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Summary and Conclusions

Chapter 9

SUMMARY AND CONCLUSIONS

BIOPRODUCTION OF TRITERPENOIDS (AZADIRACHTIN-A, SALANNIN, NIMBIN) AND FLAVONOIDS (QUERCETIN AND KAEMPFEROL) IN CALLUS CULTURES

- Callus cultures derived from internodal segments of mature tree accumulated both triterpenoids (azadirachtin-A, nimbin and salannin) and flavonoids (quercetin and kaempferol) after 120 days of growth. 5 mg/l BA along with 10 mg/l each of IBA and NAA (tri-PGR) served as the best growth regulator combination that could evoke faster callus initiation and proliferation when supplemented to WPM. However secondary metabolite accumulation was better in MS tri-PGR.

- Accumulation of azadirachtin-A was highest in nodal segment derived callus from mature tree (11790 μg/g dry wt.); while in field grown plant maximum azadirachtin-A accumulation was noticed in penultimate falling seeds (1346 μg/g dry wt.). Nimbin accumulation was maximal in roots mature tree (41 μg/g dry wt.) cultured callus tissues raised from internodal segments of mature tree accumulated highest amount (67 μg/g dry wt.). Highest salannin production was recorded in callus

tissues grown from leaflets of *in vitro* seedlings (5.8 mg/g dry wt.) while in the field conditions, penultimate falling seeds exhibited highest amount (73 μg/g dry wt.).

- Of the flavonoids, considering the field grown plants, quercetin and kaempferol accumulation was highest in inflorescence (11 μg/g dry wt. and 32 μg/g dry wt. respectively). However, in the callus cultures, quercetin accumulation was highest in internode derived callus from mature tree (12 μg/g dry wt) and kaempferol accumulation was highest in callus tissues grown from leaflet of mature tree (12 μg/g dry wt.). Therefore kaempferol accumulation was lower in callus culture compared to that of field grown plants.

- Callus cultures of neem triggered accumulation of triterpenoids (azadirachtin-A, nimbin and salannin) compared to flavonoids (quercetin and kaempferol).

- Accumulation of secondary metabolites studied was not detected in the first 90 days of callus growth, however appreciable accumulation was noticed in 120 days old callus tissue suggesting that some sort of tissue organization is required for the efficient synthesis of these compounds.

BIOPRODUCTION OF TRITERPENOIDS (AZADIRACHTIN-A, SALANNIN, NIMBIN) AND FLAVONOIDS (QUERCETIN AND KAEMPFEROL) IN CELL SUSPENSION CULTURES

- Compared to callus cultures (grown for 120 days) accumulation of both triterpenoids and flavonoids studied was very low in cell suspension cultures (grown for 21 days).
- Since triterpenoids and flavonoids are not detected in the early stages (until 90 days) of callus growth and their yield was lower in cell suspension cultures, it may be concluded that a cell to cell contact and some sort of tissue organization is favouring triterpenoid and flavonoid biosynthesis in neem callus cultures.
- Growth curve of cultured cells from internodal callus tissue exhibited typically a sigmoid curve that can be classified into a lag phase, log phase, linear phase, progressive deceleration phase and stationary phase. Stationary phase was attained only in cells grown in MS medium supplemented with tri-PGR.
- Biosynthesis of azadirachtin-A as witnessed only in MS medium (basal as well as growth regulator supplemented) and not in WPM. Production curve shows an enhanced accumulation with the onset of progressive deceleration phase and stationary phase clearly indicating that azadirachtin-A biosynthesis is stress dependent.

- Salannin production was higher around 15th day of the culture period indicating that salannin bioproduction is triggered late in the culture.
- Nimbin biosynthesis was activated after 9th day of the culture before salannin biosynthesis was activated.
- Quercetin and kaempferol production could be detected only in growth regulator supplemented media and early in the culture period (3rd day). Quercetin accumulation was highest on the 15th day. Kaempferol production pattern was different in growth regulator supplemented MS and WPM.

EXTENDED STATIONARY PHASE CELL SUSPENSION CULTURES AND PRODUCTION OF TRITERPENOIDS (AZADIRACHTIN-A, SALANNIN AND NIMBIN) AND FLAVONOIDS (QUERCETIN AND KAEMPFEROL)

- By feeding cultivated cells of neem in suspension with 5 ml sucrose solution without subculturing, it possible to extend the stationary phase until 60 days without much loss of viability. This technique favoured cultivated cells to be grown in a steady stress state, so as to trigger and enhance the secondary metabolite synthesis.
- Triterpenoid and flavonoid biosynthesis triggered several fold in the extended stationary phase (60 days of incubation) compared to cell suspension grown for 21 days.

