DISCUSSION
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Generally the Albizia chinensis is propagated through seeds only and the genetic variation crept in the seedlings. It is therefore, difficult to obtain uniform seedlings with desired genotype. But till date vegetative propagation was not at all successful in the genus. Therefore, as a matter of fact, to get uniform planting materials from selected disease free and elite plants, only tissue culture method backed by micro-clonal propagation can provide the answer to the large scale propagation of *A. chinensis* tea industry.

For the obvious reason in the present investigation emphasis has been given on axillary bud culture from selected plants with a view to get disease free plantlets and maintain genetic homogeneity throughout the process of multiplication and also tried to get plantlets from callus derived from different plant parts to get diversity for selection with respect to morphology.

Many workers reported the *in vitro* propagation of leguminous spp. (Tommer and Gupta 1988a, 1988b; Phukon and Mitra, 1983; Jaiwal and Gulati, 1991). But most of them however, worked on *in vitro* grown seedlings only. The present study has several advantages as well as complete *(i.e. multiplication to establishment in the field) one with respect to simplicity, high rate of multiplication, quick rooting and finally establishment in the field.
5.A. Direct Regeneration Of Multiple Shoots From Axillary Buds Through Micropropagation:

5.A.1. Sources Of Explant:

The study shows that it is possible to established *in-vitro* culture of *A. chinensis* from axillary buds of matured field grown plants selected from well developed trees. For *in-vitro* culture of explants from matured tree, Bonga (1985) emphasized that it is important to choose the explants from the plant parts of the tree that remain more or less juvenile. In *A. chinensis* the axillary shoots which start growing, juvenility is retained in those new shoots.

Shoot tips were collected for this experiments from the selected disease free trees at the first flush season, when a number of new shoots were available. The other workers had taken explants from the *in-vitro* grown seedling, where the possibility of having wide variations among the explants exists. But in the present investigation the possibility of occurrence of variation is very limited, because the explants cultured was collected from a single mother plant, and the commercial saplings derived from such restricted number of mother plants are called clones.

Generally the size of the explant determines the survival of the culture (Murashige, 1974). In our trial, this problem has been overcome removing the stipules and leaf primordia in maintaining bud size at 1 cm. But survival rate was very low only 50% . In small size explants, the apical
domes lack developmental capacity due to their dependence on the adjacent leaf and stem tissues for hormonal resources (Hu and Wang, 1983).

5.A.2. Contamination Problems:

The surface of the field grown plant carry dust and a number of microflora, which although do not affect the plants, can multiply rapidly in the culture medium and it become very difficult during the monsoon season due to rapid bacterial contamination as was earlier reported by Zimmerman (1986), Sarwar (1985), Fritch and Camper (1987) Hadlemen et al. (1987), Demand et al. (1982). Therefore, it is very difficult to avoid external contamination caused by endogenous bacteriological infection, although it is relatively easy to avoid by surface contamination of the field grown explant. During the rainy season water borne pathogen on the buds and their contamination was found to be high.

De Ropp (19-191, Philips et. al (1981), Chen et al. (1983) has used antibiotics solution to avoid bacterial contamination. Haldman et al. (1987) used rifampicin to control the bacterial contamination. However, using antibiotics some time caused damaged of the inoculum (Piezik, 1988). In the present study, almost 30% of the explants were free from contamination of bacterial and fungi due to surface sterilization of 0.05% (W/V) mercuric chloride 0.5 (W/V) bavistin and 0.01% (W/V) streptomycin sulphate.
5.B.3. Browning Of The Explant:

Browning on discolouration of the explant when placed in culture medium was reported in different waxy
sp. Jacquot (1964), Nitsch and Strain (1969) and Pryor (1961), due to its phenolic exudation. The browning of the
explant in Eucalyptus, Cressweil & Nitsch, (1974), (1975),
(1976), Sarwar (1985), Arulpragasam and Latiff (1986) have
also reported it. Zimmerman (1985) reported that repeated
transfer to fresh medium daily for a periods up to several
months gave good result. In this experiment we used Zimmer-
man's advice to overcome the problem. Due to the delicacy of
the shoot tips only axillary buds were used for the experimen-

5.A.4. Basal Medium:

There is no general purpose medium for shoot tip
culture. Phukon and Mitra (1983) used MSBM for \textit{In Vitro}
regeneration of \textit{A. Guareaissima},Jaiwal and Sulati (1991) on
\textit{Tamarindus indica} (L), and Tomar & Gupta (1986a & 1986b)
used B5BM and achieved success. In this experiment also
MSBM was found to be the best for multiplication of shoot
bud & callus initiation and proliferation and B5BM was best
for regeneration of shoot bud from cotyledon callus. In
this experiment MSBM, B5BM and WBM was tried but MSBM and
B5BM actually augmented the growth due to high nitrogen
content.

5.A.5. Effect Of Different Cytokinin:
Generally cytokinin is important for the morphogenesis and growth of the in-vitro culture (George and Sherrington 1984). In case of field growing tea plant, the cytokinin activity is more during the intensive growth of shoots (Margvelashvili, 1986). The present investigation proved that the type and concentration of cytokinins affect the number of shoot produced (Table No. 4, 5 and 6). Similar observation was reported on rose (Hasegawa, 1980; Raout et al., 1989a and 1989b) and crab apple (Sinha, 1982). In the present study BA at 2 mg/l induced maximum axillary shoot proliferation, which was more than that obtained from Kn. In apple, Lengerers and Janich (1980) compared BA, Kn and 2iP, while Hutchinson (1982) compared BA, Kn, Zeatin and 2iP. In both the cases, BA was found to be the best. In Gardenia jasminoides (Economou and Spanoudaki, 1986) more number of shoots were produced in cultures grown in the medium supplemented with BA then that with 2iP and Kn. They have also found in shoot tip cultures of Elaeagnus angustifolia, that BA at 0.5μM resulted 3.5 fold multiplication rate over control while, Kn at 0.5μM resulted in 1.3 fold multiplication rate over control. 2iP at 0.5, 5.0 and 50.0μM failed to induce multiple shoot formation.

The effect of BA on shoot multiplication was observed on A.odoratissima (Phukon and Mitra, 1983), Leucana (Dhawan and Bhogwani, 1985) in Phaseolus vulgaris (Mallik & Saxena 1991) in Albizia (Borthakur, 1992) in Acacia furtifiz (Nandwani 1995 in Accacia auroculiformis while in Tamarin-
*dus indica* culture (Mascarenhas et al. 1982) used mixture of Kn and BA. In the present investigation BA at 2mg/l in MSBM produced 10-12 shoots per explants in 2 months. The number of shoots produced was not strictly related to the number of axillary buds of the explants, that could be induced to grow. Shoot first developed from axillary buds and then from the basal buds of the new shoots. Shoot proliferation resulted from continuous action of the cytokinin during culture. The basitonic growth would indicate that the effect of cytokinin was more directly on the buds in contact with the media.

High concentration of BA induced shoot proliferation but inhibited elongation and quality of the buds. This problem has been encountered in in-vitro studies of number of species like *Cordyline terminalis*, *Quercus shumardii*, *Castanea mollissima*, *Camellia japonica*, *Carya illinoensis*, *A. odoratissima* and *C. sieamea*. In *Cordyline terminalis* when the BA concentration was increased to 2mg/l, the shoot size diminished with increasing shoot number (Evaldson and Welander, 1985). Bennett and Davies (1986) reported in in-vitro studies of *Quarucus shumandii* the shoots produced at 5mg/l were stunted, swollen and exhibited abnormalities in leaf morphology. Optimum concentration of BA was found to be 1mg/l which yielded multiple shoots without any callus formation. In *Castanea mollissima*, BA at 0.1 mg/l induced shoot formation and elongation and promoted callus growth (Qi Guang et al., 1986). In *Camellia japonica* BA at 1 mg/l was found optimum for shoot proliferation, while 2 mg/l
produced the highest shoot number but the length of the shoot was much less. At 5 mg/l of BA shoots were very short and became succulent with thick and brittle leaves (Samartin et al., 1984). In the case of *Carya illinoensis* the optimum concentration of BA for shoot multiplication was 4 mg/l. At 8 mg/l of BA the shoot number decreased while at the highest (32 mg/l) concentration all the explants died (Wood, 1982). Phukon and Mitra (1983) used 1 mg/l of BA for obtaining multiple shoots in *A. odoratissma*. Increasing the BA concentration resulted in the decreased number of shoot buds but formation of callus at the basal end.

Gibberallin or Gibberelic acid (GA$_3$) are synthesized by most explants. When GA$_3$ was supplemented its function is primarily for bud elongation (Schnabdranch and Sink, 1979). Only few workers use GA$_3$ in multiplication medium (stage II medium). The concentration range used was exceedingly low compared with other *in-vitro* culture system. Its role is essentially for axillary bud elongation (Schnabdranch and Sink, 1979). Wochok and Sluis (1980) observed that treatments of GA$_3$ on *Atriplex* shoot explant stimulated not only shoot elongation but also enhancing cytokinin combination. In the *in-vitro* culture of apple when supplemented with GA$_3$ (0.29uM), no positive effect was observed by Lindergan and Janick in 1980. Ide et al. (1987) observed the presence of GA$_3$ overcome inhibition of bud break in *Betula grossa* culture but Rajekkar (1994) has observed no such effect of GA$_3$ in *Grevillea robusta* culture. In the present observation presence of 0.25 mg/l of GA$_3$ in culture medium
showed highest number of shoot multiplication rate in low concentration of BA (1 mg/l) and bigger bud size.

5.A.6. Shoot Elongation

As a result of frequent transfer of the explants into cytokinin medium, formation of stunted shoots were observed. Further, it was difficult to induce rooting in small/stunted shoots in culture. These necessitated to elongate the stunted shoots in cultures. To elongate the stunted shoots, several ways are normally practised (i) addition of growth hormones like GA₃ in the medium which would induce the cell elongation, (ii) addition of weak cytokinins, (iii) alteration of light intensity and (iv) reducing the basic salts concentrations (Maene and Debergh, 1986). In the present study reduced concentration of BA (1 mg/l) was used to get sizeable shoots from stunted shoots as well as from in-vitro nodal cuttings. Similar results were observed by San Jose et al. (1984) in chestnut and Penuelar et al. (1988) in walnut.

Further, size and in-vitro history of the shoots are important factors in determining the rooting of shoots especially in-vivo condition (Maene and Debergh, 1986). All our attempts to induce roots on excised buds from the stunted shoot clusters were vain. These necessitated a transfer of these isolated buds to elongation medium (MSBM + 1 mg/l of BA) for a period of three to four weeks. Pretreatment on such a medium allowed the elongation of shoot buds which facilitated easier isolation as well as subsequent root
initiation and further growth of roots when transferred to a rooting medium.

5.A.7. Rooting

Shoots which were inoculated in IBA (1 mg/l) containing MSBM were rooted but the number of roots and percentage of rooting were very low. Type of auxin and its concentration played a major role in induction of rooting in A. chinensis. NAA had no effect on rooting except callus formation whereas lower concentration of IBA (0.5 mg/l in 1/2 strength of MSBM) induced roots in 20 -25% of the shoots. Supplement of activated charcoal in the medium containing 1 mg/l of IBA in 1/2 strength of MSBM induced roots in 50 -60% of the shoots. The shoots transferred to WBM without IBA induced roots in 50 -60% of the shoots whereas, the treatments with 1mg/l of IBA in WBM induced roots in 100% of the shoots.

In the method of rooting, the shoots were grown in *in-vitro* conditions, and therefore, they were sterile. As the cuticle in the leaves were very thin fungal infection was a problem. This was overcome by treating the shoots with suitable fungicides (Bavistin) after transplanting in the polythene sleeves. The establishment of microcuttings from culture vessels to soils was often difficult. This is because the growth conditions inside the culture vessels induce normal morphology and physiology in the plants (Ziv, 1986). Hence, the acclimatisation phase is the most important final step in tissue culture programme. Great care
is needed with respect to maintenance of relative humidity and composition, texture and pH of the rooting medium (soil) in order to obtain consistent results. Once rooted, the leaves of the *A. chinensis* plantlets would not normally be prone to dessication, since they are considerably cutinised. In the present study we have obtained survival rate more than 80% plantlets in the field.

5.B. Indirect regeneration Of Shoots Through Callus Culture:

5.B. Indirect Regeneration Of Shoot From Callus Or Organogenesis:

5.B.1. Basal Medium:

There is no such general medium for induction of callus from different plant parts and organogenesis from the callus. Phukon and Mitra (1983) used MSBM for callus induction and differentiation of shoot bud from the callus. Tommer and Gupta (1988a, 1988b) used BgBM for induction of callus and regeneration of shoot buds from the callus of *A. amara, A. lucida, and A. richardiana*. Jaiwal and Gulati (1991) used B5BM for regeneration of shoot bud from *Tamarindus indica* (L) and achieved success. In the present investigation MSBM and BgBM were tried and MSBM was found to be better in induction of callus. BgBM showed best result with IAA and BA in induction of shoot bud from cotyledon callus only.
5.B.2.Effect Of Auxin:

The auxins have major effect on the growth and metabolism of plants. In some cases it is stimulatory and in some cases inhibitory. The stimulatory effect of auxins on growth vary considerably between tissues.

The application of wide range of concentration in the media, often lead to identification of an optimum concentration for growth. Gauthert (1955) used NAA for cultivation of entire tissue, this auxin stimulates proliferation of callus, at a same time inhibits the bud initiation. Levine (1947) showed that carrot (Daucus sps.) plantlets have been formed in presence of NAA. In Dauglus fir for adventitious buds formation require both cytokinin and auxin, NAA is more effective than IBA or IAA (Cheng, 1947).

Formation of organogenesis from cultured tissue (callus) is controlled by three factors, i). the inoculum, ii) the medium (auxin cytokinin ratio) and iii). culture condition (Thrope, 1982). In a given callus a few cells are involved in the initiation process. Skoog(1944) reported that to some extent in in-vitro organogenesis could be chemically regulated. He found that addition of auxin to the medium could stimulate root formation, whereas shoot initiation was inhibited.

The high auxin concentration commonly used for callus induction and maintainence are generally inhibitory to morphogenesis and the most cases have to be replaced by a precise auxin - cytokinin balance to obtain proper root and
shoot primordia. However, inspite of extensive trials, varying the auxin - cytokinin ratio did not produce morphogenesis (Cheons et al., 1982). For example, Lee and Defossard (1974) tested 175 different auxin - cytokinin combinations on callus culture of *E. bancroffii* with no distinctive morphogenetic response.

Though many workers reported callus induction from the different parts of the leguminous plants. Phukon and Mitra (1983) observed callus formation in every part of seedling cultured in NAA and 2,4-D in addition with BA or Kn at 1 mg/l level in MS medium. Tommar and Gupta (1988a) got callus on Hypocotyl explant of *A. richardiana* using BA, Sankhala et al. (1993) in *A. julibrissin* hypocotyl using Uniconazole, paclobutazone etc. with B5BM. In the present study callus was observed in all the explants of the seedling e.g. Root, Hypocotyl, leaf and cotyledon in 2,4-D & NAA in addition with BAP or Kn. High concentration 4 mg/l cytokinin inhibit callus formation in root and leaf explant. IAA and IBA also produced callus on Hypocotyl and cotyledon explant, but in leaf IBA produced root initiation in combination with 1mg/l cytokinin.

5.B.3.Root Explant

Root culture technique was devised to study the infection of legume roots with *Rhizobium* (Raggio and Raggio, 1956; Raggio et al., 1957). Isolated roots from *Phaseolus vulgaris* and *Glycine max* was tried for symbiotic studies by
Cartwright (1967) and Torrpy (1978). The first successful root culture was reported by Whites (1934) with tomato roots, less success has been achieved in starting root cultures.

Phukon and Mitra (1983) obtained callus from root explant of A. odoratissima and obtained callus in the treatments containing 2,4-D combind with BA or Kn but achieved no success in regeneration of shoot from leaf callus. In IBA containing media with low (1 mg/l) cytokinin developed secondary roots from the root explants. Cellarova et al. (1982) tried on Matricaria chamomilla and obtained roots only, no shoot production has been achieved. In the present investigation callus was obtained in the treatments containing 2,4-D and NAA. IAA had failed to produced callus on root explant, but IBA produced secondary and tertiary roots from the root explant, in presence of 1 mg/l of BA or Kn. Presence of high level (4 mg/l) of cytokinin (BA or Kn) inhibited root development or callus growth.

5.B.4. Leaf Explant:

Several workers obtained callus from the leaf explants of different genera. Raman (1983) reported to get callus from leaf disc of Salpiglossis callus. Phukon and Mitra (1983) obtained callus from A. odoratissima leaf using 2,4-D and NAA in combination with BA or Kn in MSBM. But they failed to differentiated shoot bud and from the same callus. In 1991, Singh and Mallick observed callus development from the leaflets of woody legume Sesbania biglobosa (Jacq.) using 0.5 mg/l of BA and 2 mg/l of 2,4-D in MSBM
and successfully regenerated shoot buds in 2 and 4 mg/l of BA with 15% V/V coconut milk in MSBM. Callus was observed from the leaf of *Turnera diffusa* using 2,4-D and BA in MSBM and B5BM and successfully regenerated shoot bud from the leaf callus by Dias Rondro and Alcarz - Melendaz in 1987.

In the present investigation leaf callus was obtained in the MSBM and B5BM with 2 and 4 mg/l of 2, 4-D or NAA in combination with BA or Kn (at 1mg/l). Shoot bud was differentiated from the leaf callus in MSBM containing IAA (0.5 mg/l) or NAA (0.5 mg/l) in addition with 2 and 4 mg/l of BA. Kn had failed to differentiated shoot bud from leaf callus.

5.8.5. Hypocotyl Culture

Various workers obtained callus and differentiated shoot bud from hypocotyl explant of different genera. In *A. odoratissima* Phukan and Mitra (1983) obtained callus using NAA, IBA and 2,4-D in combination with BA or Kn in MSBM or B5BM and successfully regenerated shoot bud in the MSBM containing NAA and BA. They also reported that Kn had failed to differentiate shoot bud. Barua and Wakhlu (1989) obtained morphogenesis of 8 days old callus of *Plantago ovata*. Frossak, Tommer and Gupta (1988a) observed that sucrose was having a major role in differentiation of organogenesis or embryogenesis. They noticed 2% sucrose was optimum for production of somatic embryo, but 4% sucrose inhibited embryogenesis. Tommer and Gupta (1988b) also reported that they
had successfully differentiated shoot bud from hypocotyl explants of *A. lucida*, *A. amara* and *A. richardiana*. They observed that higher concentration of auxins in the medium enhanced rooting and callusing, but BA enhanced the differentiation of Shoot-Bud.

In the present study callus production was observed in all the treatments containing IAA, IBA, NAA and 2,4-D in combination with BA or Kn. But 2,4-D had failed to differentiated shoot bud from hypocotyl callus. NAA showed best result in shoot bud differentiation, IBA also produced shoot bud to a lesser extent, but in combination with the lower concentration (1 mg/l) of cytokinin (BA or Kn) produced 1-2 roots from the callus. Kn Differentiated shoot bud only in the presence of NAA, in other cases it had failed to differentiated shoot bud from hypocotyl callus.

5.B.6. Cotyledon Culture

A few workers successfully differentiated shoot bud from the cotyledon explant. Sinha and Mallick (1991) obtained callus from Cotyledon explant of *Sesbania bispinosa* (Jacq.) in the medium containing 2,4-D singly or in combination with BA. Use of low level of BA (0.2-0.5 mg/l) with 2,4-D (2 mg/l) supported better differentiation and callus growth. Addition of 15% CH in the callusing medium produced green and compact calli and also differentiated shoot bud in MSBM containing BA (2mg/l) and 15% V/V CH. Ozcan et al. (1992) successfully regenerated adventitious shoot directly
from the immature cotyledon of two different cultivars (Orb. and Consort) of pea (*Pisum sativum*) using 0.5 mg/l of BA and 4 mg/l of NAA. Ganga and Allaveana (1991) noticed morphogenesis in immature cotyledon of pea using 2iP and NAA in 1/2 strength of MSBM. Substitution of sucrose with glucose gave strongest enhancement of the morphogenetic process. In 1983, Phukon and Mitra obtained callus from cotyledon explant of *A. odoratissima* using 2,4-D or NAA in combination with BA or Kn. But they have failed to differentiated shoot bud from cotyledon callus.

In the present investigation callus was obtained in MSBM or BgBM containing 2,4-D, NAA, IBA or IAA in combination with BA or Kn. Presence of IBA in low concentration (1 mg/l) of cytokinin (BA or Kn) induced roots from the callus. 2,4-D showed best result in production of callus. Callus was soft, friable and white in colour. IAA and NAA produced green and compact callus. Best shoot bud differentiation was observed in the treatments of 0.5 mg/l of IAA in combination with 2mg/l of BA in BgBM and 0.5 mg/l of NAA with 2 and 4 mg/l of BA in MSBM. Kn has failed to differentiated shoot bud in MSBM or in BgBM. Presence of 2,4-D in the medium failed to differentiated shoot bud from cotyledon callus.