DISCUSSION

The presence of phenolic compounds causing death of explants is an important problem in tissue cultures of woody perennials (Compton and Preece 1986). Addition of anti-oxidants/reducing agents like ascorbic acid, in the medium or before surface sterilization, reduces the redox potential of explants and stops oxidation reaction (Marks and Simpson 1990; Poudyal et al., 2008). Due to the oxidation of externally released polyphenols, explants (young stem and leaf) as well as the nutrient medium became brown and explants did not respond in vitro. A strategy to control phenolic oxidation has therefore become a necessity. In control experiments of the present study, all explants of *D. falcata* died due to phenolic browning and the exudates appeared as a reaction in injury/ infection. Tissue blackening has been reported due to action of copper containing oxidase enzymes polyphenoloxidases like tryosinases. These are released or synthesized in oxidative conditions after tissue wounding. They oxidize o-diphenols released due to cellular wounding to o-quinones (Scalbert et al., 1988; Marks and Simpson 1990). The onset of tissue browning has been found to be associated with changes in pattern, amino acid content, ethylene production, the occurrence of saccharose and accumulation of starch (Lindfors et al., 1990). These changes eventually lead to the growth inhibition/death of explants. Other types of phenolic exudates appear at the end of incubation period and are apparently the products of dying cells (Seneviratne and Wijesekara 1996). He et al., (1995) treated banana explants with ascorbic acid before culturing and lessened the percentage of browning. Use of 280 mmol/L of ascorbic acid also reduced browning of the medium in cashew (Aliyu, 2005). Similarly, in an experiment conducted by Peng et al., (2007) on *Bromelia* spp., they found more severe problems of browning in *Aechmea fasciata* than in *Guzmania* and *Vriesea charlotte* during in vitro culturing. The phenolic exudation is aided by light and is autocatalytic. In present study, the problem of phenolic oxidation in *D. falcata* was overcome completely by incorporation of 40mg/l ascorbic
acid in nutrient media and accounted for healthier and faster growth of the callus of *D. falcata*. The problem of phenolic oxidation was less when the embryo was used as an explant and the *in vitro* grown seedlings was juvenile.

The diversity among the nutrient compositions of media and their application for a variety of plant species might be the reason for variable responses of these nutrient media supplemented with different auxins and cytokinin concentration for callus induction from different explants of *D. falcata*. The MS enriched with higher basal salts was found most suitable for the highest degree of callus induction and proliferation (Baskaran and Jayabalan 2005).

**Effect of auxins and cytokinin on callus induction and its growth:**

The variable concentrations of auxins (NAA, IBA or 2, 4-D) and cytokinins (BA or Kin) alone or in combinations affected the callus induction and its proliferation. The auxin/cytokinin ratio plays an important role in initiation and proliferation of callus from different types of explants. The higher auxin/cytokinin ratio generally initiates callus induction, whereas, higher cytokinin to auxin ratio was involved in the direct organogenesis or indirect organogenesis through development of callus. However, the concentrations of auxins or cytokinins alone directly initiated the callus formation, direct organogenesis or indirect organogenesis. The results of present investigation followed the regulation of impact of different concentrations of auxins and cytokinins alone or in combination on callus induction and proliferation from different types of explants.

Among the various types of auxins (NAA, IBA or 2, 4-D) and their concentrations (0.2 to 7.5 mg) incorporated alone in the media for callus induction and proliferation, 2, 4-D at 3 mg concentration was found most suitable for highest induction and proliferation of callus from embryo explants of *D. falcata* as compared to other types and concentrations of auxins. Apart from 2, 4-D, NAA was also effective for callus induction as compared to IBA. Callus obtained from embryo explants on 2, 4-D and IBA showed less browning as compared to callus obtained on NAA. The similar effect for
highest callus growth was recorded in callus cultures of Gymnema sylvestre on medium supplemented with 2, 4-D as well as NAA (Gopi and Vatsala 2006). Thus, it is worthwhile to note that, addition of 2, 4-D and IBA to the medium separately initiated and promoted the growth of healthy callus from different types of embryo explants. In the present investigation, incorporation of 2, 4-D alone in the medium responded well for higher amount of callus formation and proliferation from different types of explants of D. falcata. Many report shows maximum callus induction and proliferation from different types of explants of different species (Shah et al., 2003; Islam et al., 2005; Uddin et al., 2006; Ozyigit et al., 2007; Hasan et al., 2008).

