International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 17-01-2021; Accepted: 17-02-2021: Published: 23-02-2021
www.botanyjournals.com
Volume 6; Issue 1; 2021; Page No. 581-587

Effect of 2, 4-D in combination with cytokinins on somatic embryogenesis of *Thevetia peruviana* leaf explants

EA Nesy[1*], Lizzy Mathew[2]
[1] Department of Botany, KKTM Govt. College, Pullut, Thrissur, Kerala, India
[2] Department of Botany, St. Teresa's College, Ernakulam, Kerala, India

Abstract
Studies have been conducted using *Thevetia peruviana* (Apocynaceae) leaf explants to evaluate the effect of auxin 2.4-D on varying concentrations and combinations of cytokinins (KIN, BA) for the induction of somatic embryos, as the taxon is a rich treasure of many natural cardiac glycosides. Soft, friable creamy yellow calli was obtained in 2, 4-D + KIN combination, where as it was creamy white and granular in 2,4D + BA. Results indicated that most of the tested concentrations were effective, but lower concentrations of 2, 4-D + KIN (0.5 - 1.0 mg/L^{-1}) were more conducive than higher (2.0 - 3.0 mg/L^{-1}) combinations for the induction of somatic embryos. Among the cytokinins, KIN is more efficient than BA. An increase in KIN concentration from 0.5 to 3.0 mg/ L^{-1}, keeping 2.4-D constant (1.0 mg/ L^{-1}) resulted in greater callus biomass with lesser frequency of somatic embryos, and the frequency of embryogenic masses decreased as the KIN concentration increased. Mature detached bipolar embryos with well-developed cotyledons and radicles developed into fully matured plantlets in 68% of culture tubes successfully in full strength MS medium having IBA + BA (0.1 - 0.5 mg/L^{-1}) for regeneration. In order to prevent natural erosion of genetic resources, this successful regeneration protocol is useful for large scale multiplication within a short period of time and to minimize the impact of over exploitation by therapeutic industrialists.

Keywords: auxin, callus induction, embryos, regeneration, sub culture

Introduction
Thevetia peruviana, synonymously called *T. neriifolia* is one among the most important medicinal shrubs in the family Apocynaceae, with a wide range of pharmacological activities. Various plant parts were reported to have diuretic, cardiotonic, emetic and purgative properties, hence, traditionally used to cure cardiac disorders, arthritis, rheumatism, hemorrhoids, acne, constipation and nausea[1, 2]. Apart from this, the plant deserves an important and significant place in the therapeutic industry as it contains a higher percentage of cardiac glycosides, including the most valuable cardiotonic drug Peruvoside [3]. The plant grows up to a height of 12 - 16 ft and is ornamented with evergreen foliage and large attractive terminal or axillary cymes that flourish in different colours.
Genus *Thevetia* is conventionally propagated by seeds in their natural habitat. Exploitation of richest medicinal flora by industrialists from their habitats leads to the destruction of natural resources, loss of biodiversity and extinction of endangered species [4, 5]. To compensate this loss, it is necessary to establish an alternate source of biological materials for the continuous supply for the successful production of valuable therapeutics, without depleting natural resources. Plant tissue culture technique plays an important role in the vast and continuous production of enough plantlets throughout the year, independent of the seasons. At the same time they can be used as raw materials for their regular supply to therapeutic industries [6, 7].
Studies have been conducted earlier in the establishment of callus cultures [8] and plantlet regeneration from the young leaves of this plant [9, 10]. Similarly, calli and regenerated microshoots were developed by Taha *et al*., [11] from the immature seeds, leaves, stem and roots of *Thevetia* sps growing in Egypt. *In vitro* regeneration studies have been successfully carried out with other members of the family Apocynaceae including *Rauvolfia serpentina* (L.) Benth. [12, 13], *Tabernaemontana fuchsiaefolia* L. [14], *Nerium oleander* L. [15, 16], *Vinca rosea* L. [17, 18], *Carissa carandas* L.[19] and *Cryptolepis grandiflora* Wight [20].
In vitro propagation is beneficial to conserve and maintain plants with immense medicinal values by effective regeneration of micro-shoots via indirect organogenesis and somatic embryogenesis. Therefore, present study was designed to develop an efficient and reproducible protocol for the vast multiplication of micro-propagules from 4 - 5 week old seedlings.

Materials and methods
Studies were conducted using various plant growth regulators (Sigma Aldrich, USA), analytical grade chemicals (E-Merck India Pvt Ltd) and quality glass wares (Borosil Pvt Ltd) following the standard tissue culture protocol.

Preparation of culture media
Based on Murashige Skoog[21] media composition, stock solutions were mixed together by adding 3.0% sucrose (w/v), 0.75% agar (w/v), different auxins 2,4-D (2,4 Dichloro phenoxyacetic acid), IBA (Indole Butyric Acid) and cytokinins KIN (Kinetin), BA (6-Benzyl Adenine) in varying ratios as growth regulators. The pH of the medium was adjusted to 5.8 ± 0.02 using 0.1 N HCl or 0.1 N NaOH before autoclaving at 121 ^{o}C for 15 min.

