DISCUSSION

Plant materials are used throughout the developed and developing world as home remedies. They are the traditional source for many chemicals used as pharmaceuticals, biochemicals, fragrance, food colours and flavours. Numerous drugs derived from plants are currently used in the world today. Due to various reasons, the natural genetic resources are depleting slowly leading to reduction or extinction of medicinal plants. Several approaches are proposed to conserve these threatened / endangered plant species. Micropropagation is an advanced vegetative propagation technique for producing a large number of genetically uniform and pathogen-free transplants in a limited time and space (Zobayed and saxena, 2003). Micropropagation technique offers an alternative method for cloning plants (Unander, 1991; Santos et al., 1994). The application of mycorrhizal associations and phytochemical approaches coupled with medicinal plants would also benefit from the development of cell, tissue and organ culture systems for in vitro growth and regeneration of medicinal plants. In addition, such tissue culture systems could also prove useful for large scale biotechnological production of phytochemicals (Briksin, 2000).

The advantage of micropropagation through tissue culture is particularly relevant in agriculture, horticulture, floriculture, arboriculture and also in forest management. In order to produce, standardize plant material for secondary metabolite production, tissue culture system for Drymaria cordata was initiated and established. In vitro plantlets were micropropagated in order to provide enough uniform explants for further studies. Significance of somaclonal variations in the genetic improvement of the economically important plants is now well established (Larkin and Scowcroft, 1982).
In vitro studies

Plant tissue culture may be applied to the rapid propagation and ex situ conservation of rare, endemic and endangered medicinal plants (Purohit et al., 1994). Recently, in vitro methods have found increasing significance as alternative means to in situ plant conservation, especially for vegetatively propagated and recalcitrant seed-producing species (Withers and Engelmann, 1997). Although the development of successful in vitro propagation and storage protocols are reliable methods that make possible the establishment of extensive germplasm collections, the specific culture conditions can cause changes in plants and lead to progenies with modified characteristics, both heritable (genetic) or non-heritable (epigenetic) (Jain, 2001). Variability has been documented to occur at physiological, morpho-anatomical, karyotypic, and biochemical levels (Apostolo & Lorente 2000; Kitin et al., 2005; Sarasan et al., 2006; Zhao et al., 2006). Maintaining the genetic identity of the mother plant and an entirely viable acclimatised plant yield after in vitro storage are the crucial prerequisites for using in vitro culture techniques for ex situ conservation purposes.

An adequate supply of nutrients and growth regulators are required for continuous growth of cultures. These requirements of plant tissues grown in vitro are similar in general to those of plants growing in nature. The amounts of organic and inorganic nutrients required for morphogenetic response vary with every plant. The different tissues of the same plant may require different growth regulators or different combinations of growth regulators for the same physiological response. Exposure to various growth substances, probably initiate a programmed sequence of events within the cells that ultimately results in a highly regulated patterns of division leading to the formation of callus /root / shoot.
A regular feature of the most suitable medium is the concentration of nitrates. The most widely used high salt medium is Murashige and Skoog's media (1962) which is highly nutritious compared to other media having high level of sodium, potassium and nitrogen source which have been found to be critical for the differentiation of entire plants from cultured tissues (Halperin and Wetherell, 1964). Philip's and Collin's (1979) is a slow salt media popularly used in leguminous plant cultures. It is also known as L2 media. In this media the inorganic and organic salts concentrations are moderate. Nicotinic acid is absent in L2 medium. Presence of nicotinic acid in the medium is reported to have a promotory effect on the callus formation (Murashige and Skoog, 1962; Tejavathi et al., 2012). Nicotinic acid has been shown to induce embryogenesis (Barwale et al., 1986). However myoinositol is relatively rich in this media which improves the cell growth (Chawla, 2002).

In the present studies, both MS and L2 media were used. MS basal medium was found to be the most favourable for organogenesis in *Drymaria cordata*. MS medium has been frequently used for micropropagation of large number of plants (Feyissa et al., 2005b). The explant variability has been linked to explant's physiological status based on genetic constitution in many species (Benson, 2000). Concentration and combination of plant growth regulators are the key factors significantly affecting indirect and direct organogenesis of explants from *in vitro* cultures of *Drymaria cordata*.

In the present study, shoot proliferation occurred either in the presence of auxin or cytokinin individually and in conjunction. Hormones play an important role in controlling the morphogenic response of the cultures. Balance between the endogenous and exogenously supplied hormones determine the morphogenetic response. The results have indicated that *Drymaria cordata* grew better on MS medium in comparison to L2 medium. The explants in
MS medium appeared healthy and grew vigorously. This is in conformity with the earlier studies reported by Ghimire et al., (2010) and Tejavathi and Indira (2011 and 2013) in the same taxon and hence was extensively used. Sharma et al., (2007) have reported the effectiveness of MS medium for shoot multiplication in different Bacopa species. Similar response of MS over other media was observed by Rathore et al., (2008) in Terminalia bellerica. Karuppusamy et al., also have reported better response of MS medium for shoot regeneration in V. Peadata than B5 (Gamborg et al., 1968) and Woody Plant Medium (WPM: Lloyd and McCown, 1981). However, the superiority of L2 medium over MS medium has been reported in Majorana hortensis by Tejavathi and Padma (2012).

Various combinations and concentrations of growth regulators have been used in tissue culture studies for raising multiple shoots directly from different explants. Several reports have stated that the success of in vitro shoot formation may depend upon the type of explant used (Hong et al., 2004; Koroch et al., 2002). In the present studies, node, internode and leaf explants were tried for direct shoot regeneration. Among the different explants tried, the nodal explants were ideal for mass proliferation of shoots directly from Drymaria cordata. Regeneration of shoots from nodal explants have been reported in many plants by several workers (Band, 2011; Kumar, 2011).

Large scale rapid production of clonal plants through in vitro culture of single node stem segments and shoot to shoot proliferation was achieved in several medicinal plants (Chaturvedi et al., 2007). Kulkarni et al., (2000) have obtained direct shoots from node, internode, hypocotyl and embryo explants obtained from in vitro raised seedlings of Withania somnifera. Direct shoot regeneration from leaf explants of Digitalis lamarckii was reported by Verma et al., (2011). Cirak et al., (2007) found that internodal explants of
*Hypericum bupleuroides* were more responsive than leaf tissues to direct plant regeneration. Genotype, explant type and medium composition are considered three main factors affecting the percent regeneration and number of shoots per explant (Afshar *et al.*, 2011).

**Effect of auxins on morphogenesis from cultures**

Growth and morphogenesis in *in vitro* are regulated by the interaction and balance between the growth regulators supplied to the medium and growth substances produced endogenously by cultured cell. Auxins are involved with elongation of stem and internodes, tropism, apical dominance, abscission and rooting. Auxins also promote adventitious root development on stems. Syntheic auxins such as NAA and IBA are usually more effective than IAA, apparently because they are not destroyed by IAA oxidase or other enzymes and therefore persist longer (Salisbury and Ross, 2004. However, in plant tissue culture auxins have been used for cell division and root differentiation (Bhojwani and Razdan, 2004).

In the present investigation, auxins like IBA, NAA, IAA, and 2,4-D were used. The most widely used method of *in vitro* plant propagation is the stimulation of axillary bud development. Nodal segments are found to be the best explants for axillary shoot proliferation (Amin *et al.*, 2002; Karim *et al.*, 2003; Waseem *et al.*, 2008). Multiple shoot induction through nodal explants were well observed in medicinal plants such as *Rauwolfia serpentina* (Roy *et al.*, 1995), *Vitex negundo* (Vadawale *et al.*, 2006) and *Emblica officinalis* (Rahman, 1999).

Results obtained in the present experiment confirm stimulative effect of auxins on adventitious shoot formation. In the present study, low concentrations of auxins were sufficient to raise more number of shoots than higher concentrations which induced
callusing. Similar response of low concentration of auxin supplemented media was recorded by Waseem et al., (2008) in Chrysanthemum morifolium L. Amongst the various auxins tested, MS + IBA (4.92 µM) on MS medium produced maximum number of (3.82±0.12) shoots per nodal explant. IBA supplemented medium showed pronounced shoot elongation with effective rooting. Presence of auxins positively influences rhizogenesis (Dabski, 2007). IBA is considered as root promoting auxin as stated by a number of research workers (Khan et al., 1994; Hoque et al., 1998; Sarkar and Shaheen, 2001). The presence of IAA and NAA at a higher concentration in the media caused callusing along the basal roots of Drymaria cordata. Dabski and Parzymies, in 2007 observed callusing of shoots in Hebe buchananii at higher concentration of NAA in the medium. Muhammad and Faheem (2012) have reported direct shoot regeneration from in vitro derived shoot tip explants of Tectona grandis on individual NAA and IBA supplemented medium.