- Ideal carbon source for secondary metabolite accumulation was found to be 5% sucrose and 1% mannitol.

- 0.1 mg/l ABA was found to be triggering maximum accumulation of all the five secondary compounds studied in extended stationary phase cultures.

- Long-term cell suspension cultures in MS medium augmented with 0.1mg/l ABA and by feeding cells with solution containing 5 ml each of 5% sucrose and 1% mannitol, on the 20^{th} and 40^{th} day was identified as the production medium. Here the stationary phase of growth curve could be extended without much loss of cell viability. The yields of azadirachtin -A, salannin, nimbin, quercetin and kaempferol in the production medium were 7, 6.6, 5.5, 7.2 and 7.2 mg/g dry wt. respectively. This technique offers the possibility of exploiting the cell's enhanced ability to synthesize secondary compound in response to stress faced during stationary phase.

- 166 mg/l IPP, 166 mg/l GPP and 166 mg/l squalene was ideal precursors for maximum accumulation of azadirachtin-A, salannin and nimbin respectively in production medium.

- Yield of azadirachtin-A, nimbin and salannin when considered together, 166 mg/l squalene was most effective, (39.66 mg/g dry

wt.) followed by 166 mg/l GPP (32.19 mg/g dry wt.) and 166 mg/l IPP 30.46 mg/g dry wt.).

- 166 mg/l of PEP was most effective in stimulating the accumulation of intracellular quercetin (18.05 mg/g dry wt.) and kaempferol (13.96 mg/g dry wt.)
- Release of quercetin and kaempferol was witnessed when the cells were grown in presence L-Phenylalanine or PVP or Shikimic acid.
- Release of triterpenoids was noticed only when permeabilizers were added. 5% DMSO was most effective in releasing all the compounds of interest except nimbin and azadirachtin-A. A lower concentration of DMSO (0.1%) induced maximum release of nimbin. Maximum extracellular accumulation of azadirachtin-A was observed in presence of 0.1% Triton X-100. Among the triterpenoids, maximum release was observed in the case of salannin in which the amount recovered from the liquid medium was even higher than the intracellular amount.
- Turbidity of the medium obtained during permeabilization is an indication of cell lysis, which might have caused the release of intracellular organelles, which is also associated with decrease in cell viability.

- Vanillin-sulphuric acid reagents apart from being an efficient TLC detector, also worked well for the histochemical localization of all the five compounds in callus, dispersed cells and explant tissues. Vanillin-sulphuric reagent was able to localize simultaneously all the five compounds giving pink shade to azadirachtin related limonoids, distinct blue shades to salannin and nimbin related limonoids, and yellow shades to flavonoids both querecetin and kaempferol all visible under ordinary light. This vanillin - sulphuric acid reaction could be utilized as a qualitative test for detecting the presence of all the five compounds selected in the present study.

- Highest yield obtained in the present study for azadirachtin-A (cells cultivated in production medium permeabilized with 0.1% Triton X-100), salannin (cells cultivated in production medium permeabilized with 5% DMSO), nimbin (cells cultivated in production medium permeabilized with 1% DMSO), quercetin (cells cultivated in production medium permeabilized with 5% DMSO) and kaempferol (cells cultivated in production medium with 166 mg/ PEP as the precursor) were 17.06 mg/g dry wt., 23.87 mg/g dry wt., 22.68 mg/g dry wt., 34.41 mg/g dry wt. and 32.97 mg/g dry wt. respectively. This yield, obtained in extended stationary phase cell suspension cultures grown for 60 days, is several times higher compared to naturally grown plants

or callus cultures or cell suspension cultures grown for 21 days. This clearly proved the feasibility of cultivating cells for long term (60 days) under stationary phase.

