GENERAL DISCUSSION

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This research endeavor had six directions of special emphasis, First: screening of *Boerhaavia diffusa* L. and *Achyranthes aspera* L. plants for the presence of alkaloids, further their isolation, identification and quantification to select `elite' superior plants amongst them. Second: screening of individual organs of the `elite' plants for locating the site of synthesis/ accumulation of the alkaloids. Third: establishment of *in vitro* excised root cultures of B. diffusa by meeting their nutritional/ hormonal requirements with an attempt to enhance the alkaloid production by precursor feeding. Fourth: establishment of callus cultures of excised organs from `elite' plants of *B. diffusa* L. and *A.aspera* L. by standardising their nutritional / hormonal requirements for maximum biomass production and observing the regenerative potential of the callus tissues with the underlying histological changes involved during the process. Fifth: screening of callus cultures for their alkaloid profile. Sixth: Identifying the regenerative ability of the excised leaf-segments of both these plants. Results of the experimental work conducted on *B. diffusa* L. and *A.aspera* L. have been discussed in light of the work on medicinal plants reported by others.

A) SELECTION OF `ELITE' PLANTS OF *B.DIFFUSA* L. AND *A.ASPERA* L.

Alkaloids are widely synthesized in plants which are useful pharmaceutically because of their physiological activities (Misawa, 1982). They are synthesized and accumulated by plants with apparent ease hence, till today, plants constitute their

main source.

Ethanolic extract of mature plant of *B.diffusa* L. when subjected to chromatographic analysis gave positive results with Dragendorff's reagent indicating the presence of alkaloids (Expt.1). Alkaloidal fraction was isolated by running the chromatogram of the extract using chloroform : alcohol (2:1) solvent system. The isolated alkaloid was co-chromatographed with the authentic sample of punarnavine (authentic sample of punarnavine was supplied by late Prof.K.N.Udupa, Institute of Medical Sciences, Banarus Hindu University, Varnasi). The identical Rf values (0.85) confirmed that the isolated compound was the punarnavine alkaloid. Thus it was evident that *B.diffusa* plants synthesized/accumulated punarnavine alkaloid. Further, for separation of punarnavine in pure form, the extract was spotted on silicagel TLC plates and eluted with chloroform : alcohol (2:1) solvent system. The purified sample was used for the chromatography, which again gave identical results indicating that the isolated punarnavine sample was in pure form. Reports of medicinal use of the extracts of *B.diffusa* against liver cirrhosis (Sudarsana and Shanthamma, 1988) and as anti-hepatotoxic agent (Chakraborti and Handa, 1989) have been known. Its cholinesterase activity at lower dose have also been reported recently. (Goswami and Sharma, 1991).

The ethanolic extract of mature *A. aspera* L. plants when subjected to chromatographic seperation gave positive results with Dragendorff's reagent. This proved that the alkaloids were isolated in the extract. The alkaloid fraction in pure form was further isolated by subjecting it to the preparatory chromatography. The spots developed were eluted, redissolved and run on silicagel coated

chromatographic plates along with the authentic sample of achyranthine (Authentic sample of the alkaloid achyranthine was procured from Prof.V.K. Kapoor, of Punjab University, Chandigarh, since it was not available with any commercial companies). The Rf values produced by the authentic sample and the extract were the same (0.72). This confirmed that the isolated compound was the achyranthine alkaloid (Expt.19).

During 1988, at the first Asian Conference on Pharmaceutical Education Research and Drug Industry and the Workshop thereafter held on medicinal and Aromatic Plants at National University of Singapore, Singapore, recommendations were made about developing drugs on priority basis from both these plants namely *B.diffusa* L.and *A.aspera* L. For this, intensive research *in vitro* studies were required which could facilitate the availability of these alkaloids in large amounts. Drug of *A.aspera* L. would be useful as anti-fertility agent which constitute the present- day need.

Extracts of *B.diffusa* L. plants collected during rainy season contained the alkaloid fraction in high quantity (Mudgal,1975). However there are not many reports on the quantitative estimation of the alkaloid/s of this plant.

At Indian Council of Medicinal Research, New Delhi, a research project was conducted with monitoring centres at various Medical Colleges such as Madras, Chandigarh, Delhi, where `Kshaarsootra' a medicated thread prepared with the application of ash of *A.aspera* plant was used against the anal fistula in about one thousand patients. Results showed that all the patients were cured without

reoccurrence of the disease and also without any side effects (Shukla *et al.*, 1991). Besides this, extract of *A.aspera* plant exhibited excellent contraceptive properties when tested in rats and hamsters (Wadhwa *et al.*,1985). Similar results were reported by Pakrashi and Bhattacharya (1977) working on rabbits. Reports of reducing blood pressure by *A.aspera* decoction dilating the blood vessels due to increased rate and amplitude of respiration are also known (Neogi *et al.*, 1970).

