CHAPTER 12

DISCUSSION
CALLUS CULTURES

In this study on Hibiscus it has been observed that callus formation could be initiated from all the vegetative explants of H. cannabinus var. 'HC 583' and 'Bangalore Local', and H. sabdariffa var. 'sabdariffa'. But in H. rosa-sinensis, with the exception of cell proliferation at the cut ends of tender stem explants, there was no callusing in the different explants used.

All the vegetative explants required some growth regulator or combinations of growth regulators to form callus. On the basal medium there was hardly any cell proliferation. Coconut milk was found to be very effective in callus initiation and brought about prolific growth of callus from stem, leaf, petiole and hypocotyl explants of H. cannabinus and H. sabdariffa. van Overbeek et al. (1941) were the first to demonstrate that CM which normally nourished the developing coconut embryo, could provide factors which encouraged small heart-stage embryos of Datura to grow and mature in vitro. CM was used as a supplement to the otherwise standard culture medium. Chaplin and Steward (1948) demonstrated that CM had a pronounced effect on the growth of differentiated cells isolated from carrot roots. Morel (1950) successfully initiated tissue cultures of monocotyledons using CM. Chaplin and Steward (1952) have shown that CM has a growth-promoting effect on carrot tissues. Steward (1958) has shown
that coconut has nutrients that make for active growth by cell division, but that the progress towards organised growth requires the growing system to acquire a measure of independence from the coconut milk stimuli which, if unregulated, leads to unorganised growth. Chemical analysis of CM has shown that it contains a non-specific fraction of reduced N\textsubscript{2} compounds, particularly amino acids and their amides. One third of the total N\textsubscript{2} is in the form of Biotin, Riboflavin, Folic acid, Thiamine and Pyrodoxine. Sugars like sucrose, glucose, fructose and mannitol, and growth activating substances like auxins, gibberellins and cytokinins are present. Besides it also contains sorbitol, myoinositol, leucoanthocyanin, 1-3-diphenyl urea enzymes like acid phosphatases, diastases RNA polymerases, dehydrogenases, peroxidases and catalases. The presence of all these compounds make CM a useful ingredient in tissue culture media and has become a much used additive. However, CM has not been active in all cases and has been found to be either inhibitory (Hall, 1943) or of no benefit in certain cell cultures (Smith, 1944; Haagen-Smit et al., 1945; Ziebur and Brink, 1951). Similar results were seen in the present study when vegetative explants of the different cultivars of H. rosa-sinensis did not respond to CM.

The combination of an auxin with CM enhanced the process of callus initiation and growth. The combination of
2,4-D and CM produced excellent callus growth. Such an effect of the combination of 2,4-D and CM was reported by Steward and Chaplin (1951). They observed indefinite growth in potato tubers cultured on a medium which contained CM plus a synergistic combination of 2,4-D or some other substance. Gayathri (1975) has reported the necessity of CM for the induction of callus in explants of Codeum variegatum. Thulajappa (1982) found that there was an increase in callus formation in stem, leaf and excised flower buds of Hibiscus 'Benazeer' and Malvaviscus arboreus, when CM and an auxin were present in the medium. When IAA or NAA was used in combination with CM there was increased callus formation. But these combinations could not produce callus from hypocotyl explants of H. sabdariffa. If IAA was present there was root formation in all the explants. IAA though linked to almost all known physiological process (Thimann, 1977), its involvement in morphogenetic processes such as root formation has been demonstrated in a variety of species (Thorpe, 1978). Most workers have obtained callus from various explants of different members of Malvaceae without the use of CM (Nataraja and Patil, 1980; 1984; Blackmon et al., 1981; Trolinder and Goodin, 1987; Finer, 1988; Zimmerman and Robacker 1988). This is because CM induces excessive callus proliferation without any morphogenetic response. From the present investigation it was clear that although CM enhances
the process of callusing, it is not necessary for callus induction and growth.

In this study, cell proliferation was seen from the cut ends of tender stem explants of *Hibiscus 'Benazeer'* in the presence of CM and 2,4-D or IAA. There was no proper callus formation on the same or other media combinations. This is in contrast to the findings of Thulajappa (1982) who reported callus formation from stem and leaf explants of this cultivar. This differential response of the same plant cultivar may be attributed to the genetic variations that are commonly seen in the *Hibiscus rosa-sinensis* complex to which *Hibiscus 'Benazeer'* belongs. The growth of cultured tissue or organs are probably influenced by the genotype than by any other factor. Although seldom thought of as being influenced by genotype, explant survival can vary significantly between closely related plants and also among members of the same variety. Hammerschlag (1982) found a big difference in the survival of shoot tips derived from different peach cultivars. There are many reports of how the ability of explanted tissues to form callus and the subsequent growth rate of callus cultivars can be genotype-dependent. Gresshoff and Doy (1972) have shown that callus cultivars could be established from only three out of the eighteen *Arabidopsis thaliana* genotypes and one out of the forty three barley lines. The genes governing the manner in
which tissues or organs grow were not evolved for this purpose and their influence on tissue cultures is only a secondary effect of their normal function. Such genes have been thought most likely to exert their influence by regulating the effective levels of growth substances (Bayliss and Dunn, 1979). Endogenous growth substance levels may vary in different genotypes.

