Bull. Pharm. Sci. Assiut University
Vol. 6, PP. 196 -211

ALKALOIDS OF HYOSCYAMUS MUTICUS TISSUE CULTURES

M.M. El-Olemy & S.M.I.Moustafa

Department of Pharmacognosy, Faculty of Pharmacy, Tanta, University., Tanta, Egypt.

Callus cultures were developed from Hyoscyamus muticus seeds and stem or leaf midrib. The culture grows and maintains well in a number of culture media. Alkaloids were produced in all cultures. Chromatographic investigation of the alkaloid composition revealed the presence of hyoscyamine and scopolamine, together with an unidentified alkaloid. Quantitative estimation of the total alkaloids was done by the acid-dye complex spectrophotometric technique.

Hyoscyamus muticus and other members of the family Solanaceae are important both medicinally and pharmaceutically for their content of various tropane alkaloids[1-3]. Of all the plant families, tissue cultures of solanaceous plants are the most studied, since White in 1934 started tissue cultures of Tomato plants[4] and Novecourt in 1938 initiated tissue cultures of Solanum [5]. Since then, tissue cultures of various solanaceous plants have been developed and several suitable media have been perfected e.g. White's Tobacco medium[6] for

Tobacco and several other media for Atropa belladonna[7-8], Datura stramonium[9], and Withania somnifera[10] tissue cultures.

Suitability of solanceous tissue sultures for producing various constituents has been studied. These include production of diosgenin in Solanum xanthocorpeem tissue cultures[11], nicotine[12-13] and acopoletin[14] in Nicotiana tabacum, tropane alkaloids both in belladonna[7-8] and Datura[9] tissue cultures and anaferine, sterols and lipids in Withania[10]

H. muticus, a shrub indigenous to Egypt, is known to produce as high as 1.5% of the alkaloid hyoscyamine[1-3]. No reports on the production of alkaloids in H.muticus tissue cultures could be traced in the literature.

It was thus found intriguing to undertake the present investigation for the development of H.muicus tissue cultures and to study their ability to produce hyoscyamine. This was undertaken in a trial to provide alternative means for the production of this important medicinal agent.

RESULTS AND DISCUSSIONS

Hyoscyamus muticus callus cultures grow well and fast in all media investigated. All media (Table 1) contained approximately equivalent macro-and micro components, though in different quantities, but differ in types and concentration of vitamins, auxins and kinetin. It has been reported that the relative concentration of auxins and kinetin in the culture medium, affect to a great extent the degree of differentiation of tissues[6] and may influence the biochemical

synthetic ability of the culture[12-13].

H.muticus tissue culture was started in the prescribed fashion[6-15], either from seed ——— sterile seedling ——— callus cultures or from stem or leaf-midrib and was maintained by serial transfer to a fresh medium every 3-4 weeks, initially for the first three generations and then every 2 weeks thereafter. Cultures grow best on NAX medium (Table 1); these also showed the most differentiation with one or two small plantlets per callus culture. The characters of the callus cultures obtained in various media are described below.

Callus Cultures from Stem or Leaf Midrib:

Best and fastest growth was observed on NAX medium. This required serial transfer every 3 weeks at the beginning . After a few transfers, it showed occasional differentiation into a small plantlet, one or two per flask. The 4X medium was also suitable, but growth of the callus cells was slower and the cells were darker, being greyish brown . After a few serial transfers, callus cultures grow a little faster.

Callus Cultures from Sterile Seedling:

Best growth was on LS medium giving a white callus that grows fast, while growth on NAX medium was slow with a little brown callus formation and almost complete differentiation into plantlets. Subsequent serial transfer of the callus crust produced on NAX medium speeded up the

growth and resulted in a culture very similar to that started from stem or leaf midrib.