2,4-D supplemented medium promoted rhizogenic callus from the nodal explants of Drymaria cordata with reduction in shoot number. The leaf explants showed significant rhizogenic callus on 2,4-D fortified medium which gradually turned brown. The internode explants showed meagre callus development along its length with effective root formation. Optimum callus induction and plant regeneration could be obtained through manipulating the 2,4-D concentration and the duration of its presence in the induction medium (Zheng and Konzak, 1999). The effect of 2,4-D on induction of callus from apical meristems of two sugarcane cultivars was reported by Tahir et al., (2011). The callus induction was found to increase with increase in the concentration of 2,4-D which is in line with the present findings.
Effect of cytokinins on direct shoot regeneration

One of the most well investigated cytokinin responses is cytokinesis, studied by Fosket et al., 1977. According to them cytokinins promote cell division by increasing the transition of cells from G2 to mitosis and that they do by increasing the rate of protein synthesis. Cytokinins are incorporated in culture media mainly for cell division and differentiation of adventitious shoots from the callus and organs. For shoot proliferation, growth regulators especially cytokinins (Lane, 1979; Stoltz, 1979; Garland and Stoltz, 1981) are one of the most important factors affecting the response. In presence of cytokinin, bud dormancy is broken and axillary branches proliferate (Thorpe, 1994). The role of cytokinins in shoot organogenesis is well established (Flick et al., 1983). According to Bhojwani (1980), an exogenous supply of cytokinins is essential for potential morphogenesis from various cultures. A range of cytokinins has been used in micropropagation work (Bhojwani and Razdan, 1992). Cytokinins such as kinetin, BAP, 2-ip and TDZ were used in the present studies.

In the media without any hormone there was no regeneration. The present studies have also shown shoot proliferation on MS medium supplemented with cytokinins individually. Leaf and internode explants did not respond at all. Maximum shoot proliferation in Drymaria cordata (19.51 ± 0.18) was best observed from nodal explants on MS medium with BAP (4.44 μM). BAP appeared more potent hormone than other cytokinins at inducing multiple shoots indicating cytokinin specificity for multiple shoot induction in Drymaria. In the present study, the stimulatory effect of a singular supplement of BAP on bud break and multiple shoot formation in Drymaria cordata was similar to that reported earlier by Beena et al., (2003) in Ceropogia candelabrum and Maharana et al., (2012) in Jatropha. A wider perusal of the literature suggests that
BAP is the most reliable and useful cytokinin for the shoot tip, meristem and bud culture. A number of plants have been successfully multiplied on medium containing BAP.

The efficiency of BAP for shoot culture initiation and multiplication has been reported in several other medicinal plants (Karthikeyan et al., 2009; Purohit et al., 1994; Sasikumar et al., 2009; Shrivastava, 1999; Tiwari et al., 2001; Tiwari et al., 2006 and Sharma et al., 2010). Even in mulberry, BAP is more effective in terms of number of multiple shoots proliferation than KN, 2-ip or Zeatin (Yamanouchi et al., 1999). However no shoots were induced on leaf explants when the growth regulators were added singly to the culture medium as reported by Ghimire et al., (2010) in Drymaria cordata.

The suppression of multiple shoots at higher concentrations of BAP were in accordance with those of Pattnaik and Chand (1996) in Ocimum americanum and Ocimum sanctum. The type of cytokinin and their concentration has a definite effect on the height of multiple shoots developed. In the present studies, BAP and TDZ supplemented medium showed stunted growth while KN or AS supplemented medium resulted in reduced number of shoots, but gave shoots with longer internodes (Beena et al., 2003). This is in contrast with the observations made by Karuppusamy et al., in Vanasushava peadata where BAP was effective than KN and 2-iP for shoot length. High concentration of cytokinin has been observed to induce stunted shoots in other species (Tavares et al., 1996; Koroch et al., 1997).
Synergistic effect of growth regulators on direct shoot regeneration.

Depending on species or cultivars, the most important achievement obtained in the propagation of many plant materials through tissue culture has been frequently based on the successful adjustment of the type and combination of plant growth regulators (Murasighe, 1990; Gurel and Gurel, 1996). Enhanced shoot multiplication by addition of auxin along with cytokinin has been reported in many plants. Both auxins and cytokinins readily form conjugates in plants. The conjugation may be a way of preserving the biological activity of plant growth regulators (Skoog and Miller, 1957).

In the present studies, BAP supplemented medium proved necessary for the direct mass proliferation of shoots from nodal explants. Maximum shoot proliferation (45.87±1.00) was observed on MS + BAP (4.44 µM) + GA3 (1.44 µM) + IBA (0.49 µM). On transferring the shoots onto fresh medium containing MS + BAP (4.44 µM) + IBA (0.49 µM), promoted mass proliferation of shoots coupled with shoot elongation as well as rooting. In contrast, Kumar et al., (2011) have reported the synergistic effect of IAA and BAP on the enhancement of shoot multiplication on nodal explants. Shoot elongation was achieved on medium containing GA3. Gibberellins have the unique ability among recognized plant hormones to promote extensive growth of many intact plants. They generally enhance elongation of intact stems. Most dicots and some monocots respond by growing faster when treated with gibberellins (Salisbury and Ross, 2004). Gibberellins promote cell division in the shoot apex especially in the more basal meristematic cells and also increase cell wall plasticity thus bringing about elongation.

According to Ghimire et al., (2010) in the absence of BAP or TDZ, no shoot regeneration was observed in the leaf explants,
implying that these compounds are critical for shoot regeneration in *Drymaria cordata*. A low auxin concentration in combination with a high concentration of cytokinin is the most suitable combination for the proliferation of shoots. Synergistic effect of auxins in combination with BAP on the enhancement of shoot multiplication was observed in *Dubrisia myoporoides* (Kukreja and Mathur, 1985). Similar effect of BAP in combination with various auxins on shoot regeneration has been observed in *Vanasushava pedata* (Karuppusamy, 2006).

According to Grattapaglia and Machado (1998), the cytokinin 6-benzylaminopurine and Kinetin are very effective in promoting proliferation. Cytokinins participate in the regulation of many plant processes that include callus cell division in the presence of auxin, leading to bud or root formation directly on the explant or from calli. Gibberellins increases both cell elongation and cell division. Mitosis increases markedly in the sub apical region of the meristem of rosette long-day plants after treatment with gibberellins (Taiz and Zeiger, 2003).

Ghimire *et al.*, (2010) and Kantia and Kothari, (2002) reported adventitious shoot bud formation achieved directly of the surface of the leaf explants in *Drymaria cordata* and *Dianthus chinesis* respectively. The different responses of the explant types are probably due to the endogenous hormonal balance in plant tissues (Grattapaglia and Machado, 1998).

**Effect of growth regulators on indirect organogenesis**

Somaclonal variant selection in *in vitro* culture is often achieved through indirect regenerations through callus cultures (Sen *et al.*, 1991). For indirect organogenesis, induction of callus from the explant is an initial step. Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances.
present in the medium (auxins and cytokinins). The general growth characteristics of a callus involve a complex relationship between the plant material used to initiate the callus, the composition of the medium and the environmental conditions during the incubation period. The specific concentration of plant growth regulators needed to induce callus, varies from species to species and even depends on the explant (Zibbu and Batra, 2010). The most important characteristic of callus from a functional point of view is that this abnormal growth has the potential to develop normal roots, shoots and embryoids which subsequently form plants. Two general patterns of in vitro embryogenic development, direct or indirect have been recognized. It has been suggested that direct embryogenesis occurs from pre-embryonic determined cells, while indirect somatic embryogenesis requires the induction of embryogenically determined cells (Sharp et al., 1980; Williams and Maheswaran, 1986).

In the present investigation, leaf explants showed a good response in terms of callus initiation on media containing different concentrations of auxins individually and in combination with cytokinins. Auxin and cytokinin combinations act synergistically to promote cell division and expansion depending upon other factors in the cell system (Setterfield, 1963). Young, expanded leaves responded better than mature leaves (Perez-Tornero et al., 2000). The presence of lamina and petiole in the explant influence shoot bud induction (Kumar et al., 1998). BAP (4.44 μM) in combination with 2,4-D (4.52 μM) / NAA (2.69 μM) promoted good amount of callus from the leaf explants. The fresh weight (1.076±0.04) and dry weight (0.177±0.03) or organogenic callus being maximum in TDZ (2.27 μM) + NAA (2.69 μM) combination. Though profuse callus was obtained on 2,4-D and BAP combinations in the initial phase of the culture, it gradually turned brown and necrotic as reported by Banerjee et al., (2011) in Arachis hypogea. Sahu and Khalkho (2012) have reported similar
browning of leaf and internode callus of *Boerhaavia diffusa* in 2,4-D supplemented media.

However, healthy non-organogenic callus was obtained on media supplemented with BAP (4.44µM) and NAA (2.69µM). Similar combinations were found to be suitable for induction of callus from the culture of *Acmella calva* (Senthilkumar et al., 2007) and *Indigofera enneaphylla* (Sindhu et al., 2011). No organogenesis was recorded on this combination even after maintaining the callus for six months. This is in contrast to reports by Ghimire et al., (2010) where adventitious shoot bud regeneration was observed in the leaf segments of *Drymaria cordata* when cultured directly on MS supplemented with NAA in combination with BAP or TDZ without intervention of callus. In the present studies, however BAP in combination with NAA promoted only callus and not shoot regeneration.