Apart from alone concentrations of auxins, cytokinins (BA or Kin) alone at specific concentrations played an important role in callus induction from different types of explants. In present investigation, different concentrations of BA or Kin when used separately produced variable degree of callus. Among BA and Kin, BA was found most suitable for higher callus formation and proliferation from embryo explants of D. falcata. BA at 5.0 mg produced extensively higher amount of callus proliferation from embryo explants as compared to different concentrations of Kin. The present results are in accordance with the earlier reports of Santos et al., (1990), Gopi and Vatsala (2006) and Shah et al., (2003) on higher callus proliferation from Gymnema sylvestre and wheat, respectively. In contrast, Nikam and Shitole (1999) in Carthamus and Angelova et al., (2001) in Nicotiana tabacum reported maximum callus induction on Kin rather than BA.

The auxins (2, 4-D, IBA or NAA) supplemented with cytokinins (BA or Kin) resulted into callus induction, direct organogenesis or indirect organogenesis which is completely dependent on the concentrations of auxins in combination with cytokinins in the medium. In present investigation, among different types and concentrations of auxins incorporated in the medium in association with various levels of BA/Kin, the medium containing 2, 4-D and Kin was most useful for higher callus induction and proliferation as compared to any other types of combination of auxins and cytokinins. In
contrast IAA and BA responses for callus induction and proliferation were observed in various types of plant species like *Ionidium suffruticosum* (Sonnapanavar and Jayaraj 2011), *Duboisia myoporoides* (Khanam et al., 2000); *Gymnema sylvestre* (Gopi and Vatsala 2006) and *Tinospora cordifolia* (Rao et al., 2008). However, in present study, the response of 2, 4-D + Kin for higher callus induction from various types of explants of *D. falcata* was variable and higher from embryo explants as compared to internode, leaf or node. Similar results for callus induction from hypocotyl explant of *Bunium persicum* on MS+2, 4- D + Kin were also reported by Valizadeh and Tabar (2009). On the other hand, auxins (NAA, IBA or 2, 4-D) in combination with BA did not respond well for callus induction and proliferation. However, NAA and Kin combinations were superior to NAA and BA or 2, 4-D and BA combinations with respect to callus initiation and proliferation. In present investigation, inhibitory effect of Kin on callus induction may have been compensated by NAA in NAA + Kin combination.

Thus, from present results it is concluded that, among auxins and cytokinins, 2, 4-D and BA, respectively, alone and in combination were most suitable for the production of large callus biomass from embryo explants of *D. falcata* as compared to any other type and concentration of auxins and cytokinins as well as types of explants. The same hormones when used for maintenance of callus will amplify the callus proliferation frequency.

**Response of explants (internode, node, embryo and leaf) to callus formation and its growth:**

The results of present investigation have shown that the types of explants (internode, node, embryo and leaf) play an important role for higher degree of callus induction and growth on the medium supplemented with various concentrations of cytokinins and auxins alone or in combinations. Some explants responded better for callus initiation on medium supplemented with alone concentrations of growth hormones (either auxins or cytokinins), whereas some explants required combinations of growth regulators (auxins and cytokinins) in variable range. In addition, the growth regulator
concentrations in the medium decided the callus formation frequency/callus induction.

