Chapter 2
Review of Literature
REVIEW OF LITERATURE

2.1 A brief background

With the discovery of the cell theory of Schleiden (1838) and Schwann (1839) which identify the cell as a structural and functional unit of the entire living organism. The cell theory got more momentum with the discovery of Virchow (1855) stating that “Omnis cellula e cellula” (all new cells arises from pre-existing cells) which leads to the concept of ‘totipotency’ of the cell. However, no sincere effort was taken to examine the effectiveness of this investigation until the birth of the 20th century. The experiment of Haberlandt (1902), a German botanist, known as father of the plant tissue culture, brings a new shape when he grew single cell of Lamium purpureum and Eichhornia crassipes on Knop’s (1865) salt solution enriched with glucose as a carbon source and witnessed the growth in palisade cells but fail to divide. The poor choice of nutrient medium might be the reason for the restricted division of the cell (Vasil and Vasil 1972). Hanning in 1904 grew embryogenic tissue and was able to culture them up to the maturity on artificial nutrient medium consist of mineral salt and sugar solutions. Kotte (1922) in Germany and Robbins (1922) in United States, during their pioneer work on plant tissue culture studies they tried to culture root and shoot tip explants. Further, improved growth in the root culture was also established by Robbins and Maneval (1924). However, White in 1934, established the first report of continuous growth using tomato root tips. The work of Went in 1926, for the discovery of the naturally occurring auxin, indole-3-acetic acid (IAA) and its effects on plant growth, makes the hormone as important supplement in nutrient media. Further, Gauthe (1939), Nobecourt (1939) and White (1939) reported growth of the plant callus culture in their experiment individually by using the natural auxin. In 1941, Van Overbeek and co-workers first identified the effective role of the coconut milk on the growth and development of an embryo in Datura.

Initially, the explants having meristematic cells were used to obtain indefinite growth and a verity of plant extract including coconut milk was used to identify the factors responsible for the growth. Among these, yeast extract was found most suitable and its components were reported as having purine-like properties. This findings leads to a concept about the role of DNA and its importance in the growth medium. The overall finding rooted the discovery and isolation of ‘Kinetin’ from herring sperm DNA by
Miller et al. (1955). Skoog and Miller in 1957 were able to understand the role of phytohormone in the regulation of shoot morphogenesis. Later various studies were carried out to identify several cytokinins occurring naturally as well as synthetic and enable the technique of plant tissue culture an effective strategy for the conservation and preservation of the plant species. Several attempts were carried out to improve the growth medium by manipulating the mineral content. Murashige and Skoog (1962) were able to meet the nutrient demand of the plant cell by using universal growth medium known as MS medium, which was twenty-five times higher in salt concentration as compare to the Knop’s solution (1865). High level of inorganic salts, chelated iron and complex of vitamins and myo-inositol are the innovative ingredients of MS medium. Nowadays, MS medium is the most extensively used growth medium with a colossal application at industrial level (Shahzad et al. 2017a&b).

The concept of androgenesis was first proposed by Guha and Maheshwari 1966, they cultured Datura anther and obtained embryos leading to the formation of the haploid plant. Steward et al. (1958) was able to achieve somatic embryos via suspension culture which was a milestone in demonstrating the totipotency of somatic cells and validating the idea of the Haberlandt. As the time advanced, Murashige in 1974 illustrated three major steps of the plant tissue culture technique: Stage I: Establishment of the aseptic culture, Stage II: Multiplication of propagules, Stage III: Re-establishing in a natural environment. Eventually, many workers introduced an additional stage that is Stage 0: care and preparation of stock plant. Further, stage III of the Murashige divide as Stage III: rooting in microshoot and Stage IV: acclimatization of the rooted plants in greenhouse or field conditions. Preece and Sutter in 1991 describe the Stage IV as most problematic steps because of the fact that the transfer of the plant from in vitro to ex vitro conditions will cause various environmental stresses to the plant and it is a challenging step during the whole process of the micropropagation. However, if ex vitro rooting was carried out for the microshoot then it will combine both steps and brings original Stage III of the Murashige’s illustration. Nowadays the techniques of plant tissue culture have been widely used as a significant tool for the vegetative propagation of the important plant species (Chu and Kurtz 1989, Dave and Purohit 2002). The present reviews dealing with the achievements and advances of plant tissue culture for the in vitro regeneration of medicinal plants from various explants.
2.2 Micropropagation or in vitro propagation as a promising tool

The technique of the micropropagation is attributed as an aseptic culture of the plant cell or tissue under controlled physical and chemical conditions in vitro (Thorpe 2007). The approach of this technique is based on the concept of the totipotency as recommended by Heberlandt (1902). The possible way by which a successful micropropagation protocol would be achieved are given as I: direct and indirect organogenesis, and II: direct and indirect somatic embryogenesis. The techniques of micropropagation has been proven more beneficial as compared to the conventional methods such as, a) a larger number of the plant can be produced in limited time, b) disease-free plant can be prepared with this technique, c) the multiplication rate can be increased at the significant level, d) the technique also helps in the production of the pathogen-free material. The technique is proven more significant for the conservation and preservation of the endangered and critically endangered plant with high medicinal value and the conserving the biodiversity.

