Bioproduction of azadirachtin-A, nimbin and salannin in callus and cell suspension cultures of neem (*Azadirachta indica* A. Juss.)

Neem (*Azadirachta indica* A. Juss.) has its origins in antiquity and is inextricably linked to the history of the Indian way of life as a symbol of health. Several compounds have been reported from the neem tree[1]. Among these, azadirachtin-A, nimbin and salannin are the major triterpenoids having several biological activities[2]. *In vitro* production of azadirachtin has been earlier reported from callus cultures[3–6] and cell suspension cultures[7–9]. Reports on the *in vitro* production of nimbin are few. Nimbin accumulated in differentiating callus cultures of neem[10] and in *in vitro* cultured shoot and root of neem[11], while there are no reports on salannin accumulation in tissue cultures. Earlier, we had reported a production medium for the efficient biosynthesis of azadirachtin from callus cultures[12]. The aim of the present work was to analyse the accumulation of azadirachtin-A, nimbin and salannin in callus cultures from 13 different explants and cell-suspension cultures raised from internodal segments.

A mature neem tree growing in the medicinal garden of the Department of Botany, University of Kerala served as the explant source with the exception of root. Root was taken from a one-year-old seedling raised from the seeds of the same mature tree. For callus culture initiation, all the explants were collected during April–May, when the tree bloomed and fruited. Vegetative parts like internode, node and leaflets (4–5 cm from the shoot tip) and floral parts (from 2 mm-sized flower buds) like sepal, petal, staminal tube, anther, ovary, style and stigma were used as explants. Cotyledon and embryo were excised from the penultimate falling seeds. Sterilization of the explants was done with 0.1% (w/v) sterile solution of mercuric chloride for 8 min. Flower buds were sterilized as a whole and separated into individual explants prior to inoculation. Callus cultures were initiated on Murashige and Skoog (MS) medium[13] supplemented with various auxins [1-napthaleneacetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)], and cytokinins [N^6-benzylaminopurine (BA) and kinetin (KIN)]. Cell suspension cultures were initiated from 120-day-old callus derived from internodal segments grown in earlier standardized production medium[12] (MS medium supplemented with 5 mg/l BA, 10 mg/l each of IBA and NAA). One gram (fresh wt) of callus tissue was transferred to 50 ml of basal medium (MS) containing 3% sucrose in 250 ml flasks. The pH of the medium was 5.8. The cultures were subjected to 12 h photoperiod under a photon flux intensity of 100 µmol m^{-2} s^{-1} at a temperature of 23 ± 1°C on a gyratory shaker at 90 RPM. Subculturing was done at the end of every 14th day. Next 700 mg fresh wt of cells on the third day of incubation at the seventh passage was used for the experimental suspension cultures (30 ml) in a 150 ml flask, which was incubated for 21 days.

Plant parts collected from mature neem tree (root, bark, leaf, inflorescence and penultimate falling seed), were shade-dried and powdered. Five grams of each sample was freeze-dried, followed by petroleum ether and water extractions and concentrated under vacuum, redissolved in methanol and stored at 4°C till analysis[5]. RP–HPLC analysis was carried as described elsewhere[14]. Column, C_{18}; eluents, methanol : water (50 : 50); flow rate, 1.0 ml/min at a pressure of 3000 psi; detector, UV-Vis $\lambda = 215$ nm. Reference samples of azadirachtin-A, nimbin and salannin were obtained from T. R. Govindachari Centre for Natural Products, Chennai.

Growth and production curve of triterpenoids (azadirachtin-A, nimbin and salannin) were calculated by measuring the dry weight of cells and the amount of respective secondary metabolite produced on every three-day interval for a span of 21 days. For measuring growth, cells were removed from the suspension by filtering with a Whatman No 1 filter paper and dried in an oven at 35°C for 24 h for recording dry cell weight. After growth evaluations the cells were subjected to extraction with twice the volume of methanol, filtered and the triterpenoid content was analysed. The liquid medium devoid of cells was also analysed for the presence of triterpenoids.