In one experiment, callus obtained from seedling on LS medium being the fastest and best from seedlings, was transferred separately to various media namely: LS, NAX, 4X and VM media. The following observations were observed:

a. LS medium:

Showed normal growth, somewhat slow, giving a greyish white callus.

b. NAX medium:

Similar to LS but somewhat slower.

c. 4X medium:

Results in a more grey callus with a faster growth than on LS medium.

d. VM:

Callus grows faster giving a yellowish grey callus.

Each of these callus cultures was maintained on its respective medium, through serial transfers every 3-4 weeks (at the beginning), then every 2-3 weeks as necessary.

Thin-layer chromatographic investigation of the alkaloidal extracts of various cultures proved that most such cultures produced hyoscyamine with a trace of scopolamine.

In addition, an unknown more polar, not yet identified, alkaloid is produced in NAX, VM and 4X media grown calluses (See Figure 1 &2). All LS cultures investigated produced only hyoscyamine. The results of the quantitative estimation using the acid-dye complex formation spectrophotometric technique (Table 2), proved that NAX and LS media provide more optimum medium composition for growth and alkaloid production. Callus collection after the third generation provides the highest alkaloidal content. Total alkaloidal content as high as 1.37% w/w on a dry weight basis is possible on the two recommended media.

The decrease in the alkaloidal content after the third generation may point out to the possibility that the low alkaloid producing cell lines grow faster than high yielding cell lines and thus the latter are taken over gradually.

Cell-line selection will be performed in future studies according to the procedure applied to Tobacco cultures by Ogino et al[16] to selct cell lines of highest alkaloid productivity. Perhaps, it would be possible to select cell-lines that produce specifically a single tropane alkaloid, either hyoscyamine or scopolamine and in relatively high concentrations. Thus H.muticus cultures may serve as a suitable technique for the commercial production of these alkaloids.

Future investigation would also be directed towards identification of the unknown alkaloid produced in H.muticum tissue cultures and to undergo investigations for improvement of the yield of the produced alkaloids.

EXPERIMENTAL

H. muticus seeds were of good viability and geminated in one week's time after being planted.

Starting Tissue Cultures from Stem or Leaf Midrib:

A part of the seeds was allowed to germinate in normal soil. Plants, one month old, were used for this study. Apical part of the plant including leaves, stem and growing tip were surface sterilized[6] by soaking in alcohol (2 min), 2% NaOCl soln. dil. HCl (10 min) and then washed thoroughly with sterile dist. water. The leaf midrib and stem were sterily separated and split lengthwise into halves. The pieces were then placed on the top of an agar plate of the respective medium (either NAX, 4X, VM or LS medium and incubated at 30°C for 3 weeks or until suitable callus (if any) is produced. The callus was then transferred into a fresh medium and allowed to grow in the incubator. Once the callus is formed, it is then maintained and propagated as usual, as in microbiological work.

Starting Tissue Cultures from Sterile Seedling:

H. muticus seeds are surface-sterilized by soaking in alcohol (5 min) and then 5% NaOCl solution/dil. HCl for 20 min. then thoroughly washed with sterile distilled water. They were then thinly distributed in a number of sterile. dishes, containing sterile tap-water. These were then incubated at 30°C till they germinate (one week). The seedlings are then transferred to appropriate agar solid medium so that seedlings lye sidewise in content with the solid agar

medium. The callus resulted in 1-2 weeks and was allowed to grow for a further two weeks before being transferred to a fresh medium for maintainance and propagation.

Callus tissue cultures of either seed or stem origin were separately maintained on each of the four media investigated (Table 1) and were propagated by serial transfer to obtain collections of tissues for alkaloidal investigations. The tissues in every case were dried immediately after collection by lyopholization.

Alkaloid Extraction:

Lyopholized tissues, (200-400 mg each), were extracted by percolation with methanol to exhaustion (100 ml). The methanilic extract was evaporated to dryness and made to volume in a 10 ml. volumetric flask and used for TLC and spectrophotometric investigation.

