GENERAL DISCUSSION

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This research endeavour has eight directions of special emphasis, First : Screening of *W. somnifera* 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 *W. somnifera* 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' plant of *W. somnifera* by standardising their nutritional/hormonal requirements for maximum biomass production. Fifth : Regenerative potential of the callus tissues. Sixth : Screening of callus cultures for their alkaloid profile. Seventh : Identifying the regenerative ability of the excised leaf/leaf segments. Eighth : To induce haploid plants through pollen culture and diploidisation of these haploids for the production of homozygous plants.

(A) SELECTION OF 'ELITE' PLANTS OF *W. SOMNIFERA*

Alkaloids are widely synthesized in plants which are useful pharmaceutically because of their physiological activities (Misawa, 1982). Plants produce these secondary compounds with an apparent ease, and so they still remain important sources of drugs.

The chloroform extracts of vegetative and fertile plants of *W. somnifera* when subjected to chromatographic analysis gave positive results with Dragendorff's reagent, indicating the presence of alkaloids (Expt.1). The alkaloidal fraction was isolated by running the chromatogram of the extract using chloroform : ethanol (9:1) solvent system. The isolated alkaloid was eluted and co-chromatographed with the authentic sample of tropine (Sigma Chemicals). The authentic sample and the isolated alkaloid fraction gave identical R_f values (0.35) which confirmed that the isolated compounds were of the tropine alkaloid. Thus, it was evident that the vegetative and fertile *W. somnifera* plants synthesized/accumulated tropine alkaloid.

The quantitative estimation of tropine alkaloid, in the vegetative and fertile plants was done according to the method described by Roberts and James (1947).

In vegetative *W. somnifera* plants, the alkaloid content accumulated was 0.10% when calculated on dry weight basis. In fertile plants, the alkaloid content reached to 0.43%. This showed that the alkaloid content varied with the developmental age of the plant. These results are similar with the studies carried out by Cramer and Turner (1967) on the alkaloids of *Baptisia*. In this genus, there was a striking variation in the specific alkaloids produced, in their relative amounts of each, and in the total quantity of alkaloids present at any given time during development. Similar results have been observed by Nowacki (1963) in the genus *Lupinus*. In case of *Hyoscyamus muticus*, maximum tropane alkaloid content was accumulated before flowering (Tyagi *et al.*, 1984). Phillipson and Handa (1975) have shown that various organs *viz.*, roots, stem,

leaves and flowers accumulate / synthesize tropine alkaloid. Wiermann (1978) has, therefore, considered accumulation of secondary metabolites as an aspect of the developmental process.

W. somnifera plants in their vegetative and fertile stages of development from various localities when screened for their alkaloid contents (Exp.2) showed a lot of variation. Highest quantity of the tropine alkaloid accumulated in the vegetative (0.22%) and fertile plants (0.55%) collected from Ellora Park area. Vegetative and fertile plants collected from Karelibag area showed lower quantity of alkaloid (0.10, 0.35%). In Chhani and Harni plant samples, lowest quantities (0.05, 0.03%; 0.10, 0.08%) of alkaloid were present. Similarly, in *Hyoscyamus muticus* strains native to India produced less tropane alkaloid than those from Egypt (Husain *et al.*, 1979). This variation in the quantities of alkaloids observed in the same age group of plants but growing in different localities, possibly might be due to the environmental factors, which agrees with Adams view (1979). According to him, environmental factors play a key role in the synthesis/accumulation of alkaloids. Matveyer (1959) while, investigating different varieties of poppies in different agriculture experiment stations throughout the Soviet Union, found the morphine level to fluctuate between 0.1 to 1%. Waller and Nowacki (1978) have shown that environmental factors such as the type of soil, light intensity, availability of nutrients etc. influences alkaloid production. Based on the highest quantity of alkaloid content, *W. somnifera* plants growing in Ellora Park area were selected as 'elite' plants.