Source of explants
Mature seeds fallen from yellow oleander plants were collected, washed thoroughly in running tap water to remove adhering soil particles. After overnight soaking, seeds were allowed to germinate in small polyethylene trays in normal environmental conditions. This 4 - 5 week old, young, healthy seedlings were used as a source of explant, in order to minimize contamination.

Surface sterilization of explants
Mature leaves above the cotyledonary node was excised from the seedling, washed thoroughly and treated with 1% Tween 20 (v/v), followed by fungicide Bavistin (1% w/v). After thorough rinsing with distilled water, it was surface sterilized with 0.1% mercuric chloride (w/v) for 1 min under Laminar Air Flow Chamber (Kemi Pvt Ltd, India) and was followed by several rinses with sterile distilled water.

Hormonal combinations
To evaluate the morphogenic potential of leaf explants, combinations of auxins (2, 4-D, IBA) and cytokinins (KIN, BA) in different concentrations (0.5 - 3.0 mg/L^{-1}) were attempted, keeping either auxin or cytokinin as constant (0.5 - 3.0 mg/L^{-1}). The effect of 2, 4-D was evaluated individually (1.0 mg/L^{-1}) and in combination (0.5 - 3.0 mg/L^{-1}) with both cytokinins BA and KIN (0.5 - 3.0 mg/L^{-1}). Basal medium (MS) without any growth regulators served as control.

Inoculation
Tender leaves below 1.5 cm of the apical shoot were used as explants. For initiating callus cultures, explants of uniform size and shape (0.4 - 0.8 cm^2) were transferred into semisolid MS medium supplemented with specific concentrations of growth regulators. After inoculation, all cultures were maintained at 26 ± 2 ºC for a photoperiod of 16 h under diffused cool white fluorescent lamps with a light intensity of 2000 Lux.

Sub culturing and acclimatization
First subculturing of all proliferated calli was done in the primary medium for 4 - 6 weeks. Thereafter, calli with somatic embryos were transferred to a lower concentration of IBA (0.1 mg/L^{-1}) + BA (0.5 mg/L^{-1}) medium for regeneration, shoot elongation and maturation. Plantlets with well-developed rooting system was transferred into paper cups containing autoclaved mixture of sand and soil diluted with 0.1% activated charcoal. Subsequently, the cultures were covered with moistened perforated polyethylene bags to maintain humidity for 15 days. Plantlets were gradually exposed to the surrounding environment and later transferred to greenhouse conditions for natural acclimatization.

Statistical analysis
The results of various treatments are expressed as Mean ± SD. One way ANOVA with post-hoc Duncan's test was used to compare significant differences between group means using SPSS software version 20. A level of $p < 0.05$ was regarded as statistically significant.

Results
In vitro studies were carried out using one month old seedlings, hence a high survival rate (90 - 95%) was achieved in most combinations, compared to field collected explants. Within 10 days of inoculation, the cut surfaces of leaf explant responded quickly for callus initiation. Initially, most of the cultures produced creamy white callus, and morphology of calli varied thereafter during subsequent transfers according to hormonal combinations. The calli was soft, friable and creamy yellow in 2,4-D + KIN combination, where as it was creamy white and granular in 2,4-D + BA. However, without any growth regulators, the explants failed to initiate cell division in MS medium even after 6 weeks of inoculation.

Effect of 2, 4-D + KIN combinations
Callus cultures initiated from healthy juvenile leaf explants of *T. peruviana* showed slight dissimilarity in morphology and behaviour in MS medium supplemented with varying concentrations of 2,4-D + KIN (0.5 - 3.0 mg/L^{-1}). When both hormones were supplied individually (1.0 mg/L^{-1}), the cut ends produced either very little callus or the newly formed cells failed to organize into callus clumps. Induction of creamy white to light greenish yellow, friable callus were achieved in all combinations, but their biomass and embryogenic potential varied considerably according to the concentrations of growth regulators in the medium. Embryogenic clumps having initial stages of development, appeared on the callus surface during the first 4 - 5 weeks of culture.
The effect of varying concentrations of 2,4-D (0.5 - 3.0 mg/L^{-1}) on leaf explants was evaluated keeping KIN constant (0.5, 1.0, 2.0, 3.0 mg/L^{-1}), and similar experiments were carried out keeping 2,4-D constant (0.5, 1.0, 2.0, 3.0 mg/L^{-1}). After 5 weeks of culture, light green meristematic patches were observed on the creamy white callus mass, in the presence of 2, 4-D. These green patches upon detailed observation, showed the presence of proembryogenic structures. At lower concentrations of 2,4-D (0.5 - 1.0 mg/L^{-1}) + KIN (1.0 mg/L^{-1}), the frequency of these proembryogenic clumps was higher as mentioned in Table 1; and almost the entire callus surface was covered with different stages of proembryos. As the concentration of 2,4-D increased (2.0 - 3.0 mg/L^{-1}), embryogenic potential retarded considerably limiting the formation of proembryogenic masses ($p < 0.01$) into 2 - 3 patches, and the results are tabulated in Table 2.