For purification of alkaloids, a normal alkaloidal extraction and purification procedure was performed with $CHCl_3$ as the solvent and NH_4OH as the alkali used.

Thin-layer chromatography (TLC):

Two solvent systems were tried, these are:

1. System I : Si gel/$CHCl_3$-MeOH-NH_4OH (90 : 10 : 1%)
2. System II: Si gel/MeOH.

Reference samples (atropine)or hyoscyamine) and scopolamine) were spotted alongside with the alkaloidal extracts. The results are shown in Figure 1 and 2.

Spectrophotometric Determination of the Alakaloidal Content in the Extracts by Acid-dye Technique:

The method previously reported for the determination of solanaceous alkaloids[17] and for other related natural products[18-19] was adopted using bromothymol blue as the acid-dye used. Calibration curves were constructed using various concentrations of hyoscyamine and the alkaloidal concentrations in the various extracts is calculated as hyoscyamine (Table 2)

ACKNOWLEDGMENT

The initial part of this work was performed at the laboratories of Prof. K. Leistner, Institür Für Pharmazeutische Biologie und Pharmacognosie, Universitat Munster, 44 munster, West Germany, during an " Alexander von Humboldt Fellowship" for M. M. E. O. This is exceptionally acknowledged.

Table 1: Composition of Various Media used in the Present Investigation.

Components	*4X*	*NAX*	*LS*[1]	*VM*[2]
Macroelements: (mg/L)				
$NaH_2PO_4 . H_2O$	150	150	-	150
Na_2HPO_4	-	-	-	20
$CaCl_2 . 2H_2O$	150	150	440	200
KCl	-	-	-	200
$MgSO_4 . 7H_2O$	250	250	370	250
$(NH_4)_2SO_4$	134	134	-	100
KNO_3	2500	2500	1900	800
KH_2PO_4	-	-	170	-
NH_4NO_3	-	-	1650	-
Microelements: (mg/L)				
KI	0.75	0.75	0.83	0.05
$MnSO_4 . H_2O$	10.0	10.0	16.9	4.0
H_3BO_3	3.0	3.0	6.2	5.0
$ZnSO_4 . 7H_2O$	3.0	3.0	10.6	1.5
$Na_2MoO_4 . 2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4 . SH_2O$	0.25	0.25	0.025	0.25
$COCl_2 . 6H_2O$	0.25	0.25	0.025	0.25
$FeSO_4 . 7H_2O$	-	-	-	13.9
Additives: (mg/L)				
Fe (EDTA)	28	28	34.6	-
Na_2(EDTA)	-	-	-	18.6
Carbon and Organic Nitrogen Source: (g/L)				
Sucrose	20	20	30	20
E-Z amine (Nitrogen Source)	2.0	2.0	-	2.0

Table 1: (Cont.)

Components	*4X**	*NAX**	*LS*[1]	*VM*[2]
Vitamins: (mg/L)				
Thiamine HCl	10.0	10.0	0.4	0.5
Pyridoxine HCl	1.0	1.0	-	0.5
Nicotinic acid	1.0	1.0	-	1.25
Ca Pantothenate	-	-	-	1.0
Mese-Inositol	100.0	100.0	100.0	100.0
Growth Factors: (mg/L)				
Kinetin	2.0	-	0.2	0.25
Indole Acetic Acid (IAA)	0.5	-	-	0.1
2,4-D (2,4-dichlorophenoxacetic acid.	2.0	-	4.0	1.5
Solidifying agent: (g/L)				
Agar — Solid medium	8	8	8	10
Agar — Suspension medium	-	-	-	-
PH value:				
Adjusted with NH_4OH/H_2SO_4	5.5	5.5	5.9	5.5

* Recommended by Prof. Leistner, Institute fur Pharmacognosie und Pharmazeutische Biologie, Universitat Munster, BRD.

1. Lindsmeter E.M. & Skoog, F., "Organic growth factor requirements of Tobacco tissue cultures. Physiologia Pl. 18; 100-127 (1965).