The combination of an auxin and cytokinin brought about good growth of callus in stem, petiole and leaf explants, but not in hypocotyl, of H. cannabinus. The combination of IAA (1mg/l) and Kin (1mg/l) produced excellent callus which became nodular in appearance and produced 'pro-embryo' like structures. Since the elegant demonstration by Skoog and Miller (1957) that the relative ratio of cytokinin (kinetin) to auxin (IAA) determined the nature of organogenesis in tobacco pith tissue, quantitative changes have been found to be decisive in a number of other plant tissue systems. Rani and Bhojwani (1977) observed that the best medium for the growth of cotton callus was MS supplemented with an auxin, a cytokinin and adenine. Price et al. (1977), Smith et al. (1977) and, Price and Smith (1979) showed that MS medium supplemented with IAA and Kin was the best to initiate callus from hypocotyl explants of cotton, while a combination of NAA and BAP or 2iP was necessary for vigorous growth of the sub-cultured callus. Friable callus was obtained from
different explants of *H. acetosella* (Reynolds et al., 1980) and from hypocotyl of *Abelmoschus esculentus* (Blackmon et al., 1981) using a combination of 2,4-D and adenine, and 2,4-D and 2iP respectively. Thulajappa (1982) observed that the growth of calli derived from leaf explants of *Hibiscus 'Benazeer'* and *Malvaviscus arboreus* was directly proportional to the auxin-kinetin concentrations. The leaf and flower bud derived callus of *Sida cordifolia* also behaved in a similar way. Davidomis and Hamilton (1983) obtained slow-growing calli from cotyledons of *Gossypium hirsutum* on LS medium with NAA and Kinetin. Reynolds and Blackmon (1983) obtained a preembryoidal callus (PEC) on a medium with 2,4-D and Kinetin. Lui et al. (1986) could induce callus formation in all seedling parts cultured on MS medium with IAA and Zeatin. Shoemaker et al. (1986) used the combination of NAA and Kinetin to obtain callus from hypocotyl segments of *G. hirsutum*, while Trolinder and Goodin (1987) observed that a combination of 2,4-D and Kinetin enhanced the embryogenic potential of the callus. For mature tissue, like stem, a combination of NAA and Kinetin, and for leaf tissue a combination of 2,4-D or NAA and 2iP was found to be very effective. Zimmerman and Robacker (1988) showed that the best callus production occurred on LS medium supplemented with 2,4-D and BAP, while Dias et al. (1989) obtained callus with intense root production from hypocotyl and cotyledons of
H. cannabinus cultivar 'Guatemala 45' on MS medium supplemented with NAA and kinetin. In the present study, NAA (0.5mg/l) and kinetin (3mg/l) produced excellent callus from stem, leaf and petiole explants but not from hypocotyl explants of H. cannabinus and H. sabdariffa. Auxin-cytokinin combination could produce very little callus in stem and petiole explants of H. sabdariffa showing that this plant requires very precise conditions for the initiation and growth of callus. Although callus has been obtained from hypocotyl explants of Gossypium sp., Abelmoschus esculentus, Hibiscus acetosella and H. cannabinus by different investigators, there was no callusing seen from hypocotyl segments using auxin-cytokinin combination in this study. Roni Aloni (1980) did not report any observations in cultured first internode explants of seedlings of H. cannabinus. This may be due to the fact that young or immature parts like hypocotyl do not contain meristems and so do not respond to cultural conditions. But since callus formation is seen in the presence of CM and 2,4-D it appears that precise stimuli is required for callus formation from hypocotyl segments.

Cytokinins - Kin, 2iP and BAP, by themselves also induced callus formation in explants other than hypocotyl. Because of its morphogenetic effects in tobacco callus (Skoog and Miller, 1957) kinetin has become a widely used hormone for shoot induction specially in combination with an

The auxin 2,4-D could induce and support growth of callus, but IAA and NAA could not by themselves support growth. In the presence of IAA root formation was seen both from the explants as well as the callus. 2,4-D a potent herbicide is known to induce cell proliferation. Perhaps its most notable use other than as a herbicide, is to cause dedifferentiation and unorganized cell growth or callus. Although 2,4-D is known to induce root formation no such response was observed in the present study with the explants of *H.cannabinus* and *H.sabdariffa*. Thulajappa (1982) observed that in the presence of 2,4-D there was callusing in stem and leaf segments of *Hibiscus 'Benazeer'* while IAA induced both callus and roots in stem and leaf segments of *Malaviscus arboreus* and in leaf segments of *Sida cordifolia*. It is thus seen that auxins can bring about different responses in explants of different plant species.

Starch accumulation was seen in callus cultures obtained on media containing an auxin-cytokinin combination. Accumulation of starch started before any observable organized development and was heaviest in the cells of the *meristemoids* which gave rise to organ primordia. Shoot forming callus is known to accumulate starch (Thorpe and
Murashige, 1968) and this biosynthetic process is thought to be required for bud initiation.

