CHAPTER 5

DISCUSSION

5.1 MICROPROPAGATION

In the past decade plant tissue culture technique has been explored tremendously for the mass propagation of high value medicinal species. However, application of the plant tissue culture technique for the commercialization of medicinal plants depends on various factors such as the present day pharmaceutical importance, presence of therapeutically high value compounds, biological peculiarities as well as biosynthetic capability of a particular species. The recent developments in micropropagation of medicinal plants have immensely helped the phytochemical industry (Anand 1993). Apart from commercialisation a rapid in vitro micropropagation system provides an excellent method for ex-situ conservation of rare and endangered or endemic medicinal plants (Mercier et al. 1992; Sharma and Chandel 1992 a; Stanilova et al., 1994). The relevance of the present work is highlighted by the drastic depletion of herbal wealth and the great demand for herbal drugs in the native system of medicine.

That the selection of proper explants has prime importance in the success of any experimental system in plant tissue culture is a well established fact. For most micropropagation work, the explant of choice would be an apical or axillary bud (George and Sherrington 1984). As far as pharmaceutical
and conservational point of view of medicinal taxa are concerned, resident meristematic explants are ideal as they could provide identical plants with desired traits (Bajaj et al. 1988). Therefore in the present study of micropropagation, shoot tip and nodal explants were used for all plants.

Best results are generally achieved when the explants are harvested during the active phase of growth (Torres 1989) and in vitro clonal propagation becomes easy when they are from juvenile stock of plants (Bonga 1987). Hence explants for the present investigation were derived either from young and actively growing shoots of juvenile stock plants raised through conventional propagation by shoot cuttings (Holostemma annulare, Janakia arayalpathra) or from active stock of one-year old plants raised from seeds (Rauwolfia micrantha).

In H. annulare, vigorous growth of the axillary shoot was observed in those explants harvested from stock of pre-monsoon season which was also similar to J. arayalpathra even though, in the latter, juvenile stock was not raised during post-monsoon season. The better response obtained during pre-monsoon season might be due to the fact that, even in nature, during this period plants are in preparation for their vegetative growth after the dormancy period of summer and in post-monsoon season, the plant's vegetative growth is on the decline in most of the perennial species. In the case of H. annulare, hard cuttings are found to be the best for the establishment of parental stocks unlike in the case of J. arayalpathra where soft cuttings gave better response. Induction of roots on stem cuttings was observed in H. annulare without any external application of auxin or other rooting hormones indicating the presence of sufficient endogenous auxin for root initiation. Another possibility is that basipetal outflow of auxin through the shoot system is arrested at the cut end of cuttings. This eventually might lead to an endogenous accumulation of
auxin as suggested by Weigel et al. (1984) in the case of *Chrysanthemum morifolium*. On the other hand, exogenous application of auxin (200 ppm IBA) was needed for root initiation on the cuttings of *J. arayalpathra*. Induction of adventitious roots on branch cuttings by synthetic auxins is one of the commonest modes practised by several authorities (Ansari et al. 1995; Sham et and Handa 1996).

In *R. micrantha*, pretreatment of seed with 50 ppm GA$_3$ and mechanical chipping could enhance the germination rate. This might be due to seed dormancy breaking efficiency of GA$_3$ as reported by Kesera and Sen (1987). Previously seeds of *R. serpentina* were treated with GA$_3$ to achieve enhanced germination (Kulkarni et al. 1993). The seedlings of *R. micrantha* thus raised were allowed to grow for one year to get a transition from juvenile to mature stage. Explants were harvested from actively growing shoots of flowering plants in this case. The reason for this explantation is that attainment and maintenance of the ability or potential of flowering is the only consistent criterion available to assess the termination of the juvenile period (Hackett 1987). Explantation from this stock has inevitable importance as it suggests that maturation involves a stability of structural, organizational and cellular differentiation.

Appropriate selection of the nutrient medium is also an essential and initial step as explant choice for the success and fulfilment of all experimental systems in plant tissue culture. MS medium has been selected for the present study after a thorough survey of published reports on related members of the plants selected. Besides, it is supported by the recommendation of Bhojwani and Razdan (1983) that in order to formulate a suitable medium for a new system it would be better to start with a well known basal medium such as MS or B5 (Gamborg et al. 1968). After the selection of the proper explants and
nutrient medium, the primary goal of establishment stage *in vitro* is to obtain a large percentage of explants free from surface pathogens. Attaining the infection free explants in culture is usually difficult and problematic when they are harvested from field-grown plants. Contamination and necrosis hampered during establishment phase in plant tissue culture might be possibly due to the under or over exposure of the explants to sterilants and the use of unsuitable types and concentrations of sterilizing agents. For all the plants studied an appropriate explant sterilization procedure which is standardized by several trial and error methods could minimize the microbial contamination rate without harm to the tissue. The present result denoted that bacterial contamination was more frequent than fungal ones in all plants. This is probably caused by endogenous pathogens associated with tissue exudates as all of them are with laticiferous tissues. Similar observations were made in *Piper nigrum* (Philip *et al.* 1992) which has tissue exudates.

In *H. annulare* and *J. arayalpathra*, basal nodes were most susceptible to contamination than shoot tip or terminal nodes which was commonly observed in other systems such as Darjeeling tea clone (Jha and Sen 1992), *Saccharum* spp. (Taylor and Dukic 1993). Unlike *H. annulare* and *J. arayalpathra*, difference in microbial contamination rates between shoot tip and nodal explants were not observed in *R. micrantha*. The possible reason for this might be that bud scales and buds on either position of shoots may be equally colonized by microbes.

In a number of cases, shoot tips were found to be best explants for shoot morphogenesis. Arrillanga *et al.* (1991) observed that shoot tips of either juvenile or adult plants were more responsive than axillary bud explants in *Sorbus domestica*. However in all plants tested in the present study, shoot tips were comparatively less desirable type of explants as reported by Stapfer and
Heuser (1985). Slow response of the shoot tip meristem compared to the axillary meristem was mainly met with in *H. annulare* and *J. arayalpathra*.

Effect of orientation of explants for bud initiation and multiplication was evaluated in many micropropagation systems by several authorities (Dolcet-Sanjuan *et al.* 1990; Perez-Parron *et al.* 1994). In the present study, shoot tip explants were placed horizontally in addition to vertically in order to promote the regeneration efficiency by activating the subjacent meristem of the apical region. During culture initiation, independent of the positioning, 100% of the shoot tip explants responded with single shoot formation in *H. annulare*. Horizontal positioning of the explants usually circumstanced the easy availability of nutrients and PGR as observed in periwinkle (Stapfer and Heuser 1985). In contrast to this, single shoot formation from apical meristem of *H. annulare* showed its strong apical dominance. Distinctly different from *R. micrantha* and *J. arayalpathra* shoot tip growth and accompanied by the induction of granular and friable callus throughout the entire portion of the stem was the response in *H. annulare*, which was bathed in the medium and this indicated the presence of more auxins at apical portion and its tissue susceptibility for callusing. Shoot tip response of *H. annulare* showed results contrary to the other members of Asclepiadaceae like *Asclepias erosa* where multiple shoots were obtained (Lee and Thomas 1985). On the other hand in *J. arayalpathra*, when shoot tips were in horizontal position, adjoining leaves of apical meristem which could not be removed without harm to the apical bud, became flaccid and prevented even the elongation of the apical meristem. This may be because of the immediate contact of the leaf with the cytokinin containing medium. Regeneration of axillary shoot from the subjacent axillary meristem of shoot tip explants of *J. arayalpathra* in negligible percentage, when it was in vertical position is also could not to be considered. The problem of single shoot formation from shoot tip explants could be alleviated
by manipulating the concentration and combination of PGR. Recently in *Capsicum* spp. the parent shoot apex elongated to form a single shoot in low concentration of BAP while at higher concentrations, subtending axillary buds were proliferated (Christopher and Rajam 1994). But none of these procedures solved the problem in any of the plants tried.