TDZ (0.91µM) supplemented medium promoted the proliferation of compact green and nodular callus from the source callus after four weeks of culture. Shoot buds (30.18 ± 1.05) have taken 3-4 months to emerge from this green nodular callus on this medium. According to Liu and Pizut (2008), TDZ was the most important factor for adventitious shoot regeneration from *in vitro* leaves of *Prunus serotina*, as no adventitious shoots developed on explants exposed to media without TDZ. Regeneration efficiency increased as TDZ concentration increased in the media. However adventitious shoots formed as a cluster, and it took a much longer time for the cluster to develop into individual shoots. Lower concentrations can induce greater axillary proliferation than many other purine-based cytokinins. However higher concentrations of TDZ can stimulate callus induction as reported in many woody species (Huetteman and Preece, 1993). Dual effect of TDZ involving proliferation of shoots from the apical meristem and differentiation of shoots from the callus
has been reported in *Nothapodytes foetida* (Tejavathi et al., 2012) and Pigeon Pea (Singh et al., 2003). Adventitious shoot regeneration from the leaves on TDZ supplemented medium has been reported in *Prunus avium* (Bhagwat and Lane, 2004) and *Prunus serotina* (Liu and Pizut, 2008).

Filter paper method proved to be faster method to enhance regeneration of shoot buds from the compact nodulated callus of *Drymaria cordata* within 25 days of culture on liquid MS medium supplemented with TDZ. Blidar et al., (2011) have described an efficient method to initiate *in vitro* cultures of wheat, using Whatman filter paper bridges for liquid culture media. According to their study the use of filter paper bridge increased the availability of nutrients to the plantlets developed from the wheat caryopsis. The wheat plantlets grown on liquid culture media have shown significant development compared to plantlets grown on solid culture medium.

It was also demonstrated that both total and embryogenic callus of wheat plants were doubled and significantly a higher number of regenerates was obtained on liquid culture media provided with filter paper bridges compared to the cultivation on solid culture medium (Mohmand et al., 1991). As for orchids, it was found that in a shake liquid culture medium the proliferation is generally faster and more extensive, but on filter-paper bridges the differentiation is always better (Arditti, 2008). For *Jasminum officinale*, it was determined that the root emergence from the shoot base and later the root elongation was facilitated by using liquid culture medium provided with filter-paper bridges in comparison with solid culture media (Bhattacharya and Bhattacharyya, 2010).

One specific advantage of using liquid culture medium provided with filter paper bridges is related to acclimatization. It is well known that in the case of the agarized culture media after removing the
roots from the nutrient substrate, follows the careful removal of agar in order to avoid retention on the surface of root. It is a complex process and requires specialized care. Moreover, if some agar is still persistent on the root surface, during acclimatization it can provide a perfect medium for bacteria or fungi entry which can impede the further development of plantlets. In addition, the removal procedure of the roots from the agarized medium and the removal of this from the surface of the roots, can cause damages to the organs. In case of using a liquid culture medium provided with filter paper bridge, the paper removal is really easy and it avoids infection and other damages at the root system level of the acclimatized plantlets. Advantage of using liquid culture medium provided with filter-paper bridges is economical compared to the solid culture medium. Agar as a solidifier agent used in the classical solid culture media has a higher price compared to the corresponding filter paper, which may be translated into an economic gain. Moreover, the process is very easy, clean and fast (Blidar et al., 2011).

A somatic embryo has extensive practical and commercial applications particularly for in vitro clonal micropropagation (Bornman, 1993). A large number of herbaceous dicots and monocots have been regenerated through somatic embryogenesis. In the present studies, TDZ at a concentration of 1.82 μM seemed effective in inducing embryogenic structures from leaf derived callus obtained on MS + BAP (4.44 μM) + NAA (2.69 μM) medium. Maximum induction of somatic embryos was observed at lowest concentration of TDZ (Khadke and Kuvalckar, 2013). Although the somatic embryo like structures possessed both root and shoot meristems, simultaneous development of root and shoot was less frequent which commensurate with the findings of Venkatachalam et al., (1999).

These embryos differentiated into individual plantlets on medium supplemented with L-glutamine (50 mg l⁻¹). Amino acids
have been shown to be efficient for somatic embryogenesis and embryo development (Chia and Saunders, 1999; Witjaksono and Litz, 1999; Wu et al., 2004). The nitrogen content of the culture medium has been reported to influence the morphogenic effects of growth substances. L-glutamine has been reported critical for embryogenesis among the amino acids used as a source of nitrogen (Price and Smith, 1979; Khliifi and Tremblay, 1995; Shoyama et al., 1995). The addition of L-glutamine to MS medium significantly enhanced maturation of hypocotyl segment derived somatic embryos to cotyledonary stage embryos was reported by Sahrawat and Chand (2001) in Psoralea corylifolia.

Development of somatic embryos of Schisandra chinensis into plantlets was completed on ½ MS medium free of plant growth regulators (Sun et al., 2013). The conversion of somatic embryos into plantlets depends on the type and concentration of auxin used in the somatic embryo regeneration medium (George and Eapen, 1993). However, BAP-mediated medium enhanced somatic embryo regeneration was also reported in leguminous plants (Bhanumathi et al., 2005; Chengalrayan et al., 2001; Ghanti et al., 2010) and other species like cotton (Ganesan and Jayabalan, 2004) and Eryngium foetidum (Ignacimuthu et al., 1999). Khadke and Kuvalakar (2013) have reported direct somatic embryogenesis from leaf and stem explants cultured on MS medium supplemented with TDZ and 20% coconut water. Gayathramma (2009) has reported the developments of plantlets from somatic embryos on the same medium that prompted the growth of embryogenic callus. Germination and conversion of Somatic embryos into plantlets were affected by the physical state of the medium. On the semi-solid medium germination was poor and no plantlet developed. However, there was a marked enhancement in germination and conversion into plantlet when liquid medium was employed in both the cultivars of Mangifera indica (Ara et al., 2000).
In the present investigation, *in vitro* grown leaf explants of *Drymaria* when cultured directly on the medium containing TDZ (0.91 µM) + NAA (2.69 µM) produced fast growing and organogenic callus which differentiated into significant number of microshoots (35.5±0.62) after 75 days. Adventitious shoot regeneration from leaf explants on medium containing TDZ and NAA have been reported in *Prunus serotina* (Liu and Pijut, 2008). Adventitious shoot regeneration using leaf explants is established for several plants, such as *Prunus padus* (Hammatt, 1993), *Lablab purpureus* var. Lignosus (Thiruvengadam and Jayabalan, 2003), *Prunus serotina* (Espinosa et al., 2006) and *Thevetia peruviana* (Zibbu and Batra, 2010).

TDZ is the most potent of the diphenyl ureas that have been evaluated for use in plant tissue culture (Mok et al., 1987). It has been reported that TDZ can induce a number of regenerative processes in plant tissue culture, such as callus formation, adventitious shoot regeneration and somatic embryogenesis (Murthy et al., 1998). A carbon isotope study showed that TDZ was very stable in the culture media and persistent in plant tissue (Mok and Mok, 1985). It has been suggested that TDZ helps to establish the internal optimum balance of cytokinin and auxin required for induction and expression of somatic embryogenesis (Saxena et al., 1992; Lu, 1993). Although TDZ is relatively costly, a very low concentration of TDZ used in the present studies circumvents its price consideration and seems to be cost effective.

**Histological studies**

**Organogenic callus**
Histological analysis provides morphological details that help explain the emergence of shoots via direct or indirect organogenesis. Histological studies in the present work revealed the origin of apical meristem from a basal callus mass without undergoing the embryonic stages. Torrey (1966) put forth the hypothesis that organogenesis in callus commences with the formation of clusters of meristematic cells or meristemoids capable of responding to factors within the system to produce a primordium. Many observations on organ formation in cultured tissues support the hypothesis that localized meristematic activity precedes the organized development of shoot. The initiation of organized development involves a shift in metabolism and that phytohormones play an important role. The process of organogenesis is brought about by mechanisms which control the production of specifically required metabolic patterns at a particular time.

**Embryogenic callus**

Histological sections prepared from embryogenic cultures of *Drymaria* showed somatic embryos at different developmental stages. According to some workers, embryoids like their zygotic counterparts arise from a single cell (Wang *et al.*, 1990; Tejavathi *et al.*, 2000) and to others it is multicellular in origin (Schumann *et al.*, 1995; Martinez-Palacios *et al.*, 2003). While others are the opinion that multicellular proembryogenic tissue may have originated form a single cell (Williams and Maheswaran, 1986). In the present studies cell differentiation was observed in the ground parenchyma where competent cells become mitotically active, generating callus and embryonic structures. Some of the resultant embryos seem to be connected to the originating tissue, suggesting multiple cell origins as previously reported in *Daucus* (Haccius and Bhandari, 1975). This type of embryogenic process was defined by Sharp *et al.*, (1980) as indirect somatic embryogenesis.
Rooting response

Rooting of plants in vitro is a very important aspect of micropropagation. Rooting response of microshoots is reported to be controlled by growth regulators in the medium (Bhojwani & Razdan 1992), basal salt composition (Garland and Stoltz 1981, Zimmerman and Broome, 1981, Skirvin & Chu 1979), genotype (Rines & McCoy 1981) as well as cultural conditions (Murashige 1977). The main growth regulator used at this stage is auxin which stimulates root formation. The most often used auxins are: IAA, IBA and NAA. Bhojwani and Razdan (1992) have reported the use of NAA and IBA in root induction. The role of various concentrations of different auxins in root induction is well documented (Mii et al., 1990; Ilahi et al., 1995 and Mehta, 2004).