Table 1: Effect of 2, 4-D (1.0 mg/L^{-1}) + KIN (0.0 - 3.0 mg/L^{-1}) and KIN (1.0 mg/L^{-1}) + 2, 4-D (0.0 - 3.0 mg/L^{-1}) on callus morphology and embryogenic responses

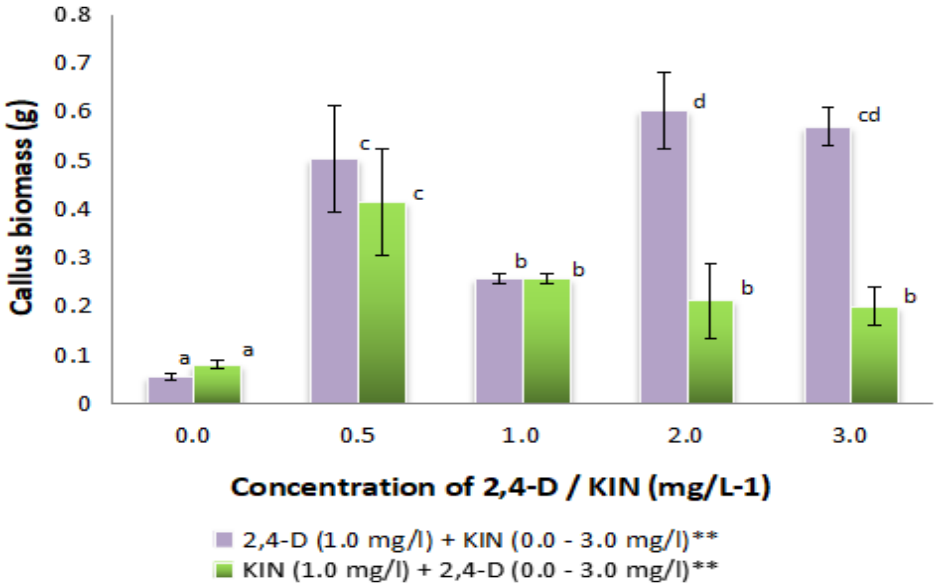
KIN/ 2,4-D (mg/L^{-1})	2,4-D (1.0 mg/L^{-1})		KIN ((1.0 mg/L^{-1})	
	Callus morphology	% of embryogenic calli	Callus morphology	% of embryogenic calli
0.0	Snowy white	0.00	Light green, granular	0.00
0.5	Creamy, light green, friable	66.66	Light green, friable	100.00
1.0	Dark green, friable	100.00	Dark green, friable	100.00
2.0	Creamy white, granular	81.18	Creamy, light brown, less compact	27.77
3.0	Creamy white, less compact	68.18	Creamy, light brown, less compact	11.76

Table 2: Effect of 2, 4-D (2.0 - 3.0 mg/L^{-1}) + KIN (0.5 - 3.0 mg/L^{-1}) on callus morphology, biomass and embryogenic responses

KIN (mg/L^{-1})	2,4-D (2.0 mg/L^{-1})			2,4-D (3.0 mg/L^{-1})		
	Callus morphology	Fresh callus weight (g)	% of embryogenic calli	Callus morphology	Fresh callus weight (g)	% of embryogenic calli
0.5	Creamy white, powdery	0.198^{a} ± 0.039	0.00	Creamy white	0.065^{a} ± 0.010	0.00
1.0	Creamy white, compact	0.211^{a} ± 0.021	17.64	Light brown, compact	0.200^{b} ± 0.024	0.00
2.0	Creamy light brown, compact	0.383^{b} ± 0.050	35.29	Light brown, compact	0.254^{c} ± 0.027	11.76
3.0	Compact, light brown	0.397^{b} ± 0.030	41.17	Light brown, compact	0.275^{c} ± 0.027	17.64

Values are mean ± SD. Superscript letters represent significant differences ($p < 0.01$) when compared using DMRT

Higher concentrations of KIN supplementation (2.0 - 3.0 mg/L^{-1}) did not make any remarkable impact on the differentiation of somatic embryos. An increase in KIN concentration from 0.5 to 3.0 mg/L^{-1}, keeping 2.4-D constant (1.0 mg/L^{-1}) resulted in greater callus biomass ($p < 0.01$) with lesser frequency of somatic embryo formation, and the frequency of embryogenic masses decreased as the KIN concentration increased. The results are expressed in Fig 1.



Values are mean ± SD; **$p < 0.01$, values with different superscript letters show significant variation

Fig 1: Effect of 2, 4-D (1.0 mg/L^{-1}) + KIN (0.0 - 3.0 mg/L^{-1}) and KIN (1.0 mg/L^{-1}) + 2, 4-D (0.0 - 3.0 mg/L^{-1}) on callus biomass

Somatic embryos growing from Proembryonal clusters tend to develop asynchronously, so that several stages were present in the culture tubes at any given time. Globular staged embryos appeared as pale yellow round bodies, heart shaped embryos as light green structures, and further enhancement led to the dark green torpedo and cotyledonary stages on the surface of calli. Various developmental stages of somatic embryogenesis is presented in Fig 2. Their formation was noticed in all combinations, but the optimum concentration for the production of maximum embryogenic callus was found in the lowest level of 2,4-D (0.5 mg/L^{-1}) + KIN (1.0 mg/L^{-1}) and equal concentration (1.0 mg/L^{-1}) of both hormone supplements. After 5 weeks of initial culture, calli were subcultured in the primary medium for further maturation.