In *B.diffusa* plant, highest punarnavine alkaloid was accumulated in the roots (2%) when calculated on dry weight basis (Expt. 2). In stem (0.56%) and leaves (0.82%), much lower quantities of alkaloids were accumulated which proved that the roots were the site of synthesis/ accumulation of the alkaloid in particular.

Earlier, use of root extract of *B.diffusa* against night blindness, eye diseases and corneal ulcer had been reported by Gupta *et al.*(1962). Also root extract exhibited broad spectrum of antiviral activity without any side effects (Verma and Awasthi, 1979). The medicinal values of *Desmodium pulchellum* lies in the alkaloids synthesized/accumulated by it (Ghosal and Bhattacharya, 1972). In the roots of young poppy plants synthesis of morphine alkaloid occurred, which was then transported to leaves and ultimately accumulated in the fruits (Fairbrain and Wossel, 1964). This indicated that the plant organ with the capacity for synthesis of alkaloid was not the site of its accumulation.

The whole plant of *A.aspera* when screened gave 0.11% of achyranthine alkaloid on dry weight basis. While individual organs of it showed that stem contained 0.05%, the highest quantity of alkaloid, while leaves, flower and roots

contained 0.03%, 0.02% and 0.01% achyranthine respectively (Expt.20). This made it clear that the whole plant needs to be extracted for the achyranthine. Shoot portion of *A.aspera* accumulated higher quantity of the alkaloid when compared to the root portion of the same plant. These results find support with the finding of Waller and Nowacki (1978). However fluctuation in alkaloid contents of the organ of a plant might be due to its *de novo* synthesis/ translocation/ degradation of the alkaloid. The diuranal variation in morphine content of *Papaver somniferum* was due to differential rate of its synthesis and catabolism. Waller and Nowacki (1978) showed that turnover of morphine alkaloid to the abaine and codeine in young poppy plant was consistent with the known biosynthetic pathway i.e. abaine -> codeine -> morphine through inter conversion reaction. This fluctuation in the alkaloid quantity might also be due to their shifts between free and bound forms, the later yielding the free form upon hydrolysis. Anderson et al.(1986) also reported variation in the quantities of alkaloids accumulated in its various organs of *Ailanthus altissima*, a tree species. In a leguminous plant, alkaloid synthesis occurs in its aerial organs however it was accumulated in the seeds (Waller and Nowacki,1978).

Root samples of *B.diffusa* plant of the same age (Expt. 3) when screened for punarnavine contents showed that its highest quantity was accumulated in the plants growing in the M.S.University Campus (2%), followed by G.I.D.C. Makarpura (0.93%) and the lowest in the Nandesari grown plants (0.88%). *A.aspera* plants from various localities showed that highest achyranthine was accumulated in the plants growing in Sama area (0.12%) followed by the M.S.University (0.11%), Makarpura (0.08%) and lastly Nandesari (0.04%) grown plants when calculated on

dry weight basis (Expt.21).

In fact, biosynthesis of alkaloid is gene governed, however, the variation in their quantities accumulated by the plants of the same age growing in different localities might possibly be due to the effects of environmental factors (Waller and Nowacki, 1978). The *Atropa belladonna* roots collected from Kashmir accumulated low levels (0.30%) of alkaloid, while those of *Atropa* plants growing in Himachal Pradesh had highest quantity of alkaloid (0.68%) Chopra *et al.*(1982). This means that the environmental factors affected the synthesis of secondary metabolites. Hamdard Pharmaceutical (1994) have also reported the impact of environmental degradation on plant forms.

B) EXCISED ROOT CULTURE

Chemical analysis of individual organs of *B.diffusa* L. plant described in previous section established that the active principle-punarnavine-alkaloid was accumulated in the roots of this plant. So far as per the literature surveyed there are no reports of the excised root cultures of *B.diffusa* in spite of their unique ability of accumulating punarnavine and other alkaloids.

The present section deals with the discussion of the results obtained on the experimental work conducted on excised root cultures of *B.diffusa.* Root tips of aseptically germinated seeds from elite plants of *B.diffusa* were used as explants for establishment of *in vitro* root cultures. The factors supporting active growth and development of these excised roots were standardized and also their ability for punarnavine synthesis/ accumulation was studied.

Experimental work for the establishment of the excised root cultures of this plant was undertaken which would provide information required for the application of advanced technologies such as hairy root cultures induced by *Agrobacterium rhizogenesis* as well as isotopic studies with radio active elements for finding out the pathway of punarnavine synthesis.

