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Indirect organogenesis and influence of benzyl adenine on shoot regeneration from *Thevetia peruviana* leaf explants

E A 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
Since the seed propagation methods are very slow, an *in vitro* protocol via indirect organogenesis has been developed using leaf explants of *Thevetia peruviana*, a potential medicinal plant having high therapeutic value. Effect of Benzyl Adenine (BA) was evaluated individually (1.0 - 5.0 mg/L^{-1}) and in combination with varying concentrations of auxins 2, 4-D (0.5 - 3.0 mg/L^{-1}) and IBA (0.5 - 2.0 mg/L^{-1}) for shoot multiplication. Explants responded very quickly with culture medium, morphologically callus appeared creamy white granular (2, 4-D + BA) and creamy light green meristematic (IBA + BA), but the callus were highly meristematic in BA with numerous shoot initials scattered over the entire surface of whole callus mass. Cytokinin BA (1.0 mg/L^{-1}) alone produced maximum number of microshoots with an average of 22.167 ± 1.518 per leaf explant, instead, in combination with IBA best response (6.444 ± 0.702) was achieved in IBA (0.5 mg/L^{-1}) + BA (1.0 mg/L^{-1}) concentration. All green meristemoid calli from other combinations were transferred into the regeneration medium (IBA - 0.5 mg/L^{-1} + BA 1.0 mg/L^{-1}) before a final change to BA (1.0 mg/L^{-1}) for further maturation. Detached microshoots were rooted in full strength MS medium fortified with 1.0 mg/L^{-1} IBA. Around 72 % of the rooted plantlets were acclimatized, so this can be utilised as an effective rapid method to conserve this indigenous medicinal plant for large scale clonal propagation.

Keywords: callus, explant, microshoots, regeneration, *Thevetia*

Introduction
Thevetia peruviana (Apocynaceae) commonly known as yellow oleander is a valuable medicinal shrub and has been used in folklore medicine since long back. Different active compounds of *Thevetia* were found its use as bactericides [1], fungicides [2], insecticides [3, 4], nematicides [5], rodenticid [6] and termiticides [7]. The reported anti-HIV [8], anthelmintic [9], antitumor [10], cytotoxic [9, 11] and genotoxic [12] properties of this plant are also noteworthy. Such varied bio-medical applications clearly indicated that the plant is a rich treasure of diverse secondary metabolites with major industrial importance.

Many ornamental plants of Apocynaceae are potent sources of natural drugs with remarkable remedial effects and are unique in origin [13]. In search of alternatives for the production of medicinal compounds from plants, biotechnological approaches, specifically plant tissue culturing techniques are widely used as a potential tool in traditional agriculture for the industrial production of bioactive metabolites for herbal and pharmaceutical industries [14]. Many plants that produces effective metabolites *viz*, catharantine, vincristine, vinblastine [15, 16], reserpine, serpentine [17, 18], nerine, oleandrin [19, 20], neriifolin and digitoxin [21, 22] have been successfully raised using tissue culture techniques. The plant selected for the present study, *T. peruviana* also have an important and significant place among the list of valuable medicinal plants with a wide array of cardiac glycosides including peruvoside. Being the repository of many cardiac glycosides and other metabolites, it will be worthwhile to propagate this taxon having wider uses which would benefit for both pharmaceutical levels as well as industrial grounds.

Calli can be regenerated by direct or indirect organogenesis and by somatic embryogenesis. Plant growth regulators play an essential role in determining the developmental pathway of plant cells and tissues in culture medium. Effect of auxin 2, 4-D on varying concentrations of cytokinins KIN and BA for the induction of somatic embryos of *Thevetia* was also well studied [23]. Cytokinins are the crucial growth substances that exerts various effects when applied to particular plants and they have proven to be the most important factor affecting shoot regeneration. Cytokinin hormones, such as BA, already play and continue to serve for a very critical role in the successful micropropagation of many crop, medicinal and ornamental plant species [24]. The healthy and proliferative callus can be subjected to many diverse applications like secondary metabolite isolation, large scale plant regeneration round the year and a stable supply of the *in vitro* raised plantlets for the manufacturers.

Material and Methods
Studies were conducted following the standard tissue culture protocol using MS [25] medium with various auxins 2, 4-D (2, 4 Dichloro phenoxyacetic acid), IBA (Indole Butyric Acid) and cytokinin BA (6-Benzyl Adenine). Culture media was prepared by adding sucrose (3.0% w/v), agar (0.75% w/v) and various growth regulators (0.1 - 3.0 mg/L^{-1}). The pH of the medium was adjusted to 5.8 ± 0.02 using 0.1 N HCl or NaOH before autoclaving at 121 ºC for 15 min [23].