In this investigation, it was disclosed that the synthetic auxin IBA was the most favourable auxin for root induction in Drymaria followed by IAA. MS + IBA (0.49 μM) proved to be efficient in producing healthy and long roots. The average root length being 6.93 ± 0.04 cm. Similar observations were made by Tejavathi and Indira (2011 and 2013) in the same taxon when the proliferated shoots were transferred to rooting medium. IBA at the same concentration was found best for in vitro rooting in Terminalia bellerica (Rathore et al., 2008) and Stevia rebaudiana (Patel and shah, 2009) while Khawar and Ozcan (2002) reported a low concentration of IBA (1.23 μM) for root induction in Lens culinaris. IBA at low concentration played a positive role during in vitro rooting without the formation of basal callus. Arockiasamy et al., (2002) have also observed callus induction from the cut portion of the shoot on increasing the concentration of IBA. However, Dabbski and Parzymies (2007) observed the rooting in microshoots of Hebe buchananii on media supplemented with IBA in higher concentrations.
The efficiency of IBA in inducing roots from in vitro cultured shoots has been reported by several workers (Logesh et al., 2011; Muthu et al., 2003; Rani et al., 2010; Sharma et al., 2010; Yeh-Jin et al., 2007). By the use of IBA many such plants such as Sesbania acculeata (Bensal and Panday, 1993), Hedychium roxburgii (Tripathi and Bitaillion 1995), carnation (Werker and Leshem, 1987), Vanasushava pedata (Karuppusamy et al., 2006), Morus indica var. Mysore Local (Tejavathi et al., 2009) and Vitex negundo (Thiruvengadam and Jayabalan, 2000) have shown in vitro rooting. While, IAA was used in rooting of microshoots of Ocimum gratissimum (Gopi et al., 2006). The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus it functions better in inducing roots (Nickell and Kirk-Othmer, 1982).

There is ample evidence that auxins from stems strongly influence root initiation. In many cases, removal of young leaves and buds, both of which are rich in auxins, inhibits the number of lateral roots formed. Substitution of those organs by auxins often restores the root forming capacity. Thus, there is an important difference in exogenous auxin effects on root elongation, in which inhibition is observed, and in root initiation and early development, in which promotion is observed (Wightman et al., 1980).

**Hardening and acclimatization**

The ultimate success of micropropagation protocol depends on rapid acclimatization and survival of plantlets under field conditions. The in vitro rooted plantlets were transferred to plastic cups containing soilrite, perlite and cocopeat (1:1:1). A mix of all three components enhanced the morphology and survival rate of the plantlets. Hardening mixtures may vary depending on the plant resone. Tejavthi et al., 2009 have established the plantlets of Morus
*indica* var. Mysore Local, in nursery pots containing sand: soil: soilrite (1:1:1), while Zibbu and Batra (2010) have used garden soil and compost for hardening of *Thevetia peruviana*. The regenerated shoots developed into plantlets within two months and yielded 90% successful acclimatization rate with normal phenotypes. The morphological characteristics of *in vitro* raised plants did not vary much from those of the normal plants. This is in conformity with studies done by Ghimire *et al.*, (2010) in the same taxon.

**Arbuscular Mycorrhizal studies**

Arbuscular mycorrhizal studies have importance in agriculture and land reiclaimations. Plant roots provide an ecological niche for many of the microorganisms that abound in soil. In natural ecosystems much of the terrestrial plants associate with root colonizing mycorrhizal fungi, which improve the fitness of both the fungal and plant associates. Two major types of mycorrhizal associations, arbuscular mycorrhiza (AM) and ectomycorrhiza (ECM) occupy the roots of the majority of plants in natural ecosystems throughout the world (Brundrett, 1991). However, exceptions exist both between and within plant families failing to associate with mycorrhizal fungi. Many plant families such as Chenopodiaceae, Brassicaceae, Caryophyllaceae, Cyperaceae and Polygonaceae appear to be non-mycorrhizal (Brundrett, 1991; Francis and Read, 1994). High incidence of mycorrhiza in weeds has been reported in other studies (Anwar and Jalaluddin 1994; Jain *et al.*, 1997; Zainab and Burni, 2005). Burni *et al.*, (2009) have observed and recorded the AM colonization in the weed *Stellaria media*, while the symbiotic response of *Dianthus caryophyllus* root stock to different mycorrhizal fungi was studied by Kerur and Lakshman (2009), thus negating the fact that caryophyllaceae members are non-mycorrhizal. Therefore, *Drymaria cordata* (Caryophyllaceae) was subjected to AM studies in order to determine its symbiotic association.
Considerable evidences suggest that AMF can affect the nature of weed communities in agro ecosystems in variety of ways. However, it is quite probable that interaction with AMF can increase the beneficial effects of weeds on the functioning of agro-ecosystem (Jordan et al., 2000, 2005; Gupta & Mukerji, 2003). AM investigation has importance in agriculture and land reclaims. AM morphology is dependent on individual plant species and the partner (Dickson, 2004). Additionally environmental factors such as temperature, light intensity & soil moisture content may influence this symbiotic association as assumed by Cavagnaro et al., (2001) because these factors affect the growth and morphology of roots.

Hardening of tissue culture plants is the most crucial step in micropropagation. Inoculation of AM fungi during an early stage of acclimatization process has become an alternative strategy for better establishment by improving the plant growth. The potentiality of using AM fungi in micropropagated plants was studied by Morandi et al., (1979) in the establishment and growth of raspberry plants. They introduced microbial activity in the root system though the soil plays a significant role in the plant growth and development by modifying their physiological activity. The mycelia network which extends from mycorrhizal root and ramifies through the soil is responsible for both enhancing nutrients and water absorption for the host plant (Sreeramulu and Bagyaraj, 1999) thus constituting a soil-root fungal continuum.

AM fungi are known to associate with plants with relatively low specificity and therefore the association is thought to be largely non-specific (Hoeksema, 1999; Smith and Read, 1997). The extent of plant growth promotion by AM fungi depends upon specific plant and fungal combinations (Adjoud et al., 1996; Van der Heijden et al., 1998). Measures of growth of AM fungal species also depends on the
associated host plant species (Eom et al., 2000). The specificity of AM fungi may have important consequences on plant ecology. The mutual interdependence of plant and AM fungal population growth rates could generate complex dynamics between the plant and fungal guilds. Host-specific changes in the AM fungal community could also lead to increase in the relative growth rate of most abundant plant species.

The present study is the foremost attempt to introduce arbuscular mycorrhizae in micropropagated plants of Drymaria cordata. Normal and regenerated plants were inoculated with Glomus mosseae and G. fasciculatum. Growth and physiological status of normal and regenerated with their controls were analysed and compared. The study revealed the influence of mycorrhiza in Drymaria cordata (Caryophyllaceae). The percent colonization was recorded over a period of 3 months of inoculation. Significant percent of mycorrhizal colonization was observed in regenerated treated plants than in normal treated plants. Maximum colonization was observed in Glomus fasciculatum treated regenerated plants while colonization was totally absent in control or untreated plants which could be attributed to sterilization of soil and absence of inoculum. The highest percent of root colonization in regenerated plants may be due to early colonization by AM fungus or may be due to increased root cell membrane permeability and more hyphal penetration. Those fungi having higher root colonization are better adapted for absorption of more nutrients and thus enhance the growth of medicinal plants (Lakshmipathy et al., 2003).

The number of arbuscules and vesicles in the root and spore count in the rhizosphere was found to be high in regenerated plants treated with Glomus fasciculatum than those treated with Glomus mosseae. Similar results were observed in Sesbania sesban (Subhan et al., 1998) Andrographis paniculata (Tejavathi et al., 2011), Agave
deserti (Cui and Nobel, 1992) and Catharanthus roseus (Satyaprasad and Shrisailaja, 1995). Increased spore count and root colonization levels in plants inoculated with AM fungi have been observed earlier by several workers (Bagyaranj et al., 1988; Edathil et al., 1994; Rajesh et al., 2011). In contrast, the percent root colonization and P uptake were also significantly higher in G. mosseae inoculated plants of Dianthus caryophyllus (Kerur et al., 2009). A low colonization percentage was recorded after one month of treatment, which may be due to the dynamic process initiated by root exudations, soil factors, environmental factors or soil microbial interactions. The concept of mycorrhizosphere explains that mycorrhiza exerts a strong influence on the microflora of the rhizosphere, stimulating the microbial activity in the soil (Oswald and Ferchau, 1968).

**Effect of AM treatment on morphological features of normal and regenerated plants.**

AM fungi treated medicinal plants have shown improved growth and development as compared to control plants (Karthikeyan, et al., 2009; Burni et al., 2013). Generally the mycorrhizal plants performed better than the non mycorrhizal plants in terms of growth performance but the degree to which the growth was affected on mycorrhization varied with the inocula used. The significant increase was observed not only in AM colonization but also in biomass production. It could be thought that mycorrhizal plants were simply better hydrated than nonmycorrhizal ones due to direct fungal water uptake and its transport to the plant (Ruiz-Lozano et al., 1995).

