme temperatures, excessive or insufficient light radiation, and anaerobiosis by waterlogging or flooding, mechanical factors such as wind or compaction soil and injuries. Chemicals include ionic stress (salinity), stress nutrition, or presence of inorganic contaminants (silicon dioxide, ozone or heavy metals) or organic (chlorofluorocarbons, polychlorinated biphenyls and polycyclic aromatic hydrocarbons). Abiotic stress is the most common and usually a combination of several of them occurs (Pérez and Jiménes, 2011). Elicitors which are frequently used in the *in vitro* culture with the aim increase the production of secondary metabolites include methyl jasmonate (Ketchum *et al.*, 1999; Wang and Zhong, 2002; Kim *et al.,* 2004; Roat and Ramawat, 2009), oligosaccharides (Prakash and Srivastava, 2008; Korsangruang *et al.*, 2010.), and fungi extracts (Namdeo *et al.*, 2002; Kim *et al*., 2004; Xu *et al.,* 2005; Arora *et al.,* 2010). Most research studies related to the use of elicitors has been developed in cell culture. To date, only a few researchers have established the use of elicitors in organ culture (Spollansky *et al.,* 2000; Kim *et al*., 2004; Liu *et al*., 2007; Orlita *et al*., 2008; Sakunphueak and Panichayupakaranant, 2010). Królicka *et al*. (2001) describe an increase of umbelliferone in the *in vitro* culture of callus, cell suspensions and roots of *Ammi majus* by abiotic elicitors (silicon dioxide and jasmonic acid) and biotic elicitor extracts of bacteria *Enterobacter sakazakii* and *Erwinia chrysanthemi*. Luo and He (2004) show a maximum of 54 mg l^{-1} of paclitaxel produced in *Taxus chinensis* cell suspension by adding a combination of different elicitors such as methyl jasmonate, silver nitrate, chitosan, precursors as phenylalanine, glycine, and was an increase from the crop does not elicited. Other studies show the positive effect of saccharin and benzotidiazol (chemical function similar to salicylic acid) in the biosynthesis of coumarins and alkaloids in *Ruta graveolens* shoots (Orlita *et al*., 2008). Studies in cell culture of *Trifolia cayratia* revealed the effect of salicylic acid, methyl

jasmonate, ethrel (growth regulator) and yeast extract in the production of polyphenols (Roat and Ramawat , 2009). The results showed an increase in the production of polyphenols with salicylic acid and methyl jasmonate. Korsangruang *et al*. (2010) demonstrated the effect of biotic and abiotic elicitors in accumulation of isoflavonoids in two varieties of *Pueraria candollei*. Methyl jasmonate was the elicitor that increased significantly the content of metabolite of interest.

On the other hand metabolic precursors have been commonly applied to the culture medium to enhance the production of secondary metabolites in plant cells (Prakash, 2002). Precursor is an intermediate compound (endogenous or exogenous), which can be converted into desired secondary metabolite. Precursors can be classified as natural (nonmember of the biosynthetic pathway) or obligatory (member of the biosynthetic pathway leading to the synthesis of desired secondary metabolite).The addition of a precursor may influence spatial orientation of enzymes, compartmentation of enzymes, and substrate accumulation for secondary metabolite biosynthesis (Srivastava, 2010).

Addition of precursors represents an attractive strategy for exploiting the potencial biosynthetic of these cells in culture enzymes. Precursors such as phenylalanine, benzoic acid or serine have been used to increase the accumulation of taxol in callus culture and cell suspensions in *Taxus cuspidata* (Fett-Neto *et al*., 1994). Studies combining precursors also have been used in cultures where estrictosidina of *Camptotheca accuminata* was biotransformed but levels of camptothecin were not readily detected (Silvestrine *et al*., 2002). Furthermore, in shoot culture of *Hypericum perforatum* have been used several precursors in order to increase the content of hypericin, and hyperforin (Liu *et al*., 2007). The results showed that hypericin biosynthesis was stimulated by L-phenylalanine but only at high concentrations favored synthesis of hyperforin while tryptophan decreased the content of interest metabolites. These results demonstrated that the secondary metabolites can be modulated by the addition of precursors of their biosynthetic pathways.

2.9. Dopamine, tyrosine, arginine and tryptamine in the biosynthesis of alkaloids

According to Speltz (2011) dopamine and 4-hydroxyphenylacetaldehyde (4HPA) are the primary metabolite precursors of many alkaloids in *Beta vulgaris*. Dopamine and 4-HPA are also primary metabolites used in the production of alkaloids in many related species. In this regard, fluorinated dopamine analogues were chosen as target precursor analogues for the production of fluorinated berberine, as well as any other alkaloids produced by *B. vulgaris*. Dopamine represents an opportunistic primary metabolite because it is used by diverse families of plants to generate many secondary metabolites.

Battersby *et al*. (1968) have reported the incorporation of tyrosine into pellotine, lophophorine and anhalonidine. Dopamine was incorporated into pellotine and that the methyl group of methionine served as a precursor of the O-methyl groups.

Leete and Braunstein (1969) have also shown that tyrosine acts as a precursor of anhalonidine. This report is realted to Lundstrom and Agurell (1969) who reported the incorporation of methyl group of methionine into the O-methyl groups of anhalonidine and anhalamine. Their data further demonstrate that tyrosine (0-34%), dopamine (1.73%) and 3,4,5-trihydroxyphenethyhtmine (1.73%) serve as efficient precursors of anhalamine.

Hawkins (2009) realized one study about the synthesis of benzylisoquinoline alkaloids (BIAs) where shows that their metabolic pathways are derived from tyrosine molecules and also indicates that dopamine is the precursor of isoquinoline moiety responsible of the chemical reactions during the biosynthesis of BIAs. According the description of the author the complex biosynthesis of BIAs in plants begins with the condensation of the backbone structure derived from two molecules of tyrosine. Along one branch, tyrosine is converted to dopamine and a second molecule of tyrosine is converted to 4-hydroxyphenylacetaldehyde (4-HPA) along a second branch. Dopamine is the precursor for the isoquinoline moiety while 4-HPA is incorporated into the benzyl component (Fig. 5). Despite extensive investigations of *Papaver somniferum*, *Eschscholzia californcia, Thalictrum flavum, Coptis japonica* and other BIAs producing plants, only two enzymes have been isolated from these very early steps involved in BIAs biosynthesis (Ziegler and Facchini, 2008). The earliest enzyme cloned in this

pathway is tyrosine decarboxylase (TYDC) which catalyzes the decarboxylation of tyrosine to tyramine or dihydroxyphenylalanine (L-DOPA) to dopamine. The other cloned enzyme in this upstream pathway is norcoclaurine synthase (NCS) which catalyzes the condensation of dopamine and 4-HPA, the first committed step in BIAs biosynthesis. These enzymes can be incorporated into a recombinant pathway but the remaining activities must be reconstituted using enzymes from other organisms.