Young healthy 4 - 5 week old seedlings of yellow oleander was used as source of explant. Surface sterilization was carried out with 1% Tween 20 (v/v), followed by fungicide Bavistin (1% w/v). After thorough rinsing with distilled water, explant was cleansed with 0.1% mercuric chloride

(w/v) for 1 min under Laminar Air Flow Chamber (Kemi Pvt Ltd, India). Effect of BA was evaluated in combination with auxins 2, 4-D and IBA (0.5 - 3.0 mg/L^{-1}) and individually (1.0 - 5.0 mg/L^{-1}). Basal MS medium without any growth regulators served as control. All inoculated cultures were maintained at 26 ± 2 ºC for a photoperiod of 16 h. After periodical subculturing, mature microshoots were transferred to rooting medium, and later moved to greenhouse conditions for natural acclimatization.

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 leaf derived explants of seedlings. All cultures produced creamy white callus, and morphology of calli varied thereafter during subsequent transfers according to hormonal combinations. Callus was creamy white and granular in 2,4D + BA; whereas it was creamy, light green and meristematic in IBA + BA supplementation. When the explants were supplied with BA alone, the callus appeared highly meristematic and the shoot initials were scattered over the entire surface of whole callus mass. However, without any growth regulators, explants failed to initiate cell division in MS medium even after 6 weeks of inoculation. Midrib regions responded more vigorously in callus initiation than leaf margins.

Effect of 2, 4-D + BA Combinations

Synergistic activity of 2, 4-D with BA was evaluated initially using leaf explants. 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}). A combination of slightly elevated concentrations of 2, 4-D (2.0 mg/L^{-1}) with BA (0.5 - 3.0 mg/L^{-1}) resulted in a significant increase in callus biomass. When this compact calli was transferred into regeneration medium (IBA - 0.5 mg/L^{-1} + BA 1.0 mg/L^{-1}), 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.

Effect of IBA + BA Combinations

When the leaf explants were cultured in IBA + BA combinations, a promising result was obtained for shoot regeneration within a short period of time. Slight enlargement and curling of leaf explants followed by callusing at cut ends was noticed after first two weeks of inoculation. Depending upon the availability of BA, a significant increase in callus biomass was observed, along with the proliferation of varying number of microshoot initials. But, their frequency decreased with increasing hormonal concentration during the first five weeks of incubation in the primary callusing medium. In lower hormone combinations of IBA (0.5 mg/L^{-1}) + BA (1.0 mg/L^{-1}), numerous shoot meristemoids were developed on the creamy green callus, which later developed to fully matured shoots of 5 - 6 cm long with 4 - 5 expanded leaves, after periodical subculturing.

However, in an inverse combination (IBA 1.0 mg/L^{-1} + BA 0.5 mg/L^{-1}), the callus was unable to develop shoots even if the meristemoids appeared in lesser frequency. Lower concentrations of IBA (0.5 - 1.0 mg/L^{-1}) in combination with BA (1.0 - 3.0 mg/L^{-1}) favoured the formation of shoot initial (Fig 1), and higher IBA content (1.5 - 2.0 mg/L^{-1}) suppressed the effect of BA in microshoot proliferation and multiplication. When the medium was fortified with a balanced hormonal combination (1.0 + 1.0 mg/L^{-1}), the advantage of BA for shoot induction was found almost suppressed by IBA. An increase in BA concentration up to 2.0 - 3.0 mg/L^{-1} did not favour the induction of microshoots as expected when compared to the results at lower concentrations (Fig 2).