SOMATIC EMBRYOGENESIS AND PRODUCTION OF SECONDARY COMPOUNDS

- Of the various explants tried, leaflets, anthers and ovaries responded to direct embryogenesis while all the others (root, internode, cotyledonary node, cotyledon, leaflets and petals) exhibited indirect embryogenesis
- Highest percentage of response regarding the embryogenic callus formation was obtained in petal explants (68%). On the other hand highest percentage of response (90%) concerned with direct embryogenesis was recorded in leaflet explants (both from mature tree and *in vitro* seedlings) followed by ovary (78%) and anther explants (67%)
- KIN served as an ideal plant growth regulator that could initiate formation of direct somatic embryoids from leaflets and anther. For ovary explants, TDZ served as an effective cytokinin for initiation of heart shaped embryoids. It was also effective for generating somatic embryoids from cotyledonary explants. ABA served to multiply the number of embryoids in the first week of initiation that was later found to be inhibitory. IAA was a

beneficial phytohormone for initiating embryogenic response from internode callus. 2,4-D was effective for root calluses and number of embryoids was high than any other hormones

- Secondary embryogenesis was witnessed often in cultures.
- Enlargement of globular and heart shaped embryoids were noticed in suspension culture derived from cotyledonary tissue.
- Scanning micrographs revealed the granular surface, notch and folding present in the globular and heart staged embryoids
- Leaf explants showed a higher potential to initiate both direct as well as indirect embryogenesis while internode and cotyledonary nodes exhibited high potential of indirect embryogenesis
- Azadirachtin was below the limit of detection in leaflet tissues exhibiting direct embryogenesis while salannin and nimbin were detected on the 25^{th}, 50^{th} and the 75^{th} day of the culture period. Highest salannin and nimbn contents were detected on the 75^{th} day.
- Leaflet explants exhibiting indirect embryogenesis showed that, all the three terpenoids (azadirachtin-A, salannin and nimbin) were below the limit of detection on the 25^{th} day, but were produced on the 50^{th} and 75^{th} day of the culture period. Highest production of all the three terpenoids was recorded on the 75^{th} day of the culture period.

- Bioproduction of all the three triterpenoids in internode and cotyledonary node explants exhibiting indirect embryogenesis could be detected only on the 50th and the 75th day and production values in both the tissues were high on the 50th day. Flavonoids- quercetin and kaempferol were below limit of detection in all the embryogenic tissues analysed

- Unorganised (cell cultures) and semi-organised cultures (callus cultures) were found to be better than organised cultures (cultures exhibiting somatic embryogenesis) in the production of secondary compounds.

EFFICACY OF PURE NEEM COMPOUNDS AND CRUDE SAMPLES FROM INTERNODE DERIVED CELL CULTURE ON HCT116 - COLORECTAL CANCER CELL LINE

- HCT 116 cells exhibited chromatin condensation, nuclear marginalization, reduction in the size of the nucleus, nuclear blebbing (chromatin fragmentation) indicating apoptosis.

- The GI_{50} value of azadirachtin-A ranged between 0.27 and 0.67μM. Cells treated with azadirachtin-A exhibited severe degree of growth inhibition. It induced chromatin fragmentation, chromosome clumping and reduction in the size of the nucleus. 5% was the active mitotic index recorded in azadirachtin-A treated HCT 116 cells

- The GI_{50} value of salannin ranged between 0.17 and 0.33 μM. Cells treated with salannin exhibited severe degree of cytotoxicity and growth inhibition
- HCT 116 cells also exhibited cytotoxicity in presence of nimbin, quercetin and kaempferol. In the case of nimbin, the GI_{50} value exceeded 0.88 μM, while it ranged between 0.58 and 140 μM for quercetin and between 0.35 and 0.69μM for kaempferol.
- Among the crude extracts, flavonoid rich fraction exhibited a higher degree of cytotoxicity compared to terpenoid rich fraction and total extract.
- The total crude cell suspension extract served both as a growth inhibitor and mild cytotoxic inducing nuclear marginalization, chromosomal aberrations like laggards and bridge formation. Its action was very much similar to azadirachtin-A and the active mitotic index in HCT 116 treated cells was 5%. The terpenoid rich crude cell suspension extract was served primarily as cytotoxic, inducing chromatin condensation and severe chromosome clumping. The active mitotic index in HCT 116 treated cells was 3%. The flavonoid rich crude cell suspension extract induced severe cytotoxicity, cell vacuolation, peripheral nuclear condensation and mild chromosome stickiness. Its action was very much similar to kaempferol and the active mitotic index in HCT 116 treated cells was 0.05%