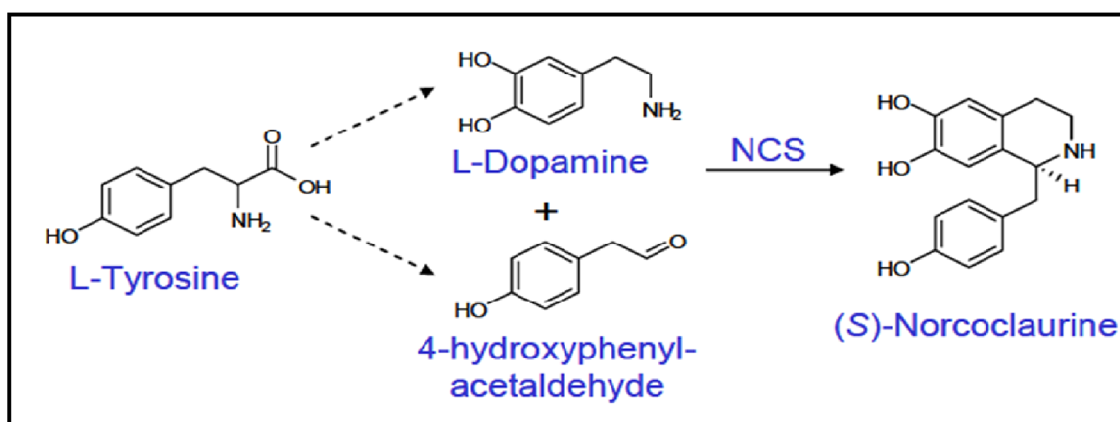


Figure 5. The native pathway for the production of the BIA precursor (S)-norcoclaurine synthesized from two molecules of tyrosine (Source: Hawkins, 2009)

Other experiments conducted by Alvarez and Marconi (2001) show the presences of arginine as part of the metabolic pathway of tropan alkaloids. They indicate that tropan alkaloids are derived from the ornintine and arginine aminoacids by a chemical reaction catalyzed by ornithine decarboxilase (ODC) and arginin decarboxilase (ADC) respectively (Fig. 6). Ornithine and arginine also are the precursors of the polyamines putrescine, espermidine and espermine, which play a critical role in plant development by the regulation of the cell division in plants (Theiss *et al*., 2002).

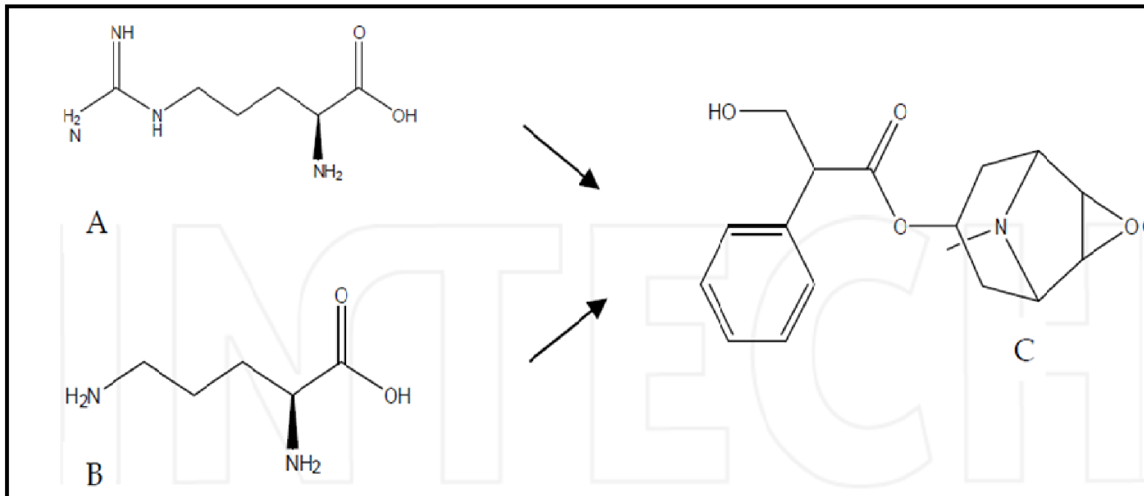


Figure 6. Primary metabolism precursors: arginine A) and ornithine B) and the tropane alkaloid final product scopolamine C) (Source: Alvarez and Marconi, 2011)

In other study, Walton *et al*. (1990) describe that two amino acids, ornithine and arginine, are involved in the biosynthesis of putrescine by alternative pathways (Fig. 7). The decarboxylation of ornithine yields putrescine directly, whereas arginine has to be transformed into agmatine to produce putrescine (Fig. 7).

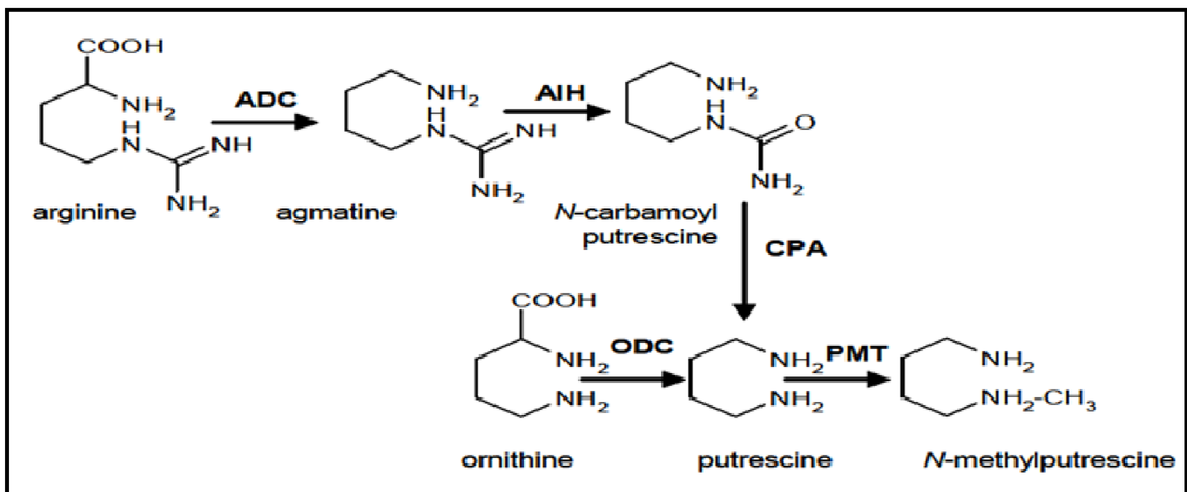


Figure 7. Biosynthesis of N-methylputrescine from arginine and ornithine. ADC: Arginine decarboxylase. AIH: Agmatine iminohydrolase. CPA: N-carmaboylputrescine amidohydrolase. ODC: Ornithine decarboxylase. PMT: putrescine N-methyltransferase (Source: Palazón *et al*., 2008)

Evans (2009) describes that tryptamine is descarboxylation product from tryptophan which is the main aminoacid give rise to the large class of indole alkaloids. Some examples of alkaloids of pharmaceutical interest, derived from tryptamine, are mainly strychnine, reserpine and ergot alkaloids (ergometrine, ergometrinine, ergotaminine, ergosine, ergocristinine, ergocryptine, ergocryptinine, ergocornine, ergocorninine) (Fig 8).