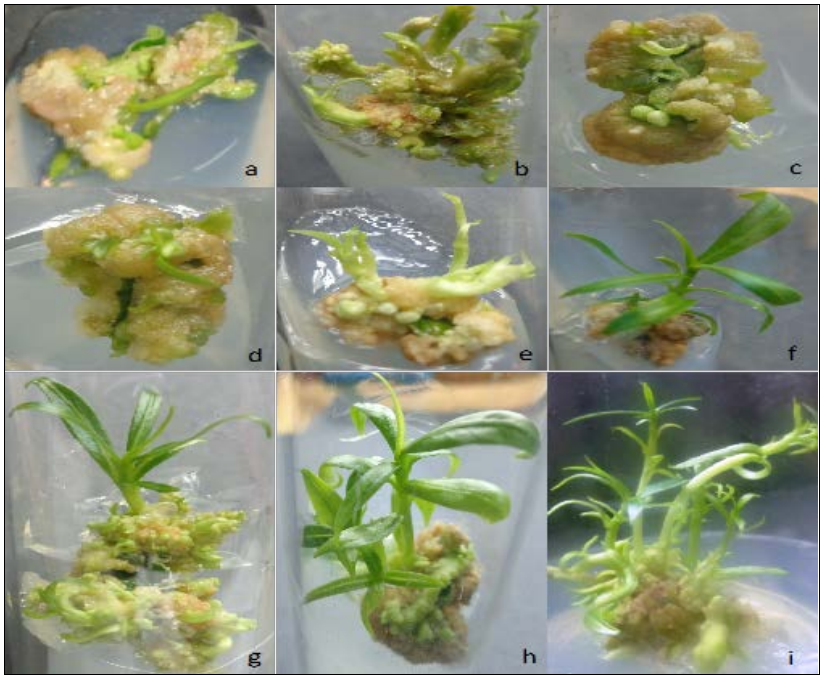
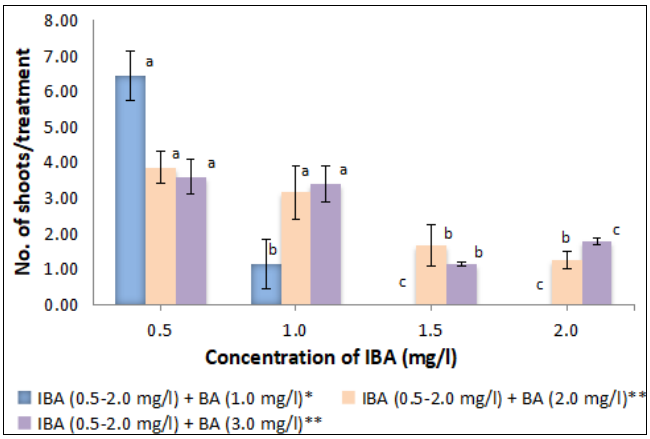


Fig 1: Effect of IBA + BA on callus morphology, shoot induction and regeneration of microshoots

a-d) Response of leaf explants during 5 weeks of culture in primary medium a) IBA + BA (0.5+1.0 mg/L^{-1}) b) 1.5 + 3.0 mg/L^{-1} c) 2.0 + 1.0 mg/L^{-1} d) 2.0 + 3.0 mg/L^{-1} e) II subculture from IBA + BA (1.0 + 1.0 mg/L^{-1}) to IBA + BA 0.5 + 1.0 mg/L^{-1}) f) III subculture from IBA + BA (2.0 + 2.0 mg/L^{-1}) to BA (1.0 mg/L^{-1}) g) II SC from IBA + BA (1.0 + 3.0 mg/L^{-1}) to IBA + BA (0.5 + 1.0 mg/L^{-1}) h) III SC from IBA+ BA (1.0 + 2.0 mg/L^{-1}) to BA (1.0 mg/L^{-1}) i) III SC from IBA + BA (0.5 + 1.0 mg/L^{-1}) to BA (1.0 mg/L^{-1})



Values are mean ± SD; * p<0.05; ** p<0.01; values with different superscript letters show significant variation when compared

Fig 2: Effect of IBA (0.5 - 2.0 mg/L^{-1}) + BA (1.0 - 3.0 mg/L^{-1}) on shoot induction

Individual Effect of BA

Leaf explants responded very quickly to MS salts supplemented with cytokinin BA (1.0 - 5.0 mg/L^{-1}) individually. Visible changes that appeared within 8 - 15 days of inoculation included enlargement, twisting, swirling of explants, whitening at the wounded ends, callus initiation and proliferation. The developed shoot initials were not localized to particular areas, but scattered over the entire surface covering the whole callus mass. Maximum number of shoot initials were obtained after 4 - 5 weeks of incubation, on medium supplemented with a lower concentration of BA (1.0 mg/L^{-1}).

Further sub culturing to the primary medium gave the best response with maximum (100 percent) efficacy (Fig 3) in shoot number (22.167 ± 1.518) (Table 1), shoot length (5.8 cm) and leaf number (5 - 8). As the concentration of BA increased from 2.0 (mg/L^{-1}) to 5.0 (mg/L^{-1}), a gradual reduction in the number of shoot bud formation was observed ($p < 0.01$), though the nature of callus was almost identical.

