Discussion

The Indo–Burmese region, including Manipur is a biodiversity hot spot region, which treasures a wide array of species that are economically important and represents a vast untapped reservoir of genetic resources. 19 genera and 88 species of Zingiberales have been reported from North–eastern Himalayan region including Manipur–Nagaland belt (Prakash and Mehrotra, 1996b). The species belonging to families Zingiberaceae and Costaceae are known to possess useful bio–active molecules. These bio–active molecules would help in designing and manufacturing important medicinal drugs. Various authors have isolated and studied the biological activities of secondary metabolites accumulated in the rhizomes, stems and seeds. The existence of many of these Zingiberales have been threatened due to deforestation, lack of awareness of its economic potential, jhum cultivation, and conversion of wetland ecosystem into agricultural land. Ex situ techniques involving conventional propagation through natural regeneration and vegetative cutting may be slow and cumbersome, in contrast to this in vitro culture offers a sustainable and viable tool for rapid propagation and storage of germplasm. Plant tissue culture has already been effectively applied to mass multiply many members of these family.

Sucrose has been widely used as carbon source for various plant tissue cells in culture but its concentration varied from 2–6 % with different tissues and plants. Most of the reports on micropropagation of Zingiberales used optimum 2–3% sucrose for shoot multiplication from shoot tip explants (Chaturvedi et al. 1984; Borthakur et al. 1999; Chirangini et
al. 2005; Thoyajaksa and Rai, 2006). In the present investigation, different concentration of sucrose was required for shoot multiplication from different explants. In case of *Costus pictus* and *Costus speciosus*, 5% sucrose was significant for shoot multiplication. However, in bud–break experiment, *Costus pictus* showed bud-break in lower sucrose concentration in comparison to *Costus speciosus*. Therefore, it may be inferred that as axillary bud of *Costus spp.* are dormant, higher concentration of sucrose is essential as the source of activation energy. In natural habitat also, one or two axillary buds of *Costus pictus* sprouted to plantlets after the main stem have flowered. In *Costus speciosus*, any such observation was not made. This may perhaps explained the requirement of higher concentration of sucrose for breaking the bud dormancy of *Costus speciosus*. In the case of *Alpinia allughas* and *Alpinia galanga*, average number of shoots increased as the concentration of sucrose increase from 1-3% sucrose, however, further increase was inhibitory. This is in agreement with earlier reports in *K. galanga*, *K. rotunda* (Chirangini et al. 2005) and *Zingiber cassumunar* (Chirangini and Sharma, 2005).

The hydrogen ion concentration of the media affected growth of the tissue by altering the pH of the cells, or by affecting the availability of nutrients to the cells because higher pH induced precipitation of phosphate, gelatination of agar and destruction of vitamins and growth regulators. Though majority of plant tissue has optimum pH from 5.0–5.5 (Butenko et al., 1984) yet the pH range is variable for individual plant tissues. During
the present investigation, it was found that the bud-break frequency of *Costus speciosus* and *Costus pictus* were highest in the pH range of 5.4–6.0. Shoot multiplication in *Alpinia allughas* and *Alpinia galanga* was significant in the pH range of 5.1–5.7.

The organogenetic route of *in vitro* cultured dormant rhizomatous bud/eye starts responding by breaking the outer thick sheath followed by emergence of shoot primordium. Induction of shoot primordium from rhizomatous eyes/buds requires either auxin or cytokinin alone or in combination. The quality and quantity of plant growth regulator required for initiation of microshoot were found to vary with the species. In *Alpinia allughas*, multiple shoots were induced after the main bud have differentiated, whereas in *Alpinia galanga*, the multiple shoots were induced along with elongation of buds. The present study shows the importance of cytokinin and auxin for microshoot induction. The frequency of microshoot induction on MS medium without growth regulators was very low in both *Alpinia allughas* and *Alpinia galanga*. Incorporation of 1-9µM BAP alone or in combination with 0.6µM NAA improved the incidence of microshoot induction. In both *Alpinia* species, high frequency of microshoot induction was obtained at 5µM BAP and 0.6µM NAA. Earlier works on *C. zedoaria* (Yasuda *et al.*, 1988), *K. galanga* and *K. rotunda* (Anand *et al.*, 1997, Geetha *et al.*, 1997, Chirangini *et al.*, 2005) and *Zingiber cassumunar* (Chirangini and Sharma, 2005) have suggested that multiple shoot
induction required a specific ratio of auxin and cytokinin that varied with species and physiological status of the explants. This is in agreement with our present findings that incorporation of auxin with cytokinin yield higher frequency of microshoot induction. With an increase in the concentration of BAP or Kn or NAA, the frequency of microshoot induction decreases in *Alpinia galanga* and *Alpinia allughas* after achieving maximum at 5µM BAP or Kn.

The result of the present study showed a higher number of average shoot in BAP and NAA supplemented medium as compared to Kn and NAA supplemented medium. Bhati *et al.* (1992) have reported that BAP, the least expensive cytokinin source, is the most suitable cytokinin for shoot multiplication followed by kinetin. In *Costus speciosus* (Chaturvedi *et al.*, 1984) and *A. microstephanum* (Thoyajaksa and Rai, 2006), higher number of multiple shoot in BAP-enriched medium as compared to Kn-enriched medium have been reported. In the present investigation, induction of multiple shoot in *Alpinia allughas* and *Alpinia galanga* was possible in medium supplemented with auxin and cytokinin. Several workers have successfully induced multiple shoot in medium incorporated with cytokinin and auxin, viz. *K. rotunda* (Anand *et al.*, 1997; Chirangini and Sharma, 2005), *K. galanga* (Anand *et al.*, 1997; Chirangini *et al.*, 2005), *C. zedoaria* (Yasuda *et al.*, 1988), *Costus speciosus* (Chaturvedi *et al.*, 1984, Malabadi *et al.*, 2005) and *Zingiber cassumunar* (Chirangini and Sharma, 2005). This is in agreement with findings in *Alpinia allughas* and *Alpinia*
galanga in which highest number of multiple shoots was induced in medium supplemented with higher BAP and lower NAA level. Borthakur et al. (1999) have reported induction of 8 shoots and shoot length of 6.7 cm in Alpinia galanga within 8 weeks in medium enriched with kinetin alone. In the present investigation, average of 4.60 ± 0.21 shoots and length of 6.7cm was achieved within 4 weeks in medium supplemented with 5μM BAP and 0.6μM NAA.

Nadgauda et al. (1978) reported that root initiation and development declined with increasing levels of BAP in turmeric tissue culture. In the present investigation, the microshoots both multiply and produce roots in medium enriched with 2μM BAP and 3–9μM NAA or IAA. IAA or NAA in conjunction with BAP was effective on root induction in Alpinia allughas and Alpinia galanga. The highest numbers of root were induced in medium supplemented with higher auxin and lower cytokinin concentration. The roots were thin and fibrous. Simultaneous production of multiple shoot and roots without additional treatment have been reported in K. galanga and K. rotunda (Chirangini et al. 2005), Zingiber cassumunar (Chirangini and Sharma, 2005) and Alpinia galanga (Borthakur et al., 1999). In our experiment also, simultaneous production of root and shoot was observed in Alpinia allughas and Alpinia galanga. However, rate of root production and elongation increases at higher level of auxin and lower level of BAP. In Alpinia allughas, only the main shoot elongated in medium supplemented with 5μM BAP and 0.3–1.2μM NAA except in 5μM
BAP and 0.6μM NAA. However, when concentration of BAP was lower and that of NAA was increased, the proliferating shoots elongated along with the main shoot. Maximum shoot length was achieved on medium supplemented with 7μM NAA, further increase was inhibitory. These results are in agreement with those of Inden et al. (1988) that high concentration of growth regulators reduced shoot elongation in ginger. In *Alpinia alllughas*, after achieving maximum root length in 2μM BAP and 5μM NAA, decrease in root length was observed with increase in NAA. It is in agreement with known fact that auxin promotes growth of intact root, but at supra–optimal concentration, it inhibits growth of roots as auxin induces the synthesis of the plant hormone ethylene, a root growth inhibitor. However, in 2μM BAP and 3-9μM IAA, increase in root length was observed with increase in IAA concentration. It has been reported that IAA is heat labile, decrease rapidly in the light and its oxidative degradation is accelerated by the presence of MS salt, whereas NAA and 2,4-D are not oxidized in light or medium (Dunlap et al. 1986). Hence, it may be inferred that the concentration of IAA was not supra-optimal due to degradation of it in presence of light, MS salt and heat.

Induction of callus was observed from root tip in MS medium supplemented with 2μM BAP and 9μM IAA. In the present experiment, when the IAA concentration was increased to above 9μM, the rate of callus proliferation increased. However, callus turned brownish in 15μM IAA. The callus produced were white–greenish, friable, granular and
embryogenic. Regeneration of plantlets through organogenesis were observed within 8 weeks. In the present work, plantlet regeneration increased when calli were subcultured on medium supplemented with higher BAP level of 5μM and lower NAA level of 0.3-1.2μM. The highest average number of adventitious plantlet (11.50 ± 0.34) were observed on 5μM BAP and 0.9μM NAA supplemented medium. This is in agreement with earlier report in *Curcuma* spp. (Yasuda et al., 1988), *K. galanga* and *K. rotunda* (Chirangini et al., 2005). Requirement of different concentration of PGRs and different PGRs for induction of callus and development of shoot primordial, in leaf base callus of *Curcuma longa* and *Zingiber officinale* have been reported (Babu et al., 1992; Salvi et al., 2001).

In the natural habitat, the axillary buds of *Costus* species are usually dormant. However, in the case of *Costus pictus*, development of axillary bud below the flowering head to plantlets was observed. These plantlets when excised and transferred to soil, developed to matured plants and proliferate vegetatively. In *Costus speciosus*, such cases were not observed. There has been no report on nodal segment culture of Costaceaous species. However, nodal segment culture have been reported in numerous tree species like *Wrightia tomentosa* (Purohit et al., 1994), *Azadiracta indica* (Arya et al., 1995) and also in bamboo species like *Dendrocalamus longispathus* (Saxena and Bhojwani, 1993) and *D. giganteus* (Ramanayake
and Yakandawala, 1997). The present studies have shown that cutting the stem to segment and culturing them on suitable medium supplemented and culturing them with suitable PGRs can break the dormancy of the bud. Literature surveys have revealed many possible reason. According to Pilate et al. (1989), auxin make the shoot apex a sink for cytokinin from the roots and decapitation increases the accumulation of cytokinin in axillary bud. Langridge et al. (1989) demonstrated with transgenic plant that contained the genes for bacterial luciferase (LUX A and LUXB), under the control of an auxin responsive promoter that auxin content of the axillary bud increases after shoot apex were decapitated. During this experiment, it was observed that bud-break frequency was higher in stem which have flowered and in nodal segment from upper portion of the stem. The completion of flowering may have led to distribution of PGRs and nutrient towards axillary buds and may be the upper portion of stem has lower level of ABA. It has already been reported that decapitation of flowering apex promoted outgrowth of axillary buds (McDaniel, 1996). Pearce et al. (1995) have also reported in *Flytrigia repens* (Quackgrass) that ABA level which is usually high in dormant lateral buds, declined to 20% of control level within 24 hours after the rhizomes were decapitated. Hence, it was possible to break bud-dormancy in cultured nodal segment due to separation from shoot apex and decapitation of rhizomes.

In *Costus pictus*, bud-break was achieved on medium supplemented with BAP and NAA. However, in *Costus speciosus*, the bud fail to break in
cytokinin and auxin supplemented medium. It required medium supplemented with either higher percentage of sucrose (4-7%) or AdS along with BAP and NAA is required for breaking bud dormancy. This may be explained by the fact that in natural habitat also one or two axillary buds of Costus pictus differentiate to plantlets. In Costus pictus, the highest percentage of bud-break was in 0.6μM NAA and 3μM BAP. In nodal segments of Costus speciosus, the highest percentage was on medium supplemented with 5μM BAP, 1μM NAA and 10 μM AdS. These findings suggest the synergism between AdS, NAA, BAP and sucrose. The possible growth regulatory effect caused by adenine was first noted by Bonner and Huagen-Smith (1939). It could induce bud formation in both tobacco stem segments and elm and tobacco callus in vitro (Bonner et al., 1939; Skoog and Tsui, 1948; Jacquot, 1951; Miller and Skoog, 1953). In Nicotiana tabacum, medium containing Kn and AdS shows marked increase in the activities of two enzymes of the oxidative pentose phosphate pathway compared to their activities in a non-shoot forming medium (Scott et al., 1964). Hence, it may be inferred that presence of BAP, NAA and AdS in the medium increases enzymes of the oxidative pentose phosphate pathway which provide activation energy for breaking bud dormancy through sucrose metabolism.

In both Costus pictus and Costus speciosus, the optimal sucrose concentration for shoot multiplication was 5% sucrose. The elongated axillary buds of Costus pictus proliferated when excised and transferred on
to medium supplemented with NAA, BAP and Kn in conjunction with 5% sucrose. However, in the case of *Costus speciosus*, only the differentiated axillary buds are able to multiply when transferred on to medium supplemented with 1μM NAA, 10μM AdS, 3-11μM BAP and 5% sucrose. The propagules of *Costus speciosus* fail to multiply in medium not supplemented with AdS in conjunction with BAP and NAA. However, the propagules of *Costus pictus* proliferated in medium supplemented with BAP and Kn only or in combination with NAA and also produced higher average number of multiple shoot in comparison to propagules of *Costus speciosus*. The present study has shown the importance of AdS and higher sucrose concentration for breaking bud dormancy and shoot proliferation. The highest average number of shoots for *Costus pictus* is 8.50±0.26 and for *Costus speciosus*, it is 3.60±0.27. This is in agreement with the findings in *Brassica campestris* in which adding adenine sulphate to medium containing Kn and IBA did not increase shoot multiplication (Paek et al., 1987). The axillary bud of *Costus speciosus* were rooted during bud-break and shoot multiplication experiment in medium supplemented with AdS in conjunction with BAP and NAA. Hence, additional treatment was not required for root induction. However, for *Costus pictus* the shoots are required to be transferred to medium supplemented with 1-12μM NAA or IAA and 8μM BAP for root induction. Start & Cumming (1976) have reported positive effect of AdS on rooting in *Saintpaulia ionantha*. Literature survey reveals that micropropagation of *Costus pictus* and
Costus speciosus using the nodal segments are the first reports. Nodal segment provides an alternative source of in vitro propagation in Costus species. It overcomes many disadvantages of using rhizomatous eye as an explants. The main disadvantage of using rhizomatous eyes are that the uprooted rhizomes usually fail to survive after rhizomatous eyes are decapitated and establishment of in vitro culture is usually difficult due to higher contamination (Balachandran et al., 1990). In nodal segment culture, the rhizomes are not uprooted. contamination was less, matured stem which have flowered was used and one matured stem usually contained 15–20 axillary buds in comparison to 4–5 rhizomatous eye in one rhizome.

Synergism between Kn and AdS was first reported by Skoog and Miller (1957). Chaturvedi et al. (1984) have reported induction of 9.2 shoots in AdS. Kn and IAA supplemented SH medium, using shoot tips of Costus speciosus. In our experiments, rhizomatous eyes/buds of C. pictus was cultured on MS, SH, and B₅ medium supplemented with 7μM Kn, 5.7μM IAA and 15μM AdS. In SH medium, induction of callus was observed, which subsequently regenerated to plantlets. Several workers have observed that AdS, in presence of other recognized cytokinin, promotes adventitious shoot formation, indirectly from callus (Thorpe and Murashige, 1968; Beach and Smith, 1979; Xiang et al., 1989). In the present study, when the shoot tips were cultured on 7μM Kn, 5.7μM IAA and 15-40μM AdS, calli were induced and the highest number of
regenerated shoot was observed on 30μM AdS. Chirangini et al. (2005) have reported induction of callus from rhizomatous eye in *Kaempferia galanga* on IAA supplemented medium. However, regeneration was on NAA and BAP enriched medium. In the present study, induction and regeneration was possible on the same medium. *Costus speciosus* shoot multiplication through shoot tip culture in medium supplemented with AdS, NAA and Kn has been already reported (Roy and Pal, 1991; Chaturvedi et al., 1984. Malabadi et al. (2005) have also reported micropropagation of *Costus speciosus* using rhizome thin sections.

Assimilate partitioning from source to sink is essential for the harvestable component of economically important plants (Wardlaw, 1990; Farrar, 1992). The harvestable yield is the result of carbon dioxide fixation and the subsequent allocation of fixed carbon and assimilates into economically important component. For Zingiberales, rhizomes are the harvestable organ of economic importance. Rhizomes serve as sink where assimilates are unloaded. In the present investigation, plantlets of *Alpinia allughas* produce microrhizomes in medium enriched with 8% sucrose, 10-30μM BAP and photoperiod of 16 h illumination, whereas plantlets of *Costus pictus* produce rhizome in ½ strength MS medium enriched with 2.40μM NAA and 32μM BAP and lower photoperiod of 8 h illumination. Several workers have reported importance of photoperiod, in addition to sucrose and temperature. Role of photoperiod in *in vitro* induction of tuber have been reported in potato (Hussay and Stacey, 1984) and tulip (Taeb and
illumination. This is in agreement with findings in *K. galanga* and *K. rotunda* (Chirangini *et al.*, 2005) and *Zingiber cassumunar* (Chirangini and Sharma, 2005).

In *Alpinia allughas*, only primary rhizome was produced, while in *Costus pictus*, both primary and secondary rhizome were produced. These rhizomes when inoculated onto fresh medium or transferred to field, produced shoots suggesting the inheritance of regeneration capacity like normal rhizome. It has already been reported that microrhizomes of *K. galanga* and *K. rotunda* (Chirangini *et al.*, 2005), *Zingiber cassumunar* (Chirangini and Sharma, 2005) and *Curcuma longa* (Sunitibala *et al.*, 2001) produced plantlets on inoculation to fresh medium.