In vivo and *in vitro* studies on *Thevetia* species Growing in Egypt
II. Establishment of *in vitro* tissue culture system and production of cardiac glycosides

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Abstract: Applicable protocol for enhancement of calli production, shootlets regeneration, evaluation and determination of cardiac glycosides from growing *Thevetia spp*. was established. Calli and regenerated shootlets were induced from immature seeds (IS), leaf, stem and root explants of *Thevetia neriifolia* Jussieu. and *T. thevetioides* Kunth. (Apocynaceae). MS medium supplemented with 1mg/l 2,4-D + 3mg/l Kin showed the best results of mass calli production. Seed explants gave the highest value of calli formation under either light or dark condition. The 3rd week of cultivation for 5 weeks recorded the significant growth dynamic of mass calli production from seed explants. *T. neriifolia* exhibited high degree of multiple shootlets proliferation on MS + 1mg/l 2,4-D + 3mg/l BAp from stem explants as compared with *T. thevetioides*. The best result of *in vitro* rootlets/ shoot formation recorded with MS + 1mg/l NAA. Qualitative and quantative determinations of thevetin B, digitoxigenin, neriifolin and peruvoside contents were carried out using HPLC technique.
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1. Introduction

Thevetia neriifolia Juss. and *T. thevetioides* Kunth. are small tree commonly used as an ornamental plant. It is belongs to the Apocynaceae family and it can be found in South and Central America, Asia, and Africa. This plant species produces several compounds with industrial application as pharmaceutical compounds, such as cardiac glucosides neriifolin, thevetoxin, peruvoside, and thevetin A and B (Arnold *et al.* 1935; Omino and Kokwaro, 1993). Among these compounds, peruvoside is particularly attractive because it uses as a digoxin substitute in allergic patients, and it commercially distributed in Germany for that purpose (Kumar 1992, Abe *et al.* 1995). In addition, these compounds usually found in low concentrations in the plant, therefore, making the direct extraction is difficult and expensive. Additionally, successful procedures for its chemical synthesis have not been estimated, probably because for their complex structures. As such, the strategy of plant cell culture is attractive for the production of these metabolites. Plant cell culture has several advantages over the traditional cultivation, such as the control of the production conditions, weather independency, and continuous production. It is still necessary to overcome some difficulties such as low productivity and low specific growth rate (Sajc *et al*. 2000).

In vitro culture of plants has gained in importance during recent years because, besides other applications, this technique used for the rapid multiplication of some plants (Tisserat 1987). The establishment of *in vitro* calli cultures were initiated from young leaves of *T. neriifolia* on MS medium supplemented with 1mg/l 2,4-D (Anjani and Pramod 1990). Anjani (1992) reported that calli cultures were induced within 30 days by culturing young leaf discs of *T. neriifoia* on MS medium supplemented with 9 µM 2,4-D and 4.6 µM Kin. Sub-culturing calli at lower 2,4-D and Kin concentrations (4.5 µM 2,4-D and 0.46 µM Kin) increased calli growth and produced numerous highly organized structures on calli surface. Furthermore, Anjani (1995) established *in-vitro* regeneration system from embryonic axis of *T. neriifolia*. He found that more than 85% of the *in vitro* derived shoots were rooted to give complete plantlets with an 80% survival rate on half-strength of MS medium containing 5.37 µM of NAA under glasshouse conditions.

Moreover, Dantas *et al.* (1993) reported that the highest cardiac glycoside contents were recorded in cell suspension cultures of *T. neriifolia* cultured on MS medium supplemented with kin + NAA, Kin+2,4-D, and IAA, respectively.

Consequently, Dantas *et al.* (1994) studied six strains of *T. neriifolia* cell cultures for cardenolides production. Even after two years of subculture, cardiac glycosides proved to be present in all these strains. The cardiac glycosides content varied from one species to another according to the nature of the original explants. Further, Lopes *et al.* (2001) reported that some compounds of cardiac glycosides found in the intact plant could be accumulate in cultured cells, cardiac

glycosides of *T. neriifolia*, accumulated in cultured cells during one year of cultivation.

This present work aim to described efficient protocol for; calli production; shootlets regeneration and, enhancement the accumulation rate of cardiac glycosides in different cultures of Egyptian *Thevetia spp*.

2. Materials and Methods

In vitro Immature seeds (IS) germination.

Immature seeds of both *Thevetia neriifolia* Juss. and *Thevetia thevetioides* Kunth were collected from Al-Orman garden, Giza, Egypt in March 2006. They were identified by Prof. Dr. K. H. El-Batanouny, Botany Department, Faculty of Science, Cairo University. Voucher specimens are deposited at the Herbarium of National Research Centre, Dokki, Cairo, Egypt. Then they were surface sterilized by immersion in 70 % ethanol for 15 sec., followed by washing with sterile distilled water for 3 times. Subsequently, they were immersed in 50 % of Clorox solution (5.25 Cl_2) containing a drop of Twin 20 for 15 min, and rinsed several times with sterile distilled water. The sterilized seeds were separated and aseptically cultured on MS basal medium (Murashige and Skoog, 1962) containing 3 % sucrose and 0.7 % agar added prior autoclaving at a pressure of 1.5 psi (1.2 Kg/cm^2) for 20 min. The pH of the culture medium adjusted to 5.8 by addition of 0.1 N HCl or 0.1 N KOH. Within 30 days, seeds germinated.

