DISCUSSION

Plant micropropagation is an efficient method of propagating disease-free, genetically uniform and massive amounts of plants. The micropropagation from cells can be achieved by direct organogenesis from hairy roots or regeneration via somatic tissue culture. The micropropagation cycle based on proliferation of vegetative tissues, includes establishment of tissue culture through introduction of shoot meristem, shoot proliferation, root induction and plant acclimatization steps. Explants such as node, shoot tips and buds are used for micropropagation protocols. During the last few years, a number of woody trees have been successfully propagated in vitro by using mature tree explants. In this study a plantlet multiplication protocol was standardized through micropropagation for Ailanthus sp. The plant growth regulators like BAP (2.0 mg/L), GA3 (1.5 mg/L) and IBA (0.8 mg/L) was confirmed as the best combination for the induction of multiple shoots from shoot tip and nodal explants and maximum of 5.1 shoots/nodal explants and 5.3 shoots/shoot tip explants were observed in this study (Tables 1,2 & 3). Bag et al., reported similar results using MS media supplemented with BAP (5 µM) and IBA (1 µM). Similar results were reported by Batra et al using MS media with 3.1 µM of BAP. BAP with or without IAA also showed a good plant growth induction. Use of NAA at 2.7 µM has shown better results. The results of Begum et al. in some way contradicts the results which we obtained by showing the maximum shoot regeneration when using 1:1 ratio of cytokinin to Auxin. In our study and in many other cases reported, higher concentration of cytokinin than Auxin showed best response whereas higher ratio of Auxin to BAP also showed
good response which highly contradicts with our data. Thus the ratio of Auxin to BAP for multiple shoot elongation depends on the plant nature.

MS Medium supplemented with B5 vitamins responded better than the other types of medium tested (Table 4). In other media’s, less than 3 shoots with minimum shoot length were observed. The use of B5 vitamins for shoot induction was also described by.42 Usually for tree species, Woody Plants Medium (WPM) was alone used for micropropagation.36 However, in this study, MS medium supplemented with B5 vitamins produced maximum number of shoots. In the present study, MS medium proved to be the best medium for the overall growth of multiple shoots.

On analyzing the combined effect of GA3 and BAP it was found that a concentration of 2 and 0.6 mg/L respectively was the optimal concentration for germinating embryos eliciting a response of 84.61% (Table 16). Similarly considering the root induction, it was found that IBA at a concentration of 1.5 mg/L evoked the maximum response inducing an average of 3.1 roots per explant having an average 4.1 cm length (Table 6).

**Elongation of shoots**

Multiple shoot elongation is an important process, in which only the shoots were allowed for maturation prior to root induction. Usually the only plant growth regulator, GA3 alone was used for elongation of induced shoots.43 Sometimes the media similar to mother medium which is used for multiple shoot induction was also used for elongation of shoots by further sub culturing.44 In some other cases, MS basal medium alone was also used for
elongation of multiple shoots. In our study contradicting the above results, combination of BAP (2.0mg/L) and GA$_3$ (1.5 mg/L) with KIN (0.8 mg/L) showed the best response for the elongation of shoots (Tables 7, 8, 9 & 10).

Carbohydrate content was found to be influenced by plant growth regulator (PGR) during shoot induction,\textsuperscript{118} which was measured during our study. Carbohydrate dependent multiplication of shoot tips and nodes were commonly observed in all plant species.\textsuperscript{46, 114 & 119} Among the different kind of carbon sources, sucrose (3\%) was the best source for tissue culture techniques.\textsuperscript{47-49} In our study effect of different types of carbon source such as glucose, fructose, maltose and sucrose were tested in various concentrations. Among them sucrose 30/gm/L was observed as the best source for micropropagation (Table 5).

**Root induction and hardening**

Root induction is usually difficult in all the woody plant species, in our studies we came across various difficulties. Finally supplementation of MS medium with IBA (1.5 mg/L) was proved as a suitable plant growth regulator for root induction from elongated shoots (Table 6). IBA is believed to be a suitable plant growth regulator for the root induction of all plant species \textit{in vitro}.\textsuperscript{50-53} Usually Indole auxins such as NAA and IAA were used for root induction process. A similar investigation was done in our study using different concentrations of NAA and IAA for root induction. After 2 weeks instead of roots, large amount of basal callus was formed. Hence these plant growth regulators were rejected for root induction.
The root induced shoots were transferred to plastic pots for hardening. About 75% of survival percentage was observed after 2 months. The fully developed plantlets under greenhouse condition were then transferred to field for further growth.

**Organogenesis**

**Organogenic callus induction**

Plant regeneration through callus induction is organogenesis process. In this study organogenic callus induction, regeneration of shoots from organogenic callus and root induction from the induced shoots were successfully obtained. For the induction of organogenic callus, combination of BAP (2.0 mg/L), GA₃ (1.5 mg/L) and KIN (0.8 mg/L) were used. Usually, individual supplementation of cytokinins and combination of one cytokinin with auxin was widely used for the organogenic callus induction. But in our study combination of two cytokinins with one auxin were used. In organogenesis also the above said callus induction medium was suitable for both leaf and rachis explants. This type of organogenic callus induction medium was not developed for different explants.

In the present study, callus induction response varied with the type of explants and hormones. Among the two types of explants tested, leaf showed maximum response followed by rachis explants. Almost both the explants responded well for the induction of organogenic callus. Combination of auxin and cytokinin improved the frequency of callusing. Greenish and compact nature of callus increased with the increase in concentrations of cytokinin. Similar observation was reported in *Mandevilla illustris* and *Plumbago*
Moreover we observed that high concentration of auxin combined with low concentration of cytokinin was more effective for callus formation. Similar results were observed in *Solanum laciniatum*.

**Multiple Shoot initiation from callus cultures**

The induced organogenic calli was then subcultured on the same mother medium for the proliferation of shoots. The continuous subculture was done for 8 weeks, at the end of 8th week around 40 shoots were derived from the callus. This type of multiple shoot induction from mother medium with change in sucrose concentration was reported in *Eragrostis curvula* leaf explants. However they used same method to obtain roots. Cytokinins are plant hormones that regulate diverse aspects of plant growth and development. BAP was reported as the most effective cytokinin for shoot bud regeneration in most of the plants. In the present investigation in contradiction to the above, the callus induced mother medium alone was used for the induction of multiple shoots from the obtained callus.

Thidiazuron is used as a substitute for BAP, and TDZ was found to be more effective than BAP. In the present study, the induction of multiple shoots from the callus cultures was contrastingly increased by the supplementation of TDZ (0.6 mg/L). In both leaf and rachis derived callus cultures 50.2 shoots and 49.8 shoots were observed respectively (Table 9). Response of cultures to other growth regulators over a range of 0.5 µM to 10 µM was 50% less than that observed with TDZ. At concentrations lower than 2.5 µM, TDZ induced shoot organogenesis, whereas at higher doses (5-10 µM) it formed somatic embryos. TDZ activity is due to TDZ-
induced regeneration and the manifestation of a metabolic cascade that includes an initial signaling event, accumulation, and transport of endogenous plant signals such as auxin and melatonin, a system of secondary messengers, and a concurrent stress response. The induced shoots with callus were then transferred to shoot elongation medium containing different concentration of GA$_3$. Among them 2.0 mg/L GA$_3$ was observed as suitable one for elongation. Like our results, shoot elongation was effectively observed in many plant species including *Ophiorrhiza mungo*. Using GA$_3$ along with BAP has also been reported in several cases.

Root induction was not a problem from the callus cultures derived shoots. Like micropropagation derived shoots here also the same results were observe during root induction and hardening process. Half strength MS medium with 1.5 mg IBA showed best response. Similar results were found with *Atractylodes macrocephala* Koidz, *Hordeum vulgare* L and *Fagopyrum esculentum* Moench. Caglar *et al.* showed that shoot exposed to IBA had better response, whereas IAA shown a better response in *Alysicarpus rugosus*.

**Somatic embryogenesis**

**Embryogenic callus induction**

Somatic embryogenesis (SE) is an asexual propagation pathway requiring a somatic-to-embryonic transition of differentiated somatic cells toward embryogenic cells capable of producing embryos in a process resembling zygotic embryogenesis. Somatic embryogenesis, in which a single cell or clusters of cells develop whole embryoids, is useful in
regeneration of many plant species. Induction and development of somatic embryogenesis in several species requires a high concentration of auxin in the culture medium. For direct somatic embryogenesis, the usual procedure was the explant transfer from an auxin supplemented induction medium to auxin free medium. In this study a simple and efficient protocol for *Ailanthus* whole plant regeneration via somatic embryogenesis is described. On analyzing the combined effect of GA₃ and BAP it was found that a concentration of 2 and 0.6 mg/L respectively was the optimal concentration for germinating embryos eliciting a response of 84.61% (Table 16). Embryogenic callus induction is an important process through which only the embryoids were regenerated. For the induction of embryogenic callus, in this study combination of 2, 4-D with TDZ was effective for embryogenic callus induction. Usually auxins were alone tested for the induction of embryogenic callus,¹¹² sometimes combination of auxins with kinetin were tested for the induction of embryogenic callus as well as embryoids.⁵¹ In our studies also combination of two auxins responded well for the induction of embryogenic callus; 2.0 mg/L of 2, 4-D and 0.6 mg/L of TDZ responded well for the embryogenic callus induction and maximum of 95.2% embryogenic callus induction was observed. It was also found that ASCA at concentration of 50 mg/L was the most effective organic additive inducing somatic embryogenesis in MS liquid medium containing 1.5 mg/L 2, 4 –D (Table 15).

**Induction and maturation of somatic embryos**

After 12-week maintenance of embryogenic callus in the liquid medium fortified with different concentrations of 2, 4 –D, 1.0 mg/L showed
best response and produced proembryoids. In *Carica papaya* 2, 4-D alone induced the formation of somatic embryos from the callus cultures through suspension culture and 4.52 μM 2, 4-D responded well for the induction of somatic embryo induction and maturation. In contradiction to the above, our results proved that combination of 2, 4-D (2.3 μM) with Kin (1.1 μM) showed best combination for the induction of embryogenic callus and embryoids. The embryo induction and maturation process required 12 months with frequent subculture. In the same way the somatic embryogenesis of *Aralia cordata* was effectively achieved by the supplementation of 3.8μM of ABA (abscisic acid) from the 2, 4-D derived embryogenic callus. In their studies, varying concentrations of 2, 4-D was tested for the induction of embryoids. Finally ABA only responded well for the induction of embryoids. Like our results, 2, 4-D mediated embryogenic callus induction and somatic embryogenesis was observed in *Prunus incisa* and *Ocimum basilicum* L. In contradiction to our results no embryo formation with NAA was observed however, NAA was used for somatic embryogenesis in *Valeriana edulis* and in *Ocimum basilicum*. Results from our study (Table 21) proved that induction of somatic embryos, number of somatic embryos/culture and percentage of response was low due to NAA supplementation.

Somatic embryos converted into plantlets were dependent on the type of plant growth regulators, embryoid induction medium used for the maturation without any alteration. In contradiction to the above, continuous formation of somatic embryos was observed in *Ocimum basilicum* on MS medium supplemented with BAP and NAA.
Germination of somatic embryos

In the present work, medium fortified with GA_3 and BAP showed good response, for the regeneration of somatic embryos into plantlets (Table 16). Similar to our results, somatic embryos at cotyledonary stage were transferred to the germination medium containing different concentrations of BAP (1.11 to 8.88 μM) in combination with GA_3 (0.72 μM). When the concentration of BAP was increased, the frequency of embryo germination also increased. Hence, the present investigation clearly showed that BAP played an important role in embryo germination. Similar results were observed in *Astragalus melilotoides*. Hu, *et al.* also reported that embryos required exposure to cytokinin for the development of normal *Amorphophallus rivieri* plants.

Effect of different media, carbon source and additives on somatic embryogenesis

Induction of somatic embryos and regeneration from the matured somatic embryos was significantly governed by the nutrients present in the media. Requirement of higher amount of nitrogenous nutrients highly controlled the somatic embryogenesis of *Picea mariana* and *Beta vulgaris*. In the present research work, MS medium with B5 vitamins was found to be highly suitable for the induction, maturation, development and regeneration of somatic embryos than MS, mMS and WPM media (Table 13). In the MS-B5 medium, supplementation with 1.5 mg/L 2, 4-D was found to be the most effective (84.6%) in inducing somatic embryogenesis (Table 13). Maximum growth of embryonic callus was recorded on modified MS medium supplemented with B5 vitamin by Kumar *et al*. 2003. Further it was
reported that low nitrogen is sufficient for initiation and development of somatic embryos.\textsuperscript{96} High level of nitrogen in MS liquid medium supported the initiation of higher number of globular embryos than LS and B5 liquid media in \textit{Vigna unguiculata}.\textsuperscript{97}

The most commonly used carbohydrate in plant tissue culture is sucrose. In our study 30 g/L of sucrose was believed to be the best concentration for the induction and regeneration of somatic embryos (table 14). In nature, carbohydrate is transported within plant tissues as sucrose and tissues may have an inherent capacity for uptake, transport and utilization of sucrose. Sucrose at 3\% produced the best result in \textit{Glycine max}.\textsuperscript{123} High sucrose concentrations induced somatic embryogenesis.\textsuperscript{124} It was reported that somatic embryos were developed from cotyledonary explant of \textit{C. melo} mature on MS medium fortified with NAA (1.0 mg/L) and adenine (33.75 mg/L).\textsuperscript{125} These results are in contradiction to the present investigation. In our current study, ASCA (50 mg/L) showed the best response as additive (Table 15). Compared with our results supplementation of charcoal and silver nitrate highly controlled the somatic embryogenesis of cotton.\textsuperscript{141} In some cases supplementation of PVP alone was believed as best for the control of phenolic exudation and oxidation from the explants and somatic embryos to medium.\textsuperscript{39}

\textit{In vitro culture of Asclepias curassavica}

\textbf{Micropropagation of \textit{A. curassavica}}

\textbf{Multiple shoot proliferation}

Micropropagation enables the mass propagation of clones and formation of uniform plants.\textsuperscript{91,32} In \textit{Asclepias curassavica} multiple shoots were induced from the combined effect of BAP (2.0 mg/L), GA\textsubscript{3} (1.0 mg/L)
and glutamine (15 mg/L) on nodal explants and shoot tip explants (Tables 17 & 18). During normal micropropagation, individual supplementation of BAP was used. In this study, combination of BAP, GA₃ and glutamine showed good response. In contradictory to the above report combination of cytokinin with one auxin produced more shoots compared with combination of cytokinin with gibberellic acid. In Prosopis chilensis similar results were obtained by the supplementation of BAP (0.05 mg L) and NAA (3 mg/L). In this study nearly 20-28 shoots were regenerated with the addition of above plant growth regulators. In Adhatoda vasica maximum of 4.2 shoots were regenerated successfully by the supplementation of BAP alone and combined effect of different plant growth regulators showed poor response on the production of multiple shoots from the nodal as well as shoot tip explants. Because supplementation of cytokinin with the multiple shoot induction medium enhanced the frequency of lateral shoot formation. Bag et al. reported that the greatest number of shoots was obtained on the medium supplemented with 4.4 μM BAP without auxins, using explants from matured plants. Among the cytokinins, BAP is the one, which is used most frequency on the majority of woody species but in our experiment combination of BAP with GA₃ and Glutamine showed best response for multiple shoot proliferation (Table 17).

**Root induction from the elongated shoots**

In this present study root induction from the elongated shoots were obtained by the supplementation of IBA (1.5 mg/L) root induction medium (Table 21). IBA enhanced root induction was observed almost in all the
plants species including Stevia rebaudiana, Capsicum frutescens, Baliospermum montanum, Saussurea obvallata and Prunus spp.

Organogenesis of Asclepias curassavica

Organogenic callus induction

Regeneration of plantlets from callus is an important process, through which the somoclonal variation was induced in plant species. In this present research work, callus induction and plant regeneration was successfully obtained for A. curassavica. Medium supplemented with IBA (1.5 mg/L) showed good response for the production of organogenic callus (Table 19). Like our results, organogenic callus induction from leaf explants was successfully obtained in Hypericum perforatum L. by the treatment of BAP and IBA. Similar to our results callus induction was clearly observed in Hypericum erectum in the medium containing IAA and IBA under darkness. It has been reported that tissues cultured in the presence of light have increased activity of IAA-oxidase altering the endogenous balance between auxin and cytokinin, consequently decreasing the callus growth. Exogenous cytokinin applications alter the endogenous auxin metabolism by reducing the activity of IAA-oxidase enzymes in Dianthus spp. The higher level of IAA induces cellular growth by increasing RNA and protein synthesis, resulting in an increase in callus proliferation. In Pennisetum glaucum also organogenic callus induction was observed in the medium consisting of 2, 4-D. According to these authors, media containing BA showed superior results in callus induction as well as growth. We observed that 2, 4-D at a concentration of 1 mg/L was very effective in inducing
somatic embryo formation from leaf derived callus on MS liquid medium (Table 23).

**Plant regeneration from the organogenic callus**

Multiple shoot induction is an important process through which shoots were initiated from callus. In the present investigation, BAP alone was used for the proliferation of shoots obtained from organogenic callus (Tables 20). Like our results, BAP was found to be the most efficient in shoot formation from the obtained callus (Chaudhuri and Ghosh et al. 2004). Similar results for the shoot regeneration from the organogenic callus were successfully obtained in *Phyllanthus caroliniensis* with BAP supplementation. The induced multiple shoots were elongated by the supplementation of BAP to the induced shoots. Usually, shoot elongation was done on the mother medium. Only in some cases, elongation of shoots was obtained in the separate medium fortified with cytokinins and gibberelic acid.

**Root induction from the matured shoots**

Root induction is an important process, after completion of root induction, the shoots were considered as fully regenerated one. In the present research work, IBA (1.5 mg/L) was found to be superior for the induction of roots from the elongated shoots cultured on half strength MS medium (Table 21). Supplementation of IBA was found to be the best one for the induction of roots from the elongated shoots. In *Photinia spp*, root induction was obtained successfully by the addition of IBA.
**Somatic embryogenesis of Asclepias curassavica**

**Embryogenic callus induction**

Regeneration of plants via somatic embryogenesis is considered to be an efficient approach for clonal plant propagation. Somatic embryogenesis also provides the basis for genetic improvement of forest grown plants. In this present study, embryogenic callus induction was obtained by the combination of 2, 4-D (2 mg/L) and KIN (0.6 mg/L) from immature leaf explants (Table 22). Somatic embryogenesis was induced up to 75% in MS-B5 liquid media containing 1.5 mg/L of 2, 4-D, (Table 24).

Like our results embryogenic callus induction from petiole explants was successfully obtained in *Dalbergia sissoo* and similar results were also observed in *Asparagus officinalis*. In leaf explants of potato also combined effect of 2, 4-D with cytokinins showed best response for the production of embryogenic callus.

**Embryoid induction, maturation and plant regeneration**

In the present investigation, induction of somatic embryos was observed on the media fortified with 2, 4-D (1.0 mg/L) alone. This type of embryoid induction by 2, 4-D supplementation was clearly observed in several plants. This type of 2, 4-D enhanced somatic embryogenesis results were clearly observed in *Arabidopsis thaliana*. Although embryoid was achieved in *Asclepias* species, this is the first report of plant regeneration via somatic embryogenesis in any members of Asclepiadaceae. Similar to our results media supplemented with MS salts and B5 vitamins responded well
for the induction of embryos, and plant regeneration. Finally the matured somatic embryos were obtained in the media fortified with GA$_3$ and BAP. Like our results, media containing BAP and GA$_3$ mediated somatic embryo germination was observed in Corydalis yanhusuo. Experiment results proved that each process of somatic embryogenesis required different phytohormones and conditions for induction of embryogenic tissues and somatic embryos. Similar results of somatic embryogenesis of Quercus suber were recently observed.

**In vitro culture of Premna latifolia**

**Micropropagation of Premna latifolia**

**Multiple shoot induction**

Micropropagation of Premna latifolia was achieved by the supplementation of MS medium with combination of BAP, NAA and IBA. Auxins IBA and IAA were not effective in inducing rooting, NAA at a concentration of 1.5 mg/L was the most effective in producing roots on 1/2 strength MS medium (Table 29). For the multiplication of nodal explants, the medium was supplemented with BAP (2.0 mg/L) + NAA (1.5 mg/L) + IBA (0.8 mg/L) (Table 26) and BAP (2.0 mg/L) + IAA (1.5 mg/L) + IBA (0.8 mg/L) for the multiple shoot initiation from shoot tip explants (Table 27). Multiple shoot induction through micropropagation was usually achieved by the addition of BAP or combination of cytokinins with auxins. In this present study multiple shoot induction was achieved by the combined effect of cytokinin with two auxins. Combined effect of cytokinin with auxin was reported for multiplication in many plants including Lagerstromia parviflora.
To our knowledge this is the first report for the multiple shoot proliferation by the combined effect of BAP with IBA, NAA and IAA.

In contradiction to the above result, potential shoot multiplication is strong in the presence of BAP alone in the culture medium. The stimulatory effect of BAP on shoot multiplication is similar to that reported in various plants. MS medium containing BAP was more effective for inducing proliferation of axillary buds as in previous reports. Caro et al. also proved that media containing combined concentration of BAP with NAA, showed best response for the production of multiple shoots from the matured explants.

**Root induction**

Root induction of elongated shoots of *Premna latifolia* is difficult due to delayed response and over phenolic oxidation process. In the present investigation supplementation of NAA showed best response for the initiation roots from elongated shoots. The well developed roots were induced without phenolic compounds on the half strength MS medium (Table 29). Like our results, enhanced root induction and root formation was observed in *Ulmus parvifolia*. In *kaempfreia galanga* also enhanced root proliferation was observed on media fortified with NAA. However addition of only NAA to the IBA containing medium increased the production rate of plantlets and root number.
Organogenesis of *Premna latifolia*

**Organogenic callus induction**

Callus induction and proliferation system are known to be very useful for the study of biosynthesis of natural products and the factors that influence it, giving some possibilities of controlled production. Optimization of cellular proliferation in semi solid medium is the first step to establish cell mass scale up. In this present investigation, combined effect of KIN, GA₃ and IBA showed the best response for production of organogenic callus from the immature leaf explants. Combination of plant growth regulators along with cytokinins and auxins was used for the organogenic callus induction, but in our study, along with auxin, cytokinin, and extra GA₃ was used for the organogenic callus induction. In contrast to our results, Bhatnagar et al.⁴¹ produced organogenic callus of *Solanum laciniatum* by the supplementation of KIN and NAA. Similarly, callus induction and plant regeneration was obtained by the supplementation of TDZ alone and in combination IBA and without BAP or any other plant growth regulators.⁴⁷

**Multiple shoot induction and elongation from organogenic callus**

In this present study supplementation of TDZ enhanced the initiation of shoots from the organogenic callus. Hormone combination of GA₃ and KIN at 1.5 and 0.8 mg/L was more effective in induction of multiple shoots from shoot tip explants of *Premna latifolia* cultured on MS medium than GA₃ alone, eliciting 100% response with a mean shoot length of 3.75 cm (Table 28). Supplementation of BAP or GA₃ to organogenic callus was used for the proliferation of shoots.⁴⁸ But in our results, TDZ showed best results. TDZ at
a concentration of 0.6 mg/L produced the maximum response, generating 52 shoots with an average length of 3.7 cm from green compact calli (Table 31), derived from MS medium supplemented with KIN (2.0 mg/L), IBA (1.5 mg/L) and GA₃ (0.8 mg/L). GA₃ at a concentration of 2 mg/L produced elongation of shoots of nearly 4.2 cm in the regenerated shoots of immature leaf explants derived green compact calli of *Premna latifolia* cultured on MS medium (Table 32). Growth regulators KIN, IBA and GA₃ together at concentrations of 2, 1.5 and 0.8 mg/L were the most effective in callus induction and regeneration of shoots from leaf explants on MS medium (Table 30).

Similarly, plant regeneration from hypocotyls cultures of *Saintpaulia ionantha* was achieved by the supplementation of TDZ to hypocotyls derived callus cultures. Haensch⁹⁴ described a similar phenomenon in *Pelargonium spp* petiole explants exposed to TDZ. TDZ has been shown to induce the accumulation of both endogenous auxins and cytokinins in legumes and herbaceous species.

**Root induction from elongated shoots**

Root induction from the elongated shoots was obtained in the half strength MS medium fortified with IBA (1.5 mg/L). However addition of IAA and NAA induced the formation of basal callus which in turn inhibited the root formation. Although rooting efficiency was less, IBA at lowest concentrations tested could induce the root formation. In contrast to the present investigation, success for root induction in auxin free basal medium was achieved by Wei *et al.*¹³⁹ It has been reported that auxin is needed for the
induction of roots in *Melia azedarach*. The incidence of highly efficient root formation on auxin free medium may be due to the availability of higher quantity of endogenous auxin in *in vitro* raised shootlets.

**Somatic embryogenesis of Premna latifolia**

**Embryogenic callus induction**

Embryogenic callus induction was effectively observed (95.2%) on the medium fortified with 2, 4-D (2.0 mg/L) and TDZ (0.6 mg/L) without any abnormalities (Tables 33 & 34). The use of 2, 4-D for the embryogenic callus induction was widely used compared with other auxins. In *Triticum aestivum* this type of enhanced induction of embryogenic callus was observed in the medium fortified with 2, 4-D alone. In this present investigation, in addition to that one of the cytokinin was used in this study, i.e. TDZ. The use of combined effect of auxins with 2, 4-D showed enhanced production of embryogenic callus. To support our results, combined effect of auxins with cytokinins was used in production of embryogenic callus and embryoids in one of the woody legume *Dalbergia sissoo*. Also ASCA at 50 mg/L was the most effective in inducing somatic embryogenesis of *Premna latifolia* in MS liquid medium containing 1.5 mg/L 2, 4 –D (Table 36).

**Embryoid induction and plant regeneration**

For the induction and maturation of embryoids 2, 4-D (2.0 mg/L) alone was used and it showed best response. Almost in all the plant species, 2, 4-D is widely used for the somatic embryogenesis. Like our results, 2, 4-D mediated embryoid induction was effectively achieved in the plants of
Daucus carota\textsuperscript{79} and Cocos nucifera.\textsuperscript{80} In some cases, 2, 4-D, BAP and NAA was used for the induction of somatic embryos.\textsuperscript{88} But, in our results 2, 4-D alone showed best response for the number of somatic embryos induction, embryo maturation percentage and percentage of embryo conversion.

**Somatic embryo germination**

Enhanced percentage of somatic embryo germination was observed in the medium fortified with various concentrations of GA\textsubscript{3} and BAP. In our previous results also GA\textsubscript{3} and BAP mediated enhanced somatic embryogenesis was observed in *Ailanthus excelsa* and *Asclepias crussavica*. Usually combination of MS medium with B5 vitamins alone was used for the germination of somatic embryos without any plant growth regulators.\textsuperscript{89} In contrast to the above in our results combination of BAP with GA\textsubscript{3} showed best response for complete germination of somatic embryos into plantlets (Table 35). During every step of somatic embryogenesis, phenolic exudation from plant was observed and it was significantly controlled by the supplementation of ascorbic acid. This type of additives enhanced somatic embryogenesis in *Carica papaya*.\textsuperscript{42}

**In vitro culture of Plumbago indica**

**Micropropagation of Plumbago indica**

**Multiple shoot induction**

Micropropagation of *Plumbago indica* was achieved by the supplementation of MS medium with combination of BAP, IBA, NAA and IAA (Table 38).
For the multiplication of nodal explants medium supplemented with BAP (2.0 mg/L) + NAA (1.5 mg/L) + IBA (0.8 mg/L) and for shoot tip explants BAP (2.0 mg/L) + IAA (1.5 mg/L) + IBA (0.8 mg/L) showed best response for the multiple shoot initiation (Tables 37 & 38). Multiple shoot induction through micropropagation was usually achieved by the addition of BAP or combination of cytokinins with auxins.\textsuperscript{120,125} Mohapatra and Rath\textsuperscript{110} showed that the MS media supplemented with BAP (2.0 mg/L) alone had better response. MS medium containing BAP was more effective for inducing proliferation of axillary buds as in previous reports.\textsuperscript{111} In contradiction to the above the potential shoot multiplication is strong in the presence of BAP with auxin.

In this present study multiple shoot induction was achieved by the combined effect of cytokinin with two auxins. Combined effect of cytokinin with auxin was reported for multiplication in many plants including \textit{Charybdis numidica}\textsuperscript{95} and \textit{Prosopis chilensis}.\textsuperscript{50} To our knowledge this is the first report for the multiple shoot proliferation by the combined effect of BAP with IBA, NAA and IAA.

We also studied the effect of GA\textsubscript{3} on shoot elongation. We found that GA\textsubscript{3} (1.5 mg/L) along with KIN (0.8 mg/L) showed 100% response and GA\textsubscript{3} alone was not as effective as the combination (Table 39).

**Root induction**

Root induction of elongated shoots of \textit{Plumbago indica} is difficult due to excess phenolic exudation process. In this present investigation
supplementation of IBA (1.0 mg/L) showed the best response for the root initiation from elongated shoots (Table 40). The well developed roots were induced without phenolic compounds on the half strength MS medium. Though Rhizogenesis is achieved mainly in the presence of auxins, it can also be carried out in the growth regulator free MS medium and also in \textit{ex vivo} using vermiculite + cocopeat mixture (1:1 v/v) as reported in \textit{Paulownia fortuneii} trees.

\textbf{Organogenesis of \textit{Plumbago indica}}

\textbf{Organogenic callus induction}

In this present investigation, combined effect of KIN, GA\textsubscript{3} and IBA showed the best response than KIN or KIN and IBA. KIN along with auxin was found to give good response. In our study using KIN and auxin a response of 86.4\% was obtained and addition of GA\textsubscript{3} to the above increased the response to 99.2 \% (Table 41). Contradicting our results, supplementation of KIN and NAA to the organogenic callus of \textit{Solanum laciniatum} The result was also better than that of using BA and GA\textsubscript{3}, where the multiplication efficiency was only 68\%.

\textbf{Multiple shoot induction and elongation from organogenic callus}

In our study MS medium supplemented with 2.0 mg/L GA\textsubscript{3} was showing highest number of elongated shoots. In many cases fresh media similar to that of the calli inducing medium is used for the elongation process however addition of GA\textsubscript{3} to the fresh medium produced better organogenic calli.
Root induction from elongated shoots

Root induction from the elongated shoots was obtained in the half strength MS medium fortified with IBA (1.5 mg/L). However addition of IAA and NAA induced the formation of basal callus which in turn inhibited the root formation. Although rooting efficiency was less, IBA at lowest concentrations tested could induce the root formation\textsuperscript{48,49} and was shown to be effective at relatively high concentrations as in our case and also in Sapindus mukorossi.\textsuperscript{116} In contrast to the present investigation, MS medium fortified with IBA (9.84 µM) combined with NAA (5.37 µM) showed best responses in Baliospermum montanum.\textsuperscript{99} Addition of auxin to the medium induces better roots.\textsuperscript{127}

Somatic embryogenesis of Plumbago indica

Embryogenic callus induction

Embryogenic callus induction was effectively observed (100%) on the medium fortified with 2, 4-D (2.0mg/L) and TDZ (0.8 mg/L) without any abnormalities (Table 43). The use of 2, 4-D for the embryogenic callus induction was widely used compared with other auxins in North American ginseng plant. Enhanced embryogenic callus induction was observed in the medium fortified with 2, 4-D alone.\textsuperscript{141} In this present investigation, in addition to the auxin 2, 4-D the cytokinin TDZ was also used. The use of combined effect of 2, 4-D with TDZ showed enhanced production of embryogenic callus. To support our results, combined effect of auxins with cytokinins was used in production of embryogenic callus and embryoids in Quercus variabilis.\textsuperscript{140}
Embryoid induction and plant regeneration

For the induction and maturation of embryoids 2, 4-D (2.0 mg/L) alone was used and it showed good response. Almost in all the plant species, 2, 4-D is widely used for the somatic embryogenesis.\textsuperscript{139} Like our results, 2, 4-D mediated embryoid induction was effectively achieved in the plants of \textit{Daucus carota}\textsuperscript{140} and \textit{Zea mays}.\textsuperscript{121} In some cases, 2, 4 D, BAP and NAA was used for the induction of somatic embryos.\textsuperscript{121} But, in our results 2, 4-D alone showed best response for the number of somatic embryos induction, embryo maturation percentage and percentage of embryo conversion (Table 44).

Somatic embryo germination

Enhanced percentage of somatic embryo germination was observed in the medium fortified with various concentrations of GA\textsubscript{3} and BAP. In our previous results also GA\textsubscript{3} and BAP mediated enhanced somatic embryogenesis was observed in \textit{Ailanthus excelsa} and \textit{Asclepias crussavica}. Usually combination of MS medium with B5 vitamins alone was used for the germination of somatic embryos without any plant growth regulators.\textsuperscript{125} In contrast to the above in our results combination of BAP with GA\textsubscript{3} showed best response for complete germination of somatic embryos into plantlets (Table 45). During each every step of somatic embryogenesis of this plant phenolic excretion was observed and it was significantly controlled by the supplementation of ascorbic acid. This type of additives mediated enhanced somatic embryogenesis was observed in Carica \textit{papaya}.\textsuperscript{42}
**Ailanthinone**

The compound extracted from *Ailanthus excelsa*, is a quassinoid. Quassinoids are known to possess antileukemic and other anticancer activity. These compounds are found to inhibit protein synthesis and hence have a high pharmacological use.

It has been shown that Ailanthinone inhibits β-lactamase activity and hence a potent compound in inhibiting protein synthesis by inhibiting transcription factor activating protein-1 (AP-1). It has also been suggested that they may serve as inhibitors for more than one transcription factor by targeting signal transduction pathways.

The possible use of the compound as a natural herbicide has also been suggested due to its allelopathic activity and its efficacy against *Plasmodium falciparum* has also been proved, hence it could also function as an antimalarial agent.

**Asclepin**

The compound present in the extract was found to be asclepin. Asclepin isolated from *A. curassavica* was chemically elucidated through spectral analysis and then evaluated for their cytotoxic activity against HepG2 and Raji cell lines. It was found that Asclepin had strong cytotoxic activity with an IC (50) value of 0.02 μM against the two cancer cell lines.

3'-O-Acetylcaltropin (asclepin), is a glycoside that was noted for its cardiotonic activity. It increases the myocardial contractility both in normal
and hypodynamic heart muscles. Various *in vitro* and *in vivo* procedures in cat, guinea pig, dog, monkey, pigeon and mouse found it to posses the properties of a cardenolide. It was more potent than digoxin and had a wider safety margin.146,147

When the cardiac effects of asclepin was studied *in vitro*, using isolated atrium and heart of guineapig, and *in vivo*, using anaesthetized cat, and the results were compared with g-strophanthin, digoxin, digitoxin, or digitoxigenin. Aselepin showed a marked positive inotropic effect as evidenced by the increase in the force of contraction and was found to be more active than the other glycosides.146

**Geniposidic Acid (GA)**

The Iridoid, geniposidic acid, from *Premna latifolia* (Verbenaceae) has been reported earlier.144

The antitumor effect of geniposidic acid (GA) was investigated in mice along with its possible effects on radioprotection after sublethal X-irradiation. Decreases in the growth of the implanted tumor ascitic cells were noticed as a result of intraperitoneal administration of GA. It was also found that GA might be a more potent tumor growth inhibitor when combined with the X-irradiation. The preliminary results of GA on hematological and blastogenic tissues also suggested that they may very well play a role in effectively decreasing undesirable radiation damage to the hematologic tissue after high dose irradiation.146, 151
The anti-inflammatory potency of a compound has also been reported. The \textit{in vitro} testing model system based on inhibition of cyclooxygenase (COX)-1/-2 enzymes, the tumor necrosis factor-alpha (TNF-alpha) formation and nitric oxide (NO) production indicated that the hydrolyzed-iridoid product with beta-glucosidase showed inhibitory activity. The inhibitory concentrations (IC-50) in each testing system were measured and H-geniposide exhibited high inhibitory effects on COX-1, revealing IC-50 values of 5.37 \textmu M and also suppressed the TNF-alpha formation with IC-50 value of 58.2 \textmu M.\textsuperscript{25}

\textbf{Plumbagin}

Prostate cancer (PCa) is the second leading cause of cancer-related deaths in men. Hormone-refractory invasive PCa is the end stage and accounts for the majority of PCa patient deaths. Plumbagin (PL), a quinoid constituent isolated from the root of the medicinal plant \textit{Plumbago zeylanica} \textit{L.}, may be a potential novel agent in the control of hormone-refractory PCa. The results indicate for the first time, using both \textit{in vitro} and \textit{in vivo} preclinical models, that plumbagin inhibits the growth and invasion of PCa.\textsuperscript{148}

Pancreatic cancer is one of the most resistant malignancies. Effect of plumbagin on the growth of human pancreatic carcinoma cells was tested. Plumbagin inhibited the growth of Panc-1 and Bxpc-3 cells in a dose-dependent and time-dependent manner. Exposure to plumbagin caused the upregulation of Bax, a rapid decline in mitochondrial transmembrane potential, apoptosis-inducing factor overexpression in cytosol. Pretreatment with caspase inhibitors did not block plumbagin-induced apoptosis.
Plumbagin may induce apoptosis in human pancreatic cancer cells primarily through the mitochondria-related pathway followed by both caspase-dependent and caspase-independent cascades. It indicates that plumbagin can be potentially developed as a novel therapeutic agent against pancreatic cancer.\textsuperscript{148}

Plumbagin chitosan-based plumbagin microspheres. Resulted in a significant tumor growth inhibition and reduced systemic toxicity in mice bearing B16F1 melanoma.\textsuperscript{148}