Results of Expt.3 showed that the individual organs of the 'elite' plant when screened for the alkaloid content, accumulation of tropine content was highest in the root (0.25%). In stem (0.10%), leaves (0.13%) and fruits (0.08%) lower quantities of alkaloids were accumulated. This proved that the roots might be the site of synthesis/accumulation of tropine alkaloid. These results find support with the findings of Endo and Yamada (1985) for *Duboisia hopwoodii* where the roots accumulated highest amount of of hyoscyamine (0.56%) followed by leaves (0.50%). Roots of young poppy plants synthesized morphine alkaloid which was then transported to leaves and ultimately accumulated in the fruits (Fairbrain and Wössel, 1964). Mothes and Engelbrecht (1956b) have confirmed that the alkaloids in *Nicotiana* are rapidly transported from the roots to the shoots and leaves. The tropane/pyridine alkaloids are synthesized predominantly in roots and converted to other compounds or degraded in aerial parts of the plants. Earlier studies regarding tropane alkaloid indicated roots to be the site of biosynthesis of solanaceous plants (Waller and Nowacki, 1978).

There are several reports that within the plant body there is variation in the constitution and in the composition of secondary products; also individual organs exhibit different alkaloid profiles possibly due to the difference in their biosynthetic potential (Wiermann, 1978).

As roots were the site of synthesis/accumulation of alkaloids in *W. somnifera*, roots of fertile plants from various localities were analysed (Expt.4). Ellora Park area roots samples accumulated maximum tropine alkaloid contents (0.25%) on dry weight basis. Next were the root samples collected from MS

University Campus plants (0.20%), and Karelibag plants (0.10%). Samples collected from Harni area plants showed lowest tropine contents (0.03%). Out of the five samples of roots, Ellora Park and M S University Campus plants possessed stout, woody and hard roots when compared with thin root samples of the same age collected from Karelibag, Chhani and Harni. It has been pointed by Wiermann (1978) that the morphology of an organ from which alkaloids are extracted accounts for their accumulation. The capacity of synthesis and accumulation of secondary compounds have been shown to vary greatly within an organ or tissue.

(B) EXCISED ROOT CULTURES

Root tips of aseptically germinated seeds from 'elite' plants of *W. somnifera* were used as explants for establishment of *in vitro* root culture.

In the present studies, out of the two known media (40 ml) *viz.*, White's (1954) and Murashige and Skoog's (1962) when tested for the growth of excised roots, in White's medium the excised roots with tips grew upto 2.9 cm in length without the production of lateral roots, whereas in the MS medium, the main root axis reached to 1.5 cm and also supported the production of lateral root. Dodds and Roberts (1985) have reported the use of liquid medium over the solid medium being ideal for excised root culture in general. Cultured roots when maintained on gyratory shaker, received aeration due to agitation and favoured active growth of the roots. Similar reports have been pointed out in tomato and *Rauwolfia serpentina* (Said and Murashige, 1979; Benjamin *et al.*, 1994).

The nutrients present in the medium support the growth and development of cultured roots. In the present study, roots were cultured in M S medium where the salt concentrations were reduced to one half strength which proved superior over standard dose of salts, as the length of main axis reached to 3.1 cm with the production of 3.3 lateral roots. Even doubling the concentration of salts of original medium failed to enhance the growth of excised roots in terms of main axis and in the production of lateral roots, proving that it was not beneficial. This confirms that the macro and microsalts, and vitamins present in MS medium in their half strength supported optimal growth of cultured roots, which is ~~supported~~ corroborated by the work of Friesen and his coworkers (1992) on the continuous culture of excised roots of *Nicotiana alata*. Butcher and Street (1964) observed that root clones ~~have~~ exhibit rapid growth rate ~~and~~ in a medium with suitable salt concentrations, and the clones are easily multiplied. For the continuous cultures of excised root of *W. somnifera*, actively growing root tips were excised and subcultured regularly in fresh medium at four weeks intervals.