In the present investigation, the normal and regenerated plants of Drymaria cordata were treated with AM fungi and their morphological characteristics compared. The regenerated plants showed good growth response to AM fungal inoculation thus bringing about a significant increase in the number of branches, number of
nodes per branch, length of internode, length and breadth of leaf, plant height. Tejavathi and Rao (1998) compared the growth of normal and micropropagated plants of *Bacopa monnieri*. They concluded that micropropagated plants showed vigorous plant growth which is in conformity with the present investigation.

The enhanced growth performance in regenerated plants of *Drymaria cordata* was due to mycorrhization of *G. fasciculatum* than *G. mosseae*. The present results are in the same line with the results obtained by Kavatagi and Lakshman (2012), where *G. fasciculatum* significantly increased shoot length, root length, dry and fresh weight of shoot and root in AM inoculated *Solanum lycopersicum*.

Gupta *et al.*, in 2002 have noted a significant increase in the plant height, fresh herbage and dry matter yield in *Mentha*. Similar findings were also documented by Gupta and Janardhan (1991) and Earanna *et al.*, (2001). Gayathramma (2009) and Tejavathi *et al.*, (2011) too have compared and reported the efficiency of *G. fasciculatum* on the growth and performance of normal and regenerated plants of *Agave* and *Andrographis paniculata* respectively.

Plant biomass is an important parameter which directly reflects the efficiency of particular fungus. The biomass (Fresh and dry weight) of the inoculated regenerated plants were found to be maximum and significant than that of untreated plants in *Drymaria cordata*. The basis may be due to the formation of external mycelium around the roots by AM fungi. The results are in agreement with the findings of earlier work by Gupta and Janardhan. Higher root biomass production in mycorrhizal plants compared to non mycorrhizal plants has been frequently reported (George, 2000). Kung'u *et al.*, (2008) reported that inoculating *Senna spectabilis* with
AM fungi increased total shoot length, shoot and root dry weight and number of leaves.

In contrast, Sowmya et al., (2004) reported increased biomass in micropropagated plants treated with *G. mosseae* compared to *G. fasciculatum*. Kerur et al., (2009) too have recorded the efficiency of *Glomus mosseae* over *G. fasciculatum* in the enhancement of plant height, stem girth and total biomass of *Dianthus caryophyllus* as compared to uninoculated plants. Yield in *Glomus* sp. treated micropropagated plants of Strawberry tended to exceed that of non-AM micropropagated plants, but AM effects differed widely with host-endophyte combinations (Chavez and Ferrera-Cerrato, 1990).

Various plant species react with an increase of auxins to inoculation with AM fungi. In maize it was shown that IBA is the major auxin induced during AM colonization (Fitze et al., 2005; Jentschel et al., 2007). Morphological changes in the root are expected to be under phytohormonal control (Selvaraj, 1998). ABA was found to be considerably enhanced in both roots and shoot of AM plants as compared to nonmycorrhizal plants (Danneberg et al., 1992). The increased levels of auxins lead to the formation of lateral roots which constitute preferential penetration sites for the AM hyphae. Together with cytokinins, they bring about an increased growth promotion of inoculated plants (Ginzberg et al., 1998).

The most prominent contribution of these AM fungi to plant growth is mainly due to uptake of phosphorus and other elements by extraradical hyphae and transference to the root tissues (Abdel-Fattah, 1997; Guissou et al., 1998; Mathur and Vyas, 1999 and Wang et al., 2008). The major function of AM fungi is phosphate uptake, because it encodes a phosphate transporter gene (Harrison and Van Buuren, 1995). AM inoculation significantly increased the uptake of N, P and K by shoot tissues of mint, but most markedly
increased the uptake of P (Gupta et al., 2002). The enhanced quantity of available phosphorus and levels of exchangeable potassium and magnesium in AM treated plant soil would certainly mean better growth in terms of number of leaves, branches and biomass in AM treated plants than control (Chandarana, 2013). Rasouli-Sadaghiani et al., (2010) found that the overall growth of Ocimum basilicum is significantly higher when inoculated with AM fungi.

**Phytochemical Studies**

**Physicochemical evaluation**

Evaluation of a drug ensures the identity of a drug and determines the quality and purity of drugs. In the present studies, physicochemical analysis was used to test and compare the quality of the normal and regenerated plant. Pharmacognostic standardization including physicochemical evaluation is meant for identification, authentication and detection of adulteration and also compilation of quality control standards of crude drugs (Kokate and Purohit, 2006).

Physicochemical analysis was carried out to establish the standard quality parameters for Drymaria which can be useful for further research, identification and authentication and also for creating drug profile. The total ash is particularly important in the evaluation of purity of drugs i.e., the presence or absence of foreign inorganic matter such as sand, soil, calcium oxalate, metallic salts or silica (Momin and Kadam, 2011; Shah and Seth, 2010). The study of total ash content, acid insoluble ash, water soluble ash, etc., have a direct bearing on the purity and strength of the medicinal plant. The quality and quantity of ash in herbaceous biomass depends on a large amount of factors including plant type, plant part, growing conditions, fertilization and time and techniques of harvest. In the current studies, about 78% of total ash content and 7.3% of moisture content was estimated in regenerated plants. As the moisture content
of the drug is not too high in plants, thus it could discourage bacterial, fungi or yeast growth (Mandali et al., 2011). In accordance to the high percentage of results obtained in Drymaria cordata, Joseph and George (2011) have reported 82% of total ash and 51.7% moisture content in Geranium ocellatum. Ash values have been recorded in a number of medicinal and aromatic plants by several workers (Sandeep et al., 2010; Vermani et al., 2010; Subash, 2013).

The drug does not have any characteristic fluorescence. Many drugs fluorescence when their powder is exposed to ultra violet radiation. For fluorescence analysis the whole plant powder of normal and regenerated plants of Drymaria were treated with different solvents for observing the characteristic radiations if there is any at 366 nm wavelength of the light which is particular for every drug and a helpful parameter for generating plant research profile. Fluorescence study of powder with different solvents also revealed distinguished colour characteristics when treated with sodium hydroxide, hydrochloric acid, sulphuric acid, ammonia, iodine solution, ferric chloride, acetic acid and picric acid. The diagnostic colour of the drug with different chemical reagents would be helpful in the characterization of crude drug (Elamathi and Ilyas, 2012; Kumari et al., 2009).

Successive isolation of botanical compounds from plant material is largely reliant on the type of solvent used in the extraction procedure. In the present phytochemical studies, the aqueous extractive yield in the regenerated plants was significantly higher than the other solvent extractive yields. The results are similar to the findings of Arya et al., (2010) in Cassia occidentalis. Anwar et al., (2013) have evaluated and reported maximum extractive yield in aqueous methanol system from Brassica oleracea.
The organoleptic properties of normal and regenerated plants were evaluated. The organoleptic properties of various solvent extracts showed a characteristic colour, texture and odour. The dry methanol extract in the normal and regenerated plant was better than corresponding petroleum ether and chloroform extract in retaining a significant odour. This may be due to the preservative ability of methanol, its enhanced extraction capability (i.e., more components extracted) or combination of both (Arya et al., 2010).

Screening of phytochemicals

When a new drug is to be discovered, qualitative phytochemical analysis is a very important step as it provides information about the presence of any particular primary or secondary metabolite in the extracts of plant which is known to posses clinical significance. The presence of various active phytochemicals (secondary plant metabolites) as revealed by phytochemical screening supports the resourcefulness of the plant extracts (Sofowora, 1993). The petroleum ether, chloroform, methanol and aqueous extract of the plants were qualitatively screened for phytochemicals.

In the present studies undertaken to screen the presence of phytochemicals in normal and regenerated plants of Drymaria cordata, the methanol extract showed positive results for majority of phytochemicals such as alkaloids, carbohydrates, glycosides, saponins, starch, proteins, phenols and flavonoids. The findings are also in line of previous reports in the same taxon (Akindele et al., 2012 and Venkatesan et al., 2003). The occurrence of tannins was not indicated in the present studies although previous workers have reported its occurrence. The absence of certain phytochemicals may be attributed to different geographic locations and climatic conditions for the growth of the plant.
Barua et al., (2009) have revealed the presence of triterpenes, diterpenes, steroids and tannins in *Drymaria cordata* hydroethanol extract (DCHE), which contribute to its anxiolytic activity. While in 2010, they also reported the anti-inflammatory properties of *Drymaria cordata* methanol extract (DCME) which could be due to the presence of flavonoids, alkaloids and steroids. The neuropharmacological effects of DCME might be attributed to these compounds (Barua, et al., 2012). Significant analgesic activity in *Drymaria cordata* has been attributed to the presence of these bioactive principles (Barua et al., 2011). Castillo and Fernando (1984) have revealed the occurrence of a polyphenolic and aromatic flavonoid in *Drymaria cordata*.

The diuretic and antibacterial activity of plant extracts containing flavonoids have been documented in other plants (Enwerem et al., 2003; Monache et al., 1996; Rao et al., 1996). The presence of phenols in the methanol extract of regenerated plants validates their anti-inflammatory and antioxidant properties. Several studies have described various biological properties of phenolic compounds (Han et al., 2007; Brown et al., 1998; Krings et al., 2001). The presence of saponins was exhibited by methanol and aqueous extract. Some of the general characteristics of saponin include haemolytic activity, cholesterol binding properties and bitterness (Okwu, 2004).