In TLC (solvents, hexane : ethyl acetate (3 : 7); thickness of the silica gel layer, 0.5 mm on 20 × 10 cm plates), *Rf* value of azadirachtin-A, nimbin and salannin was 0.54, 0.94 and 0.84, respectively. The bands tested positive to vanillin : sulphuric acid reagent (1 g vanillin in 100 ml conc sulphuric acid), which turned azadirachtin-A to pink shade, salannin to indigo and nimbin to dark teal. In RP–HPLC, retention time obtained for azadirachtin-A, nimbin and salannin was 5, 17 and 25 min respectively. Yield of azadirachtin-A, nimbin and salannin (µg/g dry wt) from the stock plant was: root (99.6, 41.9, 17.9), bark (15.4, 20.1, 21.4), leaf (1.6, 12.7, 34), flower (0.01, 23.4, 0.13) and penultimate falling seed (1346, 73.1, 35.3) respectively. Thus the yield of the individual triterpenoids analysed was higher in the penultimate falling seeds.

Accumulation of all the triterpenoids analysed was not detected in the 30-, 60-, 90-day-old callus tissues by RP–HPLC analysis. Their accumulation could be detected only towards the end of the third subculture (120th day), which was in concurrence with an earlier report[3], where azadirachtin could be detected only during the second or the third subculture. The delay in the synthesis of a secondary metabolite or its absence in callus and cell suspension cultures at the initial stages may be attributed to catabolic repression[9].

The lack of morphological differentiation in callus cells may prevent the formation of secondary metabolites and disrupt regular metabolic pathways. There is also a report stating that nimbin has positive correlation with morpho-differentiation[10]. The results of the present investigation contradict this concept. Calluses raised from all the explants synthesized and accumulated the three triterpenoids studied. Therefore, it is speculated that for the efficient synthesis of the three triterpenoids studied, a morphological differentiation is not mandatory. Flower buds exhibited low content of azadirachtin-A; however, callus tissues raised from each of the floral parts synthesized several folds of azadirachtin-A (Table 1). This is in agreement with the fact that a compound absent or accumulated in low levels in the parent plant tissue may be overproduced in cell culture[15].

Maximum amount of azadirachtin-A (11790 µg/g dry wt) was obtained from nodal segment-derived callus tissues in

Table 1. Yield of triterpenoids obtained from callus cultures raised from various explants of *Azadirachta indica* after 120 days of incubation in MS medium supplemented with 5 mg/l BA, 10 mg/l NAA and 10 mg/l IBA

Explant	Callus growth (g dry wt)	Azadirachtin-A (µg/g dry wt)	Nimbin (µg/g dry wt)	Salannin (mg/g dry wt)
Root	0.332 ± 0.01	0.180 ± 0.02	nd	0.507 ± 0.02
Internode	0.592 ± 0.01	47.380 ± 4.02	67.800 ± 1.05	3.255 ± 0.11
Node	0.661 ± 0.02	11790 ± 1075	10.900 ± 2.40	1.850 ± 0.14
Young leaf	0.775 ± 0.02	162.100 ± 0.50	5.520 ± 0.10	0.666 ± 0.03
Sepal	0.701 ± 0.01	7.002 ± 0.35	1.291 ± 0.14	1.390 ± 0.04
Petal	0.752 ± 0.01	7.610 ± 0.10	0.010 ± 0.01	0.186 ± 0.01
Staminal tube	0.213 ± 0.02	59.60 ± 2.26	0.026 ± 0.01	1.410 ± 0.03
Anther	0.267 ± 0.03	1.940 ± 0.18	0.166 ± 0.01	2.220 ± 0.05
Ovary	0.288 ± 0.01	0.320 ± 0.01	1.429 ± 0.01	1.140 ± 0.08
Style	0.339 ± 0.01	71.470 ± 1.70	1.453 ± 0.05	0.590 ± 0.03
Stigma	0.211 ± 0.01	52.750 ± 2.06	0.450 ± 0.04	3.970 ± 0.08
Embryo	0.676 ± 0.02	0.267 ± 0.018	2.290 ± 0.18	2.812 ± 0.06
Cotyledon	0.763 ± 0.01	5.800 ± 0.13	32.710 ± 0.55	1.276 ± 0.01

Results are the mean of seven replications ± standard error. nd, Not detected by RP–HPLC analysis.