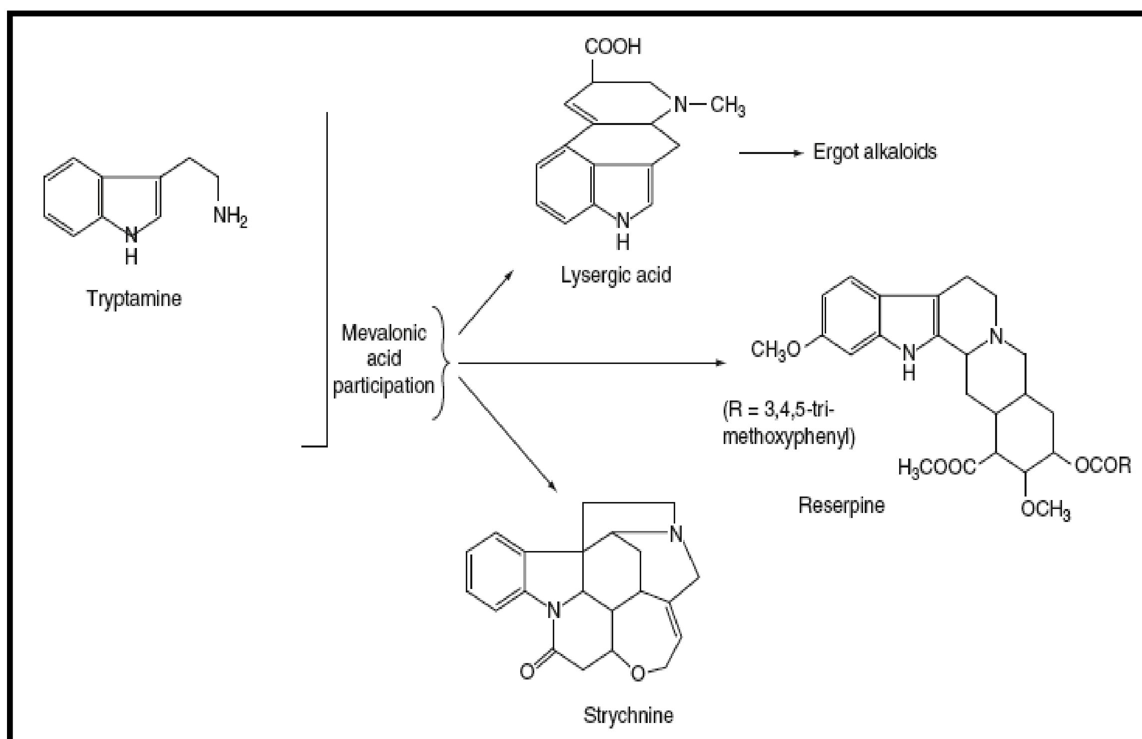


Figure 8. Tryptamine as precursor of indole alkaloids (Source: Evans, 2009)

3. Objetives

3.1. General objectives

The main aim of this thesis was to evaluate the effect of precursors and elicitors on the production of mescaline in callus culture of *T. pachanoi*. The relationship between precursor/elicitor concentration and callus growth under *in vitro* conditions was also evaluated.

3.2. Specific objectives

- Propagate initial plant material - callus of *T. pachanoi* for establishment of *in vitro* experiment
- Evaluate the effect of 100, 200 and 500 mg l^{-1} of dopamine, tyrosine, arginine and tryptamine on the production of mescaline.
- Determine the mescaline content in callus after 2 and 4 weeks of cultivation on media containing mescaline precursor/elicitors.
- Evaluate the relationship between precursor/elicitor concentration and callus growth.

Hypothesis:

- There is a significant difference in mescaline content among calli after 2 and 4 weeks of cultivation on media containing mescaline precursor/elicitors.
- Precursors/elicitors have different effects on the production of mescaline according to their position in metabolic pathway.
- Different precursors/ elicitors may significantly influence growth of callus depending on applied doses and the type of precursor.

4. Materials and methods

4.1. Plant material

The plant material of *T. pachanoi* for testing was obtained from research works realized within Department of Crop Sciences and Agroforestry in Cezch University of Lifes Sciences Prague (Appendix 1).

4.2. *In vitro* propagation of plant material

In vitro culture of *Trichocereus pachanoi* had already been established, as well as the protocol for callogenesis (Rejthar *et al*., 2013). Callus have been multiplied once a month on MS medium (Murashige and Skoog, 1962) supplemented with 100 mg l^{-1} *myo*-inositol, 30 g l^{-1} sucrose, 8 g l^{-1} agar,1 mg l^{-1} of 2,4-D and 1mg $l^{-1}$of thidiazuron (TZD). *In vitro* cultures were maintained at 25/23 °C under a 16/8 h light/dark regime with 36 µmol m^{-2} s^{-1}.

4.3. Preparation of media for the experiment

Fort the experiment, basic medium described in the previous chapter was used. The media were prepared in Erlenmeyer flasks (500 ml) and transferred to autoclave for the process of sterilization (100 kPa, 121 °C, 20 min). This process was carried out simultaneously with the preparations of the solutions of precursors because it was necessary to have both components at the same time prepared for to mixing them together in the flow box later on.

4.4. Preparation and application of precursors/elicitors.

Each precursor was diluted depending on its physical and chemical characteristics according the indications of the products. All chemicals were purchased in Sigma Aldrich. The products used were dopamine hydrochloride (catalogue No. H8502-5G), tryptamine

hydrochloride (catalogue No. 246557-5G), arginine (catalogue No. A2646) and tyrosine (catalogue No. T3754). The dilution of the precursors used is described in the table 1.

Table 1. Solubility of the precursors/elicitors.

Precursors/elicitors	Type of dilution
Dopamine hydrochloride	Water
Tyrosine	Hydrochloride acid
Arginine	Water
Trypatamine hydrochloride	Water

The application of precursors in the medium was done carefully because of their termolabile nature. This process was realized in the flow box after the solution sterilization using filtration (size of filters: pore size 0.2 μm). The culture were established in indisposable plant cell culture vessel Phytatray™ II Sigma Aldrich , W × D × H 114 mm × 86 mm × 102 mm , PETG, sterile; γ-irradiated.

4.5. Establishment of the experiment

The callus which was obtained as described in the chapter 4.2 was cutted in segments of 20 mm in size and transferred to the disposable plant cell culture vessel Phytatray™ II. In each boxes 6 callus segments were placed. Finally the boxes were labeled and classified by time, concentration and type of precursor. The table 2 showed clearly all treatments.

Table 2. General diagram of the experiment

Time (weeks)	Concentrations (mg l^{-1})			
	0	100	200	500
2	Control	Dopamine	Dopamine	Dopamine
	Control	Tryptamine	Tryptamine	Tryptamine
	Control	Tyrosine	Tyrosine	Tyrosine
	Control	Arginine	Arginine	Arginine
4	Control	Dopamine	Dopamine	Dopamine
	Control	Tryptamine	Tryptamine	Tryptamine
	Control	Tyrosine	Tyrosine	Tyrosine
	Control	Arginine	Arginine	Arginine

4.6. Mescaline analysis

4.6.1. Preparation of sample solutions

The plant material was shade dried and powdered coarsely before extraction. In order to eliminate possible interfering with lipids the cactus sample was pre-extracted using Soxhlet extraction. A 0.5-1 g portion of ground cactus was accurately weighed and extracted with 150 ml of petroleum ether for 3 hrs in a water bath at 110 °C.