Establishment of calli

One-month-old plantlets at the physiological age of 4-7 cm of shoot length, 15- 26 cm length of root and the number of leaves 4-10, were used as a source of explants (Fig.1). Leaf, stem and root explants as well as IS were cultured on MS medium supplemented with different plant growth regulators as follow:- Basal MS medium (free of PGRs) (MS_0) ; MS+1mg/l 2,4-D +1mg/l Kin (MS_1); MS +1mg/l 2,4-D + 3mg/l Kin (MS_2); MS +3mg/l 2,4-D + 1mg/l Kin(MS_3); MS +5mg/l 2,4-D + 1mg/l Kin(MS_4);MS +1mg/l 2,4-D + 5mg/l Kin(MS_5);MS +5mg/l 2,4-D + 5mg/l Kin(MS_6) ;MS+ 1mg/l 2,4-D + 3mg/l BA(MS_7) ;MS + 1 mg/l NAA (MS_8); MS + 1 mg/l IAA (MS_9);MS + 1mg/l IBA (MS_{10}).

Cultures of all treatments were maintained under photoperiod of 16 hrs/day photoperiod at intensity of 1400 Lux (80 µ mol $m^{-2}S^{-1}$) using cool light fluorescent lamps (Philips). All cultures were incubated at 26 ±1 °C for 4 weeks.

Determination of calli growth parameters

The percentage of callus formation, fresh & dry weights and dry matter contents (%) were determined for each treatment after 4 weeks of cultivation.

Shootlets regeneration

Three pieces about 250 mg/jar of leaf, stem, root and IS derived calli from MS-medium supplemented with 1mg/l 2,4-D + 3mg/l Kin were cultured onto the following regeneration media: MS basal medium (free of PGRs) (MS_0);MS + 1 mg/l 2,4-D + 1 mg/l BA (MS_1); MS + 1 mg/l 2,4-D + 3 mg/l BA (MS_2); MS + 3 mg/l 2,4-D + 1 mg/l BA (MS_3); MS + 5 mg/l 2,4-D + 1mg/l BA (MS_4); MS + 5 mg/l 2,4-D + 1mg/l BA (MS_5) ;MS + 5 mg/l 2,4-D + 1mg/l BA (MS_6); Water+ Agar medium (free of salt and PGRs) (WA)

All cultures were incubated at 26°C and 1400 Lux (80-µ mol $m^{-2}S^{-1}$) using cool light fluorescent lamps (Philips) in a controlled growth room. After one month of culturing, percentage and number of formed shootlets recorded.

Adaptation and acclimatization

In vitro rooted shoots were carefully taken out of the tissue culture jars and gently washed under tap water to remove the residual agar and medium sticking to it. Then, the obtained plantlets were dipped in 1% aqueous solution of bavistin, a systemic fungicide for 10-15 min and then washed with tap water. Subsequently, the treated plantlets were transferred in (8 cm) pots filled with peatmoss or a mixture of peatmuss: sand in (1:1) or peatmoss: sand: perlit (1:1:1) or peatmuss: sand: perlit: vermiculite (1:1:1:1). Cultures were covered with polypropelene bags and kept in green house. The cultured plantlets were watered once in a week. The top corners of polypropelene bags were cut after two weeks to gradually expose the plants to the outside environment. After 3-4 weeks, the polypropelene bags were completely removed.

Preparation of cardiac glycosides forHPLC analyses

Extraction of cardiac glycosides based on the method described by Abe *et al.* (1994). One mg of the total cardiac glycosides was re-dissolved in 1ml of methanol HPLC grade, then filtered through 0.2-0.4 mM nylon filter and subjected to HPLC techniques.

Authentic compounds

The reference cardiac glycosides (peruvoside and neriifolin) were purchased from Sigma Chemicals Co., St. louis Mo. USA. Thevetin B and digitoxigenin were chemically separated and identified throughout this work according to the described method by Bisset *et al.* (1962) and Decosterd *et al.* (1994).

General procedures

HPLC was carried out on Agilent a series 1100 interface with stationary phase (RP18), injection volume (10 µl), oven temperature (25°C), diode array detector (254 nm), flow rate (1ml / min) and mobile phase: MeOH/H_2O (1:1) under gradient conditions. This method was carried out according to Kathleen *et al.* (2007). Column chromatography was carried out on silica gel 60 (Merck; 230 - 400 mesh). TLC: pre-coated silica gel 60F_{254} plates (Merck); CC: silica gel type 60 (Merck). MS: Murashige and Skoog medium (Duchefa Biochemie The Netherlands).

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Statistical analysis

All experiments were statistically analyzed using the F-test according to Steel and Torrie (1960). ANOVA was determined and the LSD was calculated at P=0.05. The data presented are the means of five replicates ± standard error (SE).

3. Results and Discussion

Calli production

Data tabulated in Table (1) show that the effect of different combinations of 2,4-D, NAA, Kin or BA at 1mg/l on fresh weight frequency of calli production from IS, leaf, stem and root explants of *T. neriifolia* and *T. thevetioides*. In this experiment MS_2 gave the highest value of calli production (Fig. 2 A and B) from IS, leaf, stem and root explants, respectively. Moreover, the incubation under light condition was preferred as compared with incubation under dark condition. Concerning, calli production from *Thevetia spp*. on MS_2 was in agreement with Anjani (1992) who reported that the presence of 2,4-D and Kin increases the biomass of different calli culture production from leaf explants of *T. neriifolia*. Similar effect was observed with calli culture production from *Solanum aviculare* (Kittipongpatana *et al*. 1998). Further, Preece (1995) reported that when the nutrient salts are optimized for *in vitro* tissues of a plant, the nutrient level and their balance in the medium makes tissues to be under less stress and *in vitro* performance is dramatically improved. This evidence was accordance to our observation that; calli cultures of *Thevetia spp*. which cultured on MS_2 medium do not exude any phenolics, with high calli biomass. From the obtained results, it may speculate that 2, 4-D might be less antagonistic hormone interplay with combination of Kin and resulted in higher calli biomass production. The pervious results are in consistent with Dasgupta and Datta (1980); Dhru *et al.* (1990) and Anjani (1992) who reported that the presence of 2,4-D and Kin increased the biomass of different calli production from leaf explants of *T. neriifolia*..