In the present investigation, among different types (internode, node, embryo and leaf) of explants used for callus initiation and growth on various types and concentrations of growth hormones, embryo explants performed best for maximum degree of callus induction and proliferation on the media supplemented with different concentrations of cytokinins and auxins alone or in combinations. Between internode and leaf explants, leaf explant has ability to produce more calli as compared to internode explant, however, the level of callus initiation and growth was lower from both types of explants as compared to embryo explant. Similarly, Nikam and Shitole (1999), Rani et al., (1996) and Seetha (1991) reported variations in the response of different types of explants for callus initiation and growth, wherein they found that the cotyledon explants of *Carthamus tinctorius* were most suitable for maximum callus induction and proliferation as compared to root and hypocotyl explants on the medium supplemented with various levels of cytokinins and auxins. However, Da Silva et al., (2003) reported that cotyledon and hypocotyl explants of *Glycine wightii* did not show much variation for callus initiation, growth and both explants more or less performed similarly for callus induction/growth. In contrast, Thanonkeo and Panichajakul (2005) observed that among different types of explants (cotyledon, stems, leaves, shoots and roots) of *Pueraria candollei* used for callus production, the root explant produced maximum induction and growth of callus as compared to other types of explants when cultured on various levels of auxins and cytokinins. In addition, Gopi and Vatsala (2006) reported the highest callus induction from nodal explants of *Gymnema sylvestre* as compared to leaf explants on different concentrations of plant growth regulators.

Thus, it can concluded that the response of different types of explants showed variations for callus induction and it varied from explant to explant among same or different types of plant species. Further, the type of explant responded specifically for callus induction and proliferation depending upon
the composition and concentrations of plant growth regulators in the medium. The age, structure and organization of the explants also imparted the callus initiation and proliferation. Thus, the present results are in conformity with the results mentioned above for the effect of different types of explants for callus initiation and proliferation on various types of plant growth hormones.

**Effect of cytokinins and auxins on embryo growth of D. falcata after 3 weeks:**

**Effect of auxins:**
The fresh dry mature seeds did not respond for germination on media containing auxins. The inclusion of auxins in the MS medium containing 3% sucrose resulted in reduction of percentage of germination of embryo from green mature seeds and promoted the white creamy callus formation from the entire surface of the embryos. Similar observations have been reported by Rambabu et al., (2006) in seedlings of *Givotia rotteliformis* and Ghorpade et al., (2010) in *Boswellia serrata* produced in medium containing NAA and IAA, which were abnormal and associated with formation of callus. Manrique et al., (2005) have reported similar inhibitory effect on in vitro germination *Compurettia falcata* and explained that inhibition might have occurred due to high concentration of free IAA during development and resulted into intoxicification/competition with other growth regulators. The results of present study showed that, as compared to IAA, the incorporation of synthetic auxins IBA, NAA and 2, 4-D were very strong inhibitors for germination process but induced callus in large number of embryo.

**Effect of cytokinin:**
Cytokinins are regulators of cell division, and play role in breaking of bud dormancy, seed germination, callus formation in tissue culture and shoot regeneration process associated to micropropagation (Bhojwani and Razdan 1996; Taiz and Zeiger 2006). In the current study, addition of low level of cytokinins BA/Kin did not result in improvement in germination of embryo obtained from green mature seeds. However, it caused swelling and induction of callus on radical of embryo. Similar results were obtained by Ghorpade et
al., (2010) in *Boswellia serrata*. The successful *in vitro* culture of embryo using supplementation of auxins and cytokinins in *P. mume* has been reported by Ning *et al.*, (2007).

**Explants and Caulogenesis:**

The plant micropropagation success largely depends on the selection of a suitable plant part, which is to be used as the starting material for the experiment (Barik *et al.*, 2007). The *in vitro* regeneration of shoots have been reported by using different explants like root, stem, leaf, hypocotyls, cotyledons, anther, fruits and seedlings in many important plant species. The organogenesis potentiality of the explant is known to be dependent on the donor tissue (Gamborg and Shyluk, 1981; Narayanswamy, 1994; Dixon, 1985; Thorpe 1994; Bhojwani and Razdan 1996; Razdan 1995; Robert and Dennis, 2000). In the present investigation, caulogenesis was achieved from the shoot tip, axillary node, cotyledonary node, leaf node and embryo explants of *D. falcata* on MS media incorporated with various permutation and combination of growth regulator. In current study, the leaf and internode explants did not respond to shoot regeneration in MS medium used with plant growth regulators. The frequency of explants responding for shoot regeneration and the number of shoots per explant varied depending on the type of explants. Comparatively, the frequency of explant responding for shoot regeneration and the mean number of shoot per explant was highest in the cotyledonary nodal explant than other explants used. Earlier, there was no report on the comparative account of the response of different explants for caulogenesis in *D. falcata*. The results of present investigation suggested that the nodal, leaf node and cotyledonary node explants exhibited the highest potential for direct regeneration of multiple shoots in *D. falcata*.