After drying the extraction thimble, the sample was extracted with methanol-concentrated ammonia solution (99:1) (three times 30 ml; 6 h shaking) at room temperature. This solution was concentrated at reduced temperature (40 °C) on a rotary evaporator (Laborata 4000-efficient Heidoplh) and redissolved in methanol yielding a volume of 10.0 ml. Two millilitres of this solution were filtered through an LC filter and placed in an autosampler vial of 1 ml.

4.6.2. High performance liquid chromatography (HPCL)

HPLC analysis was performed on a Dionex Summit HPLC system equipped with a PDA (LC-photodiode array detection) detector. The column was a Phenomenex Gemini. Mescaline content of the callus of each treatment was determined by Soxhlet extraction with methanol, followed by acid–base extraction with water and dichloromethane, and high-performance liquid chromatography (HPLC). Authentic mescaline hydrochloride was purchased at Sigma Aldrich and used as the mescaline reference standard. The range of mescaline concentrations covered by the standard solutions was 4.59–73.3 μg m l^{-1}. The extracs were directly injected into the HPLC, system used for stimation of alkaloids was HP-A-8452A-UV-VIS-Diode Array with HP-Vectra ES/ 12-PC (more detailed specification of HPLC parameters in Appendix 2). This part of research was realized in Laboratory of Faculty of Pharmacy of Charles University in Hradec Kralove, Department of Pharmacognosy (according to regulation 243/2009 Lc. Permission to treat with psychotropic and narcotic substances, subordinated to regulation 467/2009 Lc).

5. Results

5.1. Effect of precursors/elicitors on callus growth and color

The media with dopamine as precursor had a positive effect on the growth of callus. Once this treatment was established the callus started to growth from 5th day and at the same time it was observed dark brown colorations over the callus. These characteristics remained until the 4th week, and the intensity was influenced by different doses from 100 mg l^{-1} to 500 mg l^{-1}.The concentration of dopamine with the best effect on growth was 100 mg l^{-1} and 200 mg l^{-1}. The rate of growing of these concentrations was faster than in the control treatment without any precursors or elicitors (Table 3). The dark coloration covered surface of the callus. This coloration, however, did not have negative effect on the growth of callus. The third highest concentrations of dopamine (500 mg l^{-1}) caused the most intensive dark coloration which appeared after 3 days (Fig. 9A). Later on, whitish coloration on the upper part of callus was observed (Fig. 9B). By contrats to the previous two concentrations, these colorations effected slightly on the growth. Most calli grew the same than control treatment but slower than the previous concentrations (Table 3).

Also the media with trypatamine had positive effect on the growth of callus regardless of concentration used (100, 200 and 500 mg l^{-1}). The growth of the callus under the effect of tryptamine was faster than the control treatment and after 4 weeks these calli were bigger than control treatment and even bigger than the calluses under the effect of dopamine (Table 3). The coloration of the callus surface was variable and it started to appeared after 15 days of cultivation. Whitish and greenish colorations with red points on the upper part of callus were observed (Fig. 9C). These characteristics were presented in all concentrations; there was not a particular color per dose.

The media with arginine had almost the same effect as in the case of tryptamine. The growth of callus as well as the color had the same tendency as in the previous treatments. The

coloration also started appeared after 15 days. As in the case of tryptamine there were not differences between the rate of growth and the concentration of arginine (Fig. 9D).

The media with tyrosine were the only treatments that had negative effect on the growth of callus (Table 3). The effect of tyrosine was toxic for the callus. This negative effect inhibited the growth of callus and this caused that the major part of calluses maintained small within 4 weeks. After 3 days the callus showed a blackish and reddish coloration all over callus (Fig. 9E). It was also observed that only the treatment with 100 mg l^{-1} showed growth of callus, but the growth was very slow. The effect of the other concentrations (200 and 500 mg l^{-1}) was totally toxic; there were not induction callus at all (Fig. 9F).

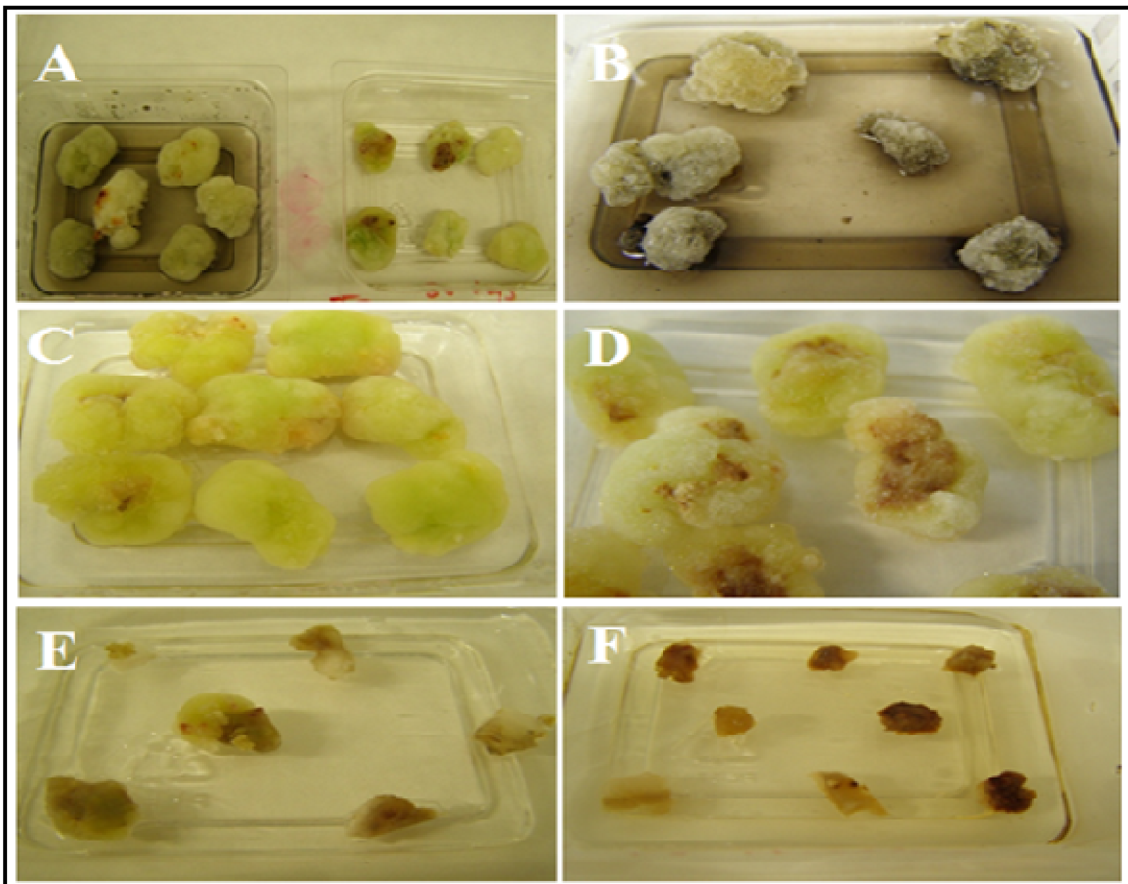


Figure 9. Different effects of precursors on callus growth in *Trichocereus pachanoi* at 500 mg l^{-1} after 4 weeks: A) Comparison between callus under the effect of dopamine and the control treatment. B) Strongest effect of dopamine on callus growth. C) Effect of tryptamine on callus growing. D) Effect of arginine on callus growing. E) Inhibitory effect of tyrosine on callus growth. F) Toxicity of tyrosine on callus growth.