2.3 Plant tissue culture studies in Decalepis spp.

The steno-endemic species of genus Decalepis are facing a high level of menace due to the destructive harvesting from the wild. The genus claimed its paramount significance for the mankind due to unique tuberous root characteristics (Mishra et al. 2017). A brief introduction of developed protocols and different strategies used has been described below;

2.3.1 Shoot induction and multiplication

2.3.1.1 D. hamiltonii

During the last few years’ considerable studies have been conducted to develope viable methods for the micropropagation and conservation of D. hamiltonii. George et al. (2000) reported indirect shoot regeneration through leaf callus. Among various concentration of cytokinin examined, a treatment of 1.9 mg L\(^{-1}\) BA + 0.05 mg L\(^{-1}\) NAA found to be optimum for the differentiation of a maximum of 4.4 shoots per culture. To achieve normal growth and development in the regenerated shoot, application of 3% sucrose to the growth medium was found optimum (George et al. 2000). The technique of axillary bud differentiation was found more applicable for the higher number of shoots production (Bais et al. 2000). They have found a treatment of
2.0 mg L\(^{-1}\) BA + 0.5 mg L\(^{-1}\) NAA most effective with a maximum of 12.8 ± 0.96 shoots per explant was reported in D. hamiltonii through shoot tip explants. However, Anitha and Pullaiah (2002) increased the concentration of cytokinin treatment to maximize the regeneration efficiency of nodal explants, but could not be able to improve the response and only 4.0 ± 0.1 shoots per explant were produced on MS medium containing 17.76 μM BA + 0.53 μM NAA. Addition of phenylacetic acid (PAA) showed the influential response on shoot multiplication through nodal segment culture and a composition of MS + 31.08 μM BA + 14.68 Mm PAA was proved to be best for the maximum number of shoot regeneration (6.4 ± 0.39 shoots per explant) (Giridhar et al. 2003).

The synergistic effect of growth additives i.e. Adenine sulphate (Ads), glutamine (Glu), phloroglucinol (PG) with the cytokinin have been reported by several workers (Gururaj et al. 2004, Giridhar et al. 2005a, Sharma et al. 2014a). Gururaj et al. (2004) found a maximum number of shoots when the shoot tip explants was inoculated on MS medium augmented 1.1 μM BA + 5.8 μM GA\(_3\) + 800 μM PG. Subculture passages on the medium comprised of MS + 5.6 μM BA + 200 μM PG + 0.11 μM TRIA produced elongated shoot with secondary shoot formation. Giridhar et al. (2005a) used shoot tip as an explants for the regeneration and the different concentrations of cytokinins have been tried for the development of efficient propagation protocols. They were successful to produce a maximum number of shoots (6.5 ± 1.2) on MS + 2-iP 4.9 μM and multiplication of shoots was achieved on MS + 2.5 μM 2-iP + 0.3 μM GA\(_3\). Similarly, Sharma et al. (2014a) applied various treatments of PGRs and growth additives, the MS medium augmented with 5.0 μM BA + 0.5 μM IAA + 30.0 μM Ads found to be significantly better and produced a maximum of 8.20 ± 0.37 shoots per explant with a mean shoot length of 6.54 ± 0.08 cm. They found that the cytokinin alone was unable to exhibit significant response for enhanced shoot multiplication but able to induce nodulation (N\(_1\)) from the basal cut end similar to cytokinin-auxin combination (N\(_2\)). N\(_2\) type nodule was found better for maximum shoot regeneration of 15.40 ± 0.67 shoots per explant with maximum shoot length of 4.56 ± 0.06 cm when subcultured on 5.0 μM BA + 0.5 μM IAA + 30.0 μM Ads + 1.0 μM GA\(_3\) (Sharma et al. 2014a). The obtained microshoot further rooted on optimum concentration of auxin i.e. NAA, a concentration of 2.5 μM α-naphthalene acetic acid supplied with MS medium was able to achieve rooting to the microshoot.
There is only one report available on somatic embryogenesis in *Decalepis* species. In the preliminary work of Giridhar et al. (2004), the somatic embryoids in the *D. hamiltonii* were obtained through leaf explant culture. The Callus obtained from the leaf when transferred to the MS + 13.68 μM Zeatin + 10.65 μM BA exhibited differentiation of somatic embryoids and subsequent maturation. Further, the embryogenesis response improved when a low concentration of Zeatin was used and a combination of MS + 4.56 μM zeatin + 10.65 μM BA was found to be more effective for the maturation of embryoids.