The effective potential of nodal explant for shoot multiplication and regeneration in *in vitro* culture is well known and documented over the years in different plant species (Gamborg and Shyluk 1981); Plant regeneration from shoot and stem meristem has been noted in *Catharanthus roseus* (Moreno and Van 1995) and nodal explant of *Catharanthus roseus* (Mitra and
Khan 1998). Similar results were obtained in other plants like Solanum paludosum (Badaoui et al., 1996) and Hybentes enneaspermus (Natarajan et al., 1999).

Multiple shoot formation from different explants has been achieved in other members of alkaloid producing plants of Solanaceae. Khanam et al., (2000) reported callus mediated shoot regeneration from leaf explants of Duboisia myoporides. Similar results were obtained from aerial parts of Duboisia leichhardtii (Yamada and Endo 1984), foliar explants of Duboisia myoporides (Kukreja et al., 1986) and shoot tips of Duboisia hybrid (Lin and Griffin, 1992). Uranbey (2005) reported multiple shoot formation in hypocotyls, cotyledon and stem explants of Hyoscyamus niger. Lorz and Potrykus (1979) reported shoot regeneration from mesophyll cell of Atropa belladonna. Corduan (1975) achieved shoot regeneration in anther culture of Hyoscyamus niger and Pandey and Chand (2004) from cotyledon explant of Hyoscyamus muticus.

In our preliminary experiment, it was noted that, in vitro raised explants were more responsive than the explants used from naturally grown plants. Similar results were reported in D. metel (Muthukumar et al., 2004) and Solanum viarum (Tejavathi and Bhuvana 1998). The results of different explants giving variation in shoot multiplication and regeneration potential, suggests that the origin of explant is an important factor that influences the shoot regeneration potential, even if the different explants may be from the same genotype or even from the same mother plant.

Physiological age of explant is another important factor in determining the morphogenetic response of different explants (Ammirato 1986). Young meristematic tissues are generally more responsive for in vitro culture treatment than, mature differentiated tissue (Bhojwani and Razdan 1996; Bhojwani and Prabhakar 1998). Earlier reports on shoot regeneration in alkaloid producing plants showed shoot regeneration potential in node, internode, leaf and hypocotyl explants but not in shoot tip explant. Shoot regeneration was evident in the present study in shoot tip, axillary node,
cotyledonary and leaf node explants. Thus by considering all the factors among the different explants, cotyledonary nodal explants were superior for maximum shoot induction in *D. falcata*. Induction of multiple shoots was however affected by the concentration of cytokinin.

**Plant growth regulator and caulogenesis**

**Effect of cytokinin**

Skoog and Miller (1957) showed that the organogenic differentiation is controlled by the proportion of auxin and cytokinin in the nutrient medium. This fundamental finding has proved true in most of the *in vitro* culture system but is not universal. Earlier reports showed that the requirement of type of auxin/ cytokinin and their concentrations for shoot-bud differentiation varies even between the explants of the same species (Long *et al*., 2010). It is also realized that the shoot differentiation is the result of relationship between the exogenous and endogenous auxins and cytokinin proportion and the influence of other growth conditions (Hu *et al*., 2005).

In the present study, shoot tip and axillary node explants of *D. falcata* cultured on MS growth regulator free medium failed to shoot formation. Similar observations were noted in apical meristems of *Ipomia batatas* which failed to respond for shoot regeneration on growth regulator free medium (Alam *et al*., 2010). The results of the present investigation and earlier reports suggest that endogenous growth regulator concentration may vary in different explants which may be responsible for variation in response of shoot regeneration. In contrast nodal explants of *D. insignis* on MS medium lacking growth regulator responded for shoot regeneration (Santos *et al*., 1990). It suggests that in the responded nodal segments, the meristematic tissue for shoot development exists and it just needed some time and nutrient supplement to develop into a complete shoot.