Table 3. Rate of callus growth in all treatments after 4 weeks

Precursor/elicitors	Concentration (mg l^{-1})	Rate of growing
Dopamine	Control	++
	100	+++
	200	+++
	500	++
Tryptamine	100	++++
	200	++++
	500	++++
Arginine	100	++++
	200	++++
	500	++++
Tyrosine	100	+
	200	**0**
	500	**0**

Note: ++++ (fast) +++ (moderate) ++ (slow) + (very slow) 0 (without growth)

5.2. Analysis of mescaline in calli cultivated on media containing precursors and elicitors

Once the callus of 2 and 4 weeks were harvested, the analysis of mescaline content was carried. The mescaline content of the different precursors/elicitors is shown in the table 4.

Table 4. Mescaline content in mg x 10^{-8} kg^{-1} of fresh callus after 2 and 4 weeks on culture media containing precursors/elicitors

Time (weeks)	**Treatments**												
	Control	**Dopamine (mg l^{-1})**			**Tyrosine (mg l^{-1})**			**Tryptamine (mg l^{-1})**			**Arginine (mg l^{-1})**		
	0	100	200	500	100	200	500	100	200	500	100	200	500
	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	0.413	ND	ND	ND	ND	ND	ND	ND	ND	ND

Note: ND not detected

As can be seen from the table 4 only dopamine at the highest concentration used (500 mg l^{-1}) and applied for 4 weeks initiated production of mescaline 0.413 mg x 10^{-8} kg^{-1} in fresh matter. Lower concentration of dopamine did not produce any mescaline in callus tissue, regardless of application time. Arginine, tryptamine and tyrosine did not cause any mescaline production. The figure 10 shows the difference between mescaline content in fresh callus and dry matter in treatment 500 mg l^{-1}, after 4 weeks of cultivation.

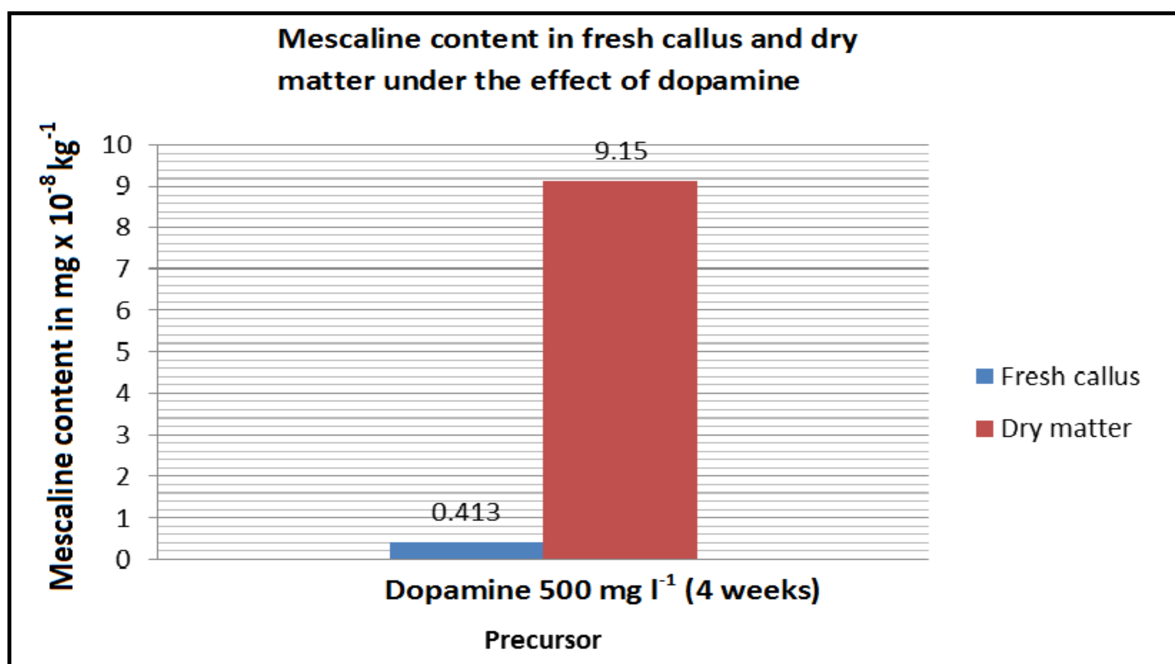


Figure 10. Mescaline content in fresh callus and dry matter under the effect of dopamine at 500 mg l^{-1} after 4 weeks

The figure 11 compares two studied parameters,, callus growth and mescaline content in treatments containing 500 mg l^{-1} precursors or elicitors. From the figure can be seen that only dopamine showed positive rusults for both parameters. Tryptamine and arginine influenced growth intensity of callus culture, but not mescaline content was detected, Tyrosine treatment due to its toxicity did not presente growth of callus and mescaline content was not detected as was described before.