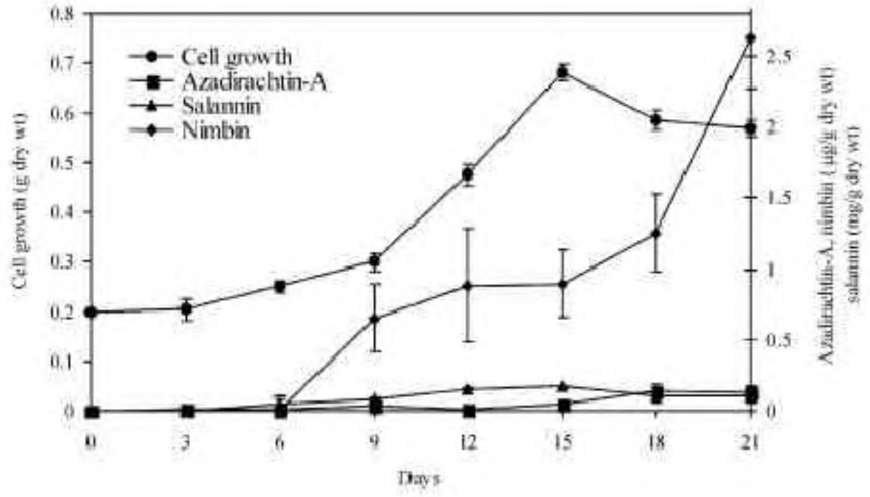

Figure 1. Time course of cell growth and triterpenoid (azadirachtin-A, nimbin and salannin) production in cell suspension cultures of *Azadirachta indica*. Vertical bars indicate standard error of seven replications.

MS medium supplemented with 5 mg/l BA, 10 mg/l each of IBA and NAA (Table 1). Callus derived from stigma accumulated maximum amount of salannin (3.97 mg/g dry wt), while the highest nimbin content (67.8 µg/g dry wt) was recorded in callus derived from internodal segments (Table 1). Among all the explants used for the study, internode-derived callus tissues accumulated appreciable amounts of azadirachtin-A, nimbin and salannin. However, in other cases, some compounds exhibited enhanced accumulation while others did not. Probably heterogeneity in the productivity of the cells has contributed to this varying ability.

Biosynthesis of azadirachtin-A, nimbin and salannin was lower in cell suspension cultures compared to callus cultures. It is expected that some sort of cell-to-cell contact had facilitated accumulation of these compounds, as observed in the case of jaceosidin production in cell cultures of *Saussurea medusa*[16].

Growth curve of cell suspension cultures derived from internodal segments exhibited five different stages: (i) lag phase 0–3rd day, (ii) log phase (3rd–9th day), (iii) linear phase (9th–15th day), (iv) progressive deceleration phase (15th–18th day) and finally (v) stationary phase (18th–21st day) (Figure 1). Accumulation of azadirachtin-A was highest towards the end of the culture. There was a rapid increase in the accumulation of nimbin between 18th and 21st day, indicating that the stress faced by the cells towards the end of the culture period might have stimulated nimbin biosynthesis. However, maximum salannin was detected on the 15th day of the culture, indicating that salannin bioproduction was triggered earlier to azadirachtin-A and nimbin biosynthesis. Azadirachtin-A and salannin were detected from the 3rd day of the cell culture, while nimbin was detected only from the 6th day. Presence of triterpenoids was not detected in the liquid medium.