Incorporation of cytokinin in MS medium improved the potential of shoot regeneration and multiplication of shoot in shoot tip and axillary node explants, while internode and leaf explant failed to respond for shoot regeneration. BA was more effective than Kin for induction of multiple shoot
regeneration. The higher number of direct shoot regeneration in axillary node explant was observed on MS containing 3.0 mg BA. Similar results were reported by Sujatha et al., (2005) that MS medium supplemented with 8.9 uM BA noted maximum shoot regeneration frequency in axillary node of J. curcas.

The results revealed that vacation in the shoot regeneration depends on the type and concentration of cytokinin present in the medium. The multiplication and regeneration effect of BA on various explants was similar with the results recorded by earlier workers in different species of Datura. Arochiasamy et al., (1999) reported maximum shoot induction in D. metel on MS supplemented with 2 mg/l BA. Similar results were reported by Muthukumar et al., (2000, 2004) in D. metel, D. innoxia (Zayed et al., 2006) and D. insignis (Santos et al., 1990). Missaleva et al., (1993) reported that modified MS supplemented with BA promoted extensive vegetative bud proliferation in callus culture of D. innoxia.

At higher concentration of BA, the shoot regeneration was callus mediated and initially induced bud formation. Similar result of budding was obtained on MS with 2.0 mg 1\(^{-1}\) BA in D. insignis (Santos et al., 1990). The formation of callus at moderate and higher concentration of BA and swelling of explants at low concentration of cytokinin (1.0 mg) suggests that the endogenous level of auxin in D. falcata is high.

The response of explant for shoot regeneration declined with the addition of high concentration of BA. These results are similar with the effect of BA in D. metel where the increase in the concentration of BA beyond 3.0 mg/l declined the frequency of shoot proliferation, the number of shoot per explant and the length of the shoot (Arochiasamy et al., 1999). Similar results were obtained by Muthukumar et al., (2004), where addition of 3.5 mg/l BA was inhibitory for shoot proliferation. Khanam et al., (2000) reported that addition of 22 uM BA in the nutrient medium inhibited shoot formation in Duboisia myoporoides. A gradual decline in the response of the explant for shoot induction was noted with increasing concentration of BA in Scopolia
parviflora (Kang et al., 2004). Addition of 16 uM BA declined the number of shoots per explant in *Hyoscyamus niger* (Uranbey 2005). According to Zayed *et al.*, (2006), addition of BA beyond 1 mg/l was unfavourable for shoot induction in *D. innoxia*.

In the present study, it was observed that the effect of Kin for shoot regeneration was less effective as compared to BA. Similar results of BA were also obtained in *Celastrum paniculatus*, in which BA induced maximum multiplication of shoot bud and Kin did not show better response for shoot multiplication (Purohit and Rao, 2006).

In contrast the promotive effects of Kin on plantlet growth for *Dioscorea bulbifera*, which increased the number of shoots per plantlet, were shown by Forsyth and Van (1982). In most cases, however, shoots were formed on media supplemented with Kin (Chaturvedi 1975; Mantell *et al.*, 1978; Forsyth and Van 1982; Poornima and Ravishankar 2007). Missaleva *et al.*, (1993) reported the shoot multiplication in MS supplemented with Kin in *D. innoxia*. In contrast to this Santos *et al.*, (1990) reported that Kin failed to induce axillary bud multiplication in *D. insignis*.

Results of the present work on *D. falcata* revealed that most suitable explant for multiple shoot formation was axillary node explant and most effective cytokinin is 3.0 mg BA.

**Interaction of combination of auxin and cytokinin**

The shoot formation response in various explants is greatly influenced by auxins and cytokinin present in nutrient media. Usually individual cytokinin or higher ratio of cytokinin to auxins favours the shoot formation and their multiplication. (Thorpe 1981, 1994; Bhojwani and Razdan 1996; Narayanswamy 1994; Gamborg and Phillips 1998; Hu *et al.*, 2005).