2. Veliky, J.A. and Martin, S.M., " A fermenter for plant cell suspension cultures", Can. J. Microbuol., 223-226 (1970).

Table 2: Percentage of the Alkaloidal. Content of Hyoscyamus muticus Tissue Culture (Different Media)

Organ	Medium	Generation	Alkaloidal percentage
Seed	LS	III	1.32
	NAX	III	0.92
		IV	0.88
	VM	III	0.70
		IV	0.42
	4X	III	0.601
Stem	LS	VI	0.74
	NAX	III	1.25
		IV	1.16
		VII	0.92
	VM	VI	0.57
	4X	IV	0.78
		V	0.51
		VI	0.33

Silica gel G.

System :Chloroform : Methanol : Ammonia (90:10:1%)

Spray : Dragendorff's reagent .

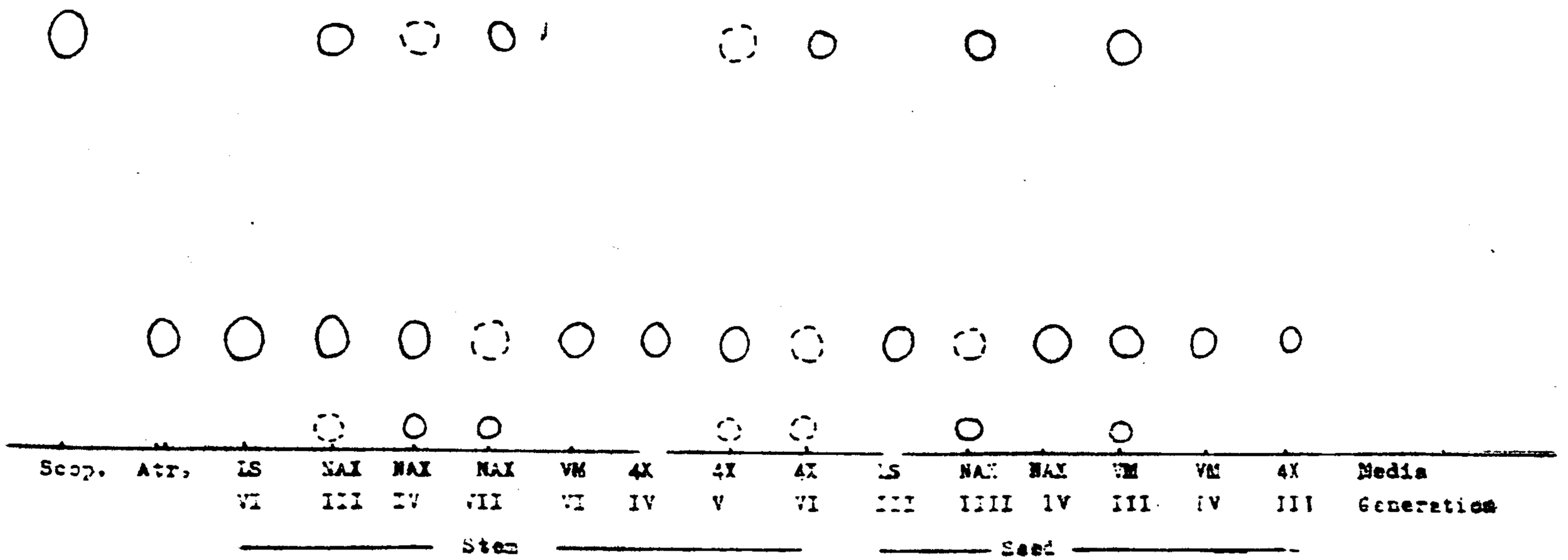

Fig. 1- TLC of the Alkaloids of Hyoscyamus muticus Tissue Cultures.

Silica gel G.

System : Methanol .

Sprey : Dragendorff's reagent.