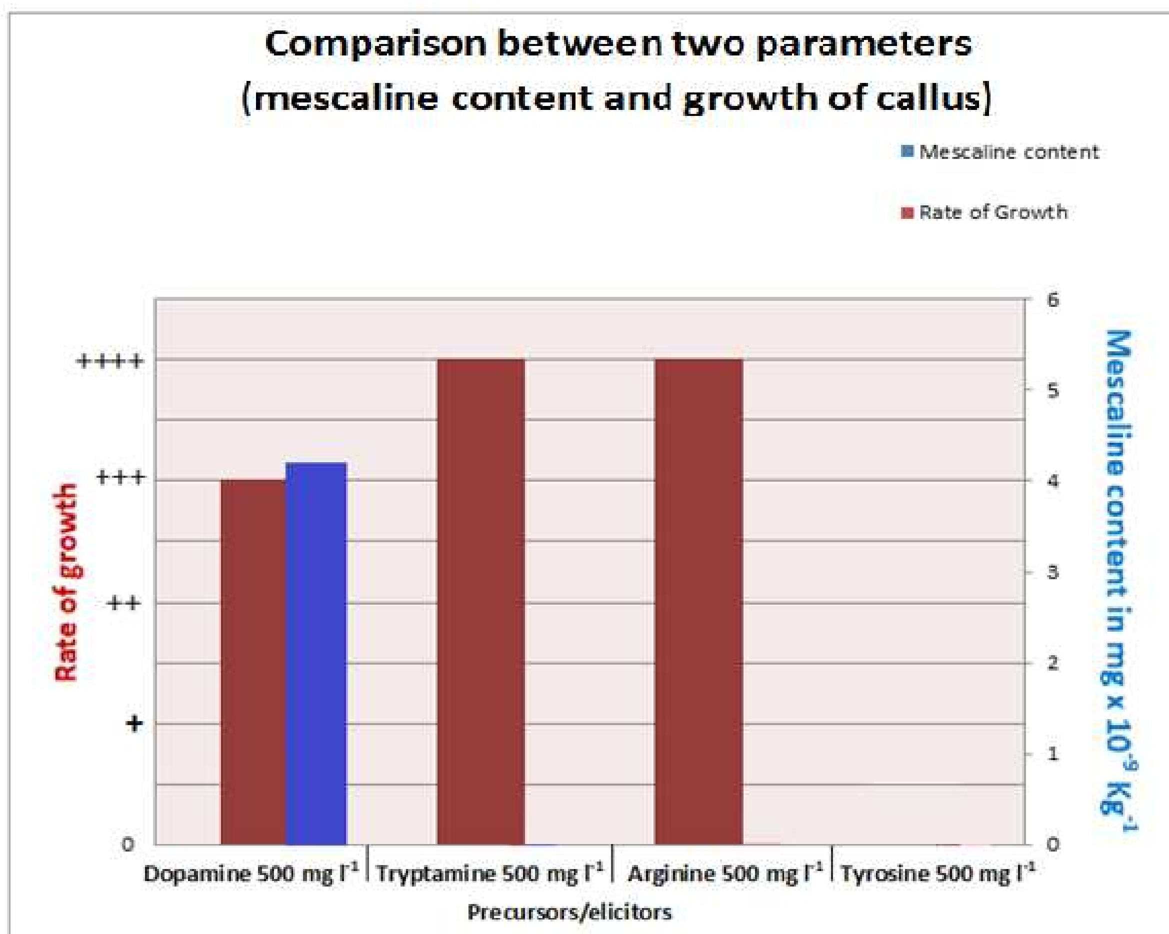
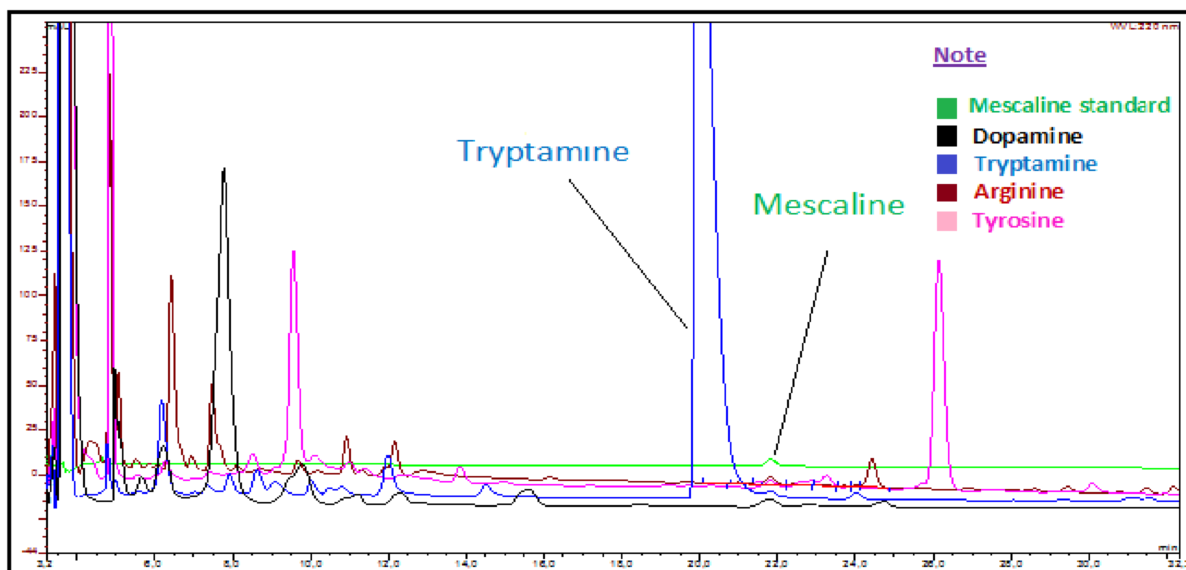


Figure 11. Comparison between studied parameters in callus induced on 500 mg l^{-1} of dopamine, tryptamine, arginine and tyrosine after 4 weeks of culture

Peak indentifying mescaline in sample extracts were confirmed by overlaying their UV absorption spectrum in extracts by comparing chromatograms with the standard mescaline. UV detection was performed at wavelengths of 220 nm. The external calibration curves from commercially mescaline standard were used for quantitative determination. Mescaline was identified by comparing chromatograms with a standard compound.

In representative chromatogram mescaline standard was characterized by the green color in the spectrum, which allowed us to recognize the presence of this component in analysis of each precursor/elicitor treatment. Simultaneously, chromatograms of dopamine (black), trypatamine (blue), arginine (red) and tyrosine (pink) treatments at 500 mg l^{-1} concentration were displayed (Fig. 12). In all the analyzed samples mescaline was only

detected in the treatment with dopamine at 500 mg l^{-1} at 220 nm (Fig. 12). Average LOQ (limit of quantification) was determined to be 7-34 mcg g^{-1}.



Note. nm: Nanometers (used to specify the wavelength of electromagnetic radiation near the visible part of the spectrum)

Figure 12. Chromatograms of mescaline in callus cultures on media enriched with precursors/elicitors (500 mg l $^{-1}$) at 220 nm. [Note. X: wavelength (nm) Y: Retention time (minuts)].

Chromatogram of sample belonging to tryptamine treatment showed one peak that was suspected to be mescaline. For this reason it was necessary to describe in detail if the compound had a relation to mescaline peak in dopamine treatments sample (Fig. 13). This chromatogram showed that the unidentified peak do not match with mescaline peak in dopamine treatment sample.