Table(1). Effect of augmented MS-medium with different combinations and concentrations of auxins and cytokinins on frequencies of callus formation from IS, leaf, stem and root explants of *T. neriifolia* and *T. thevetioides* cultured under light condition at 26 ± 1 °C.

Type of MS media	Calli production (g/jar)							
	T. neriifolia explants				*T. thevetioides* explants			
	IS	Leaf	Stem	Root	IS	Leaf	Stem	Root
MS0	-	-	-	-	-	-	-	-
MS1	-	++	++	++	++	+++	++	++
MS2	++++	++++	++++	++++	+++	+++	+++	+++
MS3	-	-	-	-	-	-	-	-
MS4	++	+++	+++	++	++	+++	+++	++
MS5	+	+	+	+	+	++	++	++
MS6	+	+	+	+	+	+++	++	++
MS7	+++	+++	+++	+++	++	+++	+++	++
MS8	++	++	++	++	+	++	++	+
MS9	++	++	++	++	+	+	+	+
MS10	++	++	++	++	+	++	++	+

Where: - =No calli induction, + =Calli FW 0.25~ 0.5 g/jar, ++ = Calli FW 0.5~ 1.5 g/jar, +++= Calli FW 1.5~ 2.0 g/jar and ++++ =Calli FW 2.0 ~ 3.0 g/jar.

Calli growth parameters

The obtained healthy calli (~250 mg/jar) from IS, leaf, stem and root of *Thevetia* spp. were sub-cultured on MS_2 to follow their growth pattern for five weeks. Data in Tables (2 and 3) show that, the highest fresh (FW), dry weights (DW) and dry matter content (%) (DMC) were recorded with IS explants of *T. neriifolia* and *T. thevetioides,* respectively.

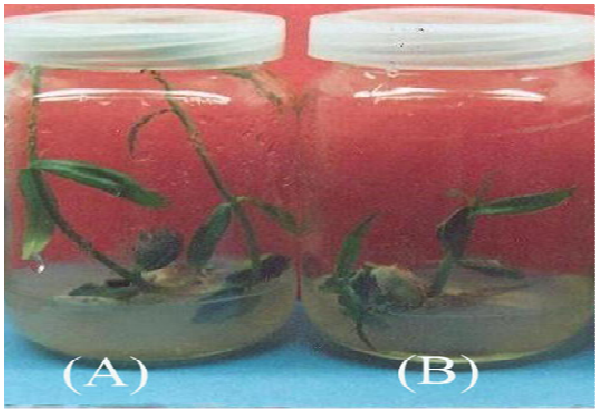


Fig. (1). One-month old seedlings of *T. neriifolia* (A) and *T. thevetioides* (B) were established on free of plant growth regulators MS medium

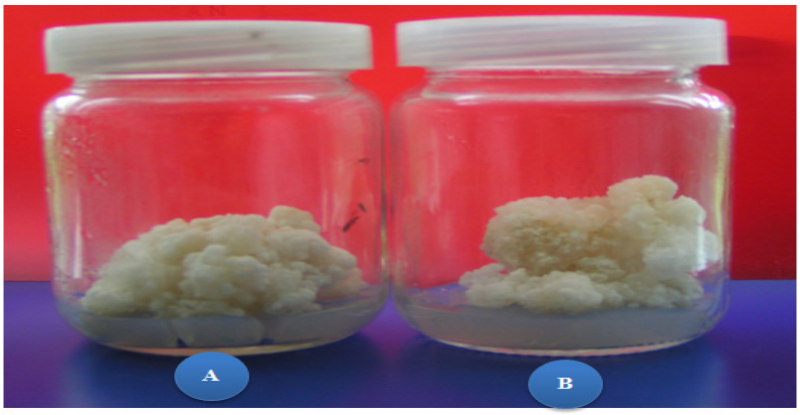


Fig.(2). Calli production from IS of *T. neriifolia* (A), and *T. thevetioides*, cultured on MS +1mg/l 2,4-D+3mg/l BA medium and incubated under light condition at 26±1°C for 3 weeks.

Concerning, *T.neriifolia* and *T. thevetioides* calli growth dynamics, the obtained results clearly showed that fresh & dry weights were weekly increased gradually up to the 3rd week of cultivation. Subsequently, the fresh & dry weights decreased in the 4th and 5th weeks of cultivation. Similar effect was observed with calli culture of *Solanum aviculare* (Kittipongpatana *et al.*, 1998). At the early stage of growth, the calli tissues were actively growing through cell multiplication and enlargement. During growth, synthesis of protein, nucleic acid, phospholipids, multiplication of organelles and utilization of energy in the form of ATP occurs (Kumar, 1999). Regarding, the morphological characters, it is interesting to mentioned that during 1st to 3rd week of cultivation, calli derived from IS, leaf, stem and root explants of the two investigated *Thevetia* spp. were healthy, friable and yellowish. However, the decline phase recorded after the log phase (i.e. 4-5th week), the growth slightly or even sharply was decreased. De and Roy (1985) attributed the decrease in the calli weights to the degradation of compounds over synthetic processes and/or the production and release of extracellular materials, which accumulated in the medium and not recovered in the cells of the senescent calli. Accordingly, the routine production of calli it was manifested by sub-culturing the initial calli on the same new fresh MS medium at the end of the 1st week to the end of the 5th week for the first month of calli cycle production.