In present investigation, the shoot regeneration was achieved in node, cotyledonal node and leaf node explant. However internode and leaf explants failed the response for shoot regeneration. The response of shoot regeneration in cotyledonal node and leaf node clearly depends on the type and concentration of cytokinin and auxins added in the nutrient medium. Low
concentrations of auxins in combination with BA/Kin induced shoot multiplication from node, cotyledonary node and leaf node explants. However, presence of higher levels of auxin IBA, IAA, NAA and 2, 4-D inhibited shoot multiplication and promoted callus formation. Among the various combinations, of BA : IBA/IAA/NAA/2,4-D and Kin : IBA/IAA/NAA/2, 4-D, the combination IBA:BA was the most effective for induction of callus mediated shoot formation from the cotyledon node and leaf node explants of *D. falcata*. Similar results were obtained by Samake *et al.*, (2011) in *Acacia nilotica*, and Prem *et al.*, (2003) in *Cyamopsis tetragonoloba*.

The combination of IAA: BA is effective as compared to NAA. Similar results were obtained by Kaewsuwan *et al.*, (2005), who reported the suppressed shoot induction rate when 0.5 uM NAA was added along with BA. In contrast to this maximum multiplication of shoots from nodal explant of *Phyllanthus fraternus* was achieved on MS medium supplemented with 1 mg/l BA + 0.3 mg/l IAA (Banu and Handique 2003).

Madhavan and Joseph (2001) also reported direct shoot formation from leaf explant of *D. metel* in cytokinin in combination with auxin IAA (MS+22.84 uM IAA+7.77 uM BA). The result of the present study in *D. falcata* and the earlier reports on *D. metel* (Madhavan and Joseph 2001), other species of *Datura* (Missaleva *et al.*, 1993) and *D. innoxia* (Zayed *et al.*, 2006) are in concurrence. It appears that natural auxins, IAA is more effective than synthetic auxin-NAA or 2, 4-D for shoot multiplication in different explants of *Datura*.

The *in vitro* shoot multiple shoot regeneration was also observed in combination of NAA with BA. Debnath *et al.*, (2006) reported multiple shoot regeneration on MS supplemented with BA (2-5 mg/l) alone and in combination of NAA (0.2-0.5 mg/l) and Kin (2-4 mg/l). The result of the present study and the above mentioned reports suggest that the auxin NAA also have the potential for induction of shoot regeneration when used in combination with cytokinin. Khanam *et al.*, (2000) reported IAA to be more
effective than NAA and 2, 4-D for induction of shoot-bud-producing callus from leaf explant in *Duboisia myoporides*.

Presence of type of cytokinin and their concentration incorporated in the nutrient medium influenced the shoot formation response in explants. In the present investigation for shoot multiplication from cotyledon node and leaf node explants, inclusion of Kin in the medium was not much effective compared to that of BA. However, the presence of Kin together with IBA, IAA, NAA or 2, 4-D induced profuse callus from the explants. This is consistent with the results reported earlier where in *Duboisia* non organogenic calli were produced with Kin in conjunctions with IAA or IBA (Badaoui *et al.*, 1996). In *Phyllanthus amarus* Bhattacharya *et al.*, (2001) reported that kinetin was superior to BA and Kin-IAA combination was more suitable than kinetin alone.

In the present study, there was remarkable change in the response of explants for shoot regeneration on BA together with low levels of IBA and IAA. However, the presence of higher levels of IBA in combination with BA counteracted the effect of BA on shoot regeneration. It appears that induction of shoot in the different explant depends on the interaction between the cytokinins and auxins as well as their concentrations.