In *R. micrantha*, the difference in shoot morphogenic potentiality has not been observed in nodal explants of different positions and therefore it was not assessed separately. But in this respect, *H. annulare* and *J. arayalpathra* behaved similarly. Rapid response of the basal nodal explants over terminal nodes for shoot initiation in the latter plants might be attributed to the increased cytokinin activity at the basal region. It is also assumed that endogenous growth regulator content differs from one meristematic part of the plant to another (Okubo *et al.* 1991) and other factors such as nutrient availability within the plant may also change as suggested by Norton and Norton (1986). In consonance with the observations of Raghavaswamy *et al.* (1992), better response of the single node explants can be interpreted as due to the presence of axillary bud at more advanced stage of development, due to the difference between the physiological status of the two explants. The present result is in consonance with the observations on *Maytenus ilicifolia*, where axillary buds at distant positions from the apical bud yielded more shoots (Pereira *et al.* 1995). Even though in the present study, nodal explants showed gradience in response between terminal and basal ones, it is important that axillary shoot culture is the best suited *in vitro* culture system for conservation purposes. This is due to the reduced risk of somaclonal variation (Larkin and Scowcraft 1981). In both explants, (shoot tip and nodes), shoot initiation sites were not affected even though callusing was observed, from the cut end and basal parts of the explants that were immersed in the medium. Callusing should be dealt with extreme caution when clonal propagation is
required, because plant regeneration through callus tissue is undesirable (Lee and Phillips 1988).

A different mode of shoot differentiation was observed from nodal explants in the plants used in the present study. In *H. annulare*, rapid micropropagation was achieved through the induction of multiple shoots from axillary meristem. Use of multiple shoot cultures (cultures where a large number of axillary buds develop simultaneously) is generally recommended for micropropagation whenever possible (De Fossard 1986). In contrast to single shoot formation, multiple shoot initiation on nodal explants has been frequently described in micropropagation systems (George *et al.* 1993; Yuan *et al.* 1994).

Clonal multiplication of *J. arayalpathra* was achieved by single shoot formation from nodal meristems. Even though nodal explants of *J. arayalpathra* has opposite axils, meristem of only one of the axils gets activated and developed into single unbranched shoot which is not a common observation as multiple shoot formation. This uncommon observation in micropropagation system was supported by the report of Mittal *et al.* (1989). The single shoot regenerated remained unbranched and this is contradictory to the observation made in *R. micrantha* of the present study where enhanced axillary branching was observed on the solitary shoot from each of the axils. Unbranched solitary shoot formation was previously reported in *Dalbergia sissoo* (Mukhopadhyay 1984), *Leucaena leucocephala* (Dhawan and Bhojwani 1985) and *Acacia senegal* (Badji *et al.* 1993). In some plants it has not been possible to induce the release of axillary buds from apical dominance by manipulating the hormonal composition of the medium, and the bud present on the initial explant grows into an unbranched shoot (Bhojwani and Razdan 1983). The rate of shoot multiplication in such cases depends on the number of nodal cuttings that can be excised from the shoot at the end of each
passage. Multiplication of *J. arayalpathra* was achieved by repeated subculture of *in vitro* nodal segments (propagules) as done in the previously mentioned plants where single shoot formation was observed. In order to achieve the differentiation of shoot bud from undeveloped or inactive meristem or multiple shoot formation from the already activated axil in *J. arayalpathra*, various attempts were made. This includes the incorporation of peptone, CH (50-200 mg l⁻¹), TCW 10%, AdSO₄ (50 mg l⁻¹, GA₃ 0.5 mg l⁻¹) into the medium, use of other basal media (SH and WPM) and inoculation of explant after a vertical splitting through the middle core. Even though supplementation of the medium with peptone, CH and TCW had positive response in many micropropagation systems, the adverse effects observed in *J. arayalpathra* and *H. annulare* after adding these organic components supported the view that for most tissue culture purposes, undefined supplements containing aminoacids, or direct aminoacids additions may be unnecessary when a correct balance of inorganic salts, particularly MS medium which contain a relatively high level of ammonium ions has been developed for a particular plant species (George and Sherrington 1984). Callus formed at the cut end of nodal explants rapidly turned brown and retarded the vigorous growth of the shoot when they were cultured in CH and peptone containing media, possibly due to the loss or maladjustment of the cells to the excessive organic nitrogen. Prolific callusing on the explant cultured in TCW was similarly observed in other systems like *Sesamum indicum* (George *et al*. 1987). 50% of the regenerated shoots in AdSO₄ supplemented media got necrosed, probably due to the toxic effect of AdSO₄ as reported in *Dalbergia latifolia* (Raghawaswamy *et al*. 1992). AdSO₄ has been used only to a limited extent in meristem or shoot tip cultures (George and Sherrington 1984). AdSO₄ inhibition of shoot formation as observed in the present study was reported in rose shoot tip cultures (Hasegawa 1980) while it had a beneficial effect in *Cephalis ipecacuanha* (Jha
and Jha 1989) and Amaryllis (Prasad and Chaturvedi 1993). Even though in low frequency, addition of AdSO₄ to the medium enhanced shoot proliferation in Piper nigrum (Philip et al. 1992) and this is also contradictory to the present observation. The net result of the regeneration experiment on J. arayalpathra also supported the view that the development of axillary bud on the explant into single or multiple shoots depends upon the species and culture medium (Torres 1989).

Use of other basal media like SH and WPM did not favour the shoot morphogenesis. Inefficiency of nodal explant for bud break in SH medium due to prolific callusing from meristematic region, observed in this study supported the view that certain species require ammonium or some other source of reduced nitrogen for cell growth (Torres 1989). The results also supported the view that ammonium/nitrate balance is one of the factors in medium composition which influence growth and morphogenesis (George and Sherrington 1984). Contradictory to the present observation, SH medium was used successfully for the micropropagation of some other plants like Penstemon serrulatus (Wysokinska 1993), Woodfordia fruticosa (Krishnan and Seeni 1994), Adhatoda beddomei (Sudha and Seeni 1994), having medicinal importance.

Fragile shoots with linear leaves of the responded explants observed in WPM medium in low frequency were not desirable for culture establishment. The low mineral salt concentration of WPM (compared to MS) and lack of potassium source might be responsible for this. A more detailed study to find the effect of various nutrient formulations has not been conducted to attempt a critical interpretation of this phenomenon.

Incorporation of 0.5 mgl⁻¹ GA₃ in the culture initiation medium (MS + 2.5 mgl⁻¹ BAP + 0.5 mgl⁻¹ 2-ip + 0.5 mgl⁻¹ NAA) was tested mainly to induce the dormant axillary bud which failed to perform. But the role of GA₃
in shoot bud differentiation in the present study is not in consonance with the bud dormancy breaking efficiency as noted in *Lycopersicum sequientum* (Catalano and Hill 1969) or promotion of elongation of buds as in *Phlox paniculata* and *P. subulata* (Schnabdrauch and Sink 1979). In the present study, GA3 could not induce the extra-axillary bud development as reported in *Saussurea lappa* (Arora and Bhojwani 1989) or shorten the time of bud initiation as in *Carica papaya* (Mondal et al. 1990).

Enhancement of regeneration during initiation of culture was brought about by culturing the split nodal halves in *Brassica alboglabra* (Pua et al. 1989) and split halves of precultured nodal explants of *Adhatoda beddomei* (Sudha and Seeni 1994). However in this study, the observation of single shoot formation from one of the halves and the other remaining without shoot bud formation is found to be an isolated report. Accessibility to the nutrient absorption by the vertical splitting of the node is not a limiting factor for the differentiation and growth of the single axillary shoot in *J. arayalpathra* as in spite of this, there was no shoot bud initiation on the bare axil and also there was no difference in number, length and vigour of the shoot which was formed from the existing axillary bud. In several other systems, it has been reported that excision or wounding of the explant may be responsible for meristem initiation (Malik and Saxena 1992). But none of these attempts could achieve the desired objective of the experiment, suggesting that the lack of shoot formation on one of the opposite axils might be due to the inhibition of bud or increased apical control of the other axillary shoot which had first differentiated.

In *J. arayalpathra*, in order to investigate whether juvenile tissues also produced single shoot as in the case of explants of field-grown plants, or whether they produced multiple shoots, various explants of *in vitro* germinating
seedlings have also been used. Most of the work dealing with seedling tissues for the regeneration performance, the response of the tissues at the different periods of growth had been investigated as in *Vigna radiata* (Gulati and Jaiswal 1994). In *J. arayalpathra*, because of the scarcity of seed availability, experiments had not been conducted elaborately as in the former system mentioned. The use of seedling explant for *in vitro* plant regeneration has several advantages because of the rapid response of these tissues over adult tissues (Bonga 1987). Moreover, in most cases, seedling tissues were harvested from aseptically raised seedlings and therefore microbial contamination of such explants is rarely a serious problem. Use of hormone free medium (BM) for raising seedling stock aseptically is the usual procedure adopted (Dewan *et al.* 1992, and Raharjo and Punja 1993). Soaking of seeds in 50 ppm GA<sub>3</sub> and its addition (0.5 mg l<sup>-1</sup>) in the BM enhanced the *in vitro* germination rate and shortened the period of germination over control. In *J. arayalpathra*, this might be due to the seed dormancy breaking efficiency of GA<sub>3</sub> as discussed previously. Incorporation of GA<sub>3</sub> in the BM was similarly employed for the seed germination of *Penstemon serrulatus* (Wysokinska 1993).

The results obtained in the present investigation by using various juvenile explants grown in identical nutrient milieu (MS + 2.5 mg l<sup>-1</sup> BAP + 0.5 mg l<sup>-1</sup> 2-ip + 0.5 mg l<sup>-1</sup> NAA), clearly revealed that endogenous PGR present in cotyledons and shoot tip played a crucial role for triggering the mechanism of shoot regeneration. Response of 100% of the cotyledons for callus formation from the cut proximal end without shoot bud differentiation when it was cultured separately as against the other systems (Arroyo and Revilla 1991; Sharma *et al.* 1991a; Sarvesh *et al.* 1993). However, it is unfair to conclude that individual cotyledons of *J. arayalpathra* are not able to initiate bud organogenesis unless tissues of different ages were analysed. This assumption might be due to the fact that the regeneration ability of the cotyledons also
depends on age of the seedlings as evidenced by the study in other systems (Gulati and Jaiwal 1990; Deleuze et al. 1994). Observation of profuse callus production on proximal end of cotyledons which eventually necrosed without shoot initials during the whole culture period observed in this system is similar to that noticed in *Sesamum indicum* (George et al. 1987).

It is assumed that cotyledons are storage organs and as such are most active in the hydrolysis of stored carbohydrates to simple sugars as well as sources of other nutrients (Burger and Hackett 1986) and it played an important role in the normal development of seedlings (Sharma et al. 1991a). In the present investigation, when both cotyledons were attached or detached from the cotyledonary nodes in the presence or absence of shoot tip, a different shoot morphogenic response was observed. Formation of callus from the meristematic portion of cotyledonary node with attached shoot tip but in the absence of cotyledon (explant type b) suggest that there might be rapid and surplus basipetal transportation of an inhibitory substance from the shoot tip which hindered the shoot organogenesis. This inhibitory substance which caused hormonal imbalance during shoot organogenesis and favoured callusing might be an auxin. Besides, rapid absorption of exogeneous PGR in the medium through the cut surface of the nodal region where cotyledons were attached might also enhance callus formation. Thus, increased callusing which resulted in slow detachment of the shoot tip portion, disturbed the extensive shoot elongation also.

Multiple shoot formation was observed from the cotyledonary node with cotyledons but without shoot tip (explant type d) On the other hand, solitary shoot formation from each axil of cotyledonary node was noticed when both cotyledons and shoot tip were removed (explant type f) The possible explanation for the multiple shoot formation from explant type d might be that
the prime factors for multiple shoot formation are diffusible promoters which emanate from the cotyledons. This is supported by the view of Burger and Hackett (1986) in Citrus. It is also an established fact that growth factor(s) for shoot morphogenesis reside in the radicular halves of the cotyledons (Rangaswamy and Rangan 1971), even though in the present experiment the effect of amount of cotyledonary tissues for shoot organogenesis was not studied to prove this. Another possibility can be attributed to the loss of apical dominance. But it is more logical to assume that emanation of some growth factors from cotyledons is important for multiple shoot formation rather than the absence of apical bud as the explant type f showed solitary shoot formation from each axil. That the presence of cotyledon on the explant stimulated shoot bud formation is well documented (Nandwani and Ramawat 1993). To some extent, the present observation is in agreement with that on Vigna radiata (Gulati and Jaiwal 1994) where rapid multiple shoot formation at high frequency from cotyledonary nodes with or without cotyledons was observed.

Another interesting observation made is that the cotyledonary nodal explant with both cotyledons and shoot tip (explant type e) virtually showed absence of shoot differentiation from the meristematic axillary portion. This suggests that some inhibitory substance emanating from the shoot apex, as hypothesised by Burger and Hackett (1986) suppressed the shoot promotive factors from the cotyledons. But on the other hand, lateral branching during the later period, after the dominant growth of the shoot tip was observed not only in this explant type but also in explant type f. This indicated a gradual decline of apical dominance. Maximum number of shoots (7 ± 0.3) observed in explant type e over explant type f (4 ± 0.0) indicated that growth factors in cotyledons, present in the former explant helped to enhance shoot formation. The observation of axillary branching at first from the basal node of the shoot of these explants suggests that the concentration of mobile auxin which inhibits
shoot organogenesis in apical cell is 10 -100 times that in basal ones (Goldsmith 1969).

Even though multiple shoot formation was observed from the explant type, the thin and flimsy nature of shoots compared to those from axillary branching probably due to the competition during tissue differentiation, makes them undesirable for mass propagation. Root formation did not occur on these explants with cotyledon suggesting that cotyledons of *J. arayalpathra* did not possess the endogenous root promoters as in *Vigna radiata* (Bassuk and Howard 1981).

Shoot morphogenic efficiency could be increased by using juvenile tissues, but they are not amenable to rapid clonal propagation as shoots from field-grown plants, due to the rapid decline of regeneration potential, and loss of culturable propagules during subculture as the shoots formed are too thin and flimsy. This fairly distinguishable difference in morphology of the shoot culture of juvenile origin (lack of sturdity, linear and small leaves) from those of mature explant suggest a possible difference in the developmental phase of some tissues.

There are many reports regarding establishment of regeneration system by using juvenile and mature tissues (Iturriaga *et al.* 1994), to achieve better regeneration and comparative account for regeneration potential is documented as in *Prosopis cineraria* (Nandwani and Ramawat 1993). In the present study, production of single shoot from mature axillary meristem and multiple shoot from juvenile (cotyledonary node) meristem of *J. arayalpathra* indicated an inhibition of one of the axillary meristems in nodal explants of mature plants. On the contrary, Badji *et al.* (1993) reported only single shoot formation from meristems of both origin of *Acacia senegal*. 
The significant difference of morphogenic efficiency among the two PGR combinations, (BAP 2.5 mg\textsuperscript{1}l\textsuperscript{-1} + 2-ip 0.5 mg\textsuperscript{1}l\textsuperscript{-1} + NAA 0.5 mg\textsuperscript{1}l\textsuperscript{-1}) and BAP 3.0 mg\textsuperscript{1}l\textsuperscript{-1} + NAA 0.5 mg\textsuperscript{1}l\textsuperscript{-1}) shown by the juvenile explant compared to mature explant which was insignificant can be attributed not only to the various physiological factors as discussed previously but also to the sensitivity and better absorption efficiency of the former ones as for both tissues origin is in uniform PGR combination. It is a well established fact that tissue sensitivity is the key factor in hormone action. Regardless of whether the explants of *J. arayalpathra* were of juvenile or mature origin, uniform requirement of PGR is in consonance with the report on *Quercus suber* (Manzanera and Pardos 1990).

Clonal multiplication of *R. micrantha* achieved through enhanced axillary branching might be due to weak apical dominance *in vitro* and it is more apparent that axillary branches that emanated were overgrown, while the axillary shoots from which they were derived remained short. Growth of the axillary branches did not stop in the presence of the terminal bud on axillary shoot, possibly because availability of exogenous cytokinins had been adequate. Shoot multiplication was at its maximum after initial 4-5 passages. This supported the statement that the enhanced axillary branching method of shoot multiplication may be initially slow but with each passage the number of shoots increases (Bhojwani and Razdan 1983). Although this is the slowest method of micropropagation based on the view of Torres (1989), it is becoming increasingly popular because the plants are least susceptible to genotypic changes under culture conditions. Enhanced axillary branching is the safest approach to *in vitro* shoot multiplication as also suggested by Dhawan and Bhojwani (1985). The relevance of this mode of multiplication is established by the recently published work on other plants like *Sorbus domestica* (Arrillanga et al. 1991), *Potentilla fruticosa* (Remphrey et al. 1993).
and *Nepenthes khasiana* (Latha and Seeni 1994). As far as the present investigation on *R. micrantha* is concerned, clonal propagation through axillary branching is beneficial as it is now recognized as a useful technique for propagation and *in vitro* conservation of threatened plants, especially those in which roots or rhizome contain the active compounds (Constabel 1990). Single shoot formation from axillary meristem observed in *R. micrantha* is in consonance with the observation on allied species, *R. serpentina* (Roja et al. 1985; Mathur et al. 1987). But single shoot formation from *in vivo* and *in vitro* nodal explants of *R. micrantha* showed a marked difference in response from that of *R. serpentina* where multiple shoot formation was observed during the subsequent subculture.

With regard to PGR, clonal propagation systems developed with the three plants investigated, in the present study the general principle that during *in vitro* culture a high concentration of cytokinin and a low concentration of auxin in a medium promotes the induction of shoot morphogenesis as generalized by Kohlenbach (1977) holds good. In a number of cases cytokinins alone are enough for optimal shoot multiplication (Garland and Stoltz 1981) but the requirement of cytokinin - auxin combination is sometimes helpful for shoot morphogenesis (Bhojawani and Razdan 1983). In the present study, even though explants showed varying apical dominance which is generally controlled by endogenous PGR, appropriate combination of PGR was needed for shoot morphogenesis. There is a hypothesis that if the explants are sufficiently rich in PGR, they do not need exogenous application of PGR. But to date there is no clear evidence to confirm this as suggested by Okubo et al. (1991). In all the plants surveyed, BAP-NAA combination was found to be the best for the rapid and active induction of axillary buds. It is perhaps because they are from closely related families, Apocynaceae (*R. micrantha)*
Asclepiadaceae (*H. annulare*) Periplocaceae (*J. arayalpathra*) and all are laticiferous.

Present results indicated that combined influence of cytokinin and auxin is indispensable for the sustenance of shoot growth. This is in agreement with other reports (Manzanera and Pardos 1990; Raghavaswamy *et al.* 1992; Bejoy and Molly 1992) But contrasting observations were made in a number of cases where the efficiency of BAP for shoot multiplication was decreased when it was supplemented with auxin (Gulati and Jaiwal 1992) or was not significant (Brandt 1992). The beneficial effect of BAP-NAA combination for direct shoot bud regeneration met with in *H. annulare* and *R. micrantha* of the present study is similarly reported in allied members in Asclepiadaceae (Sharma and Chandel 1992b) and Apocynaceae (Benjamin *et al.* 1993). In *J. arayalpathra* the influence of NAA was more pronounced with a combination of cytokinins (BAP+2-ip), even though a marginal difference in shoot morphogenesis was not observed from that of BAP alone. Synergism and quantitative interaction of two or more PGR are of common occurrence (Minocha 1987). Synergistic influence of cytokinins with auxin for shoot morphogenesis were well documented in *Adhatoda beddomei* (Sudha and Seen 1994).

Although optimal requirement of BAP-NAA used for shoot morphogenesis was varied in different plants, production of stunted shoots with short internodes at high concentration of BAP alone or with NAA is common to the three plants studied. This is consistent with the published reports (Marri *et al.* 1986; (Amin and Jaiswal 1987; Maria *et al.* 1990; Reed 1991; Hossain *et al.* 1992; Hosaki and Katahira 1994). Even for stunted shoots, their differentiation at higher concentration of BAP (5.0 mgI⁻¹) is contradictory to the report on *Gomphrena officinalis* (Mercier *et al.* 1992) where bud differentiation was lacking. Dehiscence of the bud followed by bulging,
yellowing and flacidity is more pronounced in *H. annulare* than in *J. arayalpathra* and it was mostly confined to basal nodes, whereas those abnormalities were not met with in *R. micrantha*. Dehiscence of the bud might be due to the lack of lignification under increased hyperhydricity as one of the causes of vitrification and its degree was proportional to the concentration of BAP (Phan 1991). Reduced shoot length and increased hyperhydricity at high concentration of cytokinin was similarly observed in watermelon (Compton et al. 1993).

Comparing various cytokinins, BAP possessed the highest regenerative capacity over Kn and 2-ip and this is concomitant with other reports (Frett 1987, Rathore et al. 1992; Samarajeewa et al. 1993; Wysokinska 1993). The results are firmly supported by the observation that BAP is needed for the micropropagation of latex producing plants from various explants (Lee et al. 1982, Ripley and Preece 1986). However, poor performance of Kn with *H. annulare* is contradictory to the report on *Asclepias curassavica*, an allied member of Asclepiadaceae (Pramanik and Datta 1986). In *H. annulare* restriction of the axillary shoot elongation which caused, the stumpy nature of shoot in explants grown in medium supplemented with 2-ip is probably due to the unusual overexpansion of the leaf which hindered apical bud elongation. The inefficiency of 2-ip in promoting axillary bud multiplication is reported in other systems (Stapfer and Heuser 1985; Jha and Jha 1989).

Comparing the effect of auxins with BAP, NAA was found to be more suitable than IAA and IBA, in all the systems studied eventhough its concentration varied. Better performance of NAA in multiple shoot production was also observed in other members of Asclepiadaceae, (Pramanik and Datta 1986; Roy and De 1990; Sharma and Chandel 1992b), and Apocynaceae (Roja et al. 1985, Mathur 1987; Upadhyay et al. 1992).
The tendency of callus formation at the cut end of the explant is a common observation met with as in other systems (Dhawan and Bhojwani 1985; Corchete et al. 1993). Unlike *R. micrantha* and *J. arayalpathra*, differential nature of the callus formation at the cut end of terminal and basal nodal explants of *H. annulare* probably points to the fact that endogenous PGR may vary at different positions which interact with exogenous PGR and this may lead to varying callus formation in terms of texture and quantity.

In *J. arayalpathra*, calli is devoid of green pigmentation unlike in *H. annulare* and *R. micrantha* and it became brown later. This type of browning of callus was observed in *Madhuca longifolia* (Rout and Das 1993). However, it did not interfere with the growth of the shoot, and can be considered as a nonproblematic response. It is frequently observed that greening of callus is more in *R. micrantha* than in *H. annulare* where it was occasional. This suggests that some endogenous cytokinin is responsible for green pigmentation in the latter plants, even though sucrose concentration used in all is uniform (3%). This is supported by the view that chlorophyll development in plant tissues in culture is controlled by both cytokinin and the sucrose present in culture media (Mondal et al. 1993). In *J. arayalpathra*, callusing was more extensive in juvenile explants than in mature ones. The present observation is not in agreement, with that of *Robinia pseudoacacia* (Han et al. 1993). They had suggested that unlike organogenesis, cellular growth *in vitro* is not constrained by explant age.

A comparative study of shoot regeneration potentiality between the explants of favourable (pre-monsoon) and unfavourable (post-monsoon) seasons were evaluated only for *Holostemma* as it showed seasonal dormancy for vegetative *growth*. It is a well known fact that the physiological state of the stock plant influences explant performance in culture (Litz and Cohover
1981; Sutter and Barker 1985). The lower frequency of fungal contamination of explants of post-monsoon period is possibly due to the low humidity in the climatic conditions. However, better performance of the nodal explants during the pre-monsoon period, which is the beginning of vegetative phase might be due to enhanced meristematic activity of the explants for flushing out the growth. Likewise, the observed diminished shoot morphogenesis of explants during the post-monsoon period, when the plants were entering the storage and rest period might be attributed to the fact that the metabolic rate of the plant decreased resulting in the decline of endogenous PGR and nutrients in the shoot system. For tree species, it is well documented that success in establishment of aseptic culture of field-grown plant is influenced by the season during which the explants are harvested (Hu and Wang 1983). Similar observations on seasonal effects on shoot morphogenesis was observed in another perennial plant like *Amaryllis belladonna* (De Brugn et al. 1992) with underground storage organs.

Mass propagation of shoots obtained by repeated subculturing of explants derived from aseptic shoot cultures is the usual method practiced in other systems also (Dewan et al. 1992; Sharma et al. 1993a; Krishnan and Seeni 1994; Hosaki and Katahira 1994). Subculture experiments indicated that proper *in vitro* explant selection is as important as the use of appropriate PGR. Both the factors are extremely important as far as rapid clonal multiplication is concerned and it has been documented in other studies (Sudha and Seeni 1994).

With regard to *H. annulare*, basal nodes and stumps were found to be the best *in vitro* explants. This indicated that meristems of these explants are at a more advanced stage of development. Moreover the endogenous PGR levels may also be higher. The inability of developing shoot buds for the
growth along with the regenerated shoots on nodal explants of *H. annulare* which was observed during initiation and subculture, can be explained by the suggestion of Douglas (1990) that the presence of shoots initially regenerated from the explants suppresses their further development. In such cases, reculturing the parental explant (stumps) with the removal of the existing developed shoots favoured the enormity of shoot production as in other systems (Dewan *et al.* 1992) Reculturing of the parental explants after removing the regenerated shoots in *R. micrantha* and *J. arayalpathra* did not show regeneration potentiality indicating that further activation of axillary meristem other than the existing one as in multiple shoot formation was not possible. On the other hand, axillary bud growth from the subjacent nodes of in vitro shoot tip explant of *R. micrantha* indicated weak apical dominance which was controlled by endogenous PGR and easy release of the axillary buds, possibly due to the vigorous meristematic activity at the juvenile position of the axillary buds. The remarkable difference in the caulogenic response of the shoot tip explant of field-grown plants and in vitro shoots of *R. micrantha* could be attributed to altered endogenous concentration of hormones in the latter and nourishment in high cytokinin medium at the time of culture initiation. However, both the nodal explants (*in vitro* and *in vivo* origin) responded similarly. In *J. arayalpathra* in vitro basal nodal explants were found to be the best, producing axillary shoots on either axils after a number of subcultures, indicating that tissues had attained a juvenile state as suggested by Franclet *et al.* (1987) and Gupta *et al.* (1981), that repeated subculture *in vitro* is one of the methods of maintaining juvenility *in vitro*. Unlike in *R. micrantha*, subjacent axillary buds of shoot tip (*in vitro*) explants of *H. annulare* did not develop into shoots even though they were continuously exposed to exogenous cytokinins, indicating that the plant showed strong apical dominance under *in vitro* conditions.
A substantial reduction in the concentration of the PGR during subculture favoured continuous production of healthy shoots in all the plants studied. This is in consonance with the results of other reports (Amin and Jaiswal 1987; Hossain et al. 1993). PGR requirement of propagules of *J. arayalpathra* is relatively critical as propagules derived from BAP-NAA medium required BAP alone for high frequency regeneration and *vice versa* for those derived from BAP alone. This suggested that PGR regimes employed during culture initiation were indicative of changing endogenous PGR levels and consequently arose differential sensitivity and requirement of the tissues for optimal shoot production during subculture (Okubo *et al.* 1991; Krishnan and Seeni 1994).

Unlike *J.arayalpathra* and *R. micrantha*, the intensity of callusing from cut basal end of propagules even if it has not affected the meristematic region, was pronounced in *H. annulare*. It is desirable that callus development be minimized or eliminated during micropropagation (Kantharajah and Dodd 1990). Media with a lower content of NAA reduced callusing but was accompanied by shoot deterioration. It denoted that PGR balance is an inevitable factor for continuous shoot production. In another attempt to reduce the callusing, the strength of MS salts in the medium was reduced to one half or one quarter of the normal concentration. This resulted in decreased shoot vigour, although the extent of callusing was reduced. This revealed the fact that the salt concentration of the medium is as important as the PGR balance during subculture. It has been suggested in a number of cases that low salt formulation definitely did not support adequate growth of shoots and it may be reduced when compared to growth on high salt formulation (McCown and Sellmer 1987).
In conclusion, it became apparent during the culture initiation and establishment experiments that a subtle balance of auxin or cytokinin was essential for provoking the growth of the axillary buds and the type of auxin and cytokinin are important factors for organogenesis. This was supported by the recommendation of Kohlenbach (1977). Occasional and spontaneous rooting observed in the subculture medium in all the systems is in agreement with that of Philip et al. (1992). This might be due to the habituation of cultures in the cytokinin-auxin containing medium. In all the plants studied, growth was exuberant and unlimited till the medium was exhausted without showing shoot tip necrosis, browning or any other deleterious symptoms as observed in *Passiflora edulis* (Kantharajah and Dodd 1990). Invariably shoot multiplication rate from adult explant increases with progressive subculture, probably because the shoots have became more juvenile as discussed earlier with similar observations made by Raghavaswamy et al. 1992.

On the other hand, rapid decline in the multiplication rate in the cultures of *J. arayalpathra* derived from juvenile explants might be due to the extensive juvenility formed during repeated subculture. Moreover, the delicate and flimsy nature of the shoots also contributed to this situation. The fact is that in *J. arayalpathra*, a substantial micropropagation efficiency was represented by nodal explants from field-grown explants, eventhough rapidity and profuse shoot formation was achieved for the juvenile explants. Declining in rate of multiplication has not been observed in over 3-year continuous subculture in *R. micrantha*, while it was noticed in *H. annulare* after 30 passages and after 20-25 passages in *J. arayalpathra*. Decline in the multiplication rate was common in other systems. (Dewan et al. 1992; Hossain et al. 1992).
Use of liquid medium in the present investigation for the mass propagation was undesirable due to the occurrence of varying degrees of vitrification. Even though shoots could be bulked in *R. micrantha*, as previously reported in *R. serpentina* (Roja *et al.* 1985), the present system was not useful for *in vitro* plantlet production. Lack of root initiation on such shoots probably indicate inability of vitreous tissues to absorb exogenous auxins. Inefficiency of solitary nodal segments of *R. micrantha* to induce axillary bud in liquid medium can be assumed as being due to the arrest of aeration and exchange of gas as the explant was immersed in the medium. On the other hand spectacular proliferation of shoots in liquid culture was observed when the explants were with supporting tissues like axillary branches or already developed axillary buds. The report on *Sophora toromiro* (Iturriaga *et al.* 1994) is contradictory to the present observation. Tissue vitrification, however, has been attributed to a number of factors other than the influence of the water potential of the medium (Paques and Boxus 1987). Since, in the present investigation, nutrient milieu and PGR used for the shoot cultures in solid and liquid medium were similar for all plants, vitrification was attributed to the presence of agar and this is in agreement with reports of Debergh *et al.* (1981) and Debergh (1983). Shoot tip necrosis showed by the shoot cultures of *R. micrantha* during the later period of growth in liquid medium might be related to calcium deficiency in the explants caused by the reduced transpiration and lowered calcium uptake under the high humidity in culture containers as observed by Sha *et al.* (1985). The degree of vitrification was higher in *H. annulare* than in *J. arayalpathra*, and both did not shown any symptoms of growth as tissue translucence had occurred all along the shoots. This probably indicates a loss of lignin synthesis, a biochemical condition associated with lignification (Phan and Hegedus 1986) usually met in vitrified cultures (Phan 1991).
From the experiments using the culture bottles with polypropylene caps, to assess the commercial feasibility for the rapid mass propagation of *H. annulare*, extensive axillary branching was observed which is not met with in culture tubes with cotton plugs and it might be due to loss of apical dominance. It is assumed that polypropylene covering may cause development of high humidity inside the culture bottles and this may decrease the terminal bud activity which would limit the apical dominance and facilitate easy release of axillary buds. A similar suggestion is offered by Sallanon and Maziere (1992) in rose plants. Small and narrow nature of the shoots in culture bottles is also in agreement with the suggestion of Sallanon and Maziere (1992) that it might be due to the increase of transpiration intensity under high evaporating conditions which profusely modified the plant growth and morphology. Moreover it is supported by the view that under *in vitro* conditions, growth and morphogenesis of the plantlets were influenced by the air, volume above the organs (Bateson *et al.* 1987) and some vessel characteristics, such as closure types which are known to have a strong influence on culture growth (McCown and Sellmer 1987). Shoot cultures of *H. annulare* in culture bottles remained healthy, for a long period as observed in *Rauwolfia serpentina* cultures in culture tubes with poly propylene caps (Sharma and Chandel 1992a). Besides, the present observation of death of the shoots followed by the shoot tip necrosis (discoloration), by keeping the cultures for prolonged time in culture bottles is also similar to their observation. The possible reason for this might be the high humidity of the culture vessel which depresses transpiration from the leaves which results in a slowing of the water/nutrients (especially calcium, as it is not mobilized in plant tissues from the medium to the tissues as suggested by McCown and Sellmer 1987). As the experiment has not been elaborated in detail by measuring the vapour pressure deficit, or nutrient levels and other
physiological factors, further interpretations are not attempted. Use of culture bottles in *H. annulare* was found to be beneficial in two ways.

- As profuse axillary branching was observed and the resumption of normal growth in the culture tubes with cotton plugs, it could be considered as an alternative method to scale up mass propagation.

- Since cultures were maintained alive without frequent subculture, for more than 8 months, this method can be considered as a means of short term *in vitro* conservation and subculture period could be prolonged as done in turmeric and ginger (Balachandran *et al.* 1990).

Efficient rooting of the *in vitro* regenerated plants and subsequent field establishment is the last and crucial stage of rapid clonal propagation. In all the systems studied for rooting initiation, mineral nutrients at half strength was used and this is supported by the recent report (Monier and Ochatt 1995). Results of rooting experiments in all the plants investigated showed that each plant required different types and concentrations of auxin for best quality of roots which would lead to increased rate of survival.

In consonance with the published reports (Purohit *et al.* 1994; Mao *et al.* 1995), root initiation occurred in auxin-free medium (BM) but better results were observed with the addition of an auxin (IAA, IBA, NAA) or AC. The roots formed in BM were weak, thin and few in number. Similar observations were made in *Morus laevigata* (Hossain *et al.* 1992; Islam *et al.* 1993). The present observation is not in agreement with that of Yadav *et al.* (1990) and Sharma *et al.* (1991b) where, addition of an auxin was essential for rooting. Incorporation of 500 mg l\(^{-1}\) AC alone in the BM helped to increase the length of the root, percentage of rooted shoots and its survival than that observed in BM in *R. micrantha* and *H. annulare*. The inability of AC for root initiation
was previously reported (Sharma et al. 1993a). However, the positive response of AC compared to BM in the present study can be attributed to the efficiency of AC to absorb toxic substances such as polyphenols produced by tissues during culture (Fridborg et al. 1978) and toxic compounds (1-5 hydroxy-methyl-fural) derived from sucrose dehydrations during autoclaving (Weatherhead et al. 1978) or impurities present in the culture medium (Weatherhead et al. 1979). The results of increased root length in IBA containing medium over AC in H. annulare disagrees with the observation in Psidium guajava (Mohamed-Yasseen et al. 1995).

Compared to IBA and IAA, NAA produced short and thick roots. Eventhough the roots were thick and sturdy in R. micrantha and J. arayalpathra, calloid roots were never produced with 0.5 ml$^{-1}$ NAA as observed in H. annulare. Production of thick and short roots in the presence of NAA was also observed in Penstemon serrulatus (Wysokinska 1993) and Chlorophytum borivilianum (Purohit et al. 1994). The present observation is in agreement with the observations of Demeke and Hughes (1990), that thick and short roots were accompanied with greater amount of callus in high auxin concentration and mostly high concentration of NAA in rooting medium promoted callusing (Rajasekharan 1994). A perusal of literature showed that there are no reports on abnormal cohesive roots as observed in the present study of H. annulare at high concentrations of NAA (0.5 ml$^{-1}$). The possible reason for the inefficient survival of H. annulare shoot cuttings in NAA treatments might be due to the poor vascular connection of the root with the stem by the intervention of friable callus. Long and thin roots produced in the medium with auxins (IAA and IBA) is usual in R. micrantha and J. arayalpathra. This is in consonance with report of Wysokinska (1993). Green root formation which was noticed only in H. annulare might be its species specific character.
Shoot bud differentiation on the root of *H. annulare* is a natural phenomenon of plants, depending on species. Most of the *in vitro* studies on this aspect were initiated mostly from root segments of seedlings (Sharma and Thorpe 1989; Jaiswal *et al.* 1987; Sharma *et al.* 1993b). In the present study on *H. annulare* shoot buds were observed on root segments harvested from *in vitro* raised rooted plants, parallel to the findings in *Aegle marmelos* (Bhati *et al.* 1992).

The inefficiency of the root tip segments for shoot bud and its formation uniformly from the proximal or midportion of the root, supported the view that some secondary growth is an essential factor for shoot initiation as in *Comptonia peregrina* (Goforth and Torrey 1977). Invariably, restriction of the shoot bud formation to the chlorophyllous portion of the root of *H. annulare* indicates that chloroplast development on root segments was responsible for shoot bud differentiation. Therefore it may be assumed that chlorophyll in the roots provided the necessary photosynthetic energy requirements for shoot bud differentiation on root segments of *H. annulare*. This can be correlated to the study of Thorpe (1980) that starch serves as an energy source for the high energy requiring process of shoot bud differentiation.

There are many reports that root tip meristem provides some essential ingredients(s) for the differentiation of shoot buds. Vanstaden and Smith (1978) reported that cytokinins are known to cause bud induction and the root tip has been established as a seat of cytokinin synthesis. Further it was evident in *Limnophila* that the shoot bud differentiation on root explant is obligatorily dependent on the presence of an active apical meristem. (Rao and Mohan Ram 1981) Results in the present study do not confirm this view, as there was no
bud differentiation from the root tip segment. On the other hand it is in agreement with the observation on *Citrus aurantifolia* (Bhat et al. 1992).

Exogenous application of PGR resulted in varying responses suggesting that it is an important factor to determine the shoot morphogenic efficiency of root segments in *H. annulare*. Callus formation and quality of lateral roots formed in root segments cultured, coincided with the observations on root initiation experiments of *in vitro* shoot cuttings. Presence of surplus endogenous auxin on root segments (as it was taken from auxin containing rooting medium) in addition to the exogenous auxins in the medium probably inhibits the shoot differentiation and promotes lateral roots in medium containing various auxins. This is supported by the view that relatively high concentration of auxin suppresses the inception of the shoot buds and promote the growth of the lateral root (Peterson 1975). The present observation of lateral root formation on cut root segments may also be due to the loss of apical dominance as there is a hypothesis that root apex, too, exerts dominance, in that its presence inhibits lateral root initiation. (Phillips 1969).

Addition of BAP alone at low concentrations favoured shoot bud differentiation and on the other hand localized callusing with diminished lateral roots along the root axis as was seen when BAP was combined with auxin (NAA, IAA, IBA). Both responses indicate the effect of high endogenous auxin balanced with exogenous low cytokinins. The promoting effect of BAP in inducing the shoot buds on root segments was well documented in other systems (Bhat et al. 1992, Bhati et al. 1992).

As the present study was to maximize direct shoot bud production so as to supplement the propagation technique, free from genetic variation, minor involvement of the callus, adjacent to the shoot initiation points could be retarded by transferring the responded root segments to BM. This procedure
helped to produce elongated lateral roots from shoot initiation points along with rapid growth of the shoot, thus eliminating the stage of further root initiation and this is contrary to the work on *Aegle marmelos* (Bhati 1992). This favourable response of shoot and root growth upon transferring from BAP containing medium to BM possibly indicated the development of critical growth regulator balance in it.

Bhat *et al.* (1992) suggested that low frequency of plant regeneration from the root culture speaks against using root culture for germplasm conservation until the frequency of shoot regeneration on root can be improved. However, the success in the shoot bud differentiation on excised root segments of *H. annulare* even though single shoot was differentiated per root segment is credited by the following positive points.

- Explants were derived aseptically, therefore did not face the problem of microbial contamination.
- The chlorophyllous root used for this study was harvested from the fully developed rooted plant which had two other roots sufficient for its safe field establishment.
- Shoots were produced within a short time (5-6 weeks) on a simple nutrient medium.
- Rapid elongation of the shoot on a single root segment provided 8-9 propagules. This could enhance the propagation rate by 16-18 fold from a single chlorophyllous root and can be considered as an alternative means of propagation.
- Differentiated shoot with root segment showed high percentage of survival (80%) when it was utilised for field establishment.
Since shoot bud differentiation is closely related to chloroplast development, the present system would be useful for the biochemical and physiological studies in relation to organ differentiation.

Acclimatization of the regenerated plants to the external environment is the last stage of micropropagation and its success depends upon different factors as suggested by various authorities (Bhojwani and Razdan 1983; George and Sherrington 1984).

It is a general observation that the high humidity of the environment in vitro does not allow synthesis of cuticle and epicuticular wax on the epidermis of leaves of regenerated plants (Bramerd and Fuchigami 1982). Consequently, when such plants are transferred they undergo dessication and death. Therefore it is logical to assume that rearing the in vitro plantlets of the above mentioned species in mist chamber, where high humidity is maintained gave the best survival percentage. Among the plants investigated, J. arayalpathra (86-88%) and R. micrantha (83-84%) showed best survival than H. annulare (80-82%). The ease of acclimatization of R. micrantha with the limited hardening procedure, compared to the other two might be due to the rapid and better controlled transpiration which developed in the external conditions, as the lack of stomatal closure mechanism in the plants in vitro was the main cause of rapid water loss during transfer to low RH (Bramerd and Fuchigami 1982) Besides, the sturdy nature of the stem, and leaves of this plant compared to the other two also helped in attaining easy survival.

On the other hand, the inability of rapid acclimatization of H. annulare and J. arayalpathra is presumably due to the lack of protective cuticle. Gradual exposure of plants from polythene covering which was used to conserve and develop proper balance of RH during the establishment of these plants helped to ensure weaning as far as humidity was concerned. This greatly
helped to increase the rate of survival, compared to direct exposure to mistchamber conditions. Gradual removal of the plastic cover day by day during the acclimatization was reported in *Verbena tenera* (Hosaki and Katahira 1994) for better survival.

**Clonal uniformity**

The current problem facing the regeneration system in plant tissue culture is the occurrence of an uncontrollable instability or somaclonal variations which are highly undesirable in any conservation programme. It has been overemphasised that regenerants raised from resident meristems would be true to type (Hu and Wang 1983; Bajaj *et al.*, 1988) However, in some reports variations were detected even when shoot meristems were used (Swartz *et al.*, 1983; Pramanik and Datta. 1988) Therefore it is necessary to ensure the genetic integrity of the regenerants for the conservation of the parental genotype especially in the case of rare and threatened species. This also would be relevant for exploiting the regenerants for plant breeding and improvement and other conservation practices like cryopreservation.

In the present investigation on micropropagation, various strategies have been attempted to detect the clonal uniformity, such as evaluation of morphological and growth characteristics and comparison of chemical compounds (secondary metabolites). Cytological investigation was restricted to *R. micrantha* and isozyme analysis to *H. annulare* and *J. arayalpathra*, since the attempts at preparing good root squashes were unsuccessful in the two latter plants. Esterase isozyme banding pattern could not be detected in the case of *R. micrantha*. This might be possibly due to interference by alkaloids, despite several attempts using different buffer systems.
For the three plants studied, morphological and growth characteristics measured were uniform among the regenerated plants. Measurements of morphological characters are considered as a strategy to detect the changes among the regenerants and was also reported by Oh et al., (1995). A comparative account of chemical profile (secondary metabolite) between regenerated plant and plants raised in vivo plants showed uniformity. This observation revealed that no variation arose in regenerants. These studies are in accordance with those of Benjamin et al. (1993) on Rauwolfia sepentina and Wawrosch et al. (1994) on Achillea asplenifolia.

Similarly, results of esterase isozyme pattern investigated in H. annulare and J. arayalpathra also did not show any variation. This observation substantiated the uniformity of the clonal plants. This findings are supported by the view that biochemical traits such as isozymes, provides an alternative tool to study the extent of somaclonal variation in a manner analogous to their use in elucidating genetic variation in natural population (Bhaskaran et al., 1987).

In R. micrantha, cytological analysis of randomly selected clones showed uniform chromosome number is 44 at mitotic stage of root tip cells. Plants regenerated from explants by direct organogenesis usually exhibit cytological uniformity. Similar findings were reported Mao et al. (1995). Generally the chromosome number in Rauwolfia species is based on n = 11. It was evidenced by reports on other species of Rauwolfia (R. serpentina -2n = 22 and 44 R. canescens - 2n = 44, 88 R. densiflora -2n = 44) (Kumar and Subramonium 1985).

5.2 Analysis of Alkaloids in Rauwolfia micrantha
In the present study, investigations have been carried out to detect a few therapeutically important indole alkaloids, reserpine, rescinnamine, and ajmalicine from regenerated as well as seed-derived plants of *R. micrantha*. For the alkaloid analysis, weak basic fractions of total crude alkaloid extracts were analysed, since the alkaloids looked for were all weak basic indoles as has already been reported (IWG and Court 1977).

The TLC and HPLC analysis for these alkaloids in different parts (leaf, stem and root) indicated that ajmalicine and reserpine were present in the roots of both plants also indicated its uniformity as the regenerated plants were raised without the intervention of callus.

Quantitative determination of total alkaloid content showed that the yield was slightly higher in the regenerated plants than the seed-derived plants. This observation was similar to that made in *R. serpenina* (Ruyter et al. 1991). Generally regenerated plants have been reported to accumulative more active constituents than conventionally cultivated medicinal plants. However, when the quantity of individual alkaloids were analysed by HPLC, it was seen than ajmalicine content was more in the roots of regenerated plants than the seed derived plants. A reverse trend was obtained for reserpine. It is already known that the quantity of different secondary metabolites may show variation in regenerated plants and the donar *in vivo* plants. A similar finding has been made in *Salvia miltiorrhiza* (Shimomura et al. 1991). Taking into consideration the increased accumulation of ajmalicine, which has higher commercial value (37,000/kg (Verpoorte et al. 1993) than reserpine, regenerated plants of *R. micrantha* promises to be a good source for it. Besides, this antihypertensive agent in required only in relatively small quantities (Scragg 1995). Generally industrial interest in focussed on compounds that have high economic value though required in relatively small quantities (Scragg 1995).
In addition to the above study, a brief investigation was carried out for the analysis of alkaloids in normal root culture raised from the roots of \textit{in vitro} grown plants. The two alkaloids (reserpine and ajmalicine) which are present in the root of field-grown plants have been focussed on this study.

Most of the research on the biproduction of secondary products has been carried out in the callus/cell suspension cultures. However, differentiated cultures such as roots, shoots, embryos and the transformed hairy roots have been considered as alternative sources (Signs and Flores 1990).

The optimal media requirements to ensure proper growth and maximum production of secondary compounds has to be worked out in first. The selection of liquid medium for initiation and establishment of root culture in supported by the view that liquid root culture proved better for root growth and alkaloid production (Teshima \textit{et al.} 1988). Even though other auxins, IAA and IBA were tried for initiation of root culture, NAA performed best for the increased biomass production. All the morphological peculiarities of root cultures grown in different auxins (IAA, IBA and NAA) were quite similar as that produced on \textit{in vitro} plantlets treated with above mentioned auxins. Qualitative determination by TLC and HPLC analysis indicated that reserpine was not present in root cultures, while ajmalicine showed its presence. Generally tissue/organ cultures contain basically the same chemical constituents as those of the differentiated plant (Stockigt 1995). However, it was difficult to explain the absence of reserpine in the root cultures of \textit{R. micrantha}.

The root growth showed the usual sigmoidal pattern and a rapid growth was performed by the production of lateral roots which was a common observation. In the present study, ajmalicine production was proportional to the biomass increase is supported by the view that the production of a culture
system is the sum of growth rate, yield and biomass concentration. The decrease of ajmalicine content with a decline in growth might be assumed that its production in associated with the active mitotic phase of roots. However, quantitative estimation of ajmalicine in the root culture of present study indicated a lower content in comparison to the root of field-grown plants. In many plant tissue culture system, the quantitative significance of the synthesis of secondary metabolite is low (Van der Plas et al. 1995). The possible reason for this might be due to the conversion of ajmalicine to serpentine cannot be rooted out. But an authentic interpretation can not be possible without a detailed study.

The accumulation of ajmalicine was found to be stable even after number of subcultures (6 times). But prolonged investigation in necessary to get a complete picture about the production potential of root culture for ajmalicine biosynthesis for a long period.

The IR spectrum of isolated alkaloids, ajmalicine and reserpine was found to be identical with that of respective authentic samples. This indicates its authenticity.