Table (2). Effect of fortified MS medium with 1 mg/ l 2,4-D + 3 mg/ l Kin on calli fresh, dry weights (g/jar) and dry matter content (%). Calli were derived from IS, leaf, stem and root explants of *T. neriifolia* and incubated under light conditions at 26 ± 1°C.

Explants	*T. neriifolia* calli growth parameters incubated under light condition														
	1st week			2nd week			3rd week			4th week			5th week		
	FW	DW	DMC	FW	DW	DMC	FW	DW	DMC	FW	DW	DMC	FW	DW	DMC
IS	1.25±0.011	0.056±0.2	4.48	1.63±0.04	0.095±0.01	5.83	2.95±0.2	0.211±0.06	7.15	2.83±0.04	0.187±0.04	0.187	2.75±0.02	0.175±0.03	6.36
Leaf	1.25±0.11	0.053±0.6	4.24	1.51±0.21	0.073±0.01	4.83	2.73±0.7	0.185±0.2	6.78	2.65±0.05	0.175±0.03	0.175	2.54±0.01	0.154±0.2	6.06
Stem	1.26±0.02	0.055±0.03	4.37	1.43±0.05	0.052±0.06	3.64	2.47±0.09	0.153±0.32	6.19	2.35±0.01	0.143±0.01	0.143	2.27±0.6	0.127±0.02	5.59
Root	1.27±0.03	0.050±0.07	3.94	1.35±0.3	0.041±0.07	3.04	2.34±0.03	0.142±0.3	6.09	2.25±0.03	0.125±0.1	0.125	219±0.09	0.109±0.04	4.98

Table (3). Effect of fortified MS medium with 1 mg/ l 2,4-D + 3 mg/ l Kin on calli fresh, dry weights (g/jar) and dry matter content (%). Calli were derived from IS, leaf, stem and root explants of *T. thevetioides* incubated under light conditions at 26 ± 1°C.

Explants	Calli growth parameters incubated under light conditions														
	1st week			2nd week			3rd week			4th week			5th week		
	FW	DW	DMC	FW	DW	DMC	FW	DW	DMC	FW	DW	DMC	FW	DW	DMC
IS	1.25±0.03	0.054±0.09	4.32	1.54±0.02	0.083±0.01	5.85	2.85±0.03	0.193±0.06	6.77	2.49±0.01	0.165±0.02	6.63	2.34±0.03	0.154±0.03	6.58
Leaf	1.26±0.07	0.053±0.02	4.21	1.48±0.03	0.070±0.08	2.60	2.60±0.02	0.175±0.02	6.73	2.43±0.03	0.153±0.03	6.29	2.31±0.3	0.143±0.01	6.19
Stem	1.25±0.05	0.049±0.06	3.92	1.35±0.05	0.041±0.03	2.40	2.40±0.05	0.143±0.08	5.96	2.25±0.05	0.138±0.02	6.13	2.19±0.1	0.125±0.01	5.71
Root	1.28±0.02	0.048±0.03	3.75	1.30±0.03	0.029±0.05	2.29	2.29±0.02	0.135±0.03	5.89	2.18±0.02	0.125±0.04	5.73	2.12±0.1	0.120±0.01	5.66

Shootlets regeneration

Data tabulated in Table (4) show that the best culture medium for shootlets regeneration was MS_2 as compared with other media (Figs. 3 and 4). The maximum numbers of regenerated shootlets 10.3, 6.7 and 4.0 shootlets were recorded with stem, leaf and seed calli cultures, respectively of *T. neriifolia*. However, they recorded 4.5, 3.1 and 2.2 shootlets for stem, leaf and seed calli cultures of *T. thevetioides*. The maximum value of direct shootlets regeneration was noted with shoot tip, leaf, seed, stem and root cultures, respectively. Concerning, the derived shootlets length 5.6, 3.5 and 2.6 (cm) were recorded with stem, leaf and seed calli cultures of *T. neriifolia*. However, they recorded 4.3, 3.1 and 2.5 (cm) for stem, leaf and seed cultures of *T. thevetioides* (Fig.4 A and B).

Regarding the maximum numbers of shootlets regeneration from different calli-derived explants were recorded with MS_2 as compared with other treatments. The obtained results are in consistent with the obtained results by Ratna and Misra (2005). They reported that shoot tips derived calli of mature plants of *Carissa carandas*; when cultured on MS basal medium supplemented with 0.8 mg /l IBA and 0.2 mg /l NAA produced the maximum sprouting rate.

Table (4). Effect of supplementation of MS-medium with different combinations and concentrations of auxins and cytokinins on percentage of sootlets formation from IS, leaf, stem and root explants of *T. neriifolia* and *T. thevetioides* cultured under light condition at 26 ± 1 °C.

Type of MS media	Calli production (g/jar)							
	T. neriifolia explants				*T. thevetioides* explants			
	IS	Leaf	Stem	Root	IS	Leaf	Stem	Root
MS0	-	-	-	-	-	-	-	-
MS1	-	++	++	++	++	+++	++	++
MS2	++++	++++	++++	++++	+++	+++	+++	+++
MS3	-	-	-	-	-	-	-	-
MS4	++	+++	+++	++	++	+++	+++	++
MS5	+	+	+	+	+	++	++	++
MS6	+	+	+	+	+	+++	++	++
WA	-	-	-	-	-	-	-	-

where (*): – = No response, $^{+}$ = Low regeneration value (0~20%), $^{++}$ = Medium regeneration value (20~60%), $^{+++}$ =High regeneration value (60~100%). Each treatment is the average of 5 replicates.