Fig 2: Various stages of somatic embryogenesis in 2,4-D + KIN combinations **(**mg/L^{-1}) from leaf explants of *Thevetia peruviana*

Calli from leaf explants growing in different hormonal concentrations showing a) leaf explant in 2,4-D (0.5) + KIN (1.0) after 6 weeks; b) Globular stage in primary medium 2,4-D (0.5) + KIN (1.0) after 8 weeks; c) heart shaped stages in 2,4-D (1.0) + KIN (1.0) after 8 weeks; and d) 2,4-D (1.0) + KIN (1.0) after 10 weeks e) Conversion of the torpedo stage to cotyledonary stage after 12 weeks; f) Cotyledonary stage in IBA (0.1) + BA (0.5) medium after second subculture; g) single embryo with cotyledons and radicle h) Regeneration and maturation in IBA (0.1) + BA (0.5) medium (14 weeks) i) Regenerated complete plantlet ready for hardening (18 weeks).

Results indicated that most of the tested concentrations were effective for somatic embryogenesis, but lower concentrations of 2, 4-D + KIN (0.5 - 1.0 mg/L^{-1}) were more conducive than higher (2.0 - 3.0 mg/L^{-1}) combinations. Mature detached bipolar embryos with well-developed cotyledons and radicles were collected and transferred into a full strength MS medium with very low auxin IBA (0.1 mg/L^{-1}) and cytokinin BA (0.5 mg/L^{-1}) for regeneration. Both cotyledonary leaves get thickened and expanded, apical meristem gets activated and developed into fully matured plantlets in 68% of culture tubes. Embryogenic calluses maintained without subculturing or repeated changes in primary medium, led to the proliferation of creamy white non embryogenic callus masses, and a loss or reduction in embryogenic potential was noticed. The survived embryos in these cultures lost its greenish form, and most of them were pale yellow colour in appearance with regained cell proliferation.

Effect of 2, 4-D + BA Combinations

Synergistic activity of 2, 4-D with BA was evaluated after investigating the effect of 2, 4-D + KIN combinations. Wound response within 8 - 10 days of inoculation and subsequent development of creamy white to pale yellow callusing was observed in all studied combinations. Formation of somatic embryos were noticed in a combination of lesser 2, 4-D (1.0 mg/L^{-1}) with varying concentrations of BA (0.5 - 3.0 mg/L^{-1}). Here, embryogenic clumps as well as isolated embryos were observed in relatively low frequency and a delayed formation was also noticed, as presented in Fig 3.