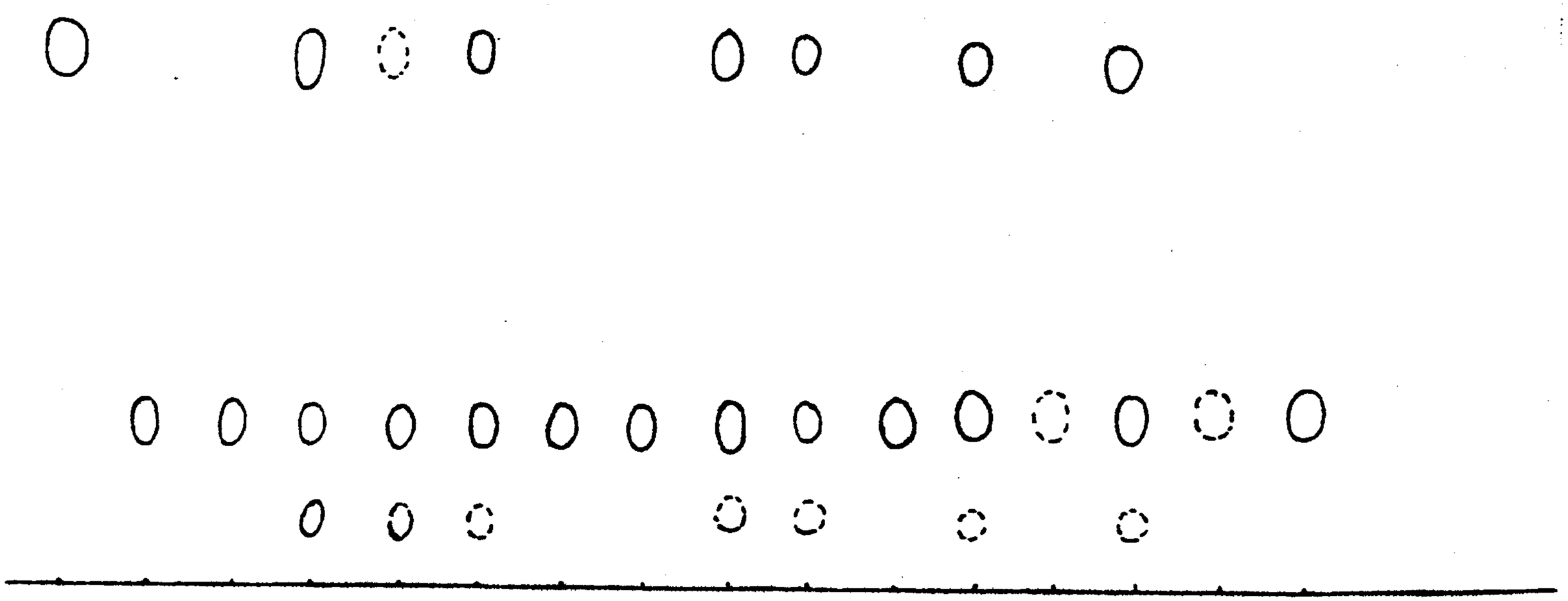

Fig. 2- TLC of the Alkaloids of Hyoscyamus Muticus Tissue Cultures .

REFERENCES

1) *E.P. Claus, V.E. Tyler, Jr and L. Brady, " Pharmacognosy", 7th Edn., Lea and Febiger, Philadelphia, (1977).*

2) *T.E. Wallis, " Textbook of Pharmacognosy", 5th Edn., The whitefrairs Press Ltd., London, (1967).*

3) *S.I. Balbaa, S.H. Hilal, and A.T. Zaki, "Medicinal Plant Constituents", 2nd Edn., Dar El-Shaab Printing House, (1976).*

4) *P.R. White, "Potentially Unlimited growth of excised tomato root tips in liquid medium", Pl. Physiol. (Lancaster) 9 : 385 (1934).*