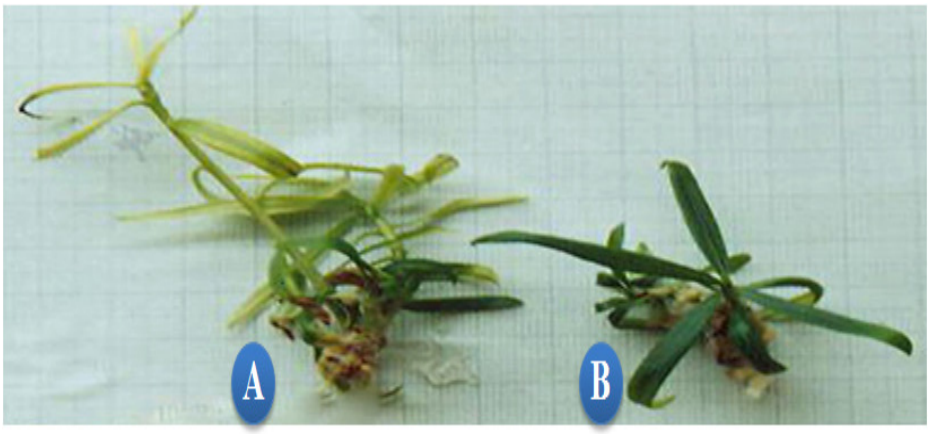
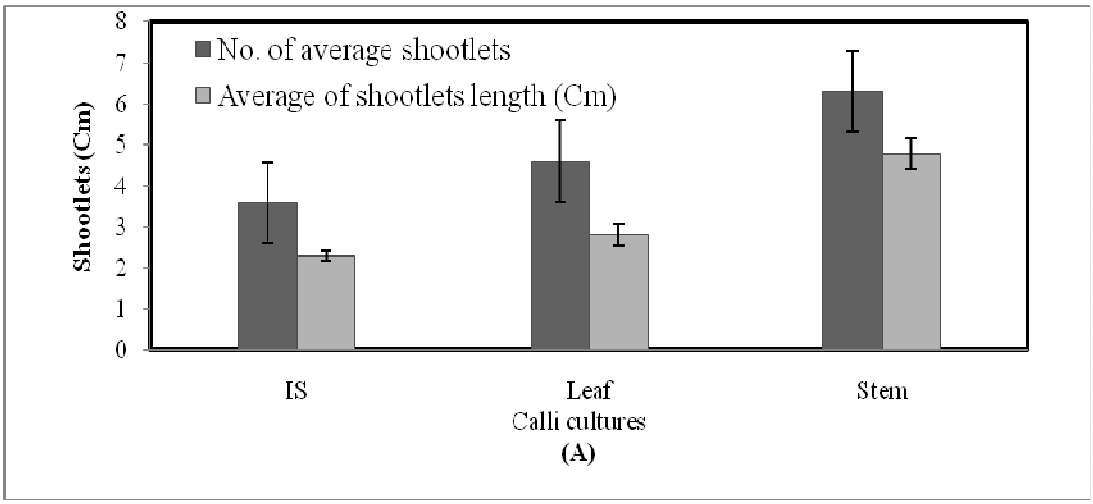


Fig.(3). *In vitro* shootlets regeneration from stem *derived* calli of *T. neriifolia* (A) and *T. thevetioides* (B) on MS_2 after 4 weeks of cultivation under light condition at 26±1°C.

Rootlets shoot formation

The effect of MS_0 or MS_8 or MS_9 or MS_{10} on achievement of rootlets formation on *in vitro* derived regenerated shootlets of stem explants of *T. neriifolia* and *T. thevetioides* is illustrated in Fig 5 (A and B). The highest percentages of root formation 63% and 52% were recorded with *T. neriifolia* and *T. thevetioides*, respectively. The maximum numbers of rootlets/shootlets formation 32 and 10 were recorded with *T. neriifolia* and *T. thevetioides* respectively. Furthermore, length of rootlets formation 17.6 and 14 (cm/shoot) were recorded with *T. neriifolia* and *T. thevetioides*, respectively. MS medium supplemented with 1mg/l NAA gave the favorable percentages of rootlets formation, number of rootlets/shootlet and length of rootlet (cm) as compared with other supplementations, as well as compared with MS basal medium.



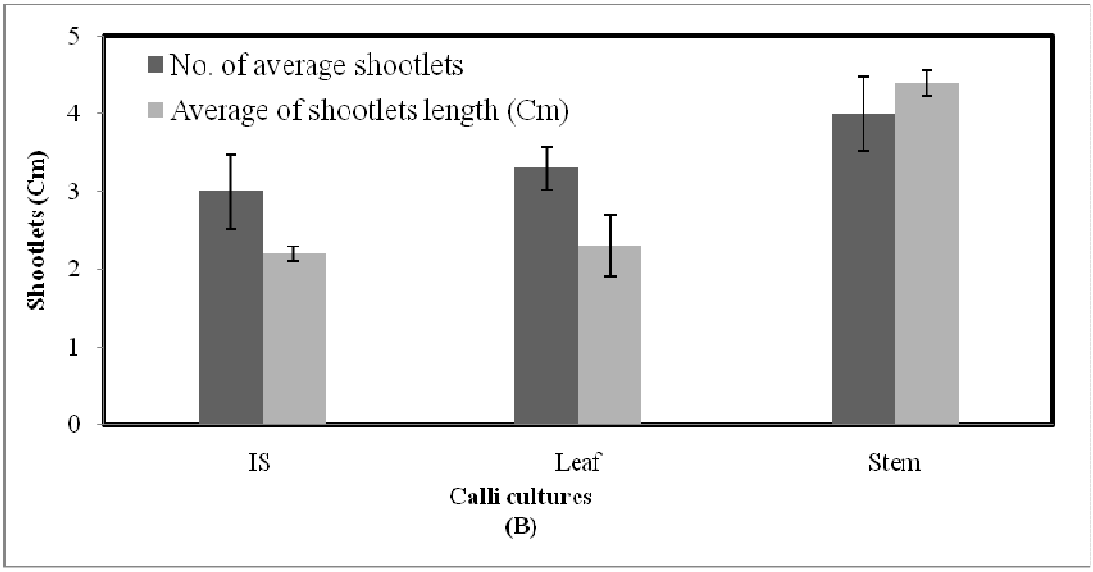


Fig. (4). Effect of MS_2 on number and length (cm) of regenerated shootlets derived from IS, leaf and stem calli-cultures of *T. neriifolia* (A) and *T. thevetioides* (B) cultured under light conditions at 26±1°C for4 weeks.

