DISCUSSION
DISCUSSION

The result of present studies on shoot bud culture, callus culture, estimation of primary metabolites, chlorophyll content and presence of secondary metabolites are being discussed below.

I. MICROPROPAGATION:

A. Shoot Bud Culture

To establish successful plant tissue culture and to select the suitable explants, it is essential to have knowledge on natural propagation system of plants.

The time of the year that the explants are collected from stock plants may have an influence on axillary shoot outgrowth. The best period of the year for initiating shoot bud cultures from nodal explants of *Vitex negundo* was found to be May-June. During these period nodal explants of *V. negundo* showed maximum bud break percentage, with very low or no contamination. Stone (1963) found better survival of carnation meristems isolated from donor plants during the active growing season of spring and autumn than those taken in summer and winter. The variation in bud break response is believed to be mediated by differences in endogenous levels of growth hormones which seem to be plausible explanation, but remained purely speculative in the absence of documented evidences in literature in support of such an explanation (Nehra & Kartha, 1994).

In this respect, the behaviour of explant in culture may be decided critically by the season and growth stage of the parent plant. In case of papaya, Litz & Conover (1981) reported that under Florida climatic regime optimum
conditions for papaya tissue cultures were either during hot summer months or during the transitional months of April and November. Similarly, the nodal explants from the donor plants of *Dioscorea alata* (yams) growing *in vitro* under a 16 hour light regime (inducing active vegetative growth) produced axillary shoot growth while the nodal explants from the same donor plants growing under 12 hour light regime showed either profuse callusing or no growth at all (Mantell *et al*., 1978).

The cultures initiated from nodal explants of *V. negundo* during July to August exhibited maximum contamination (100%). At times even 100% contamination was recorded in nodal explants of *V. negundo* during July-August. In the corresponding period, the bud break response of explant was very low.

Hohtola (1988) suggested that during winter months decreased contamination might be due to the better resistance of the tissue against microbes during active period of growth and/or susceptibility of microbes to the decontaminants. Anaz and Vijay Kumar (1997) attributed the high level of contamination during rainy month to the amount of inoculum present in the environment due to favourable condition during the rainy months.

Fluctuations in environmental factors in different seasons had a definite effect on shoot bud differentiation from explanted nodal segments in *V. negundo* and *Tinospora cordifolia* as similarly shown in other medicinal herbs including *Ocimum* species (Ahuja *et al*., 1982; Pattnaik & Chand, 1996), *Actinidia deliciosa* (Kumar *et al*., 1998), *Tridax procumbens* (Sahoo & Chand, 1998) and *Solanum surattense* (Swamy *et al*., 2004). Various workers have reported that the problem of microbial contamination of cultures is especially serious when the tissue is derived from field-grown material.
To ensure complete aseptic conditions of the explants it is essential to remove dirt and debris from the plant tissue. Failure to obtain proper sterilization is often due to the surface characteristics of the material/explant. Microorganisms lodged in crevices in the bark, in leaf axils or at the base of hairs, etc., may never come in contact with the steriliant because air bubbles entrapped in these positions prevent such contact of penetration to come in contact with microorganisms settled on explants.

To improve wetting of the tissue surface, a detergent or alcohol wash often precedes treatment with steriliants. Ethanol partially removes hydrophobic waxes and resins, which protect microorganisms from contact with aqueous steriliants (Kunneman & Faaij-Groenen, 1988). To move the steriliant into inaccessible areas it is also better to shake or stir the sterilizing solution vigorously. In the present study explants were washed in detergent solution in gently agitating conditions (to ensure good surface contact) and rinsed several times with distilled water.

For surface sterilization of nodal explants of *V. negundo* different concentrations of mercuric chloride were tried. Out of the different concentrations used, 0.10% mercuric chloride treatment for 10 min. proved to be effective, while at higher concentration of mercuric chloride (0.15%), hundred percent contamination free cultures were achieved but this concentration proved too toxic for explants and all the explants turned black and died after few days at this concentration. These findings are in confirmation with the previous reports where mercuric chloride was proved effective for sterilizing seeds (Suwal et al., 1988) as well as nodal explants of *Dalber gia sissoo* (1983). Several other workers have also reported superiority of mercuric chloride over other steriliants Anand & Bir (1984) in *Dalbergia lanceolaria*, Rai & Chandra (1988) in *Dalbergia latifolia*, Varghese & Kaur (1988) in *Albizzia lebbeck*, Mathur & Mukunthakumar, (1992) in *Bauhinia variegata* and *Parkinsonia aculeata*; Deora & Shekhawat (1995) in *Capparis*, and Joshi et
al. (2002) in Dalbergia sissoo. After surface sterilization the sterilant has to be removed by several washing in sterile water. Traces of sterilant left behind are not only toxic to the explant; they can also destroy some essential components of the nutrient medium, like thiamine is destroyed by traces of hypochlorite (Jaroensanti & Panijpan, 1981).

The frequency of regeneration varied with the dose of cytokinin and the type of explant (Saini & Jaiwal, 2000). Position of the explant within the plant as well as the size of explant also influences in vitro growth and development. The phenomenon whereby the position of the explant within the plant influences the in vitro growth and development (after excision) is known as topophysis. For instance, the higher the shoot is isolated from a tree, the lower the probability of formation of adventitious roots and that terminal buds grow faster than axillary buds.

The nodal explants of T. cordifolia showed maximum bud break percentage and minimum contamination of cultures during March to June. This indicates that the bud break response and contamination of cultures vary depending on the season of explant isolation from the mother plant. The nodal segments of T. cordifolia turned brown within a week after inoculation and then died. The browning of explants was effectively controlled by including 0.3% PVP (Poly Vinyl Pyrrolidone) in basal medium. Primary explants usually become dark and it has more or less been routinely assumed that this darkening is due to phenol oxidase activity (Vaaghan & Duke, 1984; Krikorin, 1994). PVP, being an absorbent, is generally used for the prevention of phenolic leaching from cut ends of the explants. Earlier Quraishi et al. (2004) had reported the use of PVP to control leaching from mature sources of Neem. In Tectona grandis, Devi et al. (1994) could overcome the injurious effects of exudate by rapid transfer of explants to fresh medium. Prakash et al., (2003) by the addition of PVP in MS medium, observed enhancement in shoot proliferation in female explants of Simmondsia chinensis, but along with BAP, they recorded increased
shoot proliferation response in male explants also. However, Kumar et al. (2003) in *T. cordifolia* and Hiren et al. (2004) in *Curculigo orchioides* found activated charcoal to be suitable to prevent leaching of phenolics in the medium from the cut ends of the explants, while in *Ocimum sanctum*, ascorbic acid was used to block the release of phenolics in the medium (Shahzad & Siddiqui, 2000).

Shoot tips and nodal segments have been used as explant at establishment stage. Of the two types of explants nodal segments responded better than the shoot tips. Nodal bud cultures have been found to be an efficient means of clonal multiplication of plants by several workers. Direct *in vitro* clonal propagation from nodal explants have been achieved in *Euphorbia lathyris* and *Euphorbia peplus* (Tideman & Hawker, 1982), *Euphorbia fulgens* (Zhang et al., 1987), *Jatropha curcas* (Sadana, 1998), *Wedelia chinensis* (Emmanuel et al., 2000), *Ocimum sanctum* (Shahzad & Siddiqui, 2000), *Tagetes erecta* (Rani et al., 2001), *Hyptis suaveolens* (Britto et al., 2001), *Celastrus paniculatus* (Nair & Seeni, 2001), *Dictyospermum ovalifolium* (Thoyajaksha & Rai, 2001), *Vitex negundo* (Thiruvengadam & Jayabalan, 2001, Chandramu et al., 2003), *Jatropha curcas* (Rajore et al., 2002), *Tinospora cordifolia* (Kumar et al., 2003), *Wedelia chinensis* (Martin et al., 2003), *Solanum surattense* (Seetharam et al., 2003), *Withania somnifera* (Vadawale et al., 2004) and *Centella asiatica* (George et al., 2004; Shashikala et al., 2005).

Among several media employed in tissue culture studies, the most suited formulation is Murashige and Skoog (MS medium) which has well-balanced micro and macronutrient components besides having a highest concentration of nitrogen compared to other media. In *V. negundo* and *T. cordifolia* the formulation of Murashige and Skoog's (1962) basal medium was found more suitable than B5 medium for establishment of nodal explants.
Shoots per explant, average shoot length and nodes per shoot were more on MS medium as compared to B5 medium suggesting that the MS medium can be used for establishment of the nodal explants of *V. negundo* and *T. cordifolia*. The growth additives and salt concentration requirement of medium varies from species to species and especially from one stage of culture development to another (Hu and Wang, 1983). The MS medium has been found more suitable than other media for establishment of explants of several species such as *Picrorhiza kurroa* (Upadhyay et al., 1989), *Plantago ovata* (Barna & Wakhlu, 1989), *Tylophora indica* (Chattopadhyay et al., 1992) and *Solanum suratense* (Seetharam et al., 2003). Earlier studies on members of Zingiberaceae, Hosoki & Sagawa (1977) also observed that MS medium was superior to other medium generally used for explant establishment during present investigations also, in case of *T. cordifolia*, a preliminary experiment carried out with the 2 basal medium MS and B5 for establishment of axillary bud explants, the explants placed on B5 medium did not responded well. This suggests that MS medium is useful for *T. cordifolia* as well as for *V. negundo*.

Growth regulator concentration in the culture medium is critical to control the growth and morphogenesis, as first indicated by Skoog and Miller (1957). Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus. On the other hand, low auxin and high cytokinin concentrations in the medium result in the induction of shoot morphogenesis. Auxin alone or with a very low concentration of cytokinin is important in the induction of root primordia.

Different plants are expected to respond differently to various cytokinins and auxins and this may be partly because of their endogenous hormonal levels. Supply of hormones in a proper sequence is important to achieve a particular response. Similarly, auxins stimulate root initiation on shoots but may inhibit or reduce subsequent growth of roots. Hence, these sequences are to be
standardized especially for various kinds of explants. It is well established that proper ratio of cytokinin and auxin is necessary for morphogenesis leading the formation of complete plantlets (George & Sherrington, 1984).

The different concentrations of BAP in MS medium influenced shoot number, shoot length and node number. In cultures raised from nodal explants of *V. negundo* maximum number of shoots were produced on MS medium supplemented with 1.0 mg/L BAP. These explants developed greater than 2 shoots per nodes while in all other concentrations of Kn, NAA and BAP developed either two or less than two shoots/explant.

The role of cytokinins in shoot organogenesis is well-established (Evans et al., 1983). Although, a small quantity of cytokinins may be synthesized by shoots in *in vitro* (Kodo & Okazawa, 1980), roots are the principle sites of cytokinin biosynthesis. It is unlikely that the meristem, shoot tip and bud explants have sufficient endogenous cytokinin to support growth and development (Hu & Wang, 1983). Hence, exogenous supply of cytokinins is essential for shoot morphogenesis. The most widely used cytokinin is BAP (McCown & McCown, 1987).


In *V. negundo*, explants showed low percentage of shoot bud induction on cytokinin free MS medium, but addition of BAP in MS medium increased shoot bud induction percentage, shoot length and number of nodes. Higher concentration of BAP (1.0 mg/L) was found suitable for establishment of explants. The optimum level of BAP for nodal explants of *V. negundo* appears to be 1.0 mg/L which is in contrast to the result observed by Thiruvengadam and Jayabalan (2001) where BAP (1.5 mg/L) along with NAA (0.1 mg/L) produced maximum number of shoots as also observed in *Punica granatum* (Rudra & Juwarkar, 2002), *Tinospora cordifolia* (Kumar et al., 2003) and *Centella asiatica* (Shashikala et al., 2005), Though the nodal explants of *V. negundo* gave good response with NAA and Kn and in combination of BAP with NAA and Kn but the best response were observed with BAP.

Flower induction was observed within 30 days of culture. *V. negundo* induced flowers from *in vitro* raised shoots on MS medium with BAP, NAA and Kn. After proliferation, it was observed that nodal explants produced large number of flowers on MS medium supplemented with BAP (1 mg/L) rather than on MS medium containing NAA and Kn alone. However, earlier it has been reported that MS medium with BAP (2 mg/L) along with NAA (0.1 mg/L)

In T. cordifolia, the axillary buds formed shoots at all the concentrations of BAP within a fortnight. No shoot bud induction was observed with shoot tip explants. At the concentration 0.1 mg/L BAP shoots were initiated with average shoot length of 4.4 cm. At higher concentrations of BAP shoots were formed but failed to elongate beyond 1-2 cm and resulted in a rosette. In all the explant types and positions, higher concentrations of BAP induced the formation of shoots with short internodes thereby confirming the well known inhibitory influence of cytokinins on shoot elongation and consequent rosette type crowding of shoots (Tavares et al., 1996; Koroch et al., 1997). A similar effect of BAP on shoot bud elongation has been reported in Pigeon pea (Geetha et al., 1998) and Arachis hypogea (Venkatachalam et al., 1999). Addition of cytokinin promotes precocious axillary shoot development.

It may be mentioned that BAP, its riboside and nucleotides have also been reported as naturally occurring cytokinins in plant tissues with other cytokinins. This may explain the improved response with BAP. BAP has also been found to be an efficient cytokinin for shoot multiplication in other species like Syzygium alternifolium (Shavalli Khan et al., 1997) and Swertia chirata (Wawrosch et al., 1999).

Of the two cytokinins used, BAP was found to be better for maximum multiple shoot induction when compared to kinetin. Generally, BAP alone
increased the number of shoots in comparison with kinetin alone or in combination with kinetin. In the present study the shoots produced with BAP were longer and more uniform in appearance than those produced with kinetin. Repeated subculturing of nodal segments from in vitro grown shoots on MS medium with BAP helped to achieve continuous production of callus free healthy shoots. Plants obtained by direct regeneration via repeated subculturing of the nodal explants from regenerated plants did not show any visible morphological changes. Retention of regenerability and true to typeness in long term culture derived plants was suggested to be due to their genetic uniformity (Na & Kondo, 1995) which is more assured by direct regeneration of plants than through intervening callus phase (Kukreja et al., 1986). BAP has been used in preference to other cytokinins to induce multiple shoots in Aristolochia indica (Remeshree et al., 1997) and Actinidia deliciosa (Kumar et al., 1998), Withania somnifera (Govindaraju et al., 2003), Wedelia chinensis (Martin et al., 2003).

The micronodes derived from explants of V. negundo and T. cordifolia, during present investigations showed maximum average shoot length and micronodes per microshoot on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L BAP respectively. The average shoot length and micronodes per microshoot being essential for further shoot proliferation. In this study, the microshoots were not directly inoculated on shoot proliferation medium, instead micronodes were cut from microshoots that had elongated on the mother explant and then inoculated on shoot proliferation medium. Perhaps, this reduced BAP requirement for growth and development of axillary shoots. So the same level of cytokinin in explant establishment and shoot proliferation media were used for several species such as Alnus nepalensis (Kaur et al., 1993), Cleistanthus collinus (Quraishi & Mishra, 1998) and Lagerstomia parviflora (Quraishi et al., 1997).

The cultures initiated from the nodal explants of V. negundo and T. cordifolia could be maintained upto five and six subcultures respectively. They
showed significant increase in microneode number with increase in subculture. Increase in shoot multiplication and maintenance of multiplication rate during the passage of subculturing has been found in *Morus alba* (Sharma & Thorpe, 1990), *Anogeissus sericea* (Kaur et al., 1992), *Kaempferia rotunda* (Anand et al., 1997), *Tridax procumbens* (Sahoo & Chand, 1998), *Vitex negundo* (Sahoo & Chand, 1998; Chandramu et al., 2003), *Passiflora caerulea* (Jasrai et al., 1999), *Crotalaria lutescens* (Naomita & Rai, 2000), *Acorus calamus* (Rani et al., 2000), *Hypericum patulum* (Baruah et al., 2001), *Punica granatum* (Rudra & Juwarkar, 2002) and *Rubia cordifolia* (Shrotri & Mukumdan, 2004). Boulay (1985) has suggested that repeated transfer of explants on multiplication media with cytokinin help in activating the plant material.

The microshoots derived from nodal explants of *V. negundo* showed rooting on half strength MS medium supplemented with 0.10 mg/L IBA as also observed in *Wedelia calendulacea* (Emmanuel et al., 2000) and *Solanum surattense* (Swamy et al., 2004). These roots were thick with secondary root hairs, which help in establishing the plantlets in the soil. The microshoots on half MS medium with IAA produced callus without root but IAA has been found to be optimal for root induction in *Actinidia deliciosa* (Kumar et al., 1998), *Acorus calamus* (Rani et al., 2000), *Celastorus paniculata* (Nair & Seeni, 2001) and *Centella asiatica* (Shashikala et al., 2005) and the microshoots on half MS with NAA formed roots with profuse intervening callus. During root initiation, callus formation at the base of microshoot was the main problem. Callus appeared even in microshoots on half MS medium without plant growth regulator. IBA has been used for root induction in most of the herbaceous species *Tridax procumbens* (Sahoo & Chand, 1998), *Jatropha curcas* (Batra et al., 1999), *Tagetes erecta* (Rani et al., 2001), *Celastorus paniculata* (Nair & Seeni, 2001), *Baliospermum montanum* (Johnson & Manickam, 2003), *Rauvolfia serpentina* (Tiwari et al., 2003), *Vitex negundo* (Chandramu et al., 2003), *Wedelia chinensis* (Martin et al., 2003) and *Withania somnifera* (Govindaraju et al., 2003; Vadawale et al., 2004) but many workers have
reported that NAA alone and in combination with other hormones can induce root formation as in *Alpinia galanga* (Anand & Hariharan, 1997), *Solanum nigrum* (Shahzad et al., 1999), *Gloriosa superba* (Sivakumar & Krishnamurthy, 2000), *Vitex negundo* (Thiruvengadam & Jayabal, 2001), *Hyptis suaveolens* (Britto et al., 2001), *Jatropha curcas* (Rajore et al., 2002), *Tinospora cordifolia* (Kumar et al., 2003), *Ocimum basilicum* (Sudhakaran & Sivasankari, 2003) and *Solanum nigrum* (Jabeen et al., 2005). IBA along with IAA has also found to be suitable for root induction as in *Vitex negundo* (Sahoo & Chand, 1998) and *Dictyospermum ovalifolium* (Thoyajaksha & Rai, 2001).

The microshoots derived from nodal explant of *V. negundo* showed increase in percentage of root induction and the number of roots with the passage into liquid medium. In liquid medium, the roots were easily and profusely formed. The enhanced rooting in the liquid medium also reported in such woody species as sweet gum and jujube is attributed to differences of imbibing substances viz. carbon dioxide and increased partial pressure of oxygen. This attribute is of particular significance as the expensive agar may be dispensed with to reduce the cost of production and the rooted plants were readily transferred from liquid medium to soil (Nair & Seeni, 2001).

The effectiveness of IBA in rooting has been reported in many species. IBA was also shown to be taken up better than other auxins in cell suspension culture of *Petunia hybrida* (Epstein et al., 1993). According to Jutta Ludvig-Muller (2000) transport velocity of IBA was markedly slower compared to that of IAA and NAA. The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus its better function in inducing roots (Nickell, 1982).

Rooting of in vitro derived shoots of *T. cordifolia* was achieved on MS medium supplemented with varying concentration of IBA after 4 weeks of culture. Auxin (IBA) at the concentration of 0.50 mg/L induced root formation,
which was accompanied by shoot elongation. But earlier it has been reported that NAA along with BAP promoted both root and shoot regeneration and finally, complete plantlets (Kumar et al., 2003).

B. Callus Culture

Callus cultures were raised from three types of explants of *V. negundo* namely stem segments, internodal segments and leaf segments. All the three types of explants showed callus formation on MS medium supplemented with 2,4-D. Hu & Wang (1983) proved superiority of 2,4-D over other auxins for the induction of callus and strongly antagonize any organized development. Both the internodal segments and stem segments produced good amount of callus biomass in MS medium supplemented with 2, 4-D. In case of *T. cordifolia*, the internodal segments formed more callus biomass than stem explants. In most of the dicotyledonous species 2, 4-D is the principal hormone required for the induction of callus and for the maintenance of culture (Sunderland, 1973; Britto et al., 1995 and Iyer et al., 1998), *Rauvolfia serpentina* (Parveen & Elahi, 1987), *Crotalaria rustica* (Begum & Bai, 1995), *Plumbago rosea* (Harikrishnan & Hariharan, 1996), *Ocimum sanctum* (Shahzad & Siddiqui, 2000), *Ocimum basilicum* (Anilkumar et al., 2005). However few workers have reported the use of 2, 4-D along with kinetin for callus induction Kumar & Bhavanandan (1988) in *Plumbago rosea*, Barna & Wakhula (1989) in *Plantago ovata*, Sarasen et al., (1994) in *Hemidesmus indicus* and Sudhakaran & Sivasankar (2003) in *Ocimum basilicum*. NAA either alone or in combination with other plant growth regulators have also been found optimum for callus induction e.g. Cowpea (Muthukumar et al., 1996), *Solanum nigrum* (Shahzad et al., 1999), *Withania somnifera* (Govindaraju et al., 2003) and *Andrographis alata* (Nagaraju et al., 2003) whereas in *Solanum psuedocapsicum* IBA along with Kn has been standardized for callus induction (Liang et al., 1994).
Variation in callus weight of explants from different sources may be attributed during callus formation and endogenous levels of growth regulators. Callus formation is the net result of explant source and the cellular environment (Zankowski and Rost, 1990). The proliferative activity of cells of the explant may be governed by several factors including endogenous concentration of growth regulators at the time of excision, capacity to synthesize growth regulators and essential metabolites (George & Sherrington, 1984) and sensitivity to exogenous growth regulators (Trewavas, 1982).

The reports of Krueger (1982), Yamamoto & Yamada (1986) and Brission et al., (1988) pointed out that the in vitro derived calli act as an alternative to the medicinally useful plant parts for the biosynthesis of active constituents (Nagaraja et al., 2003).

The callus derived from various types of explants of both V. negundo and T. cordifolia on medium showed fresh growth on shoot induction medium but failed to differentiate inspite of several manipulations. It appears that the calli derived from these explants of V. negundo and T. cordifolia are recalcitrant. Callus formation without subsequent differentiation has been observed in cultures of Boswellia serata (Prakash & Chand, 1999) and leaf callus of Madhuca latifolia (Singh & Bansal, 1998).

II. ESTIMATION OF PRIMARY METABOLITES:

The data obtained during present investigations revealed that the regenerates are richer in primary metabolites when compared to the normal plant suggesting the possibility of gene amplification in the regenerates. The most common precursors of alkaloids are aminoacids and other small biological molecules (Constabel, 1974). There is possibility that increase in protein level in regenerated callus compared to normal plants resulted in increased production of alkaloids. Saponins are plant glycosides, derived their names from their soap
like properties (Anneosbourn, 1996). Precursors for glycosides are carbohydrate moieties.

From the presently recorded data, it appears that regenerates have better synthesizing machinery than normal plants which is probably related with high rate of photosynthesis in regenerates as supported by higher amount of total chlorophyll in regenerates.

III TEST FOR SECONDARY CELL METABOLITES:

Root, stem and leaf of *V. negundo* and *T. cordifolia* investigated presently, exhibited differences in the presence of some of the secondary metabolites.

Studies have been made on many plants to identify important biological compounds either in parts of natural plants or in *in vitro* cultured callus. It is important to compare the rate of production of secondary metabolites under *in vivo* and *in vitro* conditions. There are many reports that in many cases *in vitro* production of biologically active compounds were higher when compared with *in vivo* as in case of *Indigofera* species where leaves of the species have higher rotenoid content but their callus cultures have still higher content than the respective *in vivo* samples (Kumar, 1995). It may be concluded that the regenerated plants and callus have proved to be better than normal plants in synthesizing primary and secondary metabolites, which can be commercially exploited.

Advantages of extracting secondary metabolites using plant tissue cultures are (i) the source of these metabolites i.e., most of the higher plants have specific agro-climatic requirements. Hence, specific metabolites can be produced all through the year even in places where crops are not grown. (ii) the already limited supply of these raw materials cannot be exhausted considering
the future needs (iii) it has also been found that cells under culture tend to produce greater amount of these metabolites than that is accumulated in nature.