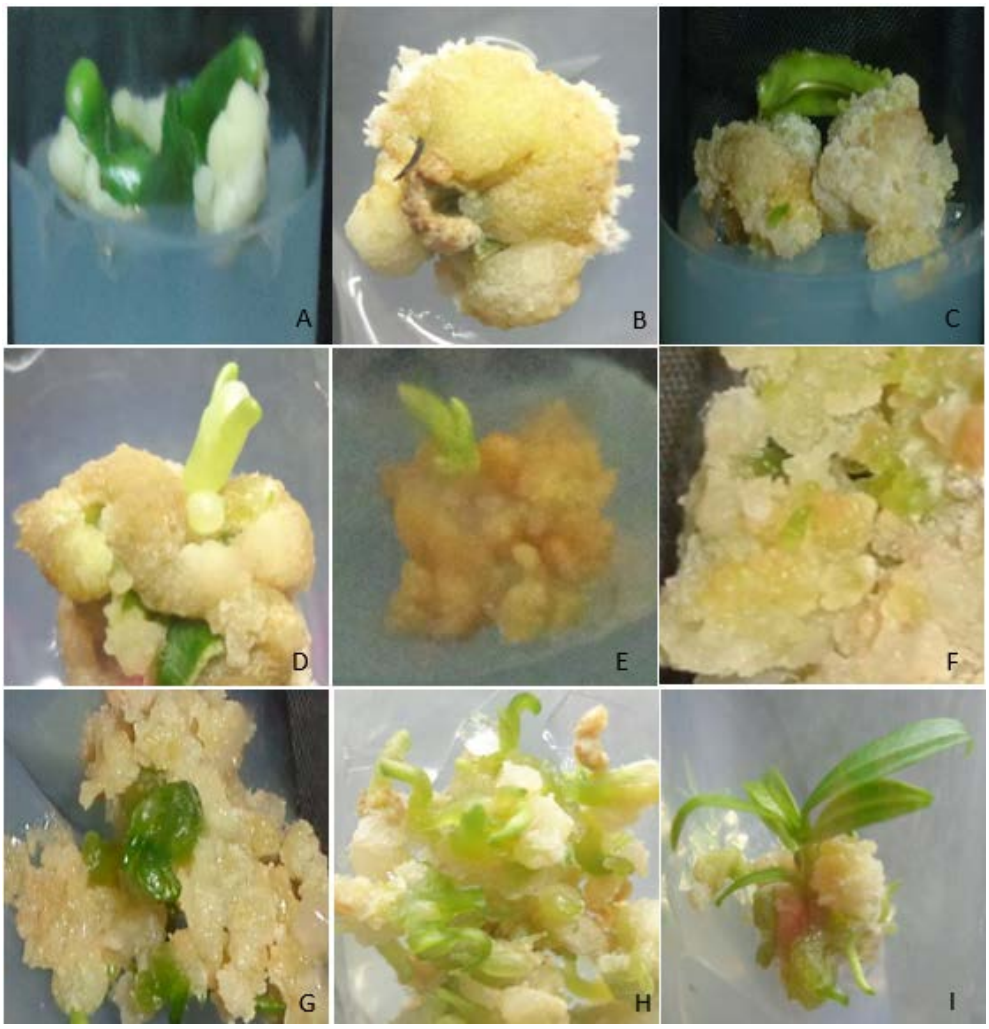


Fig 3: Somatic embryogenesis in 2, 4-D + BA combinations **(**mg/L^{-1}) of *Thevetia peruviana* leaf explants

Different stages of leaf calli from 2,4-D (1.0) + BA (1.0 - 3.0) combinations a) 5 weeks old leaf explant with creamy white callus in 2,4-D (1.0) + BA (1.0) medium b) 10 weeks old callus in 2,4-D (1.0) + BA (2.0) c) 12 weeks old callus in 2,4-D (1.0) + BA (3.0) d) globular and cotyledon stages of somatic embryogenesis in 2,4-D (1.0) + BA (2.0); and e) 2,4-D (1.0) + BA (3.0); f) calli in regeneration medium IBA (0.1) + BA (0.5) g-h) developing embryos from 2,4-D (1.0) + BA (2.0) i) fully matured somatic embryo in IBA (0.1) + BA (0.5).

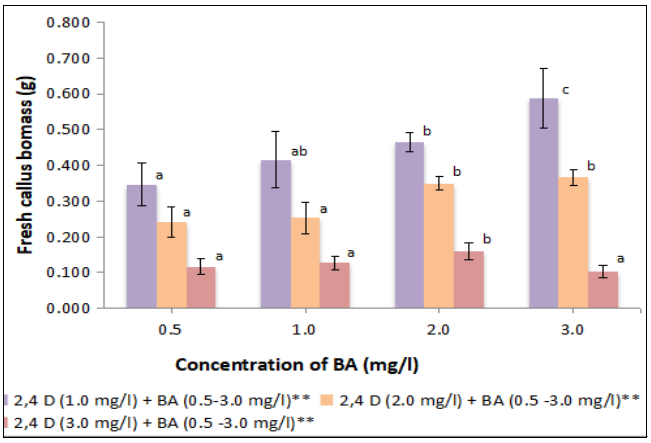
Subsequently, small amounts of calli with embryogenic potential were transferred into an IBA (0.1 mg/L^{-1}) + BA (0.5 mg/L^{-1}) medium for regeneration. During the incubation period of 6 weeks in the regeneration medium, somatic cells with regenerative potential, differentiated and germinated passing varying stages of embryogenesis.

A combination of slightly elevated concentrations of 2, 4-D (2.0 - 3.0 mg/L^{-1}) with BA did not give any positive impact on somatic embryo regeneration as presented in Table 3. However, a significant increase in callus biomass ($p < 0.01$) was observed in 2, 4-D (2.0 mg/L^{-1}) + BA (0.5 - 3.0 mg/L^{-1}) combinations during first subculture in the primary medium. When this compact calli was transferred into regeneration medium, 70% of them developed 1 - 2 shoot initials, which later grew into healthy microshoots with 4 - 6 leaves ready to be transferred into the rooting media. But cultures incubated in higher concentrations of 2, 4- D (3.0 mg/L^{-1}) failed to regain meristematic potential in the regeneration medium.

Table 3: Effect of 2, 4-D (1.0 - 3.0 mg/L^{-1}) + BA (0.5 - 3.0 mg/L^{-1}) on callus morphology and embryogenic responses

2,4-D + BA	Callus Morphology	Nature of developing callus
1.0 + 0.5	Creamy white, granular	Embryogenic calli
1.0 + 1.0	Creamy white, granular	Embryogenic calli
1.0 + 2.0	Creamy light green, friable	Embryogenic calli
1.0 + 3.0	Creamy light green, friable	Embryogenic calli
2.0 + 0.5	Creamy white, less compact	Non embryogenic calli
2.0 + 1.0	Creamy white, less compact	Non meristematic calli
2.0 + 2.0	Creamy white, less compact	Non meristematic calli
2.0 + 3.0	Creamy white, compact	Non meristematic calli
3.0 + 0.5	Creamy white, compact	Non meristematic calli
3.0 + 1.0	Creamy white, compact	Non meristematic calli
3.0 + 2.0	Creamy light brown, compact	Non meristematic calli
3.0 + 3.0	Creamy light brown, compact	Non meristematic calli

When the concentration of both hormones was increased up to 3.0 mg/L^{-1} keeping the ratio constant, a significant decrease was recorded in callus proliferation rate ($p < 0.01$). Calli subcultured in the multiplication medium increased their biomass with no signs of shoot initiation. Lower concentrations of both hormones favoured higher calli biomass, and a decline was observed as the concentration of both hormones increased, which is evident in Fig 4.