The obtained results are in agreement with the obtained results with Anjani and Abha (1994) they reported that shoots of *Catharanthus roseus* and *T. neriifolia* were rooted on MS basal medium containing 1mg/l NAA. In addition, the obtained results are in harmony with the obtained results by Soo *et al.* (2003) they reported that, MS augmented with 1mg/l NAA was proved to be the best concentration of NAA for induction of adventitious roots from base of regenerated shoots of *T. neriifolia*.

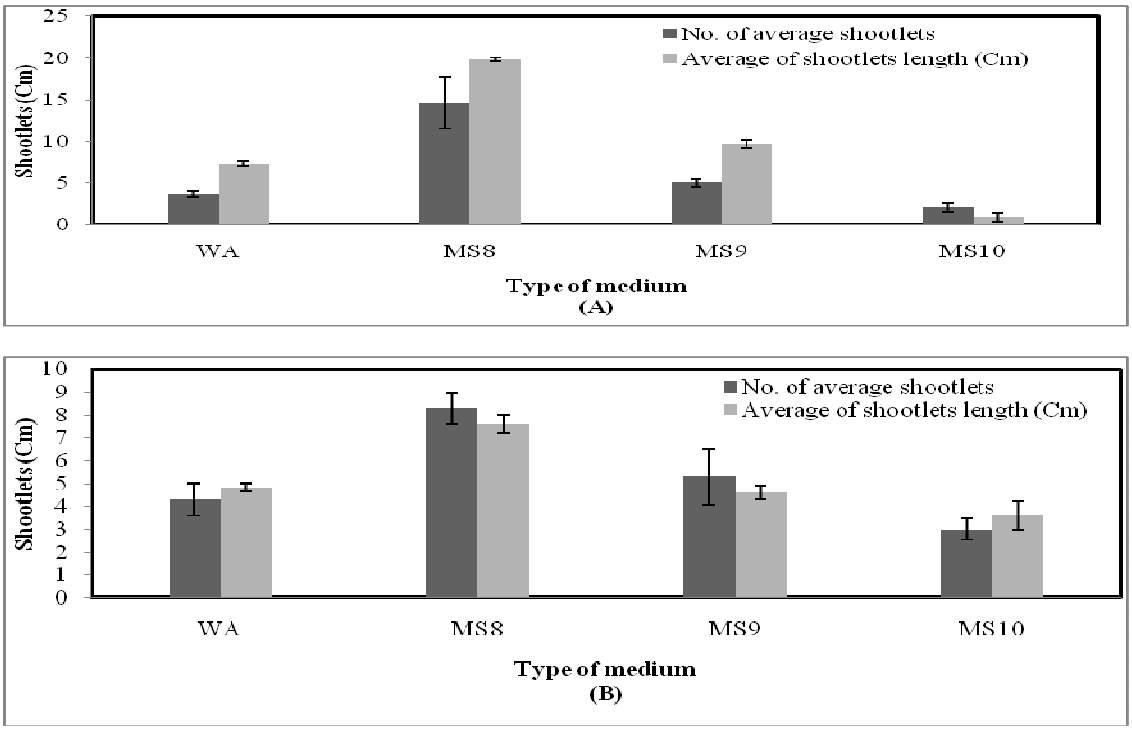


Fig. (5). Effect of WA; MS_8; MS_9 or MS_{10} media on rootlets shoots formation parameters of *T. neriifolia* (A) and *T. thevetosides* (B).

Fig.(6). In vitro rootlets/shootlets formation of *T. neriifolia* (A) and *T. thevetosides* (B) leaf explants and cultured on MS medium supplemented with 1 mg/l NAA

Adaptation and acclimatization

As shown in Table (5) the highest percentages of survival plantlets 70% and 63% were recorded with *T. neriifolia* and *T. thevetioides*, respectively. The best potting mixture medium for *in vitro Thevetia* spp. plantlets acclimatization was peatmuss, sand, perlit and vermiculite (1:1:1:1) as compared with other media structure. Furthermore, the best number of shootlets formation 6 and 5 (cm) and length of root 10.5 and 6.3 (cm) as a growth parameters were recorded with *T. neriifolia* and *T. thevetioides*, respectively, when cultured on peatmuss, sand, perlit and vermiculite (1:1:1:1) as compared with other potting mixture media (Fig. 7).