Out of the two known media *viz.*, White's (1954) and Murashige and Skoog's (1962) tested for the growth of excised roots of *B.diffusa*, the MS medium supported their active growth and development (Expt.4). The length of the main root axis was 4.6 cm with the production of four lateral roots at the end of four weeks culture period. MS liquid medium was used and the culture flasks were maintained on gyratory shaker (120 rpm) in culture room at 25±2°C in light (1000 lux). Dodds and Roberts (1985) also reported the use of liquid medium over the solid medium being ideal for excised root cultures in general. Excised root cultures when maintained on gyratory shaker received aeration due to agitation. In fact, Said and Murashige (1979) pointed out that continuous agitation to tomato roots by maintaining culture flasks on the shaker recorded length of the cultured roots to be doubled when compared with their growth in static conditions. The final form of the roots after culturing depends upon length of main axis as well as lateral root production which directly indicates nutritional status of the culture medium (Boll 1965). Butcher and Street (1964) observed that root clones have rapid growth rate and suffer no difficulties in their multiplication when they were cultured in a suitable salt combinations. For the continuous cultures of excised roots, actively growing root-tips were excised and subcultured regularly in fresh medium at four

weeks interval. Bergman and Mungae (1986) observed that for *Robus idacus*, CV Titan commonly known as red raspberry, Anderson medium (1978) supported the active growth of roots maintaining their vigour whereby they continued their growth and development during several subculturing. This proved that the requirement of mineral nutrition of one species differed from that of the other species. In *B.diffusa*, excised root cultures, MS medium was found ideal as indicated by our results (Expt.4).

Although excised roots and intact roots of a plant are alike in many ways but they differ in their metabolic activities as excised roots do not possess aerial organs. Excised root cultures where the environmental conditions are kept uniform provide information as regards to their nutritional and hormonal requirements. In order to enhance the growth rate of excised roots of *B.diffusa*, MS liquid medium was supplemented with sucrose (2%), cytokinin *viz.*, Kn/BAP and auxin *viz.*, IAA/IBA/NAA at various concentrations and combinations. The incorporation of Kn at 0.5 μM/l enhanced the growth of main axis, it was 6.5 cm in length with the production of 6 ± 1 laterals (Expt.5). It appeared that Kn supported active cell division at the root-tips which resulted in its faster growth and development. Increase in Kn level to 1 μM/l further enhanced the growth of main axis which reached to 10 cm in length simultaneously producing increased number of lateral roots (9 ± 1). But the increase of Kn to 2 μM/l all these growth parameters declined which proved that 1 μM/l of Kn to be the optimal level for the growth of the cultured roots. It has been pointed out by Street and Winter (1963) that the presence of Kn in medium enhanced the duration of meristematic activity at the root tips whereby the growth of this organ gets improved over the control. This finding

was equally supported by the work on *Rauwolfia serpentina* where addition of Kn to culture medium improved their linear growth (Mitra,1975).

Replacing Kn with BAP in MS medium led to a reduction in the growth of main axis of excised roots of *B. diffusa* when compared with growth of axis in response to Kn treatment. The main axis measured 10 cm length which was more than obtained with BAP treatment (7.2 cm). Thus Kn treatment exhibited superiority over BAP, for culturing the *B.diffusa* excised roots.

In other factoral experiment MS medium with 1 μM/l Kn was supplemented with IAA at various levels. It was observed that in *B.diffusa* root cultures, the main axis reached 12.3 cm in length with the production of 10 ± 2 lateral roots, when IAA level was 1 μM/l. This means IAA in combination with Kn produced synergistic effect whereby the growth parameters of cultured roots were enhanced over those recorded when Kn alone was used. With the increase of IAA level to 4 μM/l, there was a decline in the length of main axis and the number of laterals produced. Street and Winter (1943) have earlier reported that IAA improved the growth of main axis only at low concentration. Boll (1965) also observed similar effects of IAA, in excised tomato root cultures. Also, Bhattacharya and Gupta (1987) observed that in *Nigella sativa*, IAA (0.5 mg/l) supported the formation of lateral roots.

The second auxin IBA was tested in combination with 1 μM/l Kn for excised root cultures of *B.diffusa*. The results showed that at 0.5 μM/l, IBA, the linear length of main root axis was 9.5 cm with 8 lateral roots developed. Further

increase in IBA level to 1 μM/l, there was slight improvement in the length of main axis as well as in the number of lateral root production. However, at 2 μM/l of IBA the highest length of the main axis was 17 ± 0.2 cm with 22 ± 2 lateral roots. Further increase in IBA level to 4 μM/l, all these growth parameters declined, which proved that 2 μM/l of IBA with 1 μM/l of Kn were at their optimal levels of auxin and cytokinin for the growth of excised root cultures of *B.diffusa*. In *Rauwolfia serpentina* the beneficial effect of IBA at 0.01 mg/l on excised root culture has been reported by Mitra (1968). George and Sherrington (1984) have also reported that auxin promoted the growth of roots by inducing secretion of H^+ ion into the medium which affects RNA metabolism of plant cells. Reduction of lateral root initiation as well as decrease in the length of root axis indicates the visible symptoms associated with the deficiency of auxin availability. The essentiality of IBA for the excised root cultures of *B.diffusa* was established (Expt. 5). These findings are consistent with the finding of others. The natural auxin in the cultured cells of roots being far below the level required for their active growth and development, the supply of IBA in the culture medium became the necessity (Mitra, 1975). He working with excised roots of *Rauwolfia serpentina* observed that the greatest number of lateral roots produced were 16 with IBA (0.15 mg/l) in the culture medium. Earlier Mitra (1968) had indicated that in *Atropa bellodana*, IBA not only enhanced linear growth in each passage but also increased the proportion of lateral root formation.

With the application of NAA with 1 μM/l Kn, the excised root cultures of *B.diffusa* recorded the linear growth to 6 cm at 1 μM/l of NAA. Length of main

axis slightly improved to 6.6 cm at 2 μM/l of NAA. However, further increase in NAA level led to a decline in the growth of main axis and also in the development of lateral roots. Thus in *B.diffusa*, NAA treatment failed to produce desired results. Similarly, in *Rauwolfia serpentina* also NAA inhibited the growth of main axis with lateral root production (Mitra, 1975). But in the case of tomato roots, NAA treatment enhanced the elongation of main axis as well as lateral root formation (Butcher and Street, 1960).

When 2,4-D was incorporated in the culture medium at various levels in combination (1 μM/l) Kn, a cream coloured callus was developed from the root apices which proliferated all over the explant. At high levels of 2,4-D (2 μM/l), callus initiation occurred at much faster rate and it took only one week of culture period to become visible.

The results of experimental work proved that IBA at 2 μM/l was the optimal level required for the growth of excised roots of *B.diffusa* L. (Shrivastava and Padhya, 1991).

The effect of variation in sucrose levels was studied on excised roots of *B.diffusa* as it acts as energy source (Expt.6). The optimal concentration required varied from one species to other had earlier been reported. Incorporation of sucrose at 1% stimulated the growth of main axis with initiation of lateral roots. Increase in sucrose level to 2% in combination with Kn 1 μM/l and IBA 2 μM/l, the length of main axis was 17.1 cm with the production of 22 ± 2 lateral roots at the end of 4 weeks culture period. Further increase in sucrose level to 3% not only reduced the

length of main axis from 17.1 to 11 cm but also reduced the number of lateral roots produced from 22 to 10, thereby indicating sucrose at 2% was the optimal level as it supported highest growth in length of the main axis with the production of lateral roots. In absence of sucrose, the excised roots turned black and ultimately they died, which proved that sucrose supplied required energy for the growth and development of these roots. Dormer and Street (1949) reported that excised root cultures required energy for their maintenance and growth which was supplied by sucrose, incorporated in the culture medium. Similar results were also reported by Thomas *et.al* (1963).

An experiment was conducted to find out the effect of variation in concentration of salts of MS medium; in combination with Kn (1 μM/l), IBA (2 μM/l) and sucrose (2%) on the growth of excised cultured roots of *B.diffusa* (Expt.7). Results showed that the highest growth rate in terms of fresh weight (790 $\pm$ 15.7 mg) and dry weight (155 $\pm$ 1.5 mg) was recorded when MS medium salts were present at standard dose. The concentration of salts of MS medium when doubled keeping the same levels of the supplements as added in standard medium, the excised roots of *B.diffusa* reduced their fresh weight (348 $\pm$ 11.32 mg) and dry weight (21 $\pm$ 0.9 mg) at the end of four weeks culture period. When the MS medium salts were reduced to their half, keeping constant the levels of supplements, there was drastic reduction in fresh and dry weights (Expt.7). Thus it was evident that the salts as present in MS medium at standard dose were at optimal levels since they supported highest growth of excised roots, in terms of fresh and dry weights. Bhojwani and Razdan (1983) reported that for each species the concentration of salts differ, after

testing wide spectrum of salts of different culture media.

It has been observed that vitamins also formed important factor of the culture medium which influenced the alkaloid production (Ramawat, 1991). Even though the vitamins are added in minute quantity to root culture medium they exert profound effect on the growth of the excised cultured roots. The effect of variation in the levels of vitamins was tested on root cultures (Expt.8). The vitamin levels of the MS medium were varied to one half, standard dose, doubled and its absence, keeping Kn (1 μM/l), IBA (2 μM/l) and sucrose (2%) levels constant. Results showed that in complete absence of vitamins, excised cultured roots of *B.diffusa* recorded very poor growth. With one half dose of vitamins added to the culture medium the growth of cultured roots was improved. However, highest growth parameter of cultured roots were recorded when vitamins were present at the standard dose. The length of main axis showed linear growth upto 17 cm with the production of 25 lateral roots. Further doubling the level of vitamins, reduced their growth and development. Flores *et.al*, (1993) reported that isolated root cultures depend upon the supply of vitamins such as thiamine for their continuous growth. Earlier Torrey (1954) had pointed out that excellent growth of excised pea roots occurred in presence of vitamin B.

The rate of growth of excised roots in the MS standard medium was studied during six weeks period. It showed that in the first week, the main axis developed rather slowly but in the second week it picked up growth and reached upto the length of 17 cm with production of 25 lateral roots, at the end of four weeks culture period. The graph of growth parameters plotted against time produced sigmoid

curve (Expt.9).

The ability for alkaloid accumulation by excised roots of *B.diffusa* cultured in MS medium supplemented with Kn (1 μM/l) sucrose (2%) and auxins IAA/IBA/NAA (2 μM/l) showed that roots cultured in IAA medium, only punarnavine presence was detected after 4 weeks culture period. However, 2 μM/l IBA cultured roots of *B.diffusa* accumulated punarnavine to 0.008% while in 2 μM/l NAA cultured roots even the presence of punarnavine was not observed. Among different auxins, IBA has been found to be a promoter of alkaloid synthesis/accumulation in several cases in *Solanum eleagnifolium* (Ramawat, 1991).

At the end of eight weeks of culture period, there was enhancement in the quantity of punarnavine accumulated by *B.diffusa* roots. Roots cultured in IAA containing medium recorded 0.05% punarnavine content, while in IBA containing medium it was highest 0.06% (Shirvastava and Padhya, 1992). But punarnavine content was lowest in excised roots cultured in NAA 0.02%. It was evident that synthesis/accumulation of punarnavine occurred during 4-8 weeks culture period of the excised cultured roots. A similar correlation between the root growth and the production of tomatine was also reported by Sander (1956) and in roots of *Racinus communis* for nicotine production (Solt *et.al.*, 1960).

Amino acids are precursors of several alkaloid and when supplied in the medium they enhanced the alkaloid synthesis of the tissues. Feeding of L-tryptophan, the precursor of alkaloid to excised roots of *B.diffusa*, it was observed that at 20 μM/l of tryptophan, the biomass production of excised roots was highest in terms of fresh

and dry weights (1320 ± 25 mg,101 ± 10 mg respectively, with the punarnavine content accumulated to 0.32% (Expt. 11). At the lower level of tryptophan (10 μM/l) or its higher level (30 μM/l), the biomass production decreased with proportionate reduction in punarnavine contents. *B.diffusa* roots cultured in presence of L-tryptophan (20 μM/l) in culture medium improved punarnavine synthesis as well as the accumulation of the alkaloid (Padhya, 1993). Street and Winter (1964) in tomato roots with incorporation of tryptophan, observed improvement in alkaloid production. Roddick and Butcher (1972) have reported that the principle site of tomatine synthesis was located in the root-tips and when the number of lateral roots produced was increased naturally their alkaloid contents simultaneously increased. Zenk *et.al* (1977) found that L-tryptophan supplied at 500 mg/l medium produced three fold stimulation of serpentine level in *Catharanthus roseus* cell cultures.

C. CALLUS CULTURES AND PLANTLET REGENERATION

The third endeavour of this work involved the establishment of callus tissues from the individual organs *viz.*, stem/leaf/floral buds of `elite' plants of *B. diffusa* L. and *A. aspera* L. and further production of their optimal biomass by providing necessary nutritional/hormonal conditions. The bio-synthetic potential of these calli was determined for the production of punarnavine/achyranthine alkaloids, so also the morphogenetic potential of these calli for plantlet regeneration was evaluated.

In *B.diffusa* L. callus induction occurred from cut ends of stem segments when the culture medium was supplemented with Kn (0.5/1/2 μM/l) in combination with IAA (2/4 μM/l). Callus was cream coloured (Expt. 12). However, leaf and floral

buds cultured on Kn in combination with IAA failed to induce callus. Similarly replacing IAA by NAA at all the levels, callus initiation did not occur from any of these explants. This proved the specific auxin requirements of these tissues for the initiation of cell-division, with their proliferation leading to callus formation. In presence of 2,4-D in combination with Kn, stem/leaf explants produced profuse callus (Expt. 12).

In *A. aspera* L. excised stem/leaf/pieces when cultured on IAA, failed to induce callus however, roots regenerated from them (Expt.22). No callus was initiated from the spike explant.

Replacing IAA with NAA, failed to initiate callus tissues from stem/leaf/spike. However, these explants when cultured on media supplemented with Kn in combination with 2,4-D, (0.5, 1, 2 μM/l) induced callus from them. Callus was yellowish and friable when 2,4-D level was 2 μM/l. Floral organs such as calyx/corolla failed to initiate callus (Expt. 22). Highest amount of callus initiated from these explants when Kn was 2 μM/l in combination with 2,4-D at 4 μM/l that proved to be their optimal levels for callus initiation. Gautheret (1966) reported that the initiation of proliferating callus cultures from explants involved profound changes in developmental state, the basic architecture of the tissues was altered, certain specialized cell-types were lost, new cell-types originated and quiescent cells acquired the capacity for division. Further, each of the explant showed an absolute requirement for exogenous cytokinin and auxin for sustained proliferation (Gresshoff, 1978). In fact, Yeoman (1987) pointed out that the hormonal constitution of the culture medium was the critical factor in initiating long term

callus cultures. The most characteristic effects of cytokinins on cultured plant cells was their influence on cell-division (Szwekowska, 1974).

The stem/leaf callus cultures when grown on MS media supplemented with various levels of Kn and 2,4-D showed that their highest biomass production in terms of fresh and dry weights required specific quantities of them. In *B.diffusa*, stem callus recorded the highest biomass values (4449 ± 59 mg fresh wt. and 218 ± 20 mg dry weights) when Kn was 1 μM/l in combination with 8 μM/l of 2,4-D while in leaf callus 4104 ± 29 mg fresh wt. and 205 ± 16 mg dry weight were recorded. Stem callus proliferated profusely recording higher biomass values than leaf callus was obvious from these results.

In case of stem/leaf calli of *A.aspera* cultured on MS medium containing sucrose (2%) in combination with Kn (2 μM/l) and 2,4-D (4 μM/l) recorded highest biomass values. Their fresh weights recorded 1050 ± 35 mg, 1287 ± 40 mg and dry weights 74 ± 12 mg and 90 ± 18 mg respectively were at the end of four weeks culture period. Other combinations of Kn with 2,4-D recorded lower biomass values than were obtained at 2 μM/l Kn and 4 μM/l 2,4-D levels. However, the biomass production of stem/leaf calli of *B.diffusa* recorded were much higher than those of *A.aspera* callus tissues (Expt.23). Noughchi et al.(1977) reported that the main objective for the researcher in plant tissue culture is to devise a suitable culture medium which supported sufficient amount of biomass production of callus tissues under investigation.

Since stem/leaf callus tissues of both these plants required sucrose as the energy

source in the culture medium, the optimal level of sucrose was determined for each callus culture. It was observed that in *B.diffusa* stem and leaf calli recorded 4962 ± 40 mg and 4820 ± 39 mg of fresh weights and 253 ± 20 and 245 ± 8 mg of dry weights respectively when sucrose level was 3%. At lower or higher levels of sucrose these values recorded were much lower (Expt. 14) clearly pinpointing that 3% sucrose level was the optimal level required for the growth of these callus tissues.

In stem and leaf callus cultures of *A.aspera* the optimal level of sucrose when determined showed that at 3% highest fresh and dry weight values were recorded *viz.*, 1432 ± 35 mg, 1692 ± 42 mg, of fresh weights and 97 ± 12 mg and 115 ± 15 mg dry weights respectively at the end of four weeks culture period (Expt. 24). However, biomass production of *A.aspera* callus tissue was much less when compared to that of *B.diffusa* stem and leaf callus tissues. Mantell *et al* (1983) also reported that vast majority of cultures were grown heterotrophically using sucrose as the energy source. Rhodes *et al* (1986) have reported that sucrose was major transported carbohydrate in plants and that might be the reason for its support for highest biomass production.

Growth measurements conducted of stem/leaf callus cultured of *A.aspera* showed that the growth values in terms of fresh and dry weights when plotted against time developed typical sigmoid curves thereby indicating that it followed the typical growth pattern.

As regards the biosynthetic potentials of these callus tissues for the production of

punarnavine by *B.diffusa* cultures, it was observed that in stem callus of *B.diffusa* only presence of the alkaloid, punarnavine was detected while in leaf callus 0.007% punarnavine was accumulated at the end of four weeks. The quantity of this alkaloid increased to 0.02% (dry wt. basis) at end of eight weeks culture period (Expt. 15). Thus, it was quite evident that the stem and leaf callus cultures of *B.diffusa* have retained their biosynthetic potential for punarnavine synthesis. However, leaf callus synthesized/accumulated more of punarnavine contents when compared with the quantity produced by the stem callus (Expt. 15). These results were supported by the findings of Mantell and Smith (1983) who pointed out that IAA/NAA supported the synthesis of secondary metabolites but not 2,4-D. Dougall (1979) also reported that callus cultures established from various organs of a single plant, differ in their yields of secondary metabolites which were in agreement in the present studies on *B.diffusa* and *A.aspera* callus cultures.

Stem callus of *A.aspera* synthesized/accumulated 0.007% and 0.002% achyranthine when the media were supplemented with IAA and NAA respectively (Expt. 24). However, callus grown on 2,4-D supplemented medium just gave positive indication of the presence of achyranthine. In leaf callus, 0.003% and 0.001% were the quantities of achyranthine synthesized/accumulated when the culture media contained IAA and NAA respectively.

Thus, it was evident that both stem and leaf calli of *A.aspera* plants have retained their biosynthetic potential for the synthesis of achyranthine. In both these plants, it was observed that the use of auxin 2,4-D/NAA/IAA played role in the synthesis of the respective alkaloids. The findings of Gamborg *et al.*(1971) lend

support to these results, that both the quality and quantity of auxins administered during the course of culture development have a marked effect on secondary metabolite production by the culture. In *Nicotina tobaccum* cultures, transferred from 2,4-D medium to IAA supplemented media, nicotine synthesis was activated by IAA. Butcher (1977) also had reported that tissue cultures produced compounds identical to and similar to those present in the plant from which they were initiated. Further support to this findings was obtained by Seabrook (1980) who suggested that it was advisable to compare the cultures of various organs and their callus cultures systematically for each plant before selecting the organ or its callus cultures for the production of desired compounds. Zenk (1978) referred the biochemical totipotency of plant cells thereby meaning that all the necessary genetic and physiological potential for secondary metabolite formation was present in cultured cells. Hence they could yield identical secondary metabolites like the parent plant under uniform cultural conditions. Even in protoplast cultures of *Catharanthus roseus* in 76 cultures established, there were quantitative differences in their alkaloid contents (Mantell and Smith, 1983). It was observed that the accumulation of secondary metabolites at any stage of cultures was the result of dynamic balance between their biosynthesis and biodegradation (Mantell *et al.*,1983).

The morphogenetic potential of callus cultures of stem/leaf of *B.diffusa* and *A.aspera* when studied showed that in complete absence of 2,4-D in the culture medium, stem callus of *B.diffusa* produced roots alone. The leaf callus also differentiated into large number of white roots with plenty of root hairs (Expt. 16). It was obvious that root/shoot differentiation was a function of qualitative interaction

between growth hormones in particular cytokinins and auxin which have been reported in many plant tissue culture systems (Bhojwani and Johri, 1970). With the incorporation of 2,4-D at lower concentrations 0.01 and 0.05 μM/l, the stem callus turned compact and nodular within two weeks incubation. Histological observations revealed that a few of its cells have deposited lignin on their walls in the form of spiral/reticulate manner thereby indicating that xylogenesis occurred in them. This finding was supported by Fosket and Torrey (1969), who reported that tracheary element formation occurred, after the cell has undergone division in an environment which contains an effective level of auxin and cytokinin. Treatment of the cells with growth regulators induced the specific developmental pathway favouring tracheid formation (Yeoman, 1987). Further compact nodular callus showed that it consisted thin walled cells with prominent nucleus in each arranged compactly (Expt. 16). These nodules represent the growth centres which could develop either into roots or shoots. This growth centre became vascularised due to the appearance of tracheidal cells in them and such growth centres are the sites for the organ formation in the callus (Ross *et al.*, 1973). It has been pointed out by Mantell *et al.* (1985) that intensive studies needs to be done to optimize cultural conditions which supported efficient organogenesis in tissue cultures of several economically important plant species. These nodules when cultured on BAP and adenine sulphate containing medium, they turned green due to chlorophyll synthesis and developed into a shoot-bud. Nitsch *et al.*(1967) as well as Rao *et al.*(1973) reported that BAP with adenine promoted adventitious bud formation *in vitro* from internodal segments of *Plumbgo indica* and *Petunia* respectively. In the present studies, excised stem/leaf cultures of *B.diffusa* and *A.aspera* provided a convenient means to

elucidate the mechanism of organ differentiation in response to growth regulators.

Each of the shoot bud was isolated and cultured on NAA containing medium when it produced a plantlet. Thus complete plantlet regeneration from stem calli of *B.diffusa* was obtained. It has been reported that the biosynthetic capabilities of plant cells could be restored upon regeneration of plantlets (Mantell and Smith, 1983).

In case of *A.aspera* stem/leaf callus cultured showed that in absence of 2,4-D in the culture medium, calli turned nodular with the formation of growth centres within them. These growth centres consisted thin walled cells with prominent nuclei compactly arranged (Expt. 25). The nodular callus grown on BAP containing medium turned chlorophyllous due to the synthesis of pigments. These green nodules when cultured in presence of adenine sulphate developed shoot-buds. These shoot-buds on isolation and cultured developed into a plantlets thereby indicating that they have retained the ability of regenerating complete plants from the callus tissues. Dougall (1979) reported that the regenerated plants from callus tissues produced the same spectrum of secondary metabolites, qualitatively and quantitatively, as was produced by the plants from which they were initiated. This means that the ability of alkaloid synthesis was not lost during callus phase but was not expressed.

In the present studies, the screening of regenerated plants from stem calli of *B.diffusa* and *A.aspera* remains to be done due to insufficient amounts of the plant material.

D. REGENERATIVE ABILITY OF EXCISED LEAF SEGMENTS

The sixth endeavour of special emphasis of this research was mainly to find out the regenerative ability of excised leaves of both these plants. In *B.diffusa* L. and *A.aspera* L. when the regenerative potential of excised leaflets was examined it was observed that only roots could be produced in response to the various treatments but no where shoot-buds were developed.

Excised leaf segments in *B.diffusa* from first to fifth leaf in their serial order of development were cultured on basal medium (control) no organogenesis was observed. This clearly indicated the need of growth regulators for initiation of organogenesis in cultured leaf segments. Segments of the third and fourth leaves cultured on BM + IAA (0.5 μM/l) medium regenerated roots within one week culture period (Expt. 17). The highest number of roots, upto 17, were produced from the segments of third leaf at the end of four weeks of experimental period. The results showed that these roots reached to a length of 10 cm. Further increase in the IAA levels to 1 and 2 μM/l of the culture medium not only reduced the number of roots to 9 and 7 but also reduced their length to 5 and 3.5 cm respectively. Higher levels of IAA (4 μM/l) induced callus along with few short roots. Thus it was clearly seen that the quantity of IAA present in the culture media had pronounced effect on the morphology of regenerated roots. It appeared that in *B.diffusa* regeneration of roots from leaf segments cultured *in vitro* was an autonomous process, as observed in *Haplopappus ravnil* (Blakely *et al.*, 1972). However in *Nicotina tobaccum*, from the fourth leaf, *in vitro* rhizogenesis was observed with the application of IBA (Attifield and Evans, 1991).

Leaf segments cultured on BM + NAA failed to regenerate roots/shoots (Expt. 17). However, in presence of 2,4-D (0.5 μM/l) in the medium white callus was produced within one week culture period, which regenerated roots at the end of four weeks. Working with *Convolvulus arvensis* leaf explants, Christianson and Warnick (1984) suggested that organogenesis proceeds through three sequential stages 1) the acquisition of competence to respond to a particular inductive signal 2) induction and 3) morphogenic differentiation and development. They further observed regeneration of roots from above plant leaf explants when medium contain IBA. In presence of IAA shoot buds were induced which was contradicting with the present results of *B.diffusa*.

In the excised leaf - segments from first to fifth leaf from the apex of *Achyranthes aspera* L. when cultured on MS medium with IAA (0.5 μM/l), roots with laterals were regenerated. These roots were about 7 cm in length at end of four weeks culture period (Expt. 27). With increase in the level of IAA of the culture medium (1 or 2 μM/l) there was progressive reduction not only in the number of roots regenerated but also in the length of the regenerated roots. At 4 μM/l of IAA level, only short roots without lateral roots were regenerated from leaf segment.

In response to NAA, there was no morphogenic response (shoots/roots) from excised cultured leaf segments. When auxin used was 2,4-D, only callus was developed from leaf segments and in no case any organogenesis occurred. The variation in morphogenesis pattern in response to different auxins could be as

suggested by Gurel and Wren (1995) that our understanding of the process involved in organogenesis is still incomplete. They further added neither that how meristems are induced de novo nor the factors which regulate their development into roots or shoots is yet unknown. Earlier Boneet and Torrey (1965) have reported that in *Convolvulus arvensis* the young bud and root primordia were histologically indistinguishable and that buds could be induced to develop at presumptive root sites. Histological studies in *B.diffusa* confirmed that the roots were initiated from phloem parenchyma of the veins of leaf segments. Similar results have been reported in *Lycoperiscon esculentum* Mill (Coleman and Greyson, 1976). Vascular parenchyma cells were implicated in the petiole segments of *Brassica juncea* and *Pereskia grandifolia* (Sharma and Bhojwani, 1990) and in *Phaseolus vulgaris*, involving cell divisions in the region between the xylem and phloem leading to the formation of meristematic growth centres from where organogenesis resulted (Gramberg, 1971). It has also been stated that the absence of callus near the developing root primordia was an example of direct organogenesis (Gurel and Wren, 1995). Attfield and Evans (1991) also reported differences in the origin of adventitious roots/shoots in lamina explants of tobacco. Root formation occurred directly from leaf tissues within 24 hours while shoot developed more slowly from the callus produced.

Further, the biosynthetic potential of regenerated roots of *B.diffusa* showed that long roots produced in response to 0.5 μM/l IAA treatment accumulated maximum of 0.15% of punarnavine alkaloid (dry weight basis). However, short roots regenerated in response to higher levels of IAA (1 or 2 μM/l), there was sharp

decline in the quantity of punarnavine accumulated 0.05% and 0.02% respectively by them (Expt. 18). Further decline in the alkaloid contents in the regenerated roots were observed when IAA level was 4 μM/l. Thus, the quantity of IAA present in the culture media had pronounced effect not only on the morphology of regenerated roots but also on their alkaloid - punarnavine profile (Shrivastava and Padhya, 1995).

Leaf segments of *A.aspera* cultured on auxin - free medium turned black within four weeks culture period without regenerating any organs. In response to (0.5 μM/l) IAA, roots regenerated from the leaf segments. The length of the regenerated root was 7 cm, much less when compared to the length of the regenerated root from *B.diffusa* leaflets. At higher levels of IAA (4 μM/l) short roots without laterals were regenerated from excised leaf segments.

Leaf segments in response to NAA treatments, failed to evoke any morphogenetic response and the leaflets ultimately turned black.

Treatment with 2,4-D to excised leaflets showed the callus induction was highest at 2 μM/l level. However, none of the treatment induction of adventitious shoot-bud was observed. In case of *A.aspera* regenerated roots were not screened for the presence of achyranthine since the results of previous experiments (Expt. 20) proved that the roots of this plant were not specialized for the accumulation of achyrantine.