The report[8] available regarding the production kinetics of azadirachtin reveals that maximum azadirachtin was found on day 7, while maximum cell growth was witnessed on day 10. The result of the present study contradicts this, since maximum azadirachtin production occurred when the growth rate of the cells was minimum. This may most probably be due to difference in the growth regulators employed, explant used for the culture initiation and finally genotype specificity. In short, analysis of the growth and production curve in the present investigation revealed that azadirachtin-A, nimbin and salannin were actively synthesized after the linear growth phase of cells. Therefore, it may be concluded that accumulation of these compounds is enhanced by stress.

Results of the current investigation are significant towards studies on enhanced synthesis and accumulation of triterpenoids in callus culture. The present system is advantageous because it depicts the ability of callus raised from thirteen different explants to synthesize closely

related triterpenoids raised in a single growth hormone regime. To the best of our knowledge, this is the first report on the time course study of cell growth and the three triterpenoids azadirachtin-A, nimbin and salannin produced in the cell suspension cultures of neem. The cell suspension culture raised here helps in identifying elite cell lines overproducing the important triterpenoids, elucidation of biosynthetic pathway and production of elite neem trees. This also provides a unique opportunity for further research in different aspects on the biosynthesis of azadirachtin-A, salannin and nimbin.

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Reconfirmation of the identity and occurrence of *Phyllanthus ajmerianus* Chaudhary & Rao in Ajmer, Rajasthan, India

Chaudhary & Rao[1] in a revision of the herbaceous species of *Phyllanthus* in India described a new species namely *Phyllanthus ajmerianus* Chaudhary & Rao based on a solitary collection of V. S. Sharma 1385 (LWG) collected from a village Naikhera, Kekri tank on 30.12.1959 in Ajmer Dist, Rajasthan. However no additional collection was seen by the founding authors nor could they recollect the fresh specimens from the type locality. Parmar[2] commenting on the status of this new species remarked that it is only a mistaken identity and the specimen actually belongs to another taxon namely, *Andrachne telephioides* L. The distinguishing characters between two genera being the presence or absence of petals in male flowers. Chaudhary[3] is of the opinion that the petal character in male flowers in *P. ajmerianus* is so minute and indistinct that this character alone cannot decide the correct status of *P. ajmerianus*.

Andrachne telephioides L. originally reported from Afghanistan and Westwards to Spain[4] was reported as a new record for India by Parmar[5] in 1982 and the two taxa are quite distinct and there seems to be no ground for confusion. In order to re-examine and solve the taxonomic confusion, we undertook extensive surveys not only in the type locality but in similar other habitats in and around Ajmer. While we were able to collect the so-called *P. ajmerianus*, we could not see any specimen similar to *P. ajmerianus* in the herbarium of Botanical Survey of India, Jodhpur (BSJO). A critical examination and study of our specimens reconfirmed the occurrence of *P. ajmerianus* in Ajmer, Rajasthan and this species differs distinctly from that of *A. telephioides* as follows.

Annual, branching from the base; leaves spathulate or obovate, rounded at apex, coriaceous; sepals 5, elliptic-obovate or oblanceolate; petals absent; stamens 5, basally connate and free above; style 2-fid, deeply bilobed; capsule oblate, obtusely trilobed; seeds trigonous with 6–7 longitudinal lines on the back.

–Phyllanthus ajmerianus

Perennial, with prostrate branches arising from a rootstock; leaves ovate to elliptic or obovate, acute or subacute at apex; sepals 5–6, rounded or rhombic; petals 5–6, small, lanceolate; stamens 5–6, free, alternate with petals; style feebly 2-fid, 2-partite; capsule depressed globose; seeds triquetrous, with a convex, punctulate back.

–Andrachne telephioides

Further, the molecular profile (AFLP analysis) of *P. ajmerianus* was compared with other herbaceous *Phyllanthus* species and this study also pointed to the distinct identity of the taxon closer to *Phyllanthus kozhikodianus* Siv. & Mani and *Phyl-*