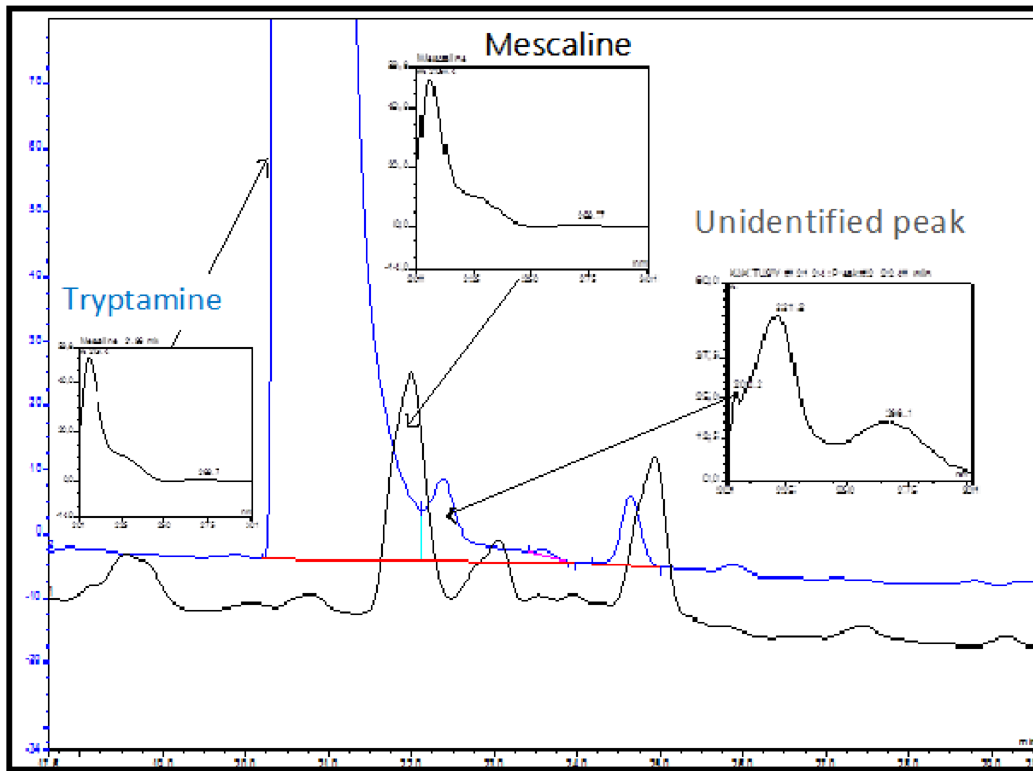


Figure 13. Comparison of the HPLC chromatograms of the treatments with dopamine and tryptamine (500 mg l^{-1}) at 220 nm and UV-Vis spectra of characteristic substances. [Note: X: wavelength (nm), Y: Retention time (min)]

6. Discussion

Positive effect of elicitors and precursors on callus growth is very beneficial, as they may simultaneously induce mescaline production and increase production of biomass, where is alkaloid contained.

Dopamine had a positive effect on the callus growth. The growth was even faster than in control treatment. This result is accordance with the study of Protacio *et al*. (1992) who observed that dopamine had a positive influence on promoting callus formation in *Nicotiana tabacum*. On the other side, Christou and Barton (1989) reported that dopamine at 1896.4 mg l^{-1} was toxic in callus culture of *Nicotiana tabacum*, *Glycine max*, *Zea mays* and *Helianthus annus.* These reports state that higher concentrations of dopamine may act toxicity in callus culture depending of the specie. In our case, it was only observed the dark coloration of callus and it was more frequent in the dose with 500 mg $l^{-1}$of dopamine. The coloration however did not negatively influence the growth of callus. Steinhart (1962) obtained callus in *Trichocereus spachianus* without the use of dopamine in the media, the growth regulators used were 2,4-D and kinetin. The callus showed red pigmentations and turned black during few days. Thus, it can be concluded that callus of *Trichocereus* tends to change colour, however it does not have to affect growth of callus.

Dopamine was the only treatment that showed positive results in mescaline production. It was necessary to applied dopamine at 500 mg l^{-1} for 30 days.This result confirms that dopamine is the real precursor of mescaline in *T. pachanoi* as was described by Lundstrom and Agurell (1969) who reported that dopamine and two derivatives (4-methoxy-3-hydroxy-phenethylamine and 3-methoxy-4-hydroxy-phenetilamine) were the best precursors of mescaline in *T. pachanoi*. Lunstrom (1971) also reported that dopamine is precursor of mescaline in *Lophophora williamsii,* another member from Cactaceae family. The amount of alkaloids produced can be influenced by many factors, such as the use of precursors, elicitors, plant growth regulators, type of culture, etc. (Ahmad*et al*., 2013). In our case we used precursors and elicitors for the production of alkaloids. Our results represent first promising step for mescaline production in *T. pachanoi* under *in vitro* conditions. Even the relation between callus growth and content of mescaline after dopamine application seems to be

satisfactory. A negative interaction between 2,4-D auxin and production of alkaloids has been reported (Merillon and Ramawat, 1999). Gantet *et al.* (1997) also observed that 2,4-D caused a dramatic inhibition on alkaloid terpenoid precursor availability in *Catharanthus roseus* cells. These reports can help us to understand that the effect of interaction between the plant growth regulators and precursors on production of mescaline is different in each species. In our experiment, this effect of 2,4-D was not studied, because the use of 2,4-D for callogenesis was indispensable. Obermeyer (1989) reported the presence of mescaline in low amounts in callus of *Lophophora willinsii* cultivated on medium with 2,4-D and BA without precursors. In this case the interaction between plant growth regulators and mescaline content porbably not so negative in contrast to our results, where the control treatment did not have mescaline content. It can be hypothesized that in our experiment auxin 2,4-D inhibited the production of mescaline, However high concentration of dopamine can overcome this negative effect.

The best result on callus proliferation in *T. pachanoi* was obtained by trypatamine and arginine. The rate of growth was faster than in the callus under the effect of dopamine and the control treatment. Zhao *et al.* (2001) similarly described positive effect of tryptamine on the callus growth in *Catharanthus roseus* from Apocynaceae family. The callus showed yellowish color that turned red. This observation confirms our results, because the callus of *T. pachanoi* formed redish points on the upper part. However, the same author mentions that the increase of tryptamine from 300 mg l^{-1} to 500 mg l^{-1} increases the rate of growth of callus culture of *C. roseus*. By contrast, our results showed no differences in concentration of tryptamine on callus growth.

Abdel-Rahim (1998) tested the effect of arginine to promote callus in *Phoenix dactylifera* from Aracaceae family. Ariginine significantly decreased growth and development of callus. These findings do not corroborate our resuls, since arginine had an excellent effect on callus production and the rate of growth. Neverthelesss arginine and tryptamine did not promote production of mescaline in callus of *T. pachanoi*. It has been demostraded that tryptamine is a precursor of indole alkaloids. Zhao *et al.* (2001) used tryptamine for the production of catharanthine and ajmalicine in callus culture of *Catharanthus roseus*. Lucumi *et al*. (2002) showed that tryptamine enhance the production of serotonine in callus culture of

Tabernaemontana elegans from Apocynaceae family. Arginine also appears as a precursor of tropane alkaloids as was showed by Osorio *et al*. (2009) who used arginine for the production of escopaline.

Clement *et al*. (1998) reported coexistence of mescaline and tryptamine in the chemical composition of *Acacia rigidula* from Fabaceae family and many others amines and alkaloids related to the mescaline. This study can be the beginning to understand that the biochemical processes of species involves many functional specific characteristics for to produce a determined secondary metabolite. For example, this explanation could be related to Evans (2009) who describes the formation of putrescine (decarboxylation product of ornithine) from ornithine, but he also mentions that putrescine can be formed from arginine without involvement of ornithine and this has led problems in understanding stereospecific incorporation, of precursors into a particular alkaloid. Tsao (1951) proposed a new synthesis of mescaline from gallic acid (organic phenolic acid) which it has not nitrogen in its structure as the mescaline. These reports allow us to make many hypotheses about alternative pathways of the alkaloids to production. However, since according to our results arginine and tryptamine did not produce mescaline, it is possible that tryptamine can produce other kind of secondary metabolites.