**Rooting of shoots**

The important aspect in tissue culture after *in vitro* regeneration of plantlets is rooting and acclimatization. Efficient rooting of *in vitro* regenerated plants and subsequent field establishment is the last and crucial stage of rapid clonal propagation. The success of plant propagation method depends on the successful development of roots in the regenerated or multiplied shoots and the acclimatization of plantlets to natural environmental condition (Thorpe 1981, 1994; Robert and Dennis 2000). In present study the MS medium was used for root induction. Out of the two types of MS media used, the MS with full strength was superior over MS half strength. Concentration of mineral salts in the medium play an important role in root
induction. High salt levels are reported to be frequently inhibitory to root initiation (Harris and Stevenson 1979; Veluchmay et al., 2009).

Generally treatment with growth regulators considerably speeds the process of rooting to produce more roots. In present study in vitro shoots were rooted maximum on full strength MS medium with auxin (IBA) as compared to growth regulator free and 1/2 strength MS medium supplemented with NAA at different concentration which showed varied effect of rooting. Auxin played a vital role in root induction. Differences were noticed in the nature of roots induced depending on the auxins tested and the IBA was found to be effective for root induction when compared to NAA. Auxins are involved in the process of adventitious root formation. IBA is a common auxin for rooting in several woody plant species (Bhatt and Dhar, 2004). In present study, the absence of IBA, in the rooting medium did not lead to root formation. Several authors have shown that auxin is only required during the initiation phase, and becomes inhibitory for root growth (Elhamdouni et al., 2000; Chalupa 2002). In the present investigation, the best rooting response (83%) was observed on MS supplemented with IBA (3.0 mg). Two to four roots were produced from regenerated shoots within two weeks of culture. The developed roots were thin whitish and attained an average length of 9 cm. The number of roots formed on each shoot often increased in proportion to the concentration of auxin applied, but when the concentration became supra optimal, callus formation was promoted and roots had an abnormal appearance and their average length and subsequent shoot growth decreased. The addition, high levels of IBA to the rooting medium sometimes caused shoot tip necrosis. The reason for the reduced survival in higher concentrations of auxin treatments may be due to poor vascular connection of the root with the stem because on the intervention of callus. Callus intervening root formation was observed in shoot subculture on NAA containing medium. This inhibition of rooting is often accompanied with callus formation. The presence of callus on the shoots increased time for rooting as well as the number of roots formed. Studies on in vitro rooting of explants of Eucalyptus (Fazal et al., 2003) also showed an
increased callus formation with increased IBA concentration. This differential requirement for auxin type may be influenced by the level of endogenous auxins in cultured shoot. Similar findings were reported by Chandrasekhar et al., (2005) in *Boswellia ovalifoliolata* where IBA 4.9 uM proved to be good for rooting as compared to the NAA and IAA. In *Prunus serotina* the highest rooting of the (70%) nodal explants derived stock cultures and number of roots per shoot (2.7 ± 0.9) was obtained with 2.5 uM IBA (Ana et al., 2006). Luis et al., (1997) showed better rooting in *Miconia* sp. a woody melastomaceae from Brazil with maximum rooting on medium supplemented with 2.5 uM IBA. Arochiasamy et al., (1999) reported that in *Datura metel* L., 2 mg/1 IBA was the most suitable concentration for root induction in regenerated shoots. Martin (2002) also reported that IBA was more suitable for root induction in *Holostemtna ada-kodien* than IAA and NAA when cultured on half-strength MS solid or liquid medium fortified with 0.05 mg l⁻¹ IBA. According to Nickell (1982), the slow movement and slow degradation of IBA facilitated its localization near the site of application and thus was better functioning in inducing roots. IBA significantly improved rooting percentage and root number in Chinese tallow tree (*Sapium sebiferum* Roxb.) (Siril and Dhar 1997). Similar results were reported by Madhavan and Joseph (2001) for rooting of shoots in *Datura metel* on half strength MS supplemented with 14.76 uM IBA. Wayne (1996) reported 83% of the *Texas madrone* microcuttings rooted within 4 weeks *in vitro* when placed on medium pulsed with 6.1 uM IBA.