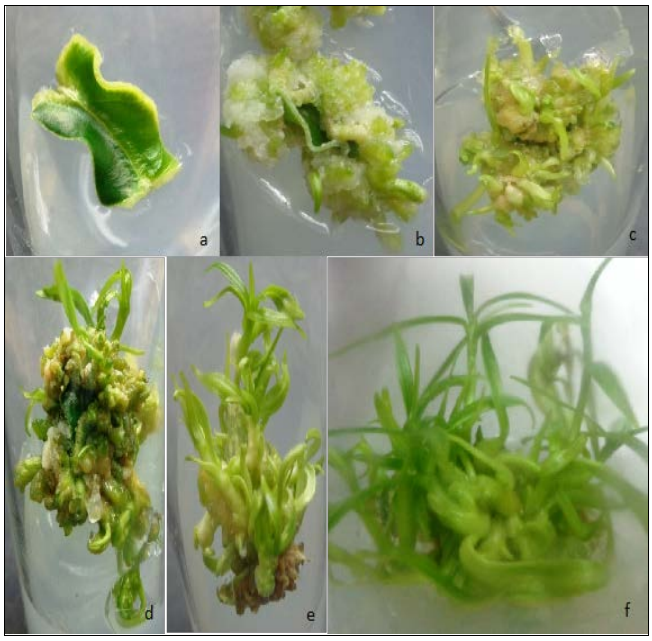


Fig 3: Effect of BA on microshoot induction and multiplication

a) Callus initiation after 1 week of incubation b) callus morphology after 4 weeks c) 6 week old callus during first subculture in the primary medium (BA 1.0 mg/L^{-1}) d) 6 weeks old callus during first subculture in the primary medium (BA 2.0 mg/L^{-1}) e) 8 weeks old callus with maximum microshoots in BA (1.0 mg/L^{-1}) f) 12 weeks old shootlets in BA (1.0 mg/L^{-1}) medium during third subculturing.

Table 1: Effect of BA (1.0 - 5.0 mg/L^{-1}) on callus morphology, biomass and number of shoots/explant

BA (mg/l)	Callus Morphology	Fresh Callus Biomass Before First Subculturing	Mean Number of shoots/Treatment
1.0	Greenish white callus	$0.813^{a} \pm 0.098$	$22.167^{a} \pm 1.518$
2.0	Greenish white callus	$0.603^{b} \pm 0.093$	$13.143^{b} \pm 2.167$
3.0	Creamy light green	$0.529^{c} \pm 0.094$	$11.000^{b} \pm 2.309$
4.0	Creamy light green	$0.307^{d} \pm 0.017$	$8.556^{c} \pm 1.832$
5.0	Creamy light green	$0.234^{e} \pm 0.036$	$4.556^{d} \pm 0.497$

Values are Mean ± SD; superscript letters represent significant differences ($p < 0.01$) when compared

Discussion

Indirect organogenesis refers to the formation of shoots indirectly from an intermediary callus that first develops on explants. Plant growth regulators are the most important inducing signal for organogenesis. Type of plant growth regulators and their interaction play a significant role in dedifferentiation, induction and development of shoots or roots [26]. It is generally believed that cytokinins are beneficial for shoot formation, while auxins stimulate callus formation, root induction and somatic embryogenesis. The ratio of cytokinins to auxins is also critical in determining shoot versus root formation, as revealed from the experiment.

Morphogenic responses of calli vary from plant to plant and even organ to organ that depend on a number of known and unknown factors. Results of different concentrations and combinations of IBA + BA showed that this blend is better for mass production of meristematic callus, shoot proliferation, multiplication and regeneration through an intervening callus phase in leaf explants. The higher success rate for maximum microshoot proliferation was achieved at lower concentrations of IBA (0.5 mg/L^{-1}) + BA (1.0 mg/L^{-1}). When the quantity of IBA exceeds (IBA 2.0 mg/L^{-1} + BA 1.0 mg/L^{-1}) or is equal to BA (IBA 2.0 mg/L^{-1} + BA 2.0 mg/L^{-1}), failure of shoot bud induction was observed as the former dominate in the medium; but in cultures with slightly

elevated concentrations of BA (2.0 + 3.0 mg/L^{-1}), subculturing in regeneration medium induced shoot initials. 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 [27].