5) *P. Nobecourt, " Sur les Proliferation in vitro du parenchyma amylifere du tubercule de Solanum tuberosum L. ", Bull. Soc. Bot. Fr. 85: 480 (1938).*

6) *H.E. Street, Ed., " Plant Tissue and Cell Culture", Univ. of California Press, Berkeley, (1973).*

7) *R. Bhondary, H.A. Collin, E.Thomas, and H.E. Street, "Root Callus and Cell Suspension Cultures from Atropa belladonna, cultivar Lutea Doll. " Ann. Bot. 33: 647 (1969).*

8) *F.R. West and S. Mika, "synthesis of atropine by isolated shoots and root callus culture of belladonna", Bot. Gaz. 119: 50 (1957).*

9) *N.N. Chan, and E.J. Staba, " Alkaloid production by Datura callus and suspension tissue cultures". Lloydia 28 : 55 (1965).*

10) *P.L.C. Yu, M.M. El-Olemy, and S.J. Stoha, " A Phytochemical investigation of Withania somnifera tissue cultures". Lloydia 37 : 593 (1974).*

11) *A.R. Heble, S. Narayanaswami, and M.S. Chadha, "Diosgenin and B-sitosterol isolated from Solanum Xanthocarpum tissue cultures", Science 161 : 114 (1968).*

12) *T. Furaya, H. Kajima, and K. Syono, " Regulation of nicotine production by auxins in Tobacco callus tissue" Phytochem. 10 : 1529 (1971).*

13) M.Tobata, H. Yamamoto, N. Hiraoka, R. Arumoto, and M. Konoshima, " Regulation of nicotin production of Tobacco tissue cultures by plant growth regulators" Phytochem. 10: 723 (1971).

14) J.A. Sargent and F. Skoog, "Effects of indolancetic acid and Kinetin on Scopoletin and scopolin levels in relation to growth of Tobacco tissues in vitro", Pl.Physiol. Lancaster, 35: 934 (1960).

15) M.M. El-Olemy ," Monographs on Modern Pharmacognosy. Par I. , Biosynthesis of Natural Products", Faculty of Pharmacy, Tanta Univ., (1976).

16) T. Ogino, N. Hiraoka, and M. Tabata, "Selection of high nicotineproducing cell lines of Tobacco callus by single-cell cloning", Phytochem. 17 : 1907 (1978).

17) S.M. Ibrahin " Study and Development of Assay Methods of Certain Alkaloid-containing Pharmacopoeial Drugs". Ph.D. Thesis, Faculty of Pharmacy, Assiut University, Assiut, Egypt. (1976).

18) S. El-Masry , and S.A.M. Khalil, " Microdetermination of hyoscyamine and scopolamine in mixtures", J. Pharm. Sci., 62, 1332 (1973).

19) S.M. Ibrahim, H.A. Kadry and M.M. El-Olemy. " Use of Acid-dye technique in the analysis of natural products. III. Spectrophotometric microdetermination of Khellin and bergaptin". J. Nat. Prod. (Lloydia 42, 366 (1979).

قلويدات الانسجة النباتية المنزرعة لنبات السكران المصرى

محمود محمد العليمى ، سوزان محمود ابراهيم مصطفى

قسم العقاقير ـ كلية الصيدلة ـ جامعة طنطا

تم تحضير أنسجة منزرعة من أوراق وسيقان والعرق الوسطى لورقة نبات السكران المصرى ، وكان ذلك بأستعمال أوساط مختلفة وقد أحتوت تلك الانسجة جميعا على قلويدات أثبتت الدراسة الكروماتوجرافية لقلويدات هذه الانسجة بعد أستخلاصها عن وجود الهيوسيامين ، السكربولامين وكذلك قلويد غير معروف.

تم تقييم نسبة القلويدات فى هذه الانسجة بأستعمال طريقة القياس الطيفى للون محلوله المركب مع الاصباغ الحمضية .

recieved in 16/8/1982 & accepted in 13/10/1982