Since the roots are excised and cultured, their hormonal requirements which are otherwise met by the intact plants, needs to be supplemented by exogenous feeding of phytohormones. In order to enhance the growth of excised roots, M S medium in its half strength was supplemented with sucrose (2%), cytokinin *viz.*, Kn/BAP and auxins *viz.*, IAA/IBA/NAA/ 2,4-D at various concentrations (Expt.7). When Kn or BAP were incorporated in the culture medium, there was no improvement in the growth of the cultured roots in terms of length of main axis or in the production of laterals. Similar

results were reported in *Rauwolfia serpentina*, where addition of kinetin failed to improve the growth of cultured roots (Mitra, 1968). In *Hyoscyamus niger* and *H. albus* too neither Kn or BAP showed any significant results in terms of growth (Hashimoto *et al.*, 1986). Dougall (1980) has explained that plant tissues when excised and placed in culture medium require an exogenous supply of phytohormones for their growth, But if the cells placed in culture are actively synthesizing sufficent auxin or cytokinin, then there is no need for that particular hormone to be provided in the culture medium. Thus, this may be the possible reason ~~that~~ the excised roots in culture of *W. somnifera* do not require an exogenous supply of cytokinins.

It was noted that ~~with~~ the incorporation of IAA at 0.5 μM/l in the medium supported growth of main axis, which ~~reached~~ grew to 2.5 cm with the production of 7.3 lateral roots. The length of the main axis improved to 3.1 cm and the number of laterals produced ~~reached~~ increased to 12.6 when IAA level was 2 μM/l. At the same concentration, highest biomass values of 12.4 mg and 0.37 mg in terms of fresh and dry weights were recorded. These growth parameters being highest, 2 μM/l of IAA was accepted as the optimal level for the development of excised roots. With the increase in IAA to 4 μM/l, there was a decline in all the growth parameters. Ball (1965) ~~have~~ observed similar effects of IAA, on excised tomato root cultures, Whereas in *Nigella sativa*, IAA at 0.5 mg/l was found to be most effective in promoting root growth (Bhattacharya and Gupta, 1987).

Incorporation of IBA in the medium improved the linear growth of main axis along with the production of lateral roots. At 2 μM/l, the length

of main axis ~~reached to~~ was 2.5 cm with the production of 15 lateral roots. At the same time, the biomass values recorded were 14.8 mg and 0.44 mg in terms of fresh and dry weights respectively. Further, at 4 µM/l and 6 µM/l level of IBA, the growth parameters showed a clear-cut decline, ~~T~~thereby showing that 2 µM/l of IBA was optimal level required to support maximum growth. Similar results have been reported for *Hyoscyamus niger*, where IBA improved the number of lateral root primordia, but ~~however~~ there was a decline in the length of main axis (Hashimoto *et al.*, 1986). Higher concentrations of IBA were found to be inhibitory for the growth of cultured roots of *Rauwolfia serpentina* (Mitra, 1968).

When NAA was tested, it neither improved the growth of main axis nor the production of laterals. Similary, in *Rauwolfia serpentina* NAA inhibited the growth of main axis and lateral root production (Mitra, 1975). However, NAA was found to be ideal for the growth of excised roots of tobacco (Butcher and Street, 1960).

Incorporating 2,4-D at various levels in the culture medium induced callus at the root tips. Similar results have been observed by Kshirsagar and Mehta (1979) in excised roots in culture of *Pteris vitata*.

With this experimental data, whether the effect of IAA in combination with IBA ~~whether had~~ was synergestic ~~effect~~ on the growth of excised roots in cultures was evaluated (Expt.8). ~~With~~ The incorporation of IAA and IBA from 0.5 to 2 µM/l in the culture medium showed a linear increase in the length of main root axis upto 4.1 cm with 30 lateral roots ~~production.~~ Increase in the number of laterals was twice as compared with any of the individual auxin as studied as seen ~~in~~ from the