### 2.3.1.2 *D. arayalpathra*

Only few reports are available on the micropropagation of *D. arayalpathra*. Initial work carried out by Gangaprasad et al. (2005) on the in vitro propagation of *D. arayalpathra* via using single node. They inoculated the nodal explant on MS medium supplied with the distinct concentration of BA (0.1-5.0 mg/L). All the concentration of BA was found to induce the shoot buds, but MS + BA 0.1 mg/L was proved to be optimum to induce rapid growth and produced the longest shoots (6.8 ± 0.42 cm) in 60 days. Thereafter, the nodal segment and shoot tip were taken from the in vitro raised plant and inoculated on MS medium supplied with BA (0.5 mg/L) on which the developed shoot attained a maximum of 8.0 ± 0.74 cm length in only 30 days (Gangaprasad et al. 2005). Sudha et al. (2005) studied the action of cytokinin on nodal segment taken from the mother plant (12-16-week-old) and inoculated on MS + 12.96 μM BA + 2.48 μM 2-iP + 2.68 μM NAA, which was able to give rise to un-branched robust solitary shoots with a shoot length of 16-17 cm in 8 weeks. The same nutrient medium was used but with other explants i.e. cotyledonary nodal segment which showed the axillary branching, however, they were thin and fragile. Single nodes of solitary shoot subcultured on MS + 2.22 μM BA + 0.24 μM 2-iP was able to produce thick shoot with 9.8 ± 0.3 nodes from 18.0 ± 0.6 cm long shoots within 5-6 weeks and basal nodes of shoots were used for subculturings purposes to enhance the stock of propagules (Sudha et al. 2005). A comparative study of node and shoot tip explants of *D. arayalpathra* conducted by Ahmad et al. (2018a) revealed a significantly improved regeneration system. Nodal segment was found to be more responsive than the shoot tip explants, however, both the explants were able to respond at different concentrations of the BA. A treatment of MS + 5.0 μM BA was found optimum for the shoot induction in both the explants. However, for higher frequency shoot
multiplication, the combination of optimized auxin-cytokinin concentration with growth additives has been found more effective and a treatment of MS + 5.0 μM BA + 0.5 μM IAA + 20.0 μM Ads was able to induce 11.8 ± 0.1 shoots/nodal segment and 5.5 ± 0.1 shoots/shoot tip with an average mean shoot length of 9.2 ± 0.1 and 4.8 ± 0.1 cm, respectively after 6 weeks of incubation period.

### 2.3.1.3 *D. salicifolia*

There is only one report available on the micropropagation of *D. salicifolia*. Ahmad et al. (2018b) developed a high frequency shoot regeneration system using nodal segment for the first time. Different concentration of cytokinin singly (BA, Kn and 2iP) has been tried and BA at a concentration of 5.0 μM was found more advantageous for shoot regeneration. However, to improve the growth, the combination of optimum cytokinin with different auxin like IAA, IBA and NAA was tried and a combination treatment of 5.0 μM BA + 0.5 μM NAA was found more effective with the production of a 4.04 ± 0.05 number of shoots with a mean shoot length of 4.99 ± 0.05 cm. Addition of different growth additives like Ads, Glu and PG at a various concentration in the optimal regeneration medium improved the regeneration efficiency significantly. A treatment consisting of MS + 5.0 μM BA + 0.5 μM NAA + 30.0 μM Ads, produced a maximum of 9.97 ± 0.01 shoots/explant and a maximum shoot length of 6.46 ± 0.1 cm was achieved after 6 weeks of incubation periods.

### 2.3.2 Rooting and acclimatization

The micropropagation protocol is unsuccessful until the effective rooting programme is not performed to the growing microshoot. Bais et al. (2000) advocated the use of silver nitrate (AgNO₃) for successful *in vitro* rooting in microshoots of *D. hamiltonii*. Another treatment like ethaphone was also tried but it brings baroque callusing at the base of microshoot. Further, they used AgNO₃ in combination with ethylene which improved the rooting response. