5. Discussion

5.1. Micropropagation

The present study has demonstrated the successful protocol for in vitro propagation of multiple shoots and complete development of plantlets.

5.1.1. Culture medium

Our results showed that of the four culture media (MS, B₅, mMS and WPM medium) used for shoot bud induction, MS medium responded well. The use of MS medium for in vitro propagation has been well documented (Dhar and Upreti, 1999 and Arumugam et al., 2003).

5.1.2. Hildegardia populifolia

5.1.2.1. Explant type

Besides media, explant type can influence the rate of explant sprouting and multiplication in woody plants. The choice of the explant is a critical factor that determines the success of most tissue culture experiments. Micropropagation of field-grown woody plants can be difficult because of contamination and exudates from cut woody stems (Brassard et al., 2003; Padilla and Encina, 2004; O’ Connor et al., 2007).

Most investigators working with trees have found that seeds and juvenile tissues are more amenable for in vitro manipulations than explants taken from trees in the adult growth phase. In vitro propagation of Sterculia urens using seedling explants has been reported (Purohit and Dave, 1996, Sunnichan et al., 1998). Callus-mediated regeneration from juvenile explants was also used as a method for rapid multiplication of the critically endangered tree Syzygium travancoricum (Anand et al., 1999) and the threatened tree Maclura tinctoria (Gomes et al., 2003).
The results obtained show that the rate of germination varies significantly depending on the treatment. Scarification with sulfuric acid proves to affect the percentage and the duration of germination, but this effect depends on the duration of the acid treatment. The effect of the sulfuric acid can be explained by the fact that it softens the teguments making them more permeable to water and gases, which overcomes the dormancy process and starts the physiological one of germination. The results are in agreement with those of Peacock and Hummer (1996) and EL Hamdouni et al. (2001) who studied the effect of sulfuric acid on the germination of Rubus and Strawberry.

The enhanced germination through *in vitro* culture is useful in securing seedlings when seeds are limited, and the germinated seeds are a good source of explants material for subsequent mass micropropagation.

5.1.2.2. Shoot bud induction and proliferation

By using this method, multiple shoots have been induced from two explants viz. axillary bud and apical bud from *in vitro* derived and *in vivo* explants. Of the two explants used, axillary bud was found to be better for shoot proliferation and multiplication when compared to apical bud explant. Propagation through axillary bud multiplication is an easy and safe method for obtaining uniformity, and it also assures the consistent production of true-to-type plants within a short span of time (George, 1993; Salvi et al., 2002). Many plant species have been propagated through axillary bud multiplication: *Santolina canescens* (Casado et al., 2002) *Aegle marmelos* (Arumugam et al., 2003).

For shoot bud induction and multiplication, growth regulators (cytokinin and auxin) have been used both individually and in combinations. Three cytokinins (BA, KN, TDZ) were tested, BA showed the highest percentage of shoot bud induction (Arumugam et al., 2003), whereas KN did not improve the number of proliferating shoots except that average shoot length was increased considerably. These results were also supported by Purohit and Dave (1996),
Handique and Bora (1999), Komalavalli and Rao (1997, 2000) and Ramulu et al. (2002). Highest mean number of shoots per explant was promoted by BA and all higher BA concentrations decreased the mean number of shoots and shoot length (Martin, 2003a) with considerable basal callusing response (Biroscikova et al., 2004). Increasing BA concentrations above a certain level does not correlate with an enhancement in shoot production. The presence of TDZ in the medium leads to the formation of fasciated shoots at the higher concentration. The need to remove this physiological abnormality by excising the fasciated shoots may slow down the micropropagation rate (Durkovic, 2003; Poovaiah et al., 2006).

In the present study combination of BA and KN was significant, however it could not supercede the effect of BA used alone. Similar results were also observed in apical bud explants of Sterculia foetida (Anitha and Pullaiah, 2002b; Rao and Purohit, 2006).

5.1.2.3. Basal callus
All of the cytokinin treatments caused basal callusing, and this problem was particularly severe in BA and TDZ treatments. The formation of basal callus has been observed frequently in shoot cultures of species with strong apical dominance (Preece et al., 1991; Saini and Jaiwal, 2000; Martin, 2002). In many cases basal callus turned brown and reduced shoot proliferation (George and Sherrington, 1984). Basal callusing has been attributed to the action of accumulated auxin at the basal ends (Marks and Simpson, 1994), which initiates cell proliferation, especially in the presence of cytokinins (Tao and Verbelen, 1996).

5.1.2.4. Hyperhydricity
Similarly the highest concentration of BA and TDZ affects the shoot morphology i.e. "vitrification" (stems thickened with shorter internodes, thick leaves frequently elongated, wrinkled and/or curled, and brittle and often accumulate decreased amounts of chlorophyll). There is also evidence that both
exogenously applied- and endogenously produced-cytokinins give responses similar to those achieved by various stresses (Harding and Smigocki, 1994; De la vina et al., 2001).

In this context, several authors have observed that isopentenyl transferase transformed shoot cultures of tobacco (which overproduce cytokinins) have fasciated stems with thick and breakable leaves, and often accumulate decreased amounts of chlorophyll (Memelink et al., 1987; Smigocki and Owens, 1988; Hamdi et al., 1995). These symptoms appear at different degrees. At extremes, vitrification leads to disfunctioning of the primary meristems (the appearance of fasciated stems is the first most clearcut visible manifestation) and even to their death through the well known phenomenon of (humid) apex necrosis (Kataeva et al., 1991; Gaspar et al., 1991).

The following factors have been listed as being responsible for hyperhydricity: the liquid medium, the low concentration of agar in solid medium, the high relative humidity in the culture vessels, osmotic shock, and superoptimal concentrations of mineral nutrients such as Cl and NH$_4^+$, or phytohormones such as cytokinins (Gaspar, 1991), Kevers et al. (1984), Gaspar (1986), Gaspar et al. (1987) and Gaspar et al. (1995) have proposed a hypothetical model which describes a sequence of reactions leading to vitrification and which integrates most of the available biochemical data. These reactions (involving soluble phenols, basic and acidic peroxidases, the metabolism of auxin and ethylene) lead to a deficiency in cellulose and lignin which may explain the anatomical change and hence part of the morphological changes. It was initially proposed that these deficiencies in structural cell wall components may be responsible for the hyperhydric transformations caused by increased water uptake via reduced cell wall pressure (Kevers et al., 1984). It was shown later that a surplus of water was located in the intercellular spaces of hyperhydric plants, while the protoplasts contained less water (Kevers and Gaspar, 1986). One of the most important difficulties in clarifying the
physiological causes of vitrification is related to the multiplicity of the factors involved in the process. Vitrification appears to result from metabolic perturbations induced by the combined action of several physical and chemical factors of the *in-vitro* environment. This complexity prevents the identification of processes which are causal to vitrification as opposed to processes which only affect vitrification in a quantitative manner.

The high amount of cytokinins (benzyladenine more than other cytokinins is efficient) or of substituted ureas (thidiazuron for instance) whose penetration in the tissue is facilitated on soft media (Bornman and Vogelmann, 1984; Leshem *et al.*, 1988; Williams and Taji, 1991; Cambecèdes *et al.*, 1991; Kataeva *et al.*, 1991). When the BA concentration was reduced in the media, the multiplication rate was maintained and the percentage of hyperhydricity decreased, indicating that the lowest BA concentration was necessary to maintain a good proliferation rate and to reduce the process of hyperhydricity (Heloir *et al.*, 1997; Abrie and van Staden, 2001 and Lopez *et al.*, 2004). Morini *et al.* (1985) and Cancellier and Cossio (1985) suggested a reduction of BA concentration to eliminate hyperhydricity but such a decrease could reduce the proliferation rate without eliminating the process of hyperhydricity. Therefore, it is important to distinguish between the establishment phase (first shoot development) and the shoot multiplication phase with respect to the concentration of BA used in the medium to maintain a good proliferation rate of shoots during subcultures.

The synergistic effect of two cytokinins (BA + KN) improved shoot proliferation efficacy when compared to individual cytokinin. Thus, the BA (1.0 mg/l) and KN (0.5 mg/l) combination was found to be efficient for maximum frequency of shoot proliferation and multiplication. When increasing the concentrations of BA and KN, shoot proliferation was also increased upto the optimum level and decreased with further increase in concentration of cytokinin. Similar results were reported by Kaur and Kant (2000), Soniya and Das (2002),
For further proliferation and elongation, the shoots were transferred to GA$_3$ and combination of cytokinin. Well proliferated and elongated shoots were observed in this treatment. The similar result was reported by Perica (2003), Mao et al. (2006), Golegaonkar and Kantharajah (2006).

The use of GA$_3$ along with cytokinins is not common for promoting shoot growth in tree species. GA$_3$ stimulates elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger, 1998).

Addition of additives enhanced the shoot number and quality. Combination of cytokinin with growth additives proved to be beneficial in Tylophora indica (Sharma and Chandel, 1992); Gymnema elegans (Komalavalli and Rao, 1997) and Decalepis hamiltonii (Anitha and Pullaiah, 2002a) as in the present study.

The elongated shoots were transferred to rooting medium containing various auxins (IAA, NAA and IBA). Among the three auxins tested, IBA showed the highest percentage of response when compared to other two auxins (Komalavalli and Rao, 2000; Soniya and Das, 2002; Nishimura et al., 2003; Durkovic, 2003; Biroscikova et al., 2004). Full-strength MS medium supplemented with IBA and NAA induced callus at the cut end of the shoots. Subsequently, roots developed from the callus. When auxin was used in the basal medium for rooting, cultures produced excessive callus at the explants base. Callus formed at the shoot base can interfere with the connection between the shoot and roots, resulting in poor root formation (Williams and Taj, 1989; Sharma and Thorpe, 1990; Quraishi et al., 1996).
Frequently, roots formed directly on the surface of the callus, and these cultures failed to become established in soil because callus separated the roots from the base of the shoots (Lee and Thomas, 1985; Hosoki and Katahira, 1994 and Sunnichan et al., 1998).

In *Hildegardia populifolia*, the highest percentage of root induction without basal callus was observed in full strength MS medium without sucrose supplemented with IBA. Similar results were also observed in *Sterculia urens* (Purohit and Dave, 1996; Sunnichan et al., 1998); *Dianthus caryophyllus* (Ghosh and Mohan Ram, 1986).

5.1.3. *Simarouba glauca*

Micropropagation contributes to forest tree improvement by multiplication of genotypes of proven value. Ease of vegetative propagation tends to diminish as trees approach a size allowing reliable evaluation of their desirable qualities; explants from adult trees are notoriously unreactive (Bonga, 1987). The *in vivo* mature explants used were not feasible for *in vitro* multiplication process due to high phenolic exudation. Even the application of antioxidants showed only negative effects by suppressing the shoot bud induction. Two month-old *in vivo* derived seedlings were selected as the explants. Among the three explants (cotyledonary node, axillary and apical bud) were tested, cotyledonary node showed the maximum percentage of shoot bud induction. The similar results were also reported by Sanchez *et al.* (1993), Quraishi *et al.* (1996) and Lakshmi and Seeni (2001).

The induction of multiple shoots from explants varied with cytokinin type and concentrations and was also influenced by explant type. The potential for shoot multiplication in *Simarouba glauca* appears to be strong in the presence of BA alone in the culture medium. The stimulatory effect of singular supplement of BA on bud burst and multiple shoot formation is similar to that reported in other medicinal plant species. Among the various concentrations tested,
BA (3.0 mg/l) showed the best response of shoot bud induction but stunted growth of the shoots was also observed in this concentration. Similar results were also reported by Martin (2003b) in *Rotula aquatica*.

Cytokinin is required optimal quantity for shoot proliferation in many genotypes but inclusion of low concentration of auxin along with cytokinin increases the rate of shoot multiplication (Shasany *et al.*, 1998; Rout *et al.*, 2000; Tiwari *et al.*, 2000; Rout, 2004). During the proliferation stage, a high cytokinin to auxin ratio is used to reduce apical dominance of the main shoot and to encourage growth of axillary buds. Shoot number increased on the media containing NAA with BA (Komalavalli and Rao, 1997; Bhatt and Dhar, 2000; Murch *et al.*, 2003). The cytokinin–auxin combination has also been widely used for shoot regeneration (Dhar and Joshi, 2005; Guo *et al.*, 2007).

The synergistic effect of NAA along with cytokinins (BA and KN) was observed in shoot multiplication and proliferation. Our results are in accordance with those of Al-Bahrany (2002) in *Citrus aurantifolia*, Arumugam *et al.* (2003) in *Aegle marmelos* to stimulate shoot multiplication.

### 5.1.3.1. Microshoots

Mostly all cytokinins tried in this experiment produced only micro shoots even with the combination of auxins and GA₃ at various concentrations. Microshoots (1-2 cm) produced in this tree were characterized by abnormal leaf morphology, short and compact shoots and difficulty in elongation and rooting of regeneration and this effect were also reported by Lu (1993), Rout *et al.* (1999) and Ramage and Williams (2004).

The microshoots were transferred to rooting medium containing different auxins and along with KN. Of the three auxins tested (NAA, IAA, IBA), NAA was the most effective with respect to *in vitro* rooting. A similar observation was made by Debnath *et al.* (2000), Sultana and Bari Miah (2003), Kongbangkerd *et al.*
Our result shows that NAA produced the highest percentage of root induction, as contrary to IBA which was also noticed in the studies of *Simarouba glauca* (Rout *et al.*, 1999).

### 5.2. Organogenesis

The organogenesis *in vitro* governed by the balance of auxin and cytokinin in the medium can not be demonstrated universally due to the explant sensitivity or the content of endogenous growth regulators (Skoog and Miller, 1957).

#### 5.2.1. *Hildegardia populifolia*

Results obtained from this experiment revealed that *Hildegardia populifolia* explants differed in growth regulator requirements for callus induction, shoot bud regeneration, shoot elongation and root induction.

##### 5.2.1.1. Callus induction and texture

In the present study, the four auxins (2, 4-D, NAA, IBA and IAA) were tested for callus induction. IBA (2.0 mg/l) was observed to be the more effective followed by 2, 4-D, IAA and NAA. Internode explant showed the highest response (100%) for callus induction followed by petiole and leaf explant. Individual auxin treatment produced only yellow, watery abnormal callus. Green compact nodular callus was observed with MS medium containing auxins (IAA and IBA) in combination with BA (2.0 mg/l). The results showed that the callusing frequency and colour were enhanced with the addition of cytokinin (BA). The beneficial effect of the presence of an auxin and a cytokinin in the culture medium for stimulating callus induction from leaflet explants of *Arachis pintoi* was demonstrated previously by Rey *et al.* (2000).

For further proliferation the callus was treated with cytokinin and auxins individually or in combination. Our result shows that high amount of cytokinin and low amount of auxin enhanced the callus (dark green compact nodular). Abubacker and Alagumanian (1999) also reported that the maximum frequency
of callusing was observed on MS medium with IAA (0.5mg/l) and BA (1.0 mg/l) in *Azadirachta indica*. Additives have a vital role in the proliferation of green compact nodular callus. Gulati *et al.* (1996) reported that the addition of amino acids in addition to auxin and cytokinin produced bright green regenerating callus.

**5.2.1.2. Shoot regeneration**

Plant regeneration via shoot and root organogenesis can be affected by culture medium, nature of the plant materials and relative concentrations of auxin and cytokinin. Callus was transferred to regeneration medium containing different cytokinin and auxin individually or in combination.

Recalcitrance is the inability of plant cells, tissues and organs to respond to tissue culture. Achieving the correct exogenous balance of the key hormones auxin and cytokinin can be critical and their appropriate application can effectively overcome certain recalcitrance problems (Benson, 2000). Callus transferred to the regeneration medium containing the varying cytokinin and auxins resulted in the negative effect of regeneration and prolonged maintenance of this callus turned its nature from green compact to friable. Green compact callus nature was maintained with the addition of glutamine and it has no role in regeneration. Even the green compact callus was subcultured frequently in the same medium leads only in the formation of habituated callus. This habituated callus bears some resemblance to crown gall tissue, which is also hormone independent. Habituation has been considered a step of neoplastic progression that can be initiated *in vitro* cultures (Gaspar, 1995). This process implicates a progressive reduction of cell-to-cell adhesion (Liners *et al.*, 1994), many morphological abnormalities (Crevecoeur *et al.*, 1992), biochemical deviations (Le Dily *et al.*, 1993), and irreversible loss of organogenic totipotency at the terminal stage.
The synergistic effect of the two cytokinins (BA and TDZ), auxin (IAA) and glutamine induced the regeneration ability and also the green compact callus nature was maintained (Franklin and Dias, 2006). In many woody plant species, callus induction and plant regeneration have been achieved using TDZ (Huetteman and Preece, 1993), which promotes efficient micropropagation of many recalcitrant woody species at relatively low concentration (<1 μM) (Huetteman and Preece, 1993; Faure et al., 1998; Te-chato and Lim, 1999; Vinocur et al., 2000). Mithila et al. (2003) reported that TDZ induced shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet. Tang and Newton (2005), Jiang et al. (2005), Gu and Zhang (2005), Poovaiah et al. (2006), Nisha Rani and Nair (2006), Amutha et al. (2006), Ahn et al. (2007), Dai et al. (2007) reported the promotive effect of TDZ in regeneration of shoots. TDZ-induced shoot regeneration from different explants of many recalcitrant species as well as from medicinal plants has been reported (Pelah et al., 2002; Schween and Schwenkel, 2002; Liu et al., 2003; Mithila et al., 2003; Thomas, 2003). Several reports suggest that TDZ results in shoot regeneration better than other cytokinins (Barna and Wakhlu, 1995; Thomas, 2003). TDZ-induced morphogenesis probably depends on the levels of endogenous growth regulators, and TDZ modulates the endogenous auxin levels (Murthy et al., 1995; Hutchinson and Saxena, 1996). In the present study BA and TDZ in combination with IAA gave better results than TDZ alone. Hence, the synergistic combination of auxin and cytokinin promoted shoot regeneration. The promoting effect of auxin and cytokinin combinations on organogenic differentiation has been well established in several systems (Lisowska and Wysonkinska, 2000; Pereira et al., 2000; Pretto and Santarém, 2000; Xie and Hong, 2001; Koroch et al., 2002).

Recalcitrance can also be mitigated by the application of potent synthetic PGRs such as thidiazuron (Murthy et al., 1998).
Thidiazuron (N-phenyl-N 0-1,2,3,-thidiazol-5-yl urea; designated TDZ) is a highly effective regulator of in vitro morphogenesis and can be used to induce a wide range of differentiated and dedifferentiated responses. To date, the mode of action of TDZ is unknown but Murthy et al. (1998) speculate that it may modulate endogenous hormonal activity, either directly or as a result of stress. TDZ can display both auxin- and cytokinin-type activities and this is most likely due to it having both phenyl and thidiazol groups. It has been most effectively used for in vitro shoot regeneration in woody species and can be far more effective in promoting morphogenetic responses compared to other cytokinins, although it must be cautioned that it can impair growth at superoptimal levels (Murthy et al., 1998).

For further enhancement of shoot regeneration, the callus was transferred to the optimal concentration of plant growth regulator and addition of antioxidant. Antioxidants can efficiently improve plant regeneration by reducing tissue browning and improving growth of cultured explants (Attree et al., 1992; Attree and Fowke, 1993; Das et al., 1996)

Activated charcoal (AC) adsors inhibitory substances accumulating in the culture medium (Weatherhead et al., 1979; Theander and Nelson, 1988) is thus often used to reduce the oxidation of phenolic compounds (Liu, 1993; Teixeria et al., 1994) in tissue culture to improve cell growth and development. The addition of activated charcoal often has a promotive effect on the growth and organogenesis of woody species (Pan and Van Staden, 1998).

5.2.1.3. Root induction
The elongated shoots were transferred to MS medium supplemented with different auxins. Of the different auxin tested, IBA showed the highest response for root induction. The similar results were observed by Ignacimuthu and Franklin (1999), Nishimura et al. (2003), Makunga et al. (2003),
Arumugam et al. (2003), Kumaresan (2006), Wang et al. (2006a), Francis et al. (2007), Tawfik and Mohamed (2007) and Ahn et al. (2007).

5.2.2. *Simarouba glauca*

5.2.2.1. Callus induction and texture

In the present study, cotyledon and internode explants were used. Cotyledon showed the highest percentage of response for callus induction followed by internode explant. These two explants were cultured on MS medium supplemented with different auxins. Of the different auxins tested, IBA showed the highest response (Ochatt and De Azkue, 1984; Kumaresan, 2006).

5.2.2.1. Shoot regeneration

Morphogenesis in tissue culture is a prior requirement for much genetic manipulation of plant cells in culture. The most important factors affecting the induction of organogenic callus and plant regeneration through organogenesis include the explant type, media formulation and growth regulators. Higher regeneration rate of young tissues has been reported to occur in other species (Baker and Bhatia, 1993; Carneiro et al., 1999).

Green compact nodular callus was transferred to regeneration medium containing different cytokinin and auxin individually or in combination. The synergistic effect of BA and KN showed the highest percentage of shoot regeneration. In *Acacia catechu, Aegle marmelos*, BA and KN concentrations resulted in an increase in bud formation from hypocotyl (Thakur et al., 2002, Arumugam et al., 2003).

Usually a combination of two or more growth regulators is required for organogenesis, either applied simultaneously or sequentially (Evans et al., 1981). A balanced ratio of growth regulators plays an important role in differentiation of shoot buds (Wilson et al., 1996). The superior effect of BA over other cytokinins for shoot bud induction has been attributed to the abilities
of plant tissues to metabolize the natural growth regulators readily than other synthetic growth regulators or to the ability of BA to induce endogenous production of zeatin (Zaerr and Mapes, 1982).

For further enhancement of shoot the callus were transferred to BA (3.0 mg/l) and KN (0.5 mg/l) with the varying concentrations of GA3 (0.1 – 2.0 mg/l). Cytokinin alone was found to be sufficient for inducing adventitious shoot regeneration whilst a medium supplemented with GA3 significantly improved shoot recovery. GA3 was found to be a requirement for the successful culture of Citrus (Gmitter et al., 1990) and Passiflora foetida (Mohamed et al., 1996).

To increase the further regeneration ability of the callus, the callus was transferred to the medium containing BA (3.0 mg/l), KN (0.5 mg/l), GA3 (1.0 mg/l) and along with different additives. Coconut water enhanced the shoot regeneration ability when compare to other additives.

5.2.2.1. Root induction
The regenerated microshoots were transferred to the medium containing different auxins. Of the three auxins tested, NAA showed the highest response for root induction. The similar results were also observed by Mohamed et al. (1996).

5.3. Somatic embryogenesis
Somatic embryogenesis offers great potential in crop improvement because it allows for efficient cloning and genetic modification. Somatic embryogenesis is a process by which the somatic cells undergo a developmental process similar to the development of zygotic embryos (Mithila et al., 2003). In general, somatic embryos are induced when cultures are exposed initially to a medium containing higher doses of auxins followed by subsequent transfer to a medium
supplemented with either lower doses of auxins or optimal levels of cytokinins (Fujimura and Komamine, 1979; Mithila et al., 2003).

Somatic embryogenesis represents a paradigm of early plant developmental process (Zimmermann, 1993; Rojas-Herrera et al., 2002). Its fundamental phases are characterized, at the morphological level, by induction of proembryogenic structures followed by somatic embryo formation, maturation and desiccation leading to plant regeneration. Each of these phases is regulated by various intrinsic as well as extrinsic factors, and, therefore, a number of critical chemical and physical treatments are applied with appropriate schedules for this complex regeneration system to be efficient. However, of all these factors, plant growth regulators appear to play the most crucial role in somatic embryogenesis.

The plant growth regulators auxin and cytokinin are widely used in plant tissue culture to induce somatic embryogenesis or adventitious shoot organogenesis (Flick et al., 1983; Ramage and Williams, 2004).

5.3.1. Somatic embryogenesis in solid medium
5.3.1.1. *Hildegardia populifolia*

5.3.1.1.1. Callus induction
For callus induction two types of explants tested, internode explants showed the best response than petiole. Of the two auxins tested, 2,4-D was more efficient than NAA for callus induction. In most cases embryogenic calluses were induced on medium containing 2,4-D (El Hadrami, 1995; El Bellaj, 2000; Limanton- Grevet and Julien, 2000; Stephan and Jayabal, 2001; Fki et al., 2003; Lin et al., 2004; Ramakrishnan et al., 2005).

5.3.1.1.2. Somatic embryo induction
2,4-D was the most suitable of the two auxins sources tested for induction of somatic embryos, while NAA induced somatic embryogenesis in a small number
of calli. This finding agrees with the report of Barna and Wakhlu (1993) and Ma et al. (2007) who reported 2,4-D treatment to be favourable for somatic embryogenesis in Chick pea and Burma Reed. Kawahara and Komamine (1995) reported the effect of exogenous auxin on the expression of polarities in early stages of somatic embryogenesis.

For further improvement of somatic embryo induction 2,4-D along with BA yielded highest percentage of somatic embryogenesis. The requirement of cytokinin addition to auxin has been also reported in Aegle marmelos and Cardiospermum halicacabum (Arumugam and Rao, 2000; Jeyaseelan and Rao, 2005, Pinto-Sintra, 2007).

MS medium with 2,4-D and glutamine (25 mg/l) enhanced somatic embryogenesis from internode derived callus. Addition of L-glutamine enhanced somatic embryogenesis, number of somatic embryos and the frequency of embryos per callus (Das et al., 1995). Similarly in the present study, glutamine played a vital role in enhancing somatic embryo development.

Addition of BA and glutamine slightly enhanced the frequency of embryo production. Auxin is the most important factor for the regulation of induction and development of embryogenesis and it has different effects on different phases of embryogenesis. This indicates that auxin is essential for induction of somatic embryogenesis. BA and glutamine showed a promotive effect with auxin on embryogenesis (Arumugam and Rao, 2000). The efficacy of BA in the induction of somatic embryos has been demonstrated on Simarouba glauca (Rout and Das, 1994a), sandalwood (Rai and McComb, 2002), and Quassia amara (Martin and Madassery, 2005).

5.3.1.1.3. Embryo maturation
The embryogenic mass (immature embryo) was transferred to various strengths of MS medium and different concentrations of sucrose (1- 4%) for maturation.
Full strength medium with 4% sucrose showed the highest percentage of maturation. High concentration of sucrose was found to be effective in inducing embryo maturation (De Wald et al., 1989; Tremblay and Tremblay, 1991; Xie and Hong, 2001). Normal development of somatic embryos was promoted by high osmotic treatment in Daucus carota (Ammirato, 1983; Finkelstein and Crouch, 1986) and prevents precocious germination (Xu et al., 1990).

Moderation of sucrose levels and the application of sugar alcohols, polyols and alternative sugars have a specific role in somatic embryogenesis and in assisting maturation in the final stages of embryo development (Benson, 2000). The application of ABA can greatly improve recalcitrance problems associated with embryo and organogenic development in vitro and whole plant regeneration (Kim and Moon, 2007). Thus, ABA and optimal sucrose levels increased the production of somatic embryos and shoots in sugar beet callus (Saunders and Tsai, 1999).

5.3.1.1.4. Somatic embryo germination

Clusters of somatic embryos were transferred to MS medium supplemented with ABA (0.01 - 0.5 mg/l) for germination. None of the embryos showed normal germination only abnormal proliferation and recalling was resulted instead of germination of embryos. Even somatic embryos that appear normal failed to germinate fully and thus lack conversion into plants. Application of ABA in late embryogenesis leads to stages of embryo maturation and conversion (Lecouteux et al., 1993; Mhaske et al., 1998; Ramakrishnan et al., 2005). The addition of ABA to the medium efficiently promoted somatic embryo maturation. The ABA-containing medium possibly mimics the maternal effect on the development of zygotic embryos (Belefant-Miller et al., 1994; Garcia-Martin et al., 2005).

PEG has been reported useful in promoting somatic embryo maturation and the development of well-formed cotyledon, inhibiting precocious somatic embryo germination and the formation of multiple-embryo clusters. However,
in the present study, PEG appeared to be inhibitory to somatic embryo development. Similar results were also reported (Zhang et al., 2005).

The failure and/or low frequency of conversion are often attributed to morphological abnormalities or immaturity of somatic embryos (Ammirato, 1987). In the present case, somatic embryos never germinated on a medium containing cytokinins or an auxin – free medium even after prolonged subculture, instead they began to recallus.

Maturation and germination of somatic embryos are two critical steps in eventual recovery of healthy plants (Ramanjini and Prakash, 1998). Generally, the development of embryos is attributed to the biophysical and genetic factors governed by the laws of embryogenesis.

The length of the embryo was more critical in its ability to develop into a plant. When embryos were too small their ability to grow into plants diminished (Emershad and Ramming, 1994). A major problem associated with somatic embryogenesis is the poor conversion into plants (Garg et al., 1996).

The protocols for somatic embryogenesis are often not very useful for clonal propagation and crop improvement due to limited plant recovery. Most of the literature on the conversion of the somatic embryo and survival of somatic embryo raised plantlets describe low frequencies of conversion (Baker and Wetzstein, 1992; Ozias-Akins et al., 1992; Chengalrayan et al., 1994; McKently, 1995).

Choi et al. (1998) suggest that the inability of regenerated plants to form roots might be related to embryonic structural abnormalities since morphologically abnormal somatic embryos (e.g. multicotyledonary and multiple embryos) have also been frequently observed (Butenko et al., 1968; Chang and Hsing, 1980; Lee et al., 1990; Arya et al., 1993).
Wetzstein and Baker (1993) and Motoike et al. (2007) presented that low conversion rate for *Arachis hypogea* was probably due to incomplete development of meristematic areas. Several embryos started germination but did not complete the conversion. Those different features associated with conversion of somatic embryos still pose the major challenge for many species, including *Hildegardia populifolia*, where additional investigation is needed in order to understand some of the controlling agents for conversion.

5.3.1.2. *Simarouba glauca*

5.3.1.2.1. Callus induction

Cotyledon and internode explants were grown on MS medium supplemented with 2, 4-D and NAA (0.1-3.0 mg/l) for callus induction. The intensity of callus proliferation was greater in the medium with 2, 4-D and more responsive with induction.

The influences of exogenously applied auxins, preferentially 2,4- D, on the induction of somatic embryogenesis are well documented (Dudits et al., 1991; Yeung, 1995; Kaur et al., 2006).

5.3.1.2.2. Somatic embryo induction

The callus was transferred to MS basal medium containing NAA (1.0 mg/l) and BA (0.1-3.0 mg/l). The somatic embryos were also increased in 2.0 mg/l of BA with the highest frequency of somatic embryo induction. The formation of somatic embryos on compact callus has been reported in *Paspalum notatum* (Bovo and Mroginski, 1989), *Echinochloa colona* (Samantaray et al., 1997) *Psoralea corylifolia* (Chand and Sahrawati, 2007). Globular somatic embryos were first observed 2 weeks after inoculation and they were white in colour (Khan et al., 2006).
As a general rule, embryogenic tissue is initiated in a medium containing low concentration of auxins, usually in the form of 2,4-D and NAA and cytokinins, such as BA (Tautorus et al., 1991). While a combination of cytokinin and auxin was used to stimulate cell division in proembryogenic masses (Nomura and Komamine, 1995) and in Quercus suber (El Maâtaoui and Espagnac, 1987), Q. ilex (Feraud-Keller and Espagnac, 1989) and Norway spruce (Mo and von Arnold, 1991).

NAA along with BA yielded highest percentage of somatic embryo induction. This similar result was also reported by Torne et al. (1997) and Kim et al. (2003, 2005). The combination of BA and NAA has often been used for somatic embryogenesis in immature zygotic embryo cultures of some species, e.g., Abies alba (Schuller et al., 1989), Abies nordmanniana (Norgaard and Krogstrup, 1991), Ginkgo biloba (Laurain et al., 1996).

5.3.1.2.3. Somatic embryo maturation
The somatic embryo mass was transferred to MS medium supplemented with BA (2.0 mg/l), NAA (0.5 mg/l), and additives (AdSO₄, sodium citrate, glutamine) for further maturation of embryos. Sodium citrate responds more with the highest frequency of somatic embryo maturation. Cytokinins required for plantlet development from somatic embryogenesis (Raj Bhansali et al., 1988). There are several reports where combination of auxin and cytokinin were important in fostering somatic embryogenesis development (Raj Bhansali and Manjit Singh, 2000). NAA has been demonstrated to be an effective auxin for both the initiation and maturation of somatic embryos in carrot (Ammirato, 1985).

5.3.1.2.4. Somatic embryo germination
Clusters of matured somatic embryos from the embryo differentiation medium were transferred to MS medium supplemented with BA (1.0 mg/l), ascorbic acid (10 mg/l) combination with TDZ of various concentrations for germination. BA,
ascorbic acid and GA₃ were proved to be essential for simultaneous development of shoot and root poles from the embryos (Arumugam and Rao, 2000). Instead of GA₃, TDZ was found to be the most potent in triggering differentiation in the meristems of embryos to form complete plantlet in this combination. Chengalrayan et al. (1997) reported that TDZ stimulates the conversion of embryos in to plantlets. The role of TDZ in the induction of somatic embryogenesis has also been reported in bamboo (Lin et al., 2004; Khan et al., 2006).

Although many somatic embryos turned to be normal plants, there is still, a large amount of variations in shapes, sizes, number of cotyledons, synchrony, maturation rate and germination (Vasil, 1994). The major limitation for mass propagation and field planting of somatic embryogenesis protocols for trees are related to low frequency and conversion rate, facts detected in this study. For a considerable number of species the conversion stage still represented the major challenge to be surpassed in order to determine optimum protocols where complete plants can be obtained (Chalupa, 1995; Preece and Bates, 1995). In *Simarouba glauca* only 20-25% of somatic embryos have reached germination stage as reported by Rout and Das (1994b) but in the present study, 45% of germination rate was achieved.

5.3.2. Somatic embryogenesis in liquid medium

Somatic embryo culture in liquid medium has some advantages, i.e., the multiplication rate is high, embryos grow faster in size and fresh weight, due to the easier nutrient absorption, and they detach easily from callus (Mauri and Manzanera, 2003).

The frequency of somatic embryo formation in liquid culture was much higher than in solid culture. Suspension and solid cultures displayed significant difference in the induction of embryos. Similar results have been reported in black pepper (Joseph et al., 1996), *Holostemma ada-kodien* (Martin, 2003a) and
Ceropegia candelabrum (Beena and Martin, 2003) where high-frequency embryo formation occurred in suspension cultures compared to a lower frequency in static cultures. Induction of somatic embryogenesis in suspension culture formed large clumps of globular and later stage of embryos and the embryos underwent proliferation and development in the same medium (Wang et al., 2006b).

In general, maturation of somatic embryos is achieved on solidified media, although, as demonstrated recently, vigorous embryos can also be obtained in liquid medium (Gupta and Timmis, 1999; Gorbatenko and Hakman, 2001).

In both trees (Hildegardia populifolia and Simarouba glauca), the suspension culture was not feasible due to poor conversion. Large numbers of somatic embryos can be produced in a small volume of liquid, and from them large numbers of plants can be grown (Ammirato, 1984). There are, however, a number of problems. Suspension cultures are a mixture of embryogenic and non-embryogenic cells and clusters, and these are of varying sizes and numbers. During successive subculture regimes, the cultures may become composed solely of non-embryogenic cells, thereby losing the ability to produce somatic embryos and plants. Because there is a proembryo decrease when embryo maturation begins, the resulting populations of somatic embryos develop asynchronously. In addition the normal pathway of development may be diverted (Ammirato, 1985) resulting in a range of structurally aberrant forms. In case of indirect embryogenesis, by contrast, a callus will be formed and it is from this callus culture that embryos arise. Somaclonal variation of the embryos arising through direct embryogenesis is lower than that of indirect embryogenesis, but frequency of direct embryogenesis in different plant species is lower than that of indirect embryogenesis.
Fig. 1. Schematic representation of in vitro micropropagation of *Hildegardia populifolia* through axillary and apical bud explants.

2 month-old *in vitro* derived seedling

Axillary and apical bud explants

**Sterilization**

(70% ethyl alcohol for 3 minutes, 0.1% HgCl₂ for 5 minutes and finally rinsed with sterile distilled water)

**Bud sprouting** (15 days)

MS + BA (1.0 mg/l)

**Multiple shoot induction** (25 days)

[MS + BA (1.0 mg/l) + KN (0.5 mg/l)]

**Shoot multiplication and shoot elongation** (45 days)

MS + BA (1.0 mg/l) + KN (0.5) + GA₃ (1.0 mg/l)

**Improvement of shoot quality and multiplication rate** (30 days)

MS + BA (1.0 mg/l) + KN (0.5 mg/l) + GA₃ (1.0 mg/l) + glutamine (50 mg/l)

**Rooting without basal callus** (45 days)

MS + IBA (2.0 mg/l) + without sucrose

**Acclimatization** (No. of plantlets/explant = 15)

[Red soil: sand (2:1) for 4 weeks]
Fig. 2. Schematic representation of *in vitro* organogenesis of *Hildegardia populifolia* through internode and petiole explants.

**Internode and petiole explants**

↓

**Sterilization**

(70% ethyl alcohol for 3 minutes, 0.1% HgCl₂ for 5 minutes and finally rinsed with sterile distilled water)

↓

**Callus initiation** (45 days)

MS + IBA (2.0 mg/l)

↓

**Callus proliferation** (25 days)

MS + BA (2.0 mg/l) + IAA (0.1 mg/l) + glutamine (25 mg/l)

↓

**Shoot bud regeneration** (45 days)

MS + BA (2.0 mg/l) + IAA (0.1 mg/l) + glutamine (25 mg/l) + TDZ (0.5 mg/l)

↓

**Rooting** (45 days)

MS + IBA (3.0 mg/l)

↓

**Acclimatization** (No. of plantlets/explant = 2.5)

[Red soil: sand (2:1) for 4 weeks]
Fig. 3. Schematic representation of in vitro somatic embryogenesis of *Hildegardia populifolia* through internode and petiole explants.

Internode and petiole explant

↓

**Sterilization**

(70% ethyl alcohol for 3 minutes, 0.1% HgCl₂ for 5 minutes and finally rinsed with sterile distilled water)

↓

**Callus initiation** (15 days)

MS + 2, 4-D (1.0 mg/l)

↓

**Somatic embryo initiation** (20 days)

[MS + 2, 4-D (1.0 mg/l) + BA (0.5 mg/l)]

↓

**Somatic embryo induction** (25 days)

[MS + 2, 4-D (1.0 mg/l) + BA (0.5 mg/l) + glutamine (25 mg/l)]

↓

**Somatic embryo maturation** (7 days)

MS + Sucrose (4%)
Fig. 4. Schematic representation of in vitro micropropagation of Simarouba glauca through cotyledonary node, axillary and apical bud explants

2 month-old in vivo derived seedling

↓

Cotyledonary node, axillary and apical bud explants

↓

Sterilization
(70% ethyl alcohol for 1 minute, 0.1% HgCl₂ for 3 minutes and finally rinsed with sterile distilled water)

↓

Bud sprouting (25 days)
MS + BA (3.0 mg/l)

↓

Multiple shoot induction (25 days)
[MS + BA (3.0 mg/l) + KN (0.5 mg/l)]

↓

Shoot multiplication and shoot elongation (25 days)
MS + BA (1.0 mg/l) + KN (0.5) + NAA (0.5 mg/l)

↓

Improvement of shoot quality and multiplication rate (30 days)
MS + BA (1.0 mg/l) + KN (0.5mg/l) + NAA (0.5 mg/l) + glutamine (10 mg/l)

↓

Enhancement of shoot quality and multiplication (30 days)
MS + BA (1.0 mg/l) + KN (0.5 mg/l) + NAA (0.5 mg/l) + glutamine (10 mg/l) + PVP (25 mg/l)

↓

Rooting (35 days)
MS + NAA (3.0 mg/l)

↓

Acclimatization (No. of plantlets/explant = 55)
[Red soil: sand (2:1) for 4 weeks]
Fig. 5. Schematic representation of *in vitro* organogenesis of *Simarouba glauca* through cotyledon and internode explants.

Cotyledon and internode explants

↓

Sterilization
(70% ethyl alcohol for 1 minute, 0.1% HgCl₂ for 3 minutes and finally rinsed with sterile distilled water)

↓

Callus initiation (35 days)
MS + IBA (1.0 mg/l)

↓

Shoot bud regeneration (25 days)
MS + BA (3.0 mg/l) + KN (0.5 mg/l) + GA₃ (1.0 mg/l)

↓

Effect of additives on shoot regeneration (25 days)
MS + BA (3.0 mg/l) + KN (0.5 mg/l) + GA₃ (1.0 mg/l) + CW (15%)

↓

Rooting (25 days)
MS + NAA (2.0 mg/l)

↓

Acclimatization (No. of plantlets/explant = 25)
[Red soil: sand (2:1) for 4 weeks]
Fig.6. Schematic representation of *in vitro* somatic embryogenesis of *Simarouba glauca* through cotyledon and internode explants.

Cotyledon and internode explant

↓

**Sterilization**

(70% ethyl alcohol for 1 minute, 0.1% HgCl₂ for 3 minutes and finally rinsed with sterile distilled water)

↓

**Callus initiation** (25 days)

MS + 2, 4-D (2.0 mg/l)

↓

**Somatic embryo induction** (25 days)

[MS + BA (2.0 mg/l) + NAA (1.0 mg/l)]

↓

**Somatic embryo maturation** (30 days)

[MS + BA (2.0 mg/l) + NAA (0.5 mg/l) + Sodium citrate (10 mg/l)]

↓

**Somatic embryo germination** (15 days)

MS + BA (1.0 mg/l) + TDZ (0.5 mg/l) + Ascorbic acid (10 mg/l)

↓

**Somatic embryo induction in liquid medium** (15 days)

MS + NAA (1.0 mg/l) + TDZ (0.5 mg/l)

↓

**Somatic embryo maturation** (30 days)

MS + TDZ (1.0 mg/l) + glutamine (10 mg/l)