In combination IBA and NAA showed better results than individually used. The maximum percentage of rooting (90%) and number of roots per shoot (9.66±0.46) was obtained in MS media supplemented with 2.0 mg IBA in combination with 0.6 mg NAA. Liew et al., (1999) showed that a combination of 0.5 mg/L NAA and 1.0 mg/L IBA induced the most shoots to form roots. The result is partially supported by Talukder et al., (2002) where they reported that in combined effect, root formation was found best with 1.0 mg/L IBA. This result is also partially agreed with Pathania et al., (1998)
where they found that both Vacin and Went (VW) and Knudson C (KC) media favoured rooting when supplemented with 1.0 mg/L IBA.

**Hardening**

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in the soil. Acclimatization of *in vitro* plantlets to greenhouse (or) field condition is a critical step for many plant species. Prior to hardening plantlets rooted *in vitro* have well proportioned shoots and roots that are capable of supporting each other. The removal of the plantlets from *in vitro* conditions damaged the delicate roots (or) more likely as they will have incomplete vascular system (Mc Cown and Lloyd 1981). High humidity is essential for successful acclimatization. Usually, the most difficult step during micropropagation is the recovery of plants from the culture vessels into the soil. *In vitro* grown plantlets require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil. Micropropagated plantlets cannot survive in the environmental conditions when directly placed in a greenhouse or field because they lack necessary anatomical features to withstand variations in the natural conditions. Also, *in vitro* grown plantlets are continuously exposed to unique micro environment that has been selected to provide minimal stress and optimum condition for plant multiplication (Sutter et al., 1992; Thakur et al., 1998). In present study, the plantlets were allowed to acclimatize in glasshouse and transferred to the field condition after one month.

**Antimicrobial bioassay**

Medicinal plants have been used for centuries as remedies for human diseases as they contain components of therapeutic value. There are numerous natural plant products which have antifungal, antibacterial and antiprotozoal activities that could be used either systemically or locally (Heinrich et al., 2004). Several plants containing volatile oils, polyphenols and alkaloids as active constituents are utilized as popular folk medicines, while others gained popularity in the form of finished products collectively named phytomedicines.
In present investigation, the antimicrobial activity of crude plant extracts of *D. falcata* was tested against the bacteria and fungi. The ethanolic extract of leaf showed promising antimicrobial activity. Similarly, various plant extracts showed antimicrobial activity as reported by Valarmathy et al., (2010), who investigated the antimicrobial activity of ethanolic extracts of various leaves such as *Moringa oleifera* (Murungai), *Musa paradisiaca* (Bananana), *Azadiratica indica* (Neem), *Solanum melongena* (kathirikai), *Cynodon dactylon* (Grass), *Alternanthera sessilis* (Ponnangkani), and *Anisochilus carnosus* (Karpooravalli), Hassan et al., (2006), reported leaf extracts of *Bosica angustifolia* against *Staphylococcus aureus*, *Pseudomonas, aeruginosa, Escherichia coli*, Arora et al., (2004), studied leaves and roots of *Withania somnifera* which showed activity against *Salmonella typhi, Escherichia, coli*. Prasad and Dhanapal, (2010) recorded extract of leaves, stem and root of *Argemone mexicana* active against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi, Bacillus cereus, Aspergillus niger* and *C. albicana*.

In present study, ethanolic leaf extract of *D. falcata* exhibited promising activity against all sets of microorganisms used. While aqueous extract showed more potent activity against *Bacillus subtilis* and *Aspergillus niger* than that of standard drug.

**HPTLC analysis of chrysin from various leaf samples**

Flavonoids have been described as health-promoting, disease preventing dietary supplements and have activity as cancer preventive agents (Moon et al., 2006). Chrysin (5, 7-Dihydroxyflavone), extensively distributed in plants, has been reported to have many biological activities, including antioxidant, antibacterial, anticancer, anti-inflammatory, anti-allergenic, and anxiolytic activities (Hecker et al., 1996; Qais et al., 1996; Habtemariam 1997; Fishkin 1997; Pearce et al., 1984; Wolfman et al., 1994).

In the present investigation, the method developed for HPTLC fingerprinting provided a quick quantitative analysis for the methanolic extract of various leaf powders of *D. falcata*. The conditions used have led a
good separation of the peaks which could be identified in the chromatogram for chrysin.