results of Expt.7. ~~As well as~~ Also, the length of laterals was three times as compared with that of the main axis. This phenomenon has also been observed with excised roots of tomato (Dawson and Street, 1959). The meristems of the main root axis ceased activity as compared with recently initated lateral meristem which grew actively thus accounting for the increased length of laterals (Street and Roberts, 1952). Further, increase in the concentrations of IAA and IBA showed a decline in both the growth parameters. Street and Winter (1961) after reviewing the physiological studies with respect to the hormonal control of excised roots, have postulated that IAA was not the only auxin controlling root growth. For the maintenance and promotion of growth of excised roots in *Rauwolfia serpentina* IBA was required, which enhanced the linear growth and formation of laterals in each passage (Mitra, 1968). In the same plant, NAA and IBA in combination was found to be superior for growth in terms of length of main axis as well as number of lateral root production. George and Sherrington (1984) have also reported that auxin promoted the growth of roots by inducing secretion of H^+ ions into the medium which affects RNA metabolism of plant cells.

The growth of cultured roots in presence of various carbohydrates *viz.*, glucose/sucrose/mannitol was studied (Expt.9). In presence of glucose, the biomass production in terms of fresh and dry weights recorded were 838 ± 9 mg and 35 ± 2 mg, while in sucrose containing medium the excised roots recorded fresh and dry weights ~~as~~ of 2018 ± 20 mg and 95 ± 3 mg, ~~T~~thereby indicating that sucrose improved the rate of growth of cultured roots. ~~But~~ However, incorporating mannitol in the medium it reduced the fresh and dry weights to

276 ± 3 mg and 15 ± 1 mg respectively as mannitol is osmotically inactive. Hence, sucrose was found to be superior as a source of carbohydrate in the present study on *W. somnifera* root cultures. White has proved since 1934, that sucrose is one of the best carbohydrate for continuous cultures of excised tomato roots, which was further strengthed by Dormer and Street in 1949. In *Duboisia leichhardtii* also, sucrose proved to be the most ideal carbohydrate tested amongst glucose, fructose, mannose, maltose, xylose and rhamnose (Kitamura *et al.*, 1991). Further, the optimal concentration of sucrose required for the growth of excised roots when studied (Expt.10) showed that sucrose at 2% was found to be ideal, recording highest biomass values (2012 ± 20 mg fresh weight and 95 ± 7 mg dry weight). In *Cephalotaxus harringtonia* similar results were recorded where 2% sucrose supported the biomass production in terms of fresh and dry weights (Wickremesinhe and Arteca, 1993).

The rate of growth of excised roots in the root culture medium when studied at weekly intervals during four weeks period showed that there was a linear increase in the biomass in the first two weeks, thereafter a sharp increase in the biomass values occurred at the end of three weeks (Expt.11). Maximum biomass production of 2100 ± 20 mg and 102 ± 3 mg in terms of fresh and dry weights were recorded at the end of fourth week. These values reached to 2200 ± 15 mg and 108 ± 5 mg at the end of eight weeks. The graph of the growth parameters plotted against time showed a typical sigmoid curve. Simultaneously, the tropine contents were quantified and only its presence was detected in the third week. In the fourth week, there was a slight enhancement of tropine content to 0.001% which improved to 0.002% at the end of eight

weeks. The results collaborates with those of the cultured roots of *Datura stramonium* where the highest alkaloid content was observed in the stationary phase (Robins *et al.*, 1991). In tobacco root cultures, anabasine production accelerated with the culture period (Solt *et al.*, 1960). Thus, it became evident that the accumulation of alkaloids in cultured roots was not uniform during the culture period (Payne *et al.*, 1987; Rhodes *et al.*, 1989). Therefore, cultural conditions favouring rapid growth are seldom those that are best for the biosynthesis of secondary metabolites (Luckner, 1980).