The results obtained in this study thus suggest that the identified phytochemicals may be bioactive constituents responsible for the usefulness of whole plant of *Drymaria cordata*. These classes of compounds are known to have curative activity against several pathogens and therefore could validate and recommend the use traditionally for the treatment of various illness (Hassan et al., 2004; Usman and Osuji, 2007).
Phytochemical studies in normal, regenerated and AM treated plants

Effects of treatments on the chlorophyll content

The inoculation of regenerated plants with *G. fasciculatum* resulted in enhanced morphological features coupled with an improvement of chl-a, chl-b and total chlorophyll contents over un-inoculated plants. Increase in chlorophyll content due to inoculation with AM fungi in plants has been reported by earlier workers (Bian *et al.*, 2001; Feng *et al.*, 2002).

*G. fasciculatum* markedly improved the chlorophyll content in the leaves of regenerated plants of *Drymaria cordata* than *G. mosseae* inoculated normal and regenerated plants. Similar influence of *G. fasciculatum* in increasing the chlorophyll contents has been recorded by Selvaraj (1998) in *Prosopis juliflora*, Bhattacharjee and Sharma (2012) in Pigeon Pea, Karthikeyan *et al.*, (2009) in few medicinal plants, Tejavathi *et al.*, (2011) in *Andrographis paniculata* and in several other plants (Abdel and Mohamedin, 2000; Franco and Cano, 2006 and Zuccarni, 2007). Increased chl-a, chl-b and total chlorophyll content were also recorded by Mathur and Vyas, (2000); Bhoopander *et al.* (2003) and Kate *et al.*, (2005). Increased chlorophyll content in micropropagated plants is directly co-related with increased surface area of the leaves and photosynthetic rate in terms of fresh and dry weight (Thakur and Panwar, 1995 and Tejavathi and Rao, 1998). Krishna and Bagyaraj (1984a), reported that the increase in chlorophyll content in the inoculated plants might be due to presence of large number of chloroplast bundle sheath in the leaves. According to Karthikeyan *et al.*, (2009), the increase in total chlorophyll content and protein content in inoculated plants may be due to increased uptake of phosphorus, which increases the chlorophyll content in plants and ultimately the
photosynthetic activity of the plants. Taiz and Zeiger (1998) correlated the enhancement of chlorophyll to copper uptake since it is involved in the electron transport system and a component of chlorophyll-proteins. Enhancement in the rate of chlorophyll synthesis may be a consequence of stress by either abiotic or biotic factors.

Though AM fungal colonization results in symbiosis, the association might exert a sort of stress on the host. AM fungi inoculated plants of *Lactuca sativa* under salt stress condition had higher photosynthetic capacity because of high chlorophyll content than those of non stressed plants indicating the counter balance of salt stress by mycorrhization (Zuccarini, 2007). Tejavathi and Rao (1998) and Sowmya *et al.*, (2004) have reported high chlorophyll content in micropropagated plants treated with *G. mosseae*. However, in *Artemisia annua*, chlorophyll contents remained the same in both control and AM treated plants (Kapoor *et al.*, 2008).

**Effects of treatments on the biosynthesis of primary metabolites**

Primary metabolites are considered as building blocks and needed for general growth and primary physiological function and are also the precursors of secondary metabolites. AMF supports the host plant in increasing the uptake of nutrient and phosphates. It can contribute to plant growth and survival by reducing stresses associated with water, soil, pH, salt, toxic metals and pathogen (Sylvia and Williams, 1992). Lovato *et al.*, (1996) have reviewed various functions performed by AMF as bioregulators, bioprotector and biofertilizers.

In the present investigation, the mycorrhizal inoculation significantly affected the physiological status of *Drymaria cordata*. Considerable variations were observed in the primary metabolites
like carbohydrates, reducing sugars, proteins and amino acids in control and AMF treated plants of *Drymaria*. In the present studies, the whole plants of AMF inoculated normal and regenerated plants of *Drymaria cordata* exhibited less carbohydrate and reducing sugars than the control plants. The symbiosis is characterized by bi-directional movement of nutrients where carbon flows to the fungus and inorganic nutrients move to the plants, thereby providing a critical linkage between the plant root and soil (Sylvia, 2003). Mycorrhizal plants differ from non-mycorrhizal plants in the significantly higher carbon translocation from the shoot to root system (Snellgrove *et al.*, 1982).

Carbon drains in mycorrhizal plants give the decreased level of total carbohydrates in leaves of both normal and regenerated plants inoculated with *Glomus mosseae* and *Glomus fasciculatum*. Untreated regenerated plants show significantly higher carbon translocation from shoot to root system in *Agave* (Gayathramma, 2009). A decrease in the reducing sugars was noticed in the AM treated normal and regenerated plants. The decreased level of reducing sugars further reinforces the evidences given by several workers who showed that AM alters sink for metabolites by mobilizing sugar in the roots (Nemac and Guy, 1981). In contrast, *G. fasciculatum* inoculated plants have shown increased level of reducing sugars as reported by Snellgrove *et al.*, (1982) and Suzzane *et al.*, (1984).

A noticeable increase in the protein and amino acid contents were recorded in the regenerated plants of *Drymaria* inoculated with *G. fasciculatum* which is in accordance to the studies done by Tejavathi *et al.*, (2011) in *Andrographis paniculata*. The carbon compounds form amino acids resulting from the incorporation of ammonium into carbon skeletons derived from fungal trehalose and mannitol. Lewis (1975) suggested that the carbon moves from host to fungus as carbohydrates and could return back to host as other
compounds, through catabolism which provide necessary skeletal material for transfer of amino acids between fungus and host. Cooper (2000) is of the opinion that changes in the contents of reduced sugars and amino acids depends on the phosphorus status of the host plant.

Salvioli et al, (2012) have reported the increase in amino acid profile in fruit of tomato plants in response to mycorrhizal association, while France and Reid (1983) attributed the increase in amino acids and protein to reverse translocation of carbon compounds to the host. Increased accumulation of protein, polysaccharide and nucleic acids in the leaf samples of AM inoculated black pepper and sorghum was reported by Rajesh et al., (2011) thus indicating the direct correlation between the AM fungi and the crop response to AM fungal inoculation.

**Effects of treatments on the biosynthesis of secondary metabolites**

Secondary metabolites have medicinal and therapeutic importance and therefore are widely used in pharmaceuticals. Quantification and comparison of secondary metabolites without AM treatment were carried out in the normal and regenerated plants. The regenerated plants have proved better than normal plants in synthesizing an increased amount of primary and secondary metabolites.

The experimental results indicated that there is a fair amount of increase in the secondary metabolite content in AM treated regenerated plants. Highest amount (2.09±0.11 mg gm⁻¹) of phenolic content was recorded in *G. fasciculatum* treated regenerated plants of *Drymaria cordata*. The phenolic compounds in plants are known to play an important role in the defence mechanism against diseases (Krishna, 1981). In agreement to the present investigation, several
workers have observed that, the considerable increase in phenolic compounds in the host is a result of AM fungus inoculation (Selvaraj and Subramanian, 1990; Charitha and Reddy, 2002; Ozgonen et al., 2009). There is a well established positive relationship between the intensity of solar radiation and the quantity of phenolics produced by plants. It can be seen at the intra-individual level by comparing plant parts exposed to different amounts of light (Mole, 1988). An increase in phenols of roots of *Arachis hypogaea* colonized by *G. fasciculatum* was reported by Krishna and Bagyaraj (1984b). The increase in total phenols in AM inoculated plants could be attributed to triggering of pathways of aromatic biosynthesis (Selvaraj and Chellapan, 2006).

There are several reports on the effect of AM fungal association on secondary metabolism in host plants (Peipp et al., 1997; Kapoor, 2008; Morone-Fortunato and Avato, 2008). AM fungi improved the overall plant growth and accumulation of secondary metabolites in roots of Ashwagandha plants (Chandarana and Jasrai, 2011). Similarly the difference between mycorrhizal and non mycorrhizal plants with respect to total carbohydrates, amino acids, lipids, phenols, flavonoids, tannins and saponins were reported by Krishna and Bagyaraj (1984a) and Lakshman (1996).

Selvaraj and Subramanian (1990) while studying histochemistry of AM plants of *Seasamum indicum* observed that, the higher concentrations of phenols and lipids in mycorrhizal plants is due to the accumulation of these compounds in AM fungal structures particularly in vesicles and they attributed that the higher amounts observed in mycorrhizal plants is the contribution of fungal structures.

Accumulation of secondary metabolites and root growth enhancement in *Chlorophytum borivilianum* due to mycorrhizal inoculation has been reported by Dave et al., (2011). It has been
recorded that flavonoids promote the germination of spores of AM fungi (Tsai and Phillips, 1991; Maier et al., 1999). Burni et al., (2013) and Gupta et al., (2002) have reported the improvement in the essential oil contents in Mentha due to the presence of AMF. G. intraradicus induces accumulation of significant amount of leaf sesquiterpenoids in Citrus jambrini (Nemec and Lund, 1990). Inoculation with G. mosseae enhanced the accumulation of terpenes in cucumber roots (Akiyama and Hayashi, 2002).