Differentiation of tracheary cells was observed in callus cultures grown on media containing IAA or NAA with 2,4-D or 2iP. The cells of the callus are mainly unspecialized and it is not yet possible to induce them to become of just one differentiated type. This is partly because culture systems are usually designed to promote cell growth: differentiation frequently occurs as cells cease to divide actively and become quiescent. Furthermore, the formation of differentiated cells appear to be correlated with organ development, therefore the prior expression of genes governing organogenesis may often be required. The in vitro environment can also be very different to that in the whole plant where each cell is governed by the restraint and influence of other surrounding cells (George and Sherrington, 1984). Callus cultures are more likely to contain tracheids than any other kind of differentiated cells. The proportion formed depends on the species from which the culture originated and especially upon the kind of sugar and growth regulators added to the medium. The tracheid formation may represent or be associated with an early stage in the development of shoot meristems. Nodules containing xylem elements in callus of *Pelargonium* have been observed to develop into shoots when moved to an auxin-free medium (Chen
The differentiation of vascular tissue in the absence of an organised meristem leads to the conclusion that the control of morphogenetic processes in callus tissue may be different from the control in whole plants, where meristems regulate the continued differentiation of the organs and tissues to which they give rise (Nuti Ronchi, 1981). Clutter (1960) has shown that vascular differentiation takes place when IAA is introduced into cultured stem with sections of *N. tobaccum*. Wetmore and Rier (1963) and Jeffs and Northcote (1966) have shown that an auxin (IAA or NAA) and sucrose are necessary for the induction and complete differentiation of xylem and phloem in callus tissues. Roni Aloni (1980) observed that the presence of IAA had a controlling effect on the differentiation of sieve and tracheary elements in callus of *Syringa, Daucus* and *Glycine*. Although the first internode explants of seedlings of *H. cannabinus* were also cultured, no results were reported. Thulajappa (1982) reported the presence of tracheids in callus derived from stem, leaf- and flower bud explants of *Malvaciscus arboreus*.

In the present study, starch accumulation was seen before the callus because nodular in appearance. Localized regions in the callus started accumulating starch and became dark regions. These regions of starch accumulation did not show any further development. Starch accumulation also oc-
curred, as the callus turned nodular and 'meristemoids' were formed. It is thus clear that starch has a role to play in organogenesis occurring in callus. Tracheary cells likewise start forming as the callus turns nodular indicating that their presence marks the onset of organogenesis in callus.

The nodule-like nest of cells produced in the callus grown on IAA and Kin, and 2,4-D, Kin and BAP, resemble the 'meristemoids' described by Torrey (1966). The thin-walled cells had densely staining cytoplasm and prominent nuclei and were densely filled with starch grains. 'Meristemoids' are the meristem-like aggregation of small, non-polar cells which appear to be non-vacuolate at the light microscope level and from which primordia and ultimately leafy vegetative shoots are formed (Murashige, 1964; Thorpe and Murashige, 1970). A similar situation was described in cultured tobacco callus by Thorpe and Murashige (1968). Gautheret (1935) was the first to report the presence of nodule-like growing centres in callus cultures. Steward et al. (1958) also reported nodule-like growing centres which formed in cultured mass of carrot roots. According to Steward (1958) this mass of cultured cells behaves like a pro-embryo forming both roots and shoots. Torrey (1966) put forth the hypothesis that organogenesis in callus commences with the formation of clusters of meristematic cells or 'meristemoids' capable of responding to factors within the system to
produce a primordium. Depending on the nature of the internal factors, the stimuli can initiate either a root, a shoot or an embryoid. Localized meristematic activity generally precedes the organized development of roots and shoots. Thorpe and Murashige (1970) found zones of preferential division in the basal portion of the stem with tissue of Nicotianum tobaccum L. var. Wisconsin 38, after eight days in culture, which led sequentially to meristemoids, primordia and shoots. During the initial stages of meristemoid formation, protein inclusions and large accumulations of plastid starch were present in the cells, while vacuoles were filled with membranous and cytoplasmic protrusions. At later stages, the cells were smaller in size and possessed small peripheral vacuoles. It is suggestive that the storage material supply the energy and other reserves needed for the organogenetic process. Ross et al. (1973) suggested that the physiological gradients of substances diffusing from the medium into the tissue may play a role in determining the loci at which meristemoids are formed. The meristematic region may also act as a sink and withdraw essential metabolites from the surrounding cells, thus localizing the meristematic zones (Street, 1977). Nuti Ronchi (1981) observed that organized development begins with parenchyma cells which divide and precociously differentiate tracheidal cells forming a growth centre with nodular structures consisting of xylem in the centre and phloem outside. The vascular
tissue is precociously separated from the surrounding callus by a layer of cells which are shown to endodermal by position and histochemical reactions. Further growth leads to the formation of a mound of meristematic tissue which later forms either shoot or root apical meristems.

It is known that callus has no predictable organization pattern, although localized centres of meristematic activity are present and often rudimentary cambial region appears with zones of vascular differentiation (Dodds and Roberts, 1985). Organs are formed in callus tissues from single cells or several cells which divide to give rise to groups of small meristematic cells filled with densely staining cytoplasms and containing large nuclei. These specialized cells or cell groups have been termed 'meristemoids' by many workers (Hicks, 1980). Cells of this kind may need to be formed through de-differentiation unless the callus has arisen from tissues which are already meristematic. This may explain why highly organogenetic cultures are most frequently obtained from young actively dividing parts of plants. Sometimes it appears that 'meristemoids' may have a different origin to other cells of the callus. Callus cultures derived from sections of sweet potato tubers originated meristematic centres of this kind from the cells of anomalous cambia within the tissue of the explant (Hwang et al., 1981). In callus, meristemoids and hence root and shoot
formation, may occur superficially (exogenously) as seen in this study, or within the interior (indigenous). The former situation is the most common and in many cases as in this study, form distinct 'neo-structures' which get detached from the callus mass. They resemble 'proembryo', as suggested by Steward (1958), giving rise to shoots and roots. Pallares (1984) obtained 'neoformations' on the periphery of ovular callus of cotton which resembled somatic embryos. The apical meristem and leaf primordia were perfectly developed and the procambium extended to the root. However, the presence of contiguous meristematic areas does not make it possible to determine precisely whether this formation is a somatic embryo or an adventitious bud. A similar situation exists in the neoformations obtained in callus cultures of H. cannabinus making it difficult to determine if they are somatic embryos or adventitious buds. Thulajappa (1982) reported that meristematic clumps of cells which are destined to produce embryoids or roots are of common occurrence in stem and leaf explants of Hibiscus 'Benazeer'.

It is not known whether meristemoids are formed with the capacity to give rise to one specific type of organ, i.e., do the growth regulators treatment which lead to their formation at the same time determine the type of organ that will be produced or, are meristemoid formation and organ
development separately and sequentially decided. In the present study the former seems to be the case, since different shoot and, root forming 'meristemoids' are formed simultaneously and in the 'neo formations' both types of initials are seen.

DE NOVO SHOOT FORMATION FROM STEM EXPLANTS

Direct shoot formation was obtained when tender stem explants of *H. cannabinus* var. 'HC 583' were cultured on a medium containing 2,4-D, Kin and BAP. Since the stem explants were tender and young they did not callus but produced shoots directly. It is known that organisation can be brought about in callus or explants by the controlled initiation of an organ primordium through manipulation of the nutrient and hormonal constituents in the culture medium. Shoot bud initiation in cultured plant tissues can be induced in many systems by an appropriate balance of exogenous auxin and cytokinin and in some cases either one or the other of these growth regulators must be omitted from the medium in order to produce buds (Street, 1977). Moreover, young stem explants have actively dividing cells (meristems) in them which can bring about active meristematic growth giving rise to organs. Blackmon and Reynolds (1982) obtained shoot regeneration from cut edges of cotyledon and primary leaf explants of *H. acetosella* on NH medium with NOA, 2ip and
CCC, while Reynolds and Blackmon (1983) could get adventitious shoots from leaf and root explants on the same medium.

**GROWTH STUDIES ON CALLUS**

Plant tissue cultures with novel phenotypes are of value in studying metabolic pathways and elucidating the mechanisms of action of various substances. In addition, plants with the desirable phenotype may be regenerated from culture. Biochemical analysis and regeneration procedures require substantial time expenditure. It is therefore desirable to carefully screen potential cell lines and in most cases growth on various media is the chosen means of characterization. The above facts indicate that the method of growth analysis is critical for an accurate evaluation of callus phenotype.

**Quantitative Analysis of Callus**

Plant tissue culture experiments are expressed in qualitative descriptions and also in quantitative measurements. The growth of a culture over a period of time, whether it is a callus, a suspension or an organ culture, is characterized by an increase in cell number, an increase in volume or mass, changes in biochemistry and cellular complexity, and growth of propagules like shoots and embryos. The growth kinetics of each cell line for each treatment must be established. The effect of different auxins, cytokinins and other
growth promoters and their combinations have shown that there are variations with respect to the treatments used.

Among the auxins, 2,4-D was found to be the best in terms of fresh weight of callus, while IAA produced maximum gain in dry weight. This results from the fact that while 2,4-D induces cell proliferation and hence increase in mass, IAA brings about cell elongation and subsequent increase in dry mass. NAA which is an analog of IAA also showed gain in dry weight. It is thus possible to understand the fact that there is only cell proliferation and little differentiation in callus grown on 2,4-D, while IAA or NAA produce differentiation, as seen in this study, specially in combination with cytokinins.

Kinetin is known to induce shoot buds in cultures of many plant pieces eg. Santalum album (Rao and Bapat, 1978), Populus nigra (Venverloo, 1973), Eucalyptus ficifolia (Baker et al., 1977). In this study, Kinetin produced maximum gain in fresh and dry weight of the callus as compared to BAP or 2iP. BAP though a highly effective and intensively used cytokinin, did not bring about much of callus growth. Though its most important role has been in promoting shoot formation and to a good extent in callus formation. In the present investigation, BAP has not been very effective in the initiation and growth of callus 2iP, a naturally occurring cytokinin was also not very effective.
A combination of coconut milk and 2,4-D or Kinetin produced excellent callus growth, with the combination of coconut milk and 2,4-D producing maximum gain in fresh and dry weight. This effect results from the fact that coconut milk contains cytokinins, specially Kinetin. Thus from this study it is clear that the synergistic action of coconut milk and 2,4-D produces copious amounts of callus while auxins IAA and NAA, and cytokinin Kinetin induces all elongation and differentiation. It is therefore suggestive that for callus induction and growth, the combination of CM and 2,4-D is best suited, while for cellular differentiation and morphogenesis, IAA or NAA with Kinetin is effective.

Though the growth rates of callus cultures are frequently expressed on the basis of increases in fresh weight these values should not be used in the estimation of cell numbers and cell division rates (Dodds and Roberts, 1985). Callus is heterogeneous and the callus mass has centres of high division rates and regions of low metabolic activity. It is advantageous to measure fresh weight as a growth parameter because this is a rapid method of following an increase in tissue mass. On the other hand, the measurement of the dry weight of a callus gives an acceptable estimation of the biosynthetic activity of a culture and at fresh weights below 500mg the relationship between fresh and dry weight is approximately linear (Wetter and Constabel, 1982).
One possible difficulty is the accumulation of large amounts of carbohydrate within the cells which tends to complicate the interpretation of data (Yeoman and Macleod, 1977).

CELL SUSPENSION CULTURES

Cell suspension cultures were initiated when friable callus was agitated in liquid medium supplemented with growth regulators. The presence of 2,4-D brought about separation of cells and cellular proliferation in suspension cultures of H. cannabinus. Similar results were obtained in experiments with pea root callus tissue (Torrey and Shigemura, 1957) and in carrot and Convolvulus tissue (Torrey and Reinert, 1961). Continued cultivation of either carrot or Convolvulus cell suspensions in the absence of 2,4-D ended ultimately in the cessation of growth. The presence of 2,4-D in the medium was essential for continued cellular proliferation, but had its most marked effect early in culture on the separation of cells. Of all the auxins or auxin-like plant growth regulators, 2,4-D has proven to be extremely useful being used in majority of the successful embryogenic cultures (Evans et al., 1981). In the present study, 2mg/l concentration of 2,4-D was optimum to produce maximum separation of tissues into free floating cell suspensions.
Price and Smith (1979) obtained suspension cultures from very friable, vigorously growing hypocotyl-derived callus cultures of *Gossypium klotzchianum*. The presence of 2,4-D in the medium resulted in increased vigour of the suspension cultures and was also beneficial though not necessary in the subsequent formation of embryoids. Preculture of the callus on a high cytokinin medium and inclusion of reduced nitrogen in the form of glutamine in the suspension medium was essential for somatic embryogenesis. Finer and Smith (1984) obtained similar results using petiole and stem derived callus of *G.klotzschianum*. Suspension cultures inoculated from callus derived directly from hypocotyls and not exposed to high 2iP medium did not undergo somatic embryogenesis. Finer (1988) obtained suspension cultures of cotyledon derived callus grown on a medium with NAA and Kinetin. Embryogenic lumps were formed in suspension cultures containing 0.1mg/l 2,4-D or 0.5mg/l Picloram. Embryo proliferation occurred when the concentration of 2,4-D was increased to 5mg/l. Further development of embryos occurred on an auxin-free solid basal medium. For embryo development in liquid medium, glutamine (15mM) was necessary. Mature embryos obtained from solid or liquid medium were germinated on a medium without any growth regulators. The presence of an auxin or auxin-like substance is critical for embryo initiation, and the lowering of the auxin concentration or its complete absence fostered maturation (Halperin and
Wetherell, 1964; Halperin 1966; Steward et al., 1967). Similarly, reduced nitrogen is important for both initiation (Halperin and Welterell, 1965; Halperin, 1966) and maturation (Ammirato and Steward, 1971). But this general protocol may not be applicable in all cases. Embryogenesis occurred when hypocotyl derived callus of cotton was grown on MS medium with 2,4-D and kinetin (Trolinder and Goodin, 1987). Embryogenic cell suspension cultures were also initiated when this callus was grown in the same liquid MS medium without any growth regulators. Further development of the embryos occurred on semi solid medium without growth regulators. Lakshmi Sita et al., 1979 found that GA_3 was essential for the growth and maturation of embryos in suspension cultures of Santalum album. In the present study with mature explants of H.cannabinus there was no pre-culture requirement on high cytokinin for somatic embryogenesis, but auxin requirement was a must for embryo initiation. But the absence of development beyond the globular stage, either in liquid or solid medium inspite of the presence of glutamine, as a source of reduced nitrogen may be due to many factors. Various nutrients, growth regulators and environmental factors are involved in the control of plant development from somatic embryos (Finer and Smith, 1984). A correct understanding of the various steps involved is essential before successful plant regeneration can be obtained from somatic embryos.
It is clear from studies on different plant species, including *Gossypium* and *Hibiscus* that each species has its own requirement for somatic embryogenesis to occur. In many cases even genotypic variations for embryogenesis exist (Trolinder and Chen, 1989).
SHOOT TIP AND NODAL CULTURES

SHOOT TIP CULTURES

The growing points of shoots can be cultured in such a way that they continue uninterrupted and organised growth. Following the successful rapid multiplication of orchids by shoot meristem culture (Morel, 1965) there has been an increasing interest in recent years in the application of shoot tip cultures as an alternative means of sexual propagation of economically important plants.

Most of the early workers reported callus from the shoot tips cultured in vitro (Hackett and Anderson, 1967; Walkey and Woolfitt, 1968). Haramaki (1971) described the rapid multiplication of Gloxinia by shoot tip culture and since then several plant species have been successfully micropropagated by this method. The greatest success using this technique has been achieved in herbaceous horticultural species, while woody plants pose some problems (Bonga, 1982; Jones, 1983; Wilkins and Dodds, 1983). Plants derived from shoot tip cultures are generally phenotypically homogenous thereby indicating genetic stability. An important exception, however was reported by Swartz et al. (1981) who found a number of distinct variants among strawberry plants derived from shoot tips.

In the present investigation with H. cannabinus and
**H. sabdariffa**, the response of shoot tip cultures was generally poor. Though new leaves were formed and shoot growth to some extent was achieved in shoot tips from field-grown plants, there was no proper growth of the shoots which remained short and stunted in most cases. When shoot tips from seedlings were used, growth was seen only rarely. In shoot tip cultures of cotton varied results have been obtained. Chappell and Mauney (1967) found that the presence of GA$_3$ in the medium could maintain normal growth of the apical meristems of *Gossypium hirsutum*. The presence of guanine or KNA enhanced GA$_3$ stimulated leaf expansion, while KNA brought about development of additional leaf axillaries. Although coconut milk could maintain green living conditions of meristems, auxins, cytokinins and other growth substances had essentially no effect on meristematic development. Complete shoot regeneration was not reported. Minakshi Sethi (1976) did not obtain growth in short apices of *G. hirsutum* and *G. raimondii* cultured on various media combinations and attributed it to the presence of gossypol in the tissue of the cotton plant. Bajaj and Gill (1986) reported plantlet regeneration from shoot tip and meristem cultures of *G. arboreum, G. hirsutum* and three hybrids of cotton on media supplemented with IAA and Kin. Similarly, Wang and Xi (1986) could also regenerate plantlets from shoot apex of *G. hirsutum* on a medium containing IAA and Kin. Hsi et al. (1987) used high concentrations of 2,4-D and Kin to obtain
plantlet regeneration from shoot tip cultures of *C. hirsutum* and *G. barbadense* via a callus phase.

The ability of explants to survive, multiply and regenerate is a consequence of a wide variety of factors such as the origin of cultures, history of the explants, physiological state of the explants, endogenous hormone concentrations, and general culture conditions like mineral salts, carbohydrates, light and temperature. Because of the variable responses obtained using different growth regulators and their combinations, on shoot tips obtained from seedlings and field grown plants, no particular factor can be pointed out as the reason for the limited success of shoot tip cultures in *H. cannabinus* and *H. sabdariffa*. As pointed out by Minakshi Sethi (1976), there could be some chemical factor present which may inhibit growth and development. But since growth has been achieved under some treatments, this hypothesis may not hold good entirely in the case of *Hibiscus* species studied here. The limited success achieved, nevertheless suggests that sizeable potential gains can be made when the optimal conditions have been identified. One of the major goal in applying shoot tip culture is to produce large numbers of genetically uniform clonal offsprings. In nature superior genotypes cannot be assured due to genetic differences introduced by cross fertilization.
NODAL CULTURES

Nodal cultures are potentially valuable both from the applied and the theoretical points of view. On one hand they afford single-bud starting material for the precise study of shoot multiplication rates in the micropropagation of certain species (George and Sherrington, 1984) on the other, they allow direct testing of the effects of a variety of media constituents on lateral shoot development uncomplicated by apical dominance or other correlative influences (Peterson and Fletcher, 1975 and Scarza et al., 1984).

Nodal cultures of the two varieties of *H. cannabinus* showed very good response in terms of axillary bud growth and development. The combination of CM and IAA produced very good results specially in tender nodal segments. IAA by itself did not produce vigorous growth of the axillary shoot, thus indicating that the synergistic combination of IAA and CM was responsible for the extensive growth of the axillary buds. NAA alone or with CM could not support growth while 2,4-D induced callus formation. Since the young stem apex is an active site for auxin biosynthesis, exogenous auxin is not always needed in the initial growth of the axillary bud. Although exogenous auxins do not promote axillary shoot proliferation, however, culture growth is seen to improve by their presence. Though IAA is considered as the weakest of the three auxins used, it shows maximum diversity
on organ formation as seen in this study. 2,4-D, the most potent auxin is known to stimulate callus formation and strongly antagonize organized development. NAA is one of the most widely used auxin for bud cultures. Its inability to evoke any response in axillary bud growth could be attributed to the antagonistic role that it may play in combination with the endogenous auxins present.

A small quantity of cytokinin may be synthesized by shoots grown in vitro (Koda and Orazawa, 1980), but it is unlikely that the axillary buds have sufficient endogenous cytokinin to support growth and development. Kinetin produced very good growth of axillary shoots and was the most effective cytokinin for the growth and development of axillary buds. 2iP and 4-PU were less potent than Kin, but nevertheless stimulated shoot growth. BAP though considered to be the most effective cytokinin for stimulating axillary shoot proliferation followed by Kin and 2iP (Bhojwani, 1980; Yang et al., 1981), did not support growth of shoots from axillary buds of H. cannabinus. A quite different order of cytokinin effectiveness exists in certain species such as Rhododendron (Anderson, 1975) and mountain laurel (Lloyd and McCown, 1980) where 2iP is more effective than Kin and BAP. A given cytokinin may not work well in certain species, while it may be quite effective in others. 2iP though less frequently used (Nair et al., 1979) gave the highest percent-
age of survival of nodal explants in this study and has been found to be the cytokinin of choice for plants in Ericaceae (McCown, quoted by Hu and Wang, 1983)

Combination of auxins and cytokinins proved to be of synergistic action in the growth and development of shoots in nodal cultures of H. cannabinus. At low concentrations of IAA or NAA and high concentrations of Kin or 2iP good response from axillary buds was seen. Increase in concentration of either the auxin or cytokinin brought about inhibition of shoot growth indicating that at a low concentration of auxin, IAA or NAA, with a high concentration of cytokinin, Kin or 2iP, provided the necessary impetus for axillary bud growth. Conversely, the combination of IAA or NAA and BAP or 4-PU proved to less effective in axillary shoot growth, indicating that this combination did not have a synergistic action on axillary bud growth.

The production of plants from axillary buds has proved to be the most generally applicable and reliable method of in vitro propagation. It is also the simplest method of in vitro propagation as it involves only shoot growth. Thus to establish a complete plant, only elongation and root differentiation are required. In vitro organogenesis and embryogenesis, on the other hand, must undergo developmental changes which usually involve the formation of callus with subsequent reorganisation into plantlets. This has not been
easy to achieve in most plants. The induction of axillary bud proliferation seems to be applicable in many cases like soyabean (Evans, 1981; Kartha et al., 1981), where methods of organogenesis and embryogenesis fail. Smith (unpublished, cited in Price and Smith, 1984) could obtain shoots from cultured axillary buds of mature cotton at a frequency of 20-40%. Although the rate of plantlet multiplication by means of organogenesis and embryogenesis is astonishing, their regeneration capacity usually diminishes rapidly after a number of sub-cultures and eventually this morphogenic potential is completely lost (Kehr and Schaeffer, 1976; Yie and Liaw, 1977). The initial multiplication rate for axillary bud proliferation, on the other hand, is rather slow. The rate, nevertheless, increases during the first few subcultures and eventually reaches a steady plateau during subsequent subculture cycles. The production of millions of plants from a single explant in a single year can be obtained. Moreover, once a stock multiple shoot culture is established, it can continuously serve as the source propagule instead of having to restart from fresh explant cultures.

Axillary bud cultures are preferred over meristem cultures when viral elimination is not part of the objective. The use of larger explants is desirable as they are easier to dissect and have much higher survival and growth rates
than smaller explants. The merit of using axillary bud proliferation from meristem shoot tip or bud cultures as a means of regeneration is that the incipient shoot has already been differentiated in vivo.

Genetic instability is a common feature of plant cells grown in vitro and limits the usefulness of such cultures for clonal propagation or germplasm storage (Hussey, 1978; Skirvin, 1978; Bayliss, 1980). In contrast, most plants produced from nodal cultures appear to be genetically identical to the original parental plants. (Dale, 1975, 1977; Earle and Langhans, 1975; Cheyne and Dale, 1980; Campell, 1982). Since genotypes within a species may differ in genetic stability when grown as cell cultures (Browers and Orton, 1982), it may be that some species, cultivars or genotypes are more variable than others even when propagated by nodal cultures. An understanding of the extent of variation of plants produced from such cultures is essential if the techniques are to be used for reliable clonal propagation of a given species.

The absence of proper and organized growth of axillary buds of *H. sabdariffa* may be due to the recalcitrant nature of the species. Gradual modification through serial sub culturing, of this recalcitrant state, as seen in *Betula platyphylla* (McCown and Amos, 1979) could not be achieved.
It is thus seen from the present investigation that nodal cultures of \textit{H. cannabinus} are amenable to in vitro culture and can be used in the production of shoots which can help in maintaining the genotype and in propagation as well.

**IN VITRO FLOWERING**

In vitro flowering has been obtained in a number of plant species (Caplin and Griesel, 1967; Wardell 1977; Tran Thank Van and Trinh, 1978; Handro 1983; Kerbauy 1984; Tisserat and De Mason, 1985; Dickens and van Staden, 1988).

In this study, in vitro flower formation was obtained at the nodes of shoots produced from nodal explants or directly on the node of the explant itself. The prerequisite for flower formation in vitro was that the explants had to be excised from plants in flower. Most workers have obtained in vitro flowering without such a prerequisite. In Tran Thanh Van's (1981)-elegant system of controlled morphogenesis in thin cell layer explants of tobacco, controlled combination of auxin, cytokinin and pH were used to dissect the regeneration of roots, vegetative buds, floral buds or callus on the explants. Various plant hormones like NAA (Smulders \textit{et al.}, 1988), BAP (Reddy and Narashimhalu, 1985) and combinations of auxins and cytokinins (Handro, 1983; Tisserat and DeMaron, 1985; Cousson and Tran Thank Van,
1981) have been used to induce flowering in different types of explants, like embryonated and deembryonated cotyledons (Reddy and Narashimhalu, 1985), pedicels (Smulders et al., 1988), nodes (Dickens and van Staden, 1988), leaf discs (Handro, 1983), thin cell layers (Cousson and Tran Thanh Van, 1981), embryoids (Chang and Hsing, 1980) and stem (Hillson and La Motte, 1977). But in this study flowers were produced only on the nodal region of the explant or from the nodes of axillary shoots produced in vitro.

Wardell (1977) has shown that there is both quantitative difference in DNA between stems of flowering and vegetative plants. The floral induced stems of flowering tobacco plants contain large amounts of rapidly renaturing DNA whereas noninduced stems of vegetative plants contain only small amounts. The extra DNA in stems of flowering plants seems to represent preferential synthesis of rapidly renaturing DNA. This DNA in buffer solution, when supplied to axillary vegetative buds with their periodic defoliation resulted in a change over of the axillary buds into floral ones. It has also been shown that IAA may modify in vitro bud expression by affecting DNA synthesis. In this study a similar situation may exist where by the explants excised from plants in bloom contain the required DNA for floral expression. But that IAA may affect flower bud formation by affecting DNA synthesis may not hold good since flower bud
formation directly on the nodes was seen in the presence of IAA.

OVULE CULTURE

The culture of ovules has been in use since White (1932) and La Rue (1942) first obtained calli on the ovules of Antirrhinum. Both fertilized and unfertilized ovules of many species have been grown to maturity.

In this study, both unfertilized and fertilized ovules of H. cannabinus, H. sabdariffa and H. rosa-sinensis could not be successfully cultivated. Only in the presence of CM, NAA and Kin, there was slight callus proliferation in 20-day old ovules of H. cannabinus var.'HC 583'. Callus formation was observed by several investigators—Beasley (1971), Hsu and Stewart (1976), Pallares (1984) and Song and Shen (1988), in ovule cultures of cotton. Beasley (1971) reported callus initiation from funicular tissue explants or the micropylar region of cultured ovules on White's medium. Hsu and Stewart (1976) obtained friable callus from the micropylar region in the presence of (2-chloroethyl) Phosphonic acid (CEPA). A combination of CEPA and GA3 further stimulated the process of callus formation. Callus was obtained from the internal integuments of unfertilized ovules of G. hirsutum, G. barbadense, G. arboreum and G. herbaceum on LS medium with NAA and BAP (Song and Shen, 1988).
Minakshi Sethi (1976) obtained both friable and compact callus from ovular integuments of three species of cotton cultured in the presence of IAA and GA₃. Auxin - 2,4-D, and other growth substances like casein hydrolysate, malt extract, yeast extract and zein also evoked good response. The callus thus formed did not show further growth when sub-cultured or transferred on to other media combinations. Suspension cultures of the friable callus could not be initiated. Unlike cotton ovules, those of *Abutilon indicum* did not show any growth and necrosed in all treatments (Minakshi Sethi, 1976). Normal seedlings were obtained from 5 to 10 day old ovules of cotton cultured on MS medium without any growth regulators (Eid et al., 1973). Inter-specific hybrids of cotton, through ovule culture have been successfully produced (Gill and Bajaj, 1984a, 1987; Zhang et al., 1988). Gill and Bajaj (1984a) used a combination of CM, IAA and K⁺ to bring about normal seedling development while Zhang et al. (1988) used IAA and proline, and NAA and glutamine for proliferation and development of embryos. Stewart and Hsu (1977, 1978, 1978) also obtained interspecific hybrids of cotton through *in-ovulo* embryo culture on BTP medium with ammonium ions (NH₄⁺). Normal seedlings were produced from ovules of *Abelmoschus esculentus* (Bajaj, 1964) and inter-specific hybrids of *Abelmoschus* sp. (Gadwal et al., 1968).
Minakshi Sethi (1976) observed that the presence of gossypol was the main reason for the absence of any morphogenetic responses in tissue cultures of cotton. The integument, which produced callus in few media combinations were gossypol free. Gossypol though not inhibitory to callus formation seems to inhibit the morphogenetic potentialities specially in the absence of 2,4-D. But the facts that other workers have been able to obtain both morphogenetic calli (Pallares, 1984) as well as normal seedling from cotton ovules (Eid et al., 1973; Gill and Bajaj, 1984a, 1987) shows that gossypol by itself may not be directly responsible for the inhibition, but may have some influence on morphogenesis. Song and Shen (1988) have shown that no differences existed between callus formed from high and low gossypol containing cotton. Gill and Bajaj (1984a) opine that the proliferation response of hybrid ovules is genotypically oriented.

The variable response of cotton ovules may be as a result of the effect of the genotype, the nutrient factor, and other internal factors. However, this variability in ovule response in vitro can be used to advantage in selection of superior plants for breeding programmes. The absence of good response in the ovules of H. cannabinus, H. sabdariffa and H. rosa-sinensis suggests that the genotypes of the three species are not amenable to in vitro culture under the conditions provided.
EMBRYO CULTURE

In embryo culture, embryos are individually isolated and germinated in vitro to provide new plants.

Lofland (1950) observed that very young embryos (less than 15 days) of cotton did not undergo further development. But Hauney et al. (1967) successfully cultured young embryos (12 to 14 day old) of cotton with upto 75% survival on a medium with CH, CM and Adenine Sulphate. Normal growth of young embryos of cotton was seen only in the presence of 40mg/l myo-inositol in BT medium by Azizkhodzaev and Uinarov (1985). Hybrid plants have been regenerated from in vitro cultured cotton embryos (Weaver, 1958; Gill and Bajaj 1984 a, 1984 b, 1986). Brar and Sandhu (1984) could produce fast growing seedlings through cotton ovule culture.

In present investigations only mature embryos of H. cannabinus could be cultured successfully. Mature embryos germinated and formed seedlings on MS basal medium without growth regulators. Even among these there were a number of those which exhibited abnormal growth. When IAA and kin combination was used the embryos showed growth of the cotyledons and root formation. But no further growth was observed in the same or other media combinations. Callus formation resulted from the growing embryos if retained in the same combination suggesting that it is suitable only for
initial embryo development and not for further growth. Other combinations of growth regulators did not affect embryo growth.

It is thus seen that for embryo growth, MS medium, which contains \((\text{NH}_4)^+\) ammonium ions, without any phytohormones is suitable. The absence of embryo growth and development in \textit{H. sabaariffa} and \textit{H. rosa-sinensis} may be due to their inability to respond to cultural conditions with the interaction of various factors.

**ANTHER CULTURE**

Anther cultures are invaluable for the production of homozygous diploids, stable mutants and have immense value in plant breeding programme (Griffing, 1975). The culture of anthers has been utilized to produce haploid tissue or plants in a number of species (Bajaj, 1984).

Guha and Maheshwari (1964, 1966, 1967) obtained embryoids from anthers on a medium with kinetin or coconut milk, while Nitsch and Nitsch (1969) regenerated plants from embryoids obtained on a basal medium. Nitsch and Norrel (1973) succeeded in inducing embryo formation by subjecting \textit{Datura innoxia} pollen to low temperatures \((4^\circ\text{C} \text{ for } 48 \text{ hs.})\) and growing them in a liquid medium. In the present study neither the effect of hormones or low temperatures could
induce callusing in anthers in all the cultivars and varieties. Although anther culture of many cotton species has yielded callus (Barrow 1978; Bajaj 1982; Thomas and Katterman 1984, Shamina et al., 1986; Turaev and Shamina 1986) androgenesis is yet to be reported. Barrow (1978) observed root formation in a mixture of haploid and diploid callus derived from anthers of G.barbadense and G.hirsutum. Nataraja and Patil (1984) obtained friable from anthers of Abutilon indicum in the presence of CM and 2,4-D. Pre-globular and elongated embryoids were observed in the callus which however failed to reach maturity. Although the combination of CM and 2,4-D has yielded good callus in the vegetative explants of H.cannabinus and H.sabdariffa studied here, no proliferation of anthers was seen. Although no reason can be pinpointed for the failure of anther cultures of H.cannabinus and H.sabdariffa, the effect of the genotype might have played a role.