Amino acids being the precursors of several alkaloids, their effect on the synthesis of the alkaloids when studied showed that, it brings about enhancement in the yield of the product (Hay *et al.*, 1986). The precursors to tropine are, ornithine, acetoacetate and methionine. As L-ornithine was readily available, it was used as a precursor of alkaloid tropine (Expt.12). It was observed that with the linear increase in the concentrations of L-ornithine from 5 to 20 μM/l level in the culture medium, there was a corresponding increase in the tropine content of roots. Highest tropine content (0.15%) was observed at 20 μM/l level of L-ornithine. Further, increase in L-ornithine level to 25 μM/l level declined the tropine content to 0.02%. Feeding of precursor L-tryptophan to the cultured roots of *Cinchona* improved the alkaloid content in it (Hay *et al.*, 1986). In *Nicotiana rustica*, nicotinic acid as the other precursor for nicotine formation enhanced the product yield (Robins *et al.*, 1987).

(C) CALLUS CULTURES AND PLANTLET REGENERATION

In Expt. 13 individual organs *viz.*, hypocotyl / stem / leaf were used as explants for the initiation of callus in presence of cytokinins Kn/BAP

alone or in combination with auxins IAA/NAA/2,4-D. Kn or BAP alone in the medium failed to initiate callus from any of the explants and they only remained green for a short period in high doses of Kn. Addition of IAA to Kn containing media at all levels also failed to induce callus from any of the explants. In presence of Kn and 2,4-D at 1 μM/l each, initiated yellowish callus from the cut ends of hypocotyl within a week which turned brown with the passage of time due to the depletion in the nutrition levels. Further increase in either Kn or 2,4-D levels failed to initiate callus earlier. Thus, Kn and 2,4-D each at 1 μ M/l level were optimal for initiation of callus from hypocotyl.

The leaf segments showed maximum callus induction when Kn was at 2 μM/l and 2,4-D at 6 μM/l. The callus was initiated from the midrib and proliferated all over the explants within four weeks of culture period.

Stem explants treated with Kn in combination with NAA/2,4-D at all the levels tested failed to induce callus within four weeks.

In 1966 Gautheret had reported that the initiation of proliferating callus cultures from explants involved profound changes in the developmental state, new cell types originated and quiescent cells acquired for division. The variation in the requirement of auxins and cytokinins by the explants could be explained on the basis that the callus being is heterogeneous in its cell population consisting of different cell types. Thus, with such heterogeneity of cell types in cultured explants, it could be expected that few cells would respond differently to the developmental stimuli as well as to their requirements for exogenous supply of phytohormones (Lindsey and Yeoman, 1985). The

variability found in the growth response of cultured cells of *Nicotiana tabacum* pith explants could be related to differences in their endogeneous levels of hormones (Snijman *et al.*, 1977).

For conducting experimental work on callus tissues, it was essential to have sufficient biomass. The highest biomass values recorded for hypocotyl callus were 4420 ± 50 mg fresh weight and 221 ± 18 mg dry weight which required Kn at 1 µM/l in combination with 6 µM/l of 2,4-D, whereas leaf callus recorded 4788 ± 47 mg fresh weight and 240 ± 21 mg dry weight at Kn 2 µM/l in combination with 6 µM/l of 2,4-D (Expt.14).Gusev in 1973 has pointed out that the type of explant from which the callus is originated influences growth of its biomass. In *Datura innoxia* the callus cultures of root, leaf, petals and ovaries showed optimal growth on MS medium supplemented with Kn and 2,4-D (Potoczki *et al.*, 1982). Hence, the optimisation of the culture medium is required, as the secondary metabolites are dramatically altered by minor changes in the medium (Whitaker and Hashimoto, 1985).

Cells in culture are not autotrophic and require exogenous supply of carbohydrates (Scabrook, 1980). Out of the various carbohydrates tested *viz.*, glucose/sucrose/mannitol, sucrose at 2% was found to be ideal for hypocotyl and leaf callus tissues. The biomass values recorded were 4500 ± 47 mg and 4770 ± 45 mg fresh weights; 225 ± 8 mg and 240 ± 10 mg dry weights respectively as evident from results of Expt. 15 and 16. Thus, sucrose at 2% proved to be optimal for the growth of callus tissues, whereas in *D. innoxia* sucrose at 3% proved to be optimal for growth (Hiraoka and Tabata, 1974).