The obtained results are in close with the recorded results by Ratna and Misra (2005). They reported that shoot tips derived from calli of mature plants of *Carissa carandas*; when cultured on MS basal medium supplemented with 0.8 mg /l IBA and 0.2 mg /l NAA produced maximum sprouting rate. The obtained shoots were

rooted on MS medium supplemented with 1mg/l NAA. Moreover, in agreement with the obtained results; Anjani and Abha (1994) reported that shoots of *Catharanthus roseus* and *T. neriifolia* were rooted on MS basal medium supplemented with 1mg/l NAA. Furthermore, the use of NAA, as a potential root hormone, is in agreement with those reported for rooting in microshoots of *Nerium oleander* (Hatzilazarou *et al.* 2003). The rootlet plantlets of *T. neriifolia* and *T. thevetosides* were successfully acclimatized in potting mixture peatmuss, sand, perlit and vermiculite (1:1:1:1). It was found to be in consistent with the obtained results by Ratna and Misra (2005). Moreover, Debergh and Maene (1981) reported that the induction and development of root system at the bases of *in vitro* grown shoots of *Nerium oleander* is an essential and indispensable step to establish tissue culture derived plantlets to the soil.

Qualitative determination of cardiac glycosides using HPLC technique

Standard curves corresponding to the compounds thevetin B, digitoxigenin, peruvoside and neriifolin were carried out using the described protocol by Scott (1996). The concentrations of referred compounds in the examined samples were detected using its retention time and peak area.

Table(5). Effect of different nutrient media composition on root parameters formation on isolated shootlets of *T. neriifolia* and *T. thevetioides.*

Media construct	*T. neriifolia*			*T. thevetosides*		
	(%) of survival plantlets	No. of shoot /plantlet	Length of root (cm)/plantlet	(%) of shoots formation	No. of survival plantlets	Length of root (cm)/plantlet
1.	20± 0.7	1± 0.1	2.1± 0.3	14± 1.3	2± 0.3	2± 0.3
2.	35 ± 5.3	2 ± 0.4	3.4± 0.2	25 ± 1.2	3± 0.1	2.1±0.1
3.	49± 3.3	4± 0.9	4.2± 0.4	33± 2.7	4± 0.2	4.1± 0.5
4.	70± 4.1	6± 0.1	10.5± 0.1	63± 1.3	5± 0.7	6.3± 0.2

Where: 1- Peatmoss (1); 2- Peatmoss + sand (1:1); 3- Peatmoss+ sand+ perlit (1:1:1); 4- Peatmoss+ sand+ perlit+ vermiculite (1:1:1:1).

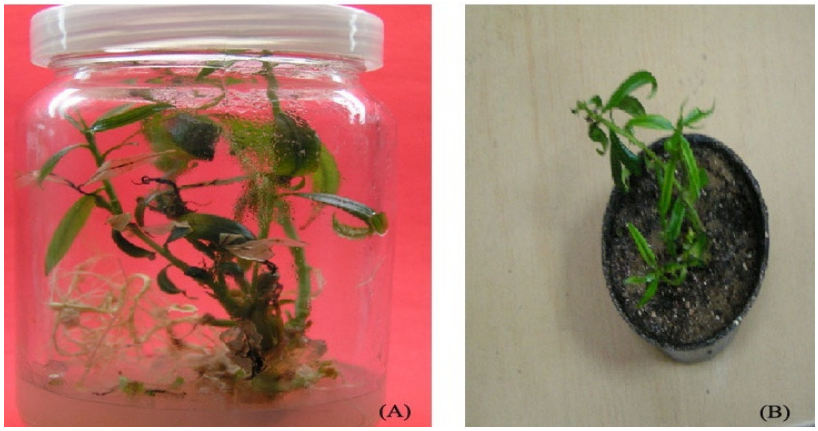


Fig. (7). Rootlets shootlets formation (derived from leaf calli cultures) on MS medium supplemented with NAA (1 mg/l), after 4 subcultures (A). Hardened *in vitro* derived plantlets of *T. neriifolia* in pots containing peatmuss, sand, perlit and vermiculite (1:1:1:1) (B).

As shown in Table (6) the percentages of thevetin B, digitoxigenin, peruvoside and neriifolin of *in vivo* IS of *T. neriifolia* plant were found to be 19, 12, 39 and 25 % , respectively. Moreover, the percentage of those compounds in *in vivo* IS of *T. thevetioides* plant were found to be 99, 15, 24 and 39 %, respectively. In addition, the maximum relative percentage of accumulated cardiac glycosides in different types of calli and regenerated shootlets cultures was recorded after 4 weeks of cultivation for IS of *T. neriifolia* and *T. thevetioides*, respectively. The highest relative percentage of thevetin B (85.7%) was found in *T. neriifolia* IS calli cultures. However the highest value of digitoxigenin (52.8 %) was found in *T. thevetioides* stem calli cultures. The maximum percentage of peruvoside (33.1%) was found in root calli cultures of *T. neriifolia*. While the maximum percentage of neriifolin (62.2 %) was found in *T. thevetioides* root calli cultures.

Moreover, the maximum percentages of thevetin B and neriifolin were found in shootlets (I) of IS calli cultures of *T. neriifolia* to be 29 % and 15.5%, respectively. While, the maximum percentage of digitoxigenin and peruvoside were found in shootlets (II) leaf calli cultures of *T. neriifolia* to be 42 % and 33%, respectively compared with *T. thevetioides.* The detail relative percentages were tabulated in Tables (7 and 8). The obtained calli from IS, leaf, stem and root explants of *Thevetia* spp. under dark condition clearly showed no cardiac glycosides formation.