An evident increase was also noticed in the amount of alkaloids, flavonoids and saponins in the regenerated plants of Drymaria than in the normal plant. Plant growth regulators affect growth and differentiation and thus affect secondary metabolite production by cultured cells. In nature, plant cells synthesize a number of compounds (Phytoalexins) in response to chemical or microbial attacks. Increased production of secondary metabolites in media supporting poor growth of cells is also regarded as a stress response (Dicosma and Towers, 1984). Consequently, a number of biotic and abiotic factors have been tested as elicitors and shown to improve the production of secondary metabolites in plant cell and organ cultures (Eilert, 1987). Simple organic and inorganic molecules can also induce product accumulation in cultured cells (Bhojwani and Razdan, 2004).

Recognition of important climatic factor(s) in relation to secondary metabolite production is required for understanding the biology of secondary metabolites in plants and to increase yields in artificial growth medium (Oloumi and Hassibi, 2011). The increased levels of primary and secondary metabolites in the regenerated plants than normal plants is in compliance with the earlier reports in Andrographis paniculata (Tejavathi et al., 2011), Agave (Gayathramma, 2009) and Bacopa monnieri (Tejavathi and Rao, 1998).
Antioxidant activity studies

Application of medicinal plants a food, preservatives and drugs is mainly due to their biological potentials such as antioxidant or antimicrobial activities (Bravo, 1998). Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS. Antioxidants through their scavenging power are useful for the management of those diseases. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract (Molyneux, 2004; Dehpour et al., 2009).

A fair correlation between phytochemical constituents and in vitro antioxidant activity was observed in Drymaria. In the present study, the methanol extracts of regenerated plants were shown to serve as potent antioxidant agent or hydrogen donor that can scavenge radicals. In DPPH and ABTS scavenging activity assay, the radical scavenging activity of methanol extract of both normal and regenerated plants increased with increasing concentration but was less than that of ascorbic acid which served as the reference compound. This could be due to the availability of methanol to extract non polar, medium polar and polar phytochemicals that act synergistically (Sintayehu et al., 2012).

The antioxidant activity of plant extracts may vary with assay methods (Yen et al., 2005). DPPH is a stable radical that has widely been used to evaluate the antioxidant activity of various natural products. The IC$_{50}$ values for DPPH and ABTS radical scavenging activity in regenerated plants was 67.4 μg ml$^{-1}$ and 193.0 μg ml$^{-1}$ respectively, while IC$_{50}$ value in the normal plants was recorded as 156.4 μg ml$^{-1}$ and 299.25 μg ml$^{-1}$ for DPPH and ABTS respectively.
The regenerated plants exhibited comparatively high antioxidant activity than normal plants. This can be correlated to increase in phenolic and flavonoid contents in regenerated plants. It is reported that phytochemicals constituents directly influences the antioxidant properties of the plant extract (Misra et al., 2008). The most important classes of natural antioxidants include tocopherols, flavonoids and phenolic acids, which are common to all plant sources (Hassas-Roudsari et al., 2009). This may be related to the presence of high amount of phytochemicals in the plant (Asaolu et al., 2010; Pourmorad et al., 2006). In contrast, Saikia et al., (2011) stated that there is no correlation between antioxidant activity and phenolic contents in certain medicinal plants.

According to Guo et al. in 2002, antioxidants display anti-inflammatory effects because the level of free radicals is increased in inflammation. Adeyemi et al., (2008), Barua et al., (2010) and Mukherjee et al., (1998) have studied the anti-inflammatory effect of various extract of Drymaria cordata while Sowmimo et al., (2011) have attributed the role of antioxidants in cancer treatment, thus justifying its inclusion in traditional recipes for cancer treatment and biopharmaceutical use.

**HPLC studies**

In the present study, the *G. fasciculatum* treated regenerated plants raised from nodal explants on medium supplemented with BAP + 2,4-D and GA3 have shown greater percent (0.39%) of isovitexin than untreated regenerated (0.33%) and normal plants (0.15%). Hsieh et al., (2004) have isolated isovitexin from the whole plants of Drymaria cordata. Isovitexin is a flavone C-glycoside, which is known to possess pharmacological activities such as antihypertensive, anti-inflammatory, antispasmodic, antimicrobial and antioxidant (Prabhakar et al., 1981; Agnese et al., 2001; Picerno
et al., 2003). The antioxidant activity of isovitexin is comparable to those of α-tocopherol and ascorbic acid (Chun-Mao et al., 2002). Veitch and Grayer (2008) have described more than 600 new naturally occurring flavonoids. They have reported the presence of isovitexin in the aerial parts of Stellaria media, a Caryophyllaceae member.

HPLC techniques have been employed in many plants to isolate and identify chemical compounds and their structures subjected to NMR and MS analysis. These studies have validated the use of the drug in the treatment of a particular ailment. Rabelo et al., (2013) have isolated and analysed isovitexin along with two other glycoside flavonoids by means of MS and NMR data. They have evaluated the anti-nociceptive, anti-inflammatory and antioxidant activities of the aqueous extract from Remirea maritima. Similarly Aderogba et al., (2011), have isolated and characterized novel antioxidant constituents from Croton zambesicus. Isovitexin was found to be a major component in Ficus deltoidea (Abdulla et al., 2009). A method based on ultrasonic extraction followed by LCMS was put forth by Fu et al., 2008 for the determination of isovitexin in pigeon pea.

It is suggested that studies may be undertaken to further bioassay experiments in order to identify, quantify and assess bioactive principles in vitro.

**Antimicrobial studies**

The presence of antibacterial substance in higher plants is well established (Srinivasan, 2001). In the present investigation, the petroleum ether, chloroform, methanol and aqueous extract of normal and regenerated plants were subjected to antimicrobial studies by well diffusion method. A number of antimicrobial studies in various plants are reported using this technique (Prabhakaran et
al., 2011; Kamalakannan et al., 2012). Similar comparative studies between normal and regenerated plants were made in other plants by Tejavathi et al., (2006) and Tejavathi and Padma (2013).

In the present investigation, the extracts of normal and regenerated plants indicated moderate to significant inhibitory activities against the bacteria tested. This suggests that the plant extract has broad spectrum in activity. However, the methanol extract of regenerated whole plants of Drymaria cordata were most effective against the tested bacterial pathogens. Higher antimicrobial activity of methanol extract was observed on Staphylococcus aureus, Salmonella typhi, while moderate activity was recorded on Klebsiella pneumonia and Proteus vulgaris.

The efficacy of methanol extract of Drymaria cordata was earlier reported by Mukherjee et al., 2009 against other pathogenic organisms. Arya et al., (2010), Kamalakannan et al., (2012), Parekh and Chanda, (2007), Raghavendra et al., (2006), Tejavathi and Padma (2013), Udhayasankar et al., 2013 and Usman et al., (2009) have recorded the efficacy of methanol extracts against several pathogenic microorganisms. 70% methanol extracts of Blepharis edulis showed best antimicrobial effect compared to ethanolic and methanolic extracts (Mahboubi et al., 2013).

The broad antibacterial activity of the methanol extract could be as a result of the plant secondary metabolites (alkaloids, flavonoids, phenols, saponins, glycosides) (Sindhu and Manorama, 2012). Extracts of various medicinal plants containing phenolic and flavonoids have been previously reported to possess antimicrobial activity (Ayaz et al., 2008; Rahman and Moon, 2007). Vaquero et al., (2007) investigated the properties of gallic acid, caffeic, vanillic acid, rutin and quercetin of different wine against pathogenic microorganisms. The presence of phenolic compounds in Drymaria
indicates that this plant might be an antimicrobial agent. This is because phenols and phenolic compounds have been extensively used in disinfection and remain the standard with which other bactericides are compared. The superior antibacterial activity of the regenerated plants over those of normal plants could be because of enhanced quantities of primary and secondary metabolites in the regenerated plants than in normal plants (Anitha et al., 2013). The presence of phytochemicals supports its use as antimicrobial agent.

Various researches have shown that gm +ve bacteria are more susceptible towards plant extracts as compared to gram negative bacteria (Lin et al., 1999; Parekh and Chanda, 2006). Gram –ve bacteria are multilayered in structure and more resistant (Yao and Moellering, 1995).

The Minimum Inhibitory Concentration (MIC) exhibited by the methanol extract of regenerated plant of Drymaria cordata on Staphylococcus aureus, Bacillus cereus, Salmonella typhi, Klebsiella pneumonia and Proteus vulgaris is of great significance more so that these multidrug resistant organisms have of great epidemiological threat. The MIC of Drymaria cordata against Staphylococcus aureus and Salmonella typhi was recorded as 450 µg ml⁻¹, while the other organisms required a slightly higher concentration for their inhibition.

There was no significant antifungal activity exhibited by any of the plant extracts. However, negative results do not mean absence of bioactive constituents nor is the plant inactive. Active compounds may be present in insufficient quantities in the extract to show activity with the dose levels employed (Taylor et al., 2001). Lack of activity can thus only be proven by using large doses (Farnsworth, 1993). Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic
effects or negating the positive effects of the bioactive agents (Jager et al., 1996).