Oliveira and Silva-Machado (2003) induced callus in *Cereus peruvianus* (Cactaceae) for the production of alkaloids using 2,4-D and kinetin. The precursor used was tyrosine at concentration 125 mg l^{-1}. After 4 weeks of callus cultivation the growth rate of callus was evaluated. Tyrosine in this experiment did not increase the growth of callus. By contrast to these results, in our experiment was evident, that tyrosine had a toxic effect on callus culture of *T. pachanoi*. This effect was even more visible in the concentrations of 200 and 500 mg l^{-1} of tyrosine. On the other hand, according to Dewick (2002) mescaline is derived from tyrosine which is the principal amino acid for the conversion of dopamine by L-DOPA precursor. Also the studies realized by Lundstrom (1971) and Lundstrom and Agurell (1969) indicate that tyrosine is the precursor of mescaline in the early steps of the biosynthesis. But according to our results the callus under the effect of tyrosine was not able to produce mescaline. By contrats Obermeyer (1989) showed a great amount of alkaloids produced in callus of

Lophophora williansii under the effect of tyrosine which means that probably mescaline could be present there as well. Probably in *T. pachanoi* tyrosine act as efficient precursor of other compounds related of mescaline but not directly for mescaline.

7. Conclusion

Dopamine at the highest concentration tested (500 mg l^{-1}) when applied for 4 weeks was able to produce mescaline in callus of *Trichocdreus pachanoi*.

- Significant effect of precursor application time on mescaline production was confiremed.
- Significant effect of precursor concentration on mescaline production was detected.
- Precursors had different effect on the production of mescaline according to their position in metabolic pathway, as confirmed by results after dopamine application. Dopamine is the closest chemical to mescaline.
- Tryptamine and arginine were the best feeding media components for the callus growth of *T. pachanoi*. However, arginine and tryptamine did not produce mescaline, They did not act as elicitors for mescaline production.
- Tyrosine showed a toxic effect on callus production in *T. pachanoi* and it did not induce production of mescaline.

This research allows us to understand more about the different factors involved in the production of alkaloids by callus culture that could be used for the effective production in large scale.

Recommendations:

- Use of suspension cultures should be tested instead of callus cultures, as the precursors in liquid medium may affect each cell separately.
- *In vitro* cultivation of organ culture on medium containing dopamine could be also recommended, as mescaline production is being associated with cortex.
- Higher concentration and longer time of dopamine application should be tested.

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Appendix

Appendix 1: Plant material, *Trichocereus pachanoi* (San Pedro)

Appendix 2: HPLC parametres

TempCtrl =	**On**
Temperature.Nominal =	**30.0 [°C]**
Temperature.LowerLimit =	**28.0 [°C]**
Temperature.UpperLimit =	**32.0 [°C]**
EquilibrationTime =	**0.1 [min]**
ReadyTempDelta =	**1.0 [°C]**
HumidityLeakSensor =	**Low**
GasLeakSensor =	**Low**
Column_A.SystemPressure =	**"Pump"**
Pressure.LowerLimit =	**10 [bar]**
Pressure.UpperLimit =	**250 [bar]**
MaximumFlowRamp =	**6.00 [ml/min2]**
%A.Equate =	**"%H2O"**
%B.Equate =	**"20 mM Amonium acetate"**
%C.Equate =	**"%AcN"**
%D.Equate =	**"%D"**
ColumnOven_Temp.Step =	**Auto**
ColumnOven_Temp.Average =	**On**
Pump_Pressure.Step =	**Auto**
Pump_Pressure.Average =	**On**
UV_VIS_1.Wavelength =	**210 [nm]**
UV_VIS_1.Bandwidth =	**1 [nm]**
UV_VIS_1.RefWavelength =	**600 [nm]**
UV_VIS_1.RefBandwidth =	**1 [nm]**
UV_VIS_1.Step =	**Auto**
UV_VIS_1.Average =	**On**
UV_VIS_2.Wavelength =	**220 [nm]**
UV_VIS_2.Bandwidth =	**1 [nm]**
UV_VIS_2.RefWavelength =	**600 [nm]**
UV_VIS_2.RefBandwidth =	**1 [nm]**
UV_VIS_2.Step = Auto	
UV_VIS_2.Average =	**On**
UV_VIS_3.Wavelength =	**254 [nm]**
UV_VIS_3.Bandwidth =	**1 [nm]**
UV_VIS_3.RefWavelength =	**600 [nm]**
UV_VIS_3.RefBandwidth =	**1 [nm]**
UV_VIS_3.Step = Auto	
UV_VIS_3.Average =	**On**
3DFIELD.RefWavelength =	**600.0 [nm]**
3DFIELD.RefBandwidth =	**1.9 [nm]**
3DFIELD.Step = 0.5 [s]	
3DFIELD.MinWavelength =	**200.0 [nm]**
3DFIELD.MaxWavelength =	**595.2 [nm]**
3DFIELD.BunchWidth =	**1.9 [nm]**

	UV_VIS_4.Wavelength =	**330 [nm]**
	UV_VIS_4.Bandwidth =	**1 [nm]**
	UV_VIS_4.RefWavelength =	**600 [nm]**
	UV_VIS_4.RefBandwidth =	**1 [nm]**
	UV_VIS_4.Step = Auto	
	UV_VIS_4.Average =	**On**
0.000	**Autozero**	
	Flow = 0.800 [ml/min]	
	%B = 95.0 [%]	
	%C = 5.0 [%]	
	%D = 0.0 [%]	
	Curve = 5	
	Wait Ready	
	Inject	
	ColumnOven_Temp.AcqOn	
	Pump_Pressure.AcqOn	
	UV_VIS_1.AcqOn	
	UV_VIS_2.AcqOn	
	UV_VIS_3.AcqOn	
	UV_VIS_4.AcqOn	
	3DFIELD.AcqOn	
	Flow = 0.800 [ml/min]	
	%B = 95.0 [%]	
	%C = 5.0 [%]	
	%D = 0.0 [%]	
	Curve = 5	
40.000	**Flow = 0.800 [ml/min]**	
	%B = 85.0 [%]	
	%C = 15.0 [%]	
	%D = 0.0 [%]	
	Curve = 5	
45.000	**Flow = 0.800 [ml/min]**	
	%B = 0.0 [%]	
	%C = 100.0 [%]	
	%D = 0.0 [%]	
	Curve = 5	
50.000	**Flow = 0.800 [ml/min]**	
	%B = 0.0 [%]	
	%C = 100.0 [%]	
	%D = 0.0 [%]	
	Curve = 5	
	UV_VIS_1.AcqOff	
	UV_VIS_2.AcqOff	
	UV_VIS_3.AcqOff	
	UV_VIS_4.AcqOff	
	3DFIELD.AcqOff	

	ColumnOven_Temp.AcqOff	
	Pump_Pressure.AcqOff	
51.000	**Flow =**	**0.800 [ml/min]**
	%B =	**95.0 [%]**
	%C =	**5.0 [%]**
	%D =	**0.0 [%]**
	Curve =	**5**
60.000	**Flow =**	**0.800 [ml/min]**
	%B =	**95.0 [%]**
	%C =	**5.0 [%]**
	%D =	**0.0 [%]**
	Curve =	**5**