In agreement, with our obtained results Gopa and Datta (1981) reported that the highest accumulation of thevetin B was detected in seed calli cultures of *T. neriifolia*. Dasgupta *et al.* (1987) reported that HPLC analysis of *in vitro* mass propagation of *T. neriifolia* revealed the presence of cardiac glycosides at different values. Furthermore, Dantas *et al.* (1993) noted that cardenolide formation was recorded in cell suspension culture of *T. neriifolia* cultures. In addition, Lopes *et al.* (2001) reported that some compounds of cardiac glycosides were accumulated in cultured cells of *T. neriifolia*. In close with our obtained results, Dantas *et al*. (1994) studied six cell suspension cultures that established from different organs of *T. neriifolia* under various growth conditions. Further, they reported that after two years of sub-culturing, cardiac glycosides was a present in all of the studied cultured organs. Moreover, Anjani and Pramod (1990) reported that the quantity of the glycoside of *T. peruviana* was increased with the age of culturing on MS medium supplemented with 1 mg /l 2,4-D, for more than 200 days. Whereas, in the present study we clearly showed that the cardiac glycosides content was decreased after 4 weeks of cultivation on supplemented of MS medium with 1mg/l 2,4-D+3mg/l Kin.

Table (6). Percentage of thevetin B, digitoxigenin, peruvoside and neriifolin in *in vivo* IS of *T. neriifolia* and *T. thevetioides.*

	Percentage cardiac glycosides in *in vivo* IS			
	Thevetin B	**Digitoxigenin**	**Peruvoside**	**Neriifolin**
T. neriifolia	19	12	39	25
T. thevetosides	99	15	24	39

Accumulation rate of cardiac glycosides during 4 weeks of cultivation

It was noted that, the 2nd week of IS of both *Thevetia* spp recorded the best time for thevetin B and neriifolin production. In addition, the 3rd week gave the maximum rate of peruvoside formation. However, the 4th week of cultivation recorded the best result of digitoxigenin accumulation under light condition (Fig. 8 A and B).

Concerning, the accumulation rates of cardiac glycosides in *T. neriifolia* calli cultures at different times were in close with Marina *et al.* (1999), they reported that the regenerated shoot cultures of *Digitalis lanata* produced cardiac glycosides reached up to 0.6 µ mol/g DW when cultured under continuous white light for 3 weeks.. Moreover they reported that the accumulation rate of cardiac glycosides was gradually decreased and reached non-detectable levels after 12 weeks of cultivation. These results are in agreement with our obtained results that clearly showed that the incubation of the different explants of *Thevetia* spp. under dark condition is not favorable for biosynthesis and accumulation of cardiac glycosides.

Table (7). Determination of cardiac glycosides (%) in different calli cultures of *T. neriifolia* and *T. thevetoside* relative to IS of intact plant using HPLC technique.

Type of *Thevetia* species	Percentage of cardiac glycosides in calli cultures relative to IS of intact plant															
	IS				Leaf				Stem				Root			
	The	Dig	Per	Ner	The	Dig	Per	Ner	The	Dig	Per	Ner	The	Dig	Per	Ner
T. neriifolia	85.7	28.9	13.2	19.2	34.7	24.9	27.5	13.71	30.01	29.43	21.56	20.3	14.3	12.33	33.1	56.4
T. thevetosides	47	27.9	17.8	32.3	42.3	17.81	6.8	5.6	42.4	52.8	6.03	60.2	37.2	23.6	26.4	62.2

Where: Not detected (-), thevetin B (R_t = 9.48) (The), digitoxigenin (R_t = 11.01) (Dig), peruvoside (R_t = 11.32) (Per) and neriifolin (R_t = 12.72) (Ner)

Table (8). Determination of cardiac glycosides (%) in different type of regenerated shootlets as compared with corresponding content of IS of *T. neriifolia* and *T. thevetosides* using HPLC technique.

	Shootlets (I)				Shootlets (II)				Shootlets (III)			
	The	Dig	Per	Ner	The	Dig	Per	Ner	The	Dig	Per	Ner
T. neriifolia	57.29	18.5	17.15	15.5	26.6	42	33	12.4	32	21.8	5.8	7.9
T. thevetosides	-	19.2	16.8	5.23	12.3	22.1	27.3	8.9	31.1	24.4	53.2	19.9

Where: Not detected (-), thevetin B (R_t = 9.48) (The), digitoxigenin (R_t = 11.01) (Dig), peruvoside (R_t = 11.32) (Per) and neriifolin (R_t = 12.72) (Ner), shootlets (I) from seed, shootlets (II) from leaf and shootlets (III) from stem calli cultures

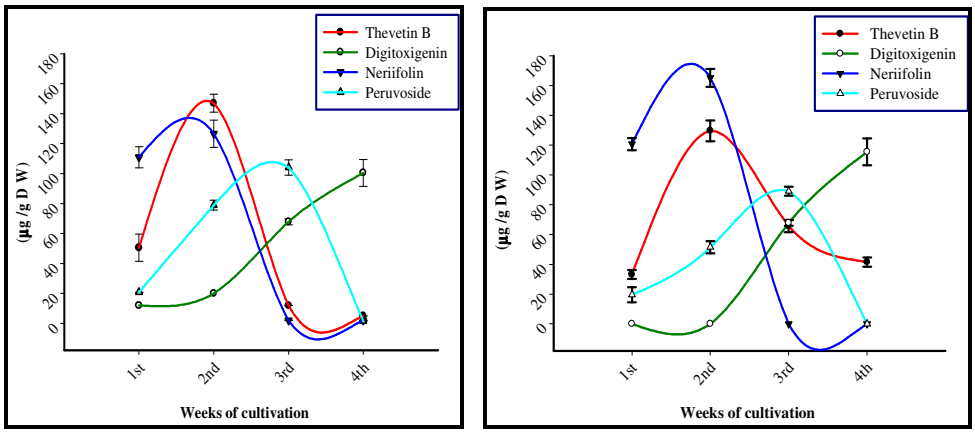


Fig. (8). Production dynamic of thevetin B, neriifolin, peruvoside and digitoxigenin (µg/g DW) of seeds derived calli *of T. neriifolia* (A) and *T. thevetioides* (B) during 4 weeks of growth stages.

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