**Future Directions**

- The potentialities of the plant could be optimised and therapeutic efficacy of the plant extract could be improved.
- The genetic fidelity of *in vitro* raised plants could be evaluated to reveal its polymorphism.
- Further investigations are required in isolating and identifying the active principle(s) of *Drymaria cordata* and to ascertain the role of individual constituents in the biological activity.
SUMMARY

The traditional people have developed remedies due to intimate relationship with nature over a long period of time. In some ailments, people prefer conventional folk medicines over modern medicines. *Drymaria cordata* (L.) Willd. ex Roem. & Schult. (Caryophyllaceae) is one such folklore medicinal herb of considerable importance. The plant though used widely in remote tribal regions of India is less known in spite of its phyto-potential. The plant is known to grow as a under storey weed and hence are weeded out of gardens and fields.

*Drymaria cordata* propagates naturally by rooting from nodes and also by seeds. Low germination rate and poor seed viability limit its propagation. Hence, an alternative propagation technique would be beneficial for the large scale multiplication, improvement and conservation of this herb. To date, there are not enough reports on *in vitro* propagation of *Drymaria cordata*. On this basis, the development of an efficient high frequency regeneration system was necessary for obtaining improved genotypes of this species. So keeping this thing in mind, micropropagation work was carried out on this plant.

Few phytochemical aspects of the plant has been worked upon and cited by several researches. Biologically active compounds have been isolated from this plant, including cyclopeptides, flavonoid glycosides, norditerpenes and norditerpene glucosides which have been used in chemotherapy against many types of diseases. Among the secondary metabolites, drymaritin reportedly exhibits anti-HIV properties while a C-glycosyl flavone – isovitexin is reported to be a potential antioxidant. As flavonoids are being considered as potent antioxidants with great healing power, it was imperative to determine and compare the antioxidant capacities of normal and *in vitro* regenerated plants. Therefore pharmacological studies were needed in *Drymaria cordata* for scientific validation of its properties. Perusal
of literature revealed that the plant is under-exploited, in all the above studies. Hence the present investigation deals with *in vitro* regeneration and phytochemical studies.

The objectives of the present study was to standardize optimum conditions for establishment of nodal/ internodal and leaf culture from elite germplasm, shoot proliferation, rooting of micro shoots, hardening and transfer of plants to soil. The objective was to evaluate the association of AMF with *Drymaria cordata* and compare the morphological and physiological parameters between them. The normal and regenerated plants were also subjected to antioxidant, HPLC and antimicrobial studies.

In the present investigation, the node, internode and leaf explants were used in the process of organogenesis studies. Healthy young plants of about 2-3 months old were chosen as the source of explants. The node and internodal region (between 3rd to 6th nodes from apical meristem) and young, fully expanded leaves (0.77-0.96 cm²) from *in vitro* shoots raised on MS basal hormone free media were used as explants. Of the two media tried *i.e.*, MS and L2, MS was found to be most suitable for multiple shoot regeneration from various cultures. These media were supplemented with auxins, cytokinins, gibberellins individually or in combinations to study their effect on morphogenesis.

Direct and maximum organogenesis was observed in nodal culture when inoculated on medium supplemented with BAP alone. The shoots obtained on auxin supplemented medium were less in number. However, IBA supplemented medium exhibited significant shoot elongation. Higher concentration of auxins promoted callusing of the explants. Synergistic effects of growth regulators too have induced direct proliferation of shoots from the nodal explants. MS medium supplemented with BAP (4.44 μM) + GA3 (1.44 μM) + IBA
(0.49 μM) resulted in mass proliferation of shoots (about 45 shoots) from nodal explants. The presence of GA3 promoted shoot proliferation and elongation.

Growth regulators have significantly influenced indirect shoot regeneration from nodal and leaf explants while the internode explants were less responsive. Emergence of maximum number of multiple shoots from basal callus in the nodal explants was recorded on MS medium supplemented with BAP (4.44 μM) + GA3 (1.44 μM) + 2,4-D (2.26 μM). *In vitro* leaf cultures responded favourably to MS + BAP (4.44 μM) + NAA (2.29 μM) and produced pale cream healthy callus which nodulated and produced shoots on TDZ supplemented medium after a considerable long period (3-4 months). TDZ (1.82 μM) supplemented medium also promoted the development of occasional somatic embryos on the nodulated callus but the frequency of conversion of these embryos into plantlets was less. The frequency of initiation of shoot buds could be enhanced if the nodular callus were cultured on a filter paper placed on the liquid medium supplemented with TDZ (2.27-4.54 μM). Filter paper method proved to a faster method to induce regeneration of shoot buds from the nodular callus within 25 days of culture.

Although hormone free MS medium was sufficient to induce rooting, MS + IBA (0.49 μM) proved highly favourable for profuse and healthy rooting as well as elongation of microshoots. Inclusion of ascorbic acid in rooting medium enhanced the morphogenic response of newly developed plantlets. The regenerated plants were hardened in cocopeat : soilrite : perlite in the ratio 1:1:1. These plantlets were well established in soil with 90% survival rate.

Histological studies provided morphological details that help to explain the process of organogenesis from *Drymaria cordata*. Both organogenic and embryogenic callus were obtained, which revealed
the origin of shoot buds and somatic embryoids. Shoot buds were seen to arise from compactly arranged meristematic cells with dense cytoplasm, small vacuoles and conspicuous nucleus. The origin was indirect as the root pole was absent and the shoots emerged from a mass of callus cells.

There were no much phenotypic changes observed in the regenerated plant in comparison with normal plants. This study showed that a dramatic increase in the rate of regeneration can be achieved from nodal and leaf explants which can be efficiently employed for different in vitro improvement methods. The plant regeneration protocol may be useful for genetic transformation studies.

The in vitro regenerated and normal plants were subjected to AMF studies. Percent colonization, occurrence of arbuscules and vesicles and morphological features of AMF treated and untreated plants were compared. From the studies undertaken, the role of AMF on Drymaria is indicative of the potential that this association is apparent for improved production. AMF treated regenerated plants have shown significant results compared to untreated plants. G. fasciculatum treated regenerated plants have shown tremendous increase in the chlorophyll, primary and secondary metabolite content. These results suggest that G. fasciculatum are better symbionts for inoculating Drymaria cordata a member of Caryophyllaceae. There is still scope for further research on efficacy of AMF species associated with Drymaria.

The present phytochemical study proved that Drymaria cordata is an amalgam of variety of important phytochemicals which contributes towards its multirole pharmacological properties like antibacterial, anti-inflammatory, antioxidant, anti-rheumatic, anti-tussive, anti-febrile etc., and justifies its wide ethnomedical usage.
Based on the results of antioxidant assays (DPPH and ABTS), *Drymaria* was found to possess free radical scavenging activity. The total phenolic content and radical scavenging potential were significantly high in regenerated plants. HPLC analysis of whole plant extracts of *Drymaria* has indicated a high content of isovitexin in mycorrhizal treated regenerated plants and a significant lower content in non mycorrhizal treated plants.

The results obtained showed that *Drymaria cordata* could be an interesting source of natural antioxidants with potential use in food supplements. Of the various solvent extracts tried for determining the antimicrobial efficacy, the methanol extract of regenerated plants exhibited good antibacterial potentials and supports the claims in folklore medicine. The methanol extract of *Drymaria* posed to be strongly effective against *Salmonella typhi*, and *Staphylococcus aureus*. A minimum concentration of 450 μg ml⁻¹ was sufficient to inhibit the growth of these two pathogenic bacteria. The results from the investigation indicates that the plant extracts offer significant potential for the development of novel antimicrobial therapies and treatments of several diseases caused by microorganisms. Some cheap, cost effective and economical herbal formulations may be prepared from the extract of *D. cordata* for certain bacterial infections.

In conclusion, the present investigation has resulted in the development and establishment of an efficient protocol which could be used for *ex situ* conservation and true to type mass propagation of this ethnobotanical medicinal herb of immense pharmaceutical relevance. Results obtained through plant tissue culture in present study could be highly promising to increase the regeneration capacity, high multiplication rate and further coupled with AMF can lead to yield improvement of *Drymaria*. The plant can be of immense use in phytomedicine and can be included in health care delivery
system particularly in developing economies. It is hoped that phytochemical study of the plant extracts will provide much needed lead for further research and new polyherbal formulations.

**HIGHLIGHTS OF THE STUDY**

- Foremost protocol for direct shoot regeneration from nodal explants was developed.
- Foremost protocol for indirect shoot regeneration from leaf explants via callus phase was developed.
- BAP was highly significant in multiple shoot regeneration in *Drymaria cordata*.
- Foremost protocol for occurrence of somatic embryos on leaf derived callus.
- Filter paper method proved to be faster method of regeneration of plants on leaf derived callus.
- Utility of low concentration of growth regulators for callus and shoot regeneration.
- Pioneer work on AMF studies in *Drymaria cordata*.
- *G. fasciculatum* a better symbiont for inoculating *Drymaria cordata*.
- Enhanced primary and secondary metabolite contents in AMF treated regenerated plants.
- Regenerated plants were found to be morphologically similar to the mother plant.
- Regenerated plants exhibited prospective antioxidant activity.
- *G. fasciculatum* inoculated regenerated plants exhibited highest amount of isovitexin.
- Methanol extract of regenerated plants exhibited significant antibacterial activity.