Values are mean ± SD; **p < 0.01, values with different superscript letters show significant variation

Fig 4: Effect of 2, 4-D (1.0 - 3.0 mg/L^{-1}) + BA (0.5 - 3.0 mg/L^{-1}) on callus biomass

Healthy matured rooted plantlets were transferred into paper cups containing autoclaved mixture of sand and soil (2:1). After 2 - 3 weeks, acclimatized plantlets were transferred to glass house conditions for further development. Around 68% of the rooted plantlets were acclimatized and they did not exhibit any morphological variations.

Discussion

Development of *in vitro* protocols will help to establish rapid propagation, tissues for germplasm conservation and for secondary metabolite production to meet the ever increasing demand of pharmaceutical industries. An efficient and reproducible protocol for somatic embryogenesis for the successful regeneration of plantlets have been developed after a series of experiments. Among *in vitro* factors, one of the most important phases of successful micro-propagation is the evaluation of the most optimal concentrations and category of plant growth regulators as medium component (Ruzic and Vujovic, 2008). These influenced the fruitful multiplication of a wide array of cells and tissues, either by direct or indirect organogenesis or by somatic embryogenesis.

Callus induction

During the study, mainly two types of calli were observed depending upon the supplementation of auxin 2, 4-D with cytokinin (KIN, BA) concentrations and combinations. Media containing 2,4-D in combination with KIN produced soft, friable creamy yellow callus with abundant proembryogenic masses, whereas 2,4-D with BA produced creamy white granular calli with less frequency of embryogenic cells. The callus induction, shoot initiation and regeneration potential vary with the developmental stages of explant and depend on the concentration and type of growth regulators supplied to the medium [22]. According to Tripathi *et al.* [23], the ratio of exogenous growth hormones added to the medium are more critical rather than their independent presence. From the present study, it can be inferred that the externally supplied growth regulators possibly influenced and directed the callus cells to compete and regain either embryogenic potential or meristematic potential.

Somatic embryogenesis

Somatic embryos could be used for proliferative propagation or for gene transfer procedures [24] as they have features like single cell origin, low frequency of malformation and production of higher number of regenerates [25]. Several investigators have worked extensively on plant regeneration through somatic embryogenesis in different plant species.

The effect of 2, 4-D in combination with cytokinins (KIN and BA), revealed that the synergistic effect with KIN was more pertinent for the induction of somatic embryos in higher frequency, than with BA combinations. Results revealed that most efficient concentration for inducing maximum embryogenic calli was lower concentrations of 2,4-D (0.5 - 1.0 mg/L^{-1}) in combination with KIN (1.0 mg/L^{-1}); and a decline in embryogenic frequency was observed as the concentration of 2,4-D increases (2.0 - 3.0 mg/L^{-1}). When leaf discs were cultured in the above hormonal combinations, identical calli with numerous unorganized structures were reported by Kumar [9]. Similarly, Sharma and Kumar[26] observed a decrease in concentration of 2,4-D from 2.0 to 1.0 mg/L^{-1}, revived the

proliferating potential and growth rate of mass calli of *Thevetia peruviana* L. leaf explants, in view of the fact that a lower 2,4-D + KIN combination (< 1.0 mg/L^{-1}) accelerated somatic embryo formation. Higher concentrations of 2, 4-D (2.0 - 3.0 mg/L^{-1}) suppressed callus growth as well as embryogenic tissue induction.

Moreover, repeated subculturing in the primary medium resulted in loss of embryogenic potential, instead it promoted callus proliferation. Studies conducted by some investigators supported the efficacy of this combination (2, 4-D + KIN) in mass callus production without embryogenic potential. Zibbu and Batra[10] found the optimum concentration for the production of mass calli as 2,4-D (2.5 mg/L^{-1}) + KIN (1.2 mg/L^{-1}); but Taha *et al.*[11] produced mass calli in MS medium with lower 2,4-D (1.0 mg/L^{-1}) + higher KIN (3.0 mg/L^{-1}) supplementation, for *T. peruviana* leaf explants. In the present study, MS medium supplemented with higher concentration of 2, 4-D (2.0 - 3.0 mg/L^{-1}) with lower amount of KIN (1.0 mg/L^{-1}) retarded callus proliferation in the leaf explant.

Somatic embryogenesis was also observed in low frequency when the leaf explants were supplied with low auxin 2,4-D (1.0 mg/L^{-1}) and varying concentrations of cytokinin BA (0.5-3.0 mg/L^{-1}). In *Medicago truncatula*, medium supplemented with 2, 4-D (1.0 mg/L^{-1}) + BA (0.2 mg/L^{-1}) favoured the induction of somatic embryos in leaf explants, but in *Rauvolfia serpentina* L. same hormonal combination 2, 4-D (2.0 mg/L^{-1}) + BA (1.0 mg/L^{-1}) favored 93.65% mass Callusing [6]. During the somatic-to-embryogenic transition, cells have to de-differentiate, activate their cell division cycle and reorganize their physiology, metabolism and gene expression patterns [27]. Formation and expression of somatic embryogenesis might be triggered by several factors, however, the most common aspect is the exclusion or reduction of auxin concentration, mainly 2, 4-D, in the medium of embryogenic cultures [28].

Embryogenic cells are extremely sensitive to minor variations in the physical and chemical environment. Some physiological stresses such as osmotic stress, dehydration stress [29], stresses applied by synthetic auxins [30] and their exogenous concentrations might trigger the induction of somatic embryogenesis [31]. Among auxins, the most frequently used one was 2, 4-D (49%), followed by cytokinins BA (57%) and KIN (37%), Reviewed by Raemakers *et al* [32].

From this study it is obvious that both classes of growth hormones in a specific ratio were required for the induction of somatic embryos as well as mass callus production. The role of growth hormones in regenerating somatic embryos from various parts of the same plant also varies considerably [33]. In *Onobrychis sativa*, combination of IBA (0.1 mg/L^{-1}) + BA (0.5 mg/L^{-1}) was effective in regenerating somatic embryos directly from leaf calli, but the hormonal ratios showed variation in regenerating embryos from stem explants[24].

Somatic embryogenesis can be induced in hormone-free media, auxin-containing media and cytokinin-supplemented media. In the present study, low IBA (0.1 mg/L^{-1}) + BA (0.5 mg/L^{-1}) combination was effective in regenerating successfully 68% of somatic embryos from leaf explant cultures. Kumar [9] observed that somatic embryos of *Thevetia peruviana* L. could have developed into complete plantlets on a medium devoid of growth regulators with 60% survival rate. On the other hand, Sharma and Kumar [26] developed somatic embryos of *Thevetia* into complete plantlets upon subsequent transfer to modified half-strength MS medium.

Replacement of one cytokinin (KIN) by another (BA) resulted in significant variation in callus morphology and shoot bud proliferation. However, variations may be due to the differences in recognition and uptake of cytokinins by the cells, or the mechanism of action of the cytokinin compounds [34]. Subsequent cultures were maintained in the maturation medium IBA (0.5 mg/L^{-1}) + BA (1.0 mg/L^{-1}). The survival rate of transplanted plantlets was moderate (68%), and the 2:1 ratio of sand-soil with 0.1% activated charcoal was found favourable for hardening of plantlets.

Conclusion

Leaf explants of *Thevetia peruviana* is capable of undergoing different morphogenic responses for somatic embryogenesis. All these changes exclusively depend on the combination and concentrations of plant growth regulators. The successful regeneration of plantlets through somatic embryogenesis has generated the possibility of large scale propagules within a short period of time. So, micro-propagation can be effectively done by the optimization of auxin-cytokinin combinations in MS media within 16 - 22 weeks, via somatic embryogenesis using lower concentrations of auxin 2,4-D (0.5 - 1.0 mg/L^{-1}) with KIN (0.5 - 1.0 mg/L^{-1}). This rapid and effective method definitely helps to promote mass multiplication of this potent medicinal plant to minimize the impact of over exploitation by therapeutic industrialists.

Acknowledgment

Authors are thankful to the Director of Amala Cancer Research Institute, Thrissur for proving enough facilities for the completion of the work.

References

1. Joshi SG. Medicinal Plants. (Oxford and IBH Publishing Company Pvt. Ltd., New Delhi), 2000.
2. Zavaleta AN. Medicinal Plants of the Borderlands: A Bilingual Resource Guide. (Author House Publications, Bloomington, USA), 2012.
3. Said IM, Rahman ZA. The cardenolides of *Thevetia peruviana* grown in Malasia. Asean J Sci Technol Develop. 1986; 3(1):83-86.
4. Tripathi L, Tripathi JN. Role of biotechnology in medicinal plants. Trop J Pharm Res, 2003; 2(2):243-253.
5. Beegum AS, Martin KP, Zhang CL, Nishitha IK, Ligimol SA, Slater a *et al.* Organogenesis from leaf and internode explants of *Ophiorrhiza prostrata*, an anticancer drug (camptothecin) producing plant. Electron J Biotechnol. 2007; 10(1):114-123.
6. Panwar GS, Attitalla IH, Guru SK. An efficient *in vitro* clonal propagation and estimation of reserpine content in different plant parts of *Rauwolfia serpentina* L. Am Eurasian J Sci Res. 2011; 6(4):217-222.
7. Petrova M, Zayova E, Yankova E, Baldzhiev G. Plant regeneration from callus culture of *Arnica Montana*. Rom Biotechnol Lett. 2011; 16(1):92-97.
8. Kumar A, Tandon P. Investigation on *in vitro* laticifer differentiation in *Thevetia peruviana* L. Phytomorphology. 1990; 40(1&2):113-117.

9. Kumar A. Somatic embryogenesis and high frequency plantlet regeneration in callus cultures of *Thevetia peruviana*. Plant Cell Tissue Organ Cult. 1992; 31:47-50.
10. Zibbu G, Batra A. Effect of adenine sulphate on organogenesis *via* leaf culture in an ornamental plant: *Thevetia peruviana* (Pers) Schum. Int J Pharma Biol Sci. 2010; 1(2):1-9.
11. Taha HS, Farag SH, Shams KA, Abdel-Azim NS, Hanna GA, Ewais EE *et al. In vivo* and *in vitro* studies on *Thevetia* species growing in Egypt I: Isolation, identification, and quantification of cardiac glycosides in *in vivo* and *in vitro* cultures of immature seeds. J Amer Sci. 2010; 6(11):390-395.
12. Sehrawat AR, Sanjogt U, Chowdhury JB. Establishment of plantlets and evaluation of differentiated roots for alkaloids in *Rauwolfia serpentina*. J Plant Biochem Biotechnol. 2002; 11(2):105-108.
13. Salma U, Rahman MSM, Islam S, Haque N, Jubair TA, Haque AKMF *et al.* The influence of different hormone concentration and combination on callus induction and regeneration of *Rauwolfia serpentina* L. Benth. Pak J Biol Sci. 2008; 11(12):1638-1641.
14. Oliveira AJB, Carvalho VM, Ferreira A, Sato FY, Machado MFPS. *In vitro* multiplication of *Tabernaemontana fuchsiaefolia* L. (Apocynaceae). Rev Arvore. 2003; 27(4):421- 425.
15. Soundararajan T, Karrunakaran CM. Micropropagation of *Nerium Oleander* through the immature pods. J Agric Sci. 2010; 2(2):181-193.
16. Vila I, Sales E, Ollero J, Munoz-Bertomeu J, Segura J, Arrillaga, I. Micropropagation of Oleander (*Nerium oleander* L.). Hort Science. 2010; 45(1):98-102.
17. Faheem M, Singh S, Tanwer BS, Khan M, Shahzad A. *In vitro* regeneration of multiplication shoots in *Catharanthus roseus*- An important medicinal plant. Adv Appl Sci Res. 2011; 2(1):208-213.
18. Haq R, Naz S, Aslam F, Manzoor F. Comparison of *in vitro* response of micropropagation and callogenesis of medicinal plant, *Vinca rosea* L. J Agric Res. 2013; 51(1):9-17.
19. Imran MA, Begum G, Sujatha K, Mallaiah B. Effect of adenine sulphate (ads) with cytokinins on multiple shoot production in *Carissa Carandas* L. Int J Pharma Biosci. 2012; 3(1):473-480.
20. Prema R, Paulsamy S, Thambiraj J, Saradha M. Indirect organogenesis of the medicinal plant species, *Cryptolepis grandiflora* Wight (Apocynaceae) by tissue culture technique. Int J Pharm Chem Biol Sci. 2013; 3(4):1001-1005.
21. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962; 15(3):473-479.
22. Ruzic DV, Vujovic TI. The effects of cytokinin types and their concentration on *in vitro* multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). Hort Sci (Prague). 2008; 35 (1):12-21.
23. Chen CC, Chen SJ, Sagare AP, Tsay HS. Adventitious shoot regeneration from stem internode explants of *Adenophora triphylla* (Thunb.) A. DC. (Campanulaceae) - An important medicinal herb. Bot Bull Acad Sin. 2001; 42(1):1-7.
24. Tripathi PK, Awasthi S, Kanojiya S, Tripathi V, Mishra DK. Callus culture and *in vitro* biosynthesis of cardiac glycosides from *Calotropis gigantea* (L.) Ait. *In vitro* Cell Dev Biol, 2013; 49(4):455-460.
25. Mohajer S, Taha RM, Khorasani A, Yaacob SJ. Induction of different types of callus and somatic embryogenesis in various explants of Sainfoin (*Onobrychis sativa*). Asian J Crop Sci. 2012; 6 (8):1305-1313.
26. Nhut DT, Giap DD, Vinh BVT, Loan NTK, Huy NP, Hai NT *et al.* Thin cell layer technology in micropropagation of *Jatropa curcas* L. In: Mohan JS, Gupta SD (Editors) Biotechnology of Neglected and Underutilized Crops. Springer, London, 2013, 33-42.
27. Sharma A, Kumar A. Somatic embryogenesis and plant regeneration from leaf-derived cell suspension of a mature tree- *Thevetia peruviana* L. Plant Cell Rep. 1994; 14:171-174.
28. Feher A, Pasternak TP, Dudits D. Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult. 2003; 74(3):201-228.
29. Jimenez VM. Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones. Rev Bras Fisiol Veg. 2001; 13(2):196-223.
30. Carman JG. Embryogenic cells in plant tissue cultures: Occurrence and behavior. *In vitro* Cell Dev Biol. 1990; 26(8):746-753.
31. Ikeda-Iwai M, Umehara M, Satoh S, Kamada H. Stress-induced somatic embryogenesis in vegetative tissues in *Arabidopsis thaliana*. Plant J. 2003; 34(1):107-114.
32. Kiyosue T, Kamada H, Harada H. Induction of Somatic Embryogenesis from carrot seeds by hypochlorite treatment. Plant Tissue Cult Lett, 1989; 6(3):138-143.
33. Raemakers CJJM, Jacobsen E, Visser RGF. Secondary somatic embryogenesis and applications in plant breeding. Euphytica. 1995; 81(1):93-107.
34. Nesy EA, Padikkala J, Mathew L. *In vitro* plant regeneration of *Thevetia neriifolia* from internode explants *via* indirect organogenesis. Int J Pharm Pharm Sci. 2015; 7(1):169-172.