Similar IBA + BA combinations were used to induce shoot bud multiplication from *Jatropha curcas* L. stem and leaf explants [28]. The highest number of buds with expanded leaves occurred on media containing different concentrations of IBA and BA in the orchid, *Cattleya walkeriana* Gardner [29]. The present study revealed that a combination of IBA + BA was necessary to regenerate *Thevetia peruviana* indirectly from leaf explants. An optimum combination for caulogenesis and rhizogenesis was found as IBA (0.5 mg/L^{-1}) + BA (1.0 mg/L^{-1}) for leaf explants, but a lesser number of microshoots were induced indirectly from internode explants of yellow oleander [30].

To explore the potential for *in vitro* propagation of *T. peruviana*, different concentrations of BA alone were evaluated for their effect on organogenesis of leaf explants. Studies revealed that individual treatment of BA (1.0 mg/L^{-1}) produced an excellent multiplication rate with an average number of microshoots in the leaf (22.167 ± 1.518 shoots/explant), than a combination with IBA (6.444 ± 0.702 shoots/explant). The results supported the findings of Haq *et al*. (2013) that BA (1.0 mg/L^{-1}) alone was proved to be very effective with an average number of shoots (7.8 ± 1.09) from nodal explants of *Vinca rosea* L., a close relative of *Thevetia*. However, NAA (1.0 mg/L^{-1}) + BA (0.5 mg/L^{-1}) combination also proved to be optimal for the production of maximum number of shoots (35.10 ± 0.74) in the same genus *Catharanthus roseus* (L.) G. Don [31].

Furthermore, BA as a cytokinin was reported to be more efficient in comparison to KIN, Zeatin, 2-isopentenyladenine or thidiazuron for axillary bud initiation and subsequent proliferation [32]. On the other hand, an addition of coconut water [19] along with BA and KIN increased the shoot bud formation from the immature pods of *Nerium oleander* L. Shoot bud initiation could be further enhanced with the continuous subculturing of induced calli in this media. The highest multiplication indexes as well as the length of axial and lateral shoots were obtained by the individual effect of BA in sweet cherry [27]. Moreover, cytokinins especially BA, were reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation [33]. However, the addition of auxin at low concentrations significantly enhanced shoot proliferation and was reported to be very important for mass scale propagation [34]. Hatzilazarou *et al.* [35] observed direct regeneration of shoots and roots occurred in nodal explants in *Nerium* on MS medium containing NAA (0.1 mg/L^{-1}) + BA (0.5 mg/L^{-1}). Moreover, a significant interaction between cytokinin type and level was observed in the shoot tip cultures of different cultivars of *Nerium oleander* L. [36].

Good amount of creamy white callus was induced by all studied hormonal combinations and the first subculturing for all calli was done in the primary medium after 4 - 6 weeks of incubation. Subsequent cultures were maintained in the maturation medium IBA (0.5 mg/L^{-1}) + BA (1.0 mg/L^{-1}) for organogenesis. Calli that showed weaker proliferation potential regained the meristematic activity partially by repeated subculturing in the BA fortified medium. During each passage, the number of shoot bud development has been increased substantially; and after two rounds of subculturing, well developed and matured individual microshoots were excised carefully and transferred to rooting medium. Studies on various medicinal plants revealed that most of the members of Apocynaceae prefer cytokinin alone or in combination for better proliferation of the shoot explants [37]. In conclusion, the microshoots were successively regenerated after 2 - 3 subcultures in presence of BA in promoting shoot formation.

Significant results for rooting were achieved in full strength MS medium fortified with 1.0 mg/L^{-1} IBA after four weeks of culture. Lower concentrations of IBA as well as half strength MS medium did not support effective rooting. A concentration of 1.0 mg/L^{-1} of NAA gave favorable results in rootlets formation in the same plant [38], but half strength MS was found as an optimal medium for rooting with lower IBA (0.5 mg/L^{-1}) in *Arnica montana* L.[39]. The transplanted plantlets showed a moderate survival rate of 72 % in the 2:1 ratio of sand-soil with 0.1 % activated charcoal was found favourable for hardening of plantlets.

Conclusion

An efficient reproducible protocol has been developed for the vast multiplication of this indigenous medicinal plant using the leaf explants from seedlings. Efficacy of BA alone and in combination was established in micro-shoot proliferation and multiplication at a higher rate via indirect organogenesis. So, *in vitro* propagation techniques is a promising way for the ex situ conservation of germplasm of important medicinal plants without depleting natural resources.

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