The growth of hypocotyl/leaf callus tissues on standard medium followed a normal pattern of growth during four week culture period (Expt.17). The tropine contents of the leaf callus tissues were 0.005%, whereas just their presence was detected in hypocotyl callus at the end of four weeks. The tropine contents of hypocotyl/leaf callus tissues increased to 0.001% and 0.018% at the end of eight weeks. Similarly, in *Datura innoxia*, leaf callus cultures accumulated 0.077% of alkaloid upto four weeks which enhanced to 0.096% with the passage of time from four to eight weeks (Szoke *et al.*, 1984). The alkaloid content of leaf callus cultures when compared with that of intact leaf showed tropine content to be nearly ten times less than it. Similar results have been quoted for intact leaves of *Datura innoxia* and leaf callus cultures (Verzar-Petri *et al.*, 1978). In the same plant Verzar-Petri and Kiet (1977) reported tropine to be in great quantity out of the eight alkaloids determined in leaf callus cultures. Thus, it was evident that the hypocotyl and leaf callus of *W. somnifera* have retained their biosynthetic potential. Callus cultures of various organs of *Datura innoxia* also showed variation in their alkaloid content (Szoke *et al.*, 1984). Dougall (1979) has reported that callus cultures established from various organs of a single plant differ in their yields of secondary metabolites which agrees with the results of the present study. Seabrook (1980), has suggested that it was advisable to compare the culture of various organs and tissues systematically for each plant before selecting a given organ or its callus cultures. It is obvious that secondary metabolites produced at any stage of culture was the result of a dynamic balance between their biosynthesis and biodegradation (Mantell *et al.*, 1983).

not listed in Ref. p.124

Further, experimental work was conducted on the suspension cultures of leaf callus (Expt. 18). The growth pattern of suspension cultures also followed a typical sigmoid curve. These cultures could accumulate maximum of 0.02% tropine contents at the end of three weeks. Suspension cultures of various genera accumulated alkaloids such as ajmalicine and serpentine in *Catharanthus roseus*, disogenin in *Dioscorea deltoidea*, and nicotine in *Nicotiana tabacum* (Fowler, 1986). Optimisation of cultural conditions, both internal and external environment, is a prerequisite for good alkaloid production (Mantell and Smith, 1983). More work needs to be carried out in case of *W. somnifera*.

Supplementing the culture medium with one of the biosynthetic presursors have been shown to increase alkaloid yield (Mantell and Smith, 1983). Misawa (1985) too has reported that the addition of appropriate precursor to the culture medium stimulates production potential of cultured cells. In the present studies (Expt. 19), out of the several precursor for tropane alkaloid, L-ornithine which is specific to tropine alkaloid was fed to suspension cultures. There was a linear increase in the tropine content with concomitant increase in L-ornithine, maximum being at 20 μ M/l, the tropine content increased to 0.030%. There was no further significant increase in the tropine content, the reason could be that in suspension cultures, cells being freely suspended, might get washed out of the intracellular precursors into the medium, which reduces the possibility of their availability to the cultured cells (Lindsey, 1986). The biosynthetic activity of the cells in suspension varies at a given time due to the variation of the cell age constituting the suspension culture (Dougall, 1987).

In Expt. 20 high yielding cell-lines were developed by cell plating method (Bergmann, 1977). Cell suspension cultures after plating on standard media developed cell colonies and their tropine content was 0.025%. The suspension of these cell colonies when replated showed that the tropine content increased to 0.035%. Ajmalicine and serpentine in *Catharanthus roseus* (Zenk *et al.*, 1977); solasodine in *Solanum laciniatum* (Zenk, 1978), and nicotine in *Nicotiana tabacum* (Ogino *et al.*, 1978) all of them have been isolated as high yielding cell lines. Cloning procedures facilitates the isolation and reisolation of high yielding cell-lines with respect to their biosynthetic activity (Dix, 1986). In *W. somnifera* high yielding cell lines were established.

The differentiation of leaf callus into roots occurred in presence of IAA (0.1 - 0.4 μM/l). But in presence of 2,4-D at lower levels the callus turned nodular (Expt.21). Histological observations of each nodule revealed meristematic growth centres consisting of embryogenic cells with a prominent nucleus. These meristematic centres are responsible for the regeneration into roots/shoots in presence of phytohormones (Ross *et al.*, 1973, Torrey, 1966). When supplied with Kn and BAP together, these nodules developed into shoot buds. Identical observations were noticed in *Vicoa indica* (Thulaseedharan and Vaidyanathan, 1990). The shoot buds were developed on fresh medium. In *Duboisia myoporoides* also the regeneration of plantlets from shoot buds has been reported when cultured on fresh medium (Kitamura *et al.*, 1980a). Yoshikawa and Furuya (1985) regenerated plantlets from callus meristemoids of *Papaver somniferum*. Rooting of shoot buds occurred onto medium with reduced dose of sucrose (1%) supplemented with IBA (1 μM/l). In case of

Cicer arietinum IBA was responsible for development of plantlets (Barna and Wakhlu, 1994).

The excised leaf segments of fourth leaf of *W. somnifera* regenerated into roots when cultured in presence of IAA/IBA (Expt.22). Highest number of 15-20 roots regenerated in presence of IBA (1 μM/l). Christianson and Warnick (1984) have reported the formation of roots in leaf explants of *Convolvolus arvensis* when cultured on medium supplemented with IBA. *In Vitro* rhizogenesis was also observed in *Nicotiana tabacum* and *Vigna unguiculata* with the application of IBA (Attfield and Evans, 1991; Ladeinde and Soh, 1991). Further, the excised leaves also showed the capacity to differentiate into adventious shoot buds when cultured in presence of Kn and BAP at 4 μM/l. Similar results have been observed in leaf explants of *Crataeva nurvala* (Sharma and Padhya, 1989). Each shoot was separable and further it grew into plantlet.

In has been stated by Christianson and Warnick (1988) that the control over the type of organ is governed by the balance of the phytohormones. The induction phase in organogenesis is a developmental process that necessarily precedes morphological differentiation (Waddington, 1966).

(D) PRODUCTION OF ANDROGENIC HAPLOIDS

Flower buds from young plants in their first flowering were used for the induction of haploids. The anthers excised from the third floral bud in serial order of development mostly with uninucleate pollens showed maximum response on MS medium. Sunderland (1971) too pointed out that the anthers from

young plants are most responsive. Dunwell and Perry (1973) have reported that the older plants produce small buds containing heterogenous mixture of microspores and young pollens which delayed and reduced androgenesis. Anthers with binucleate and late binucleate pollens from the fourth and fifth floral bud gave a poor response (Expt.23). This shows that the age of pollens bears a lot of significance in anther culture, which has been discussed by Sunderland (1974). The developmental stage of microspores was responsible for the induction of haploids which varies from species to species.

Addition of coconut milk to the MS medium influenced the morphogenic response in *W. somnifera* anthers/pollens (Expt. 24). CM at 15% improved embryogenesis. Haploids produced in *Solanum grandiflorum* and *Datura metel* were also due to the presence of CM in the medium (Jaiswal *et al.*, 1980; Babbar and Gupta, 1990). While at lower levels of CM (5-10%) callusing resulted in the anthers, as was reported in *Solanum grandiflorum*.

For diplodisation of the haploid plant, various concentrations of colchicine with different time period were tried (Expt. 25). Colchicine treatment at 0.5% diplodised the plants as the 2n = 24 and thus the plantlets produced were haploids. Sunderland (1970) and Kasperbauer and Collins (1972) have also reported similar results in case of *Nicotiana tabacum*.

The discussion in the present chapter showed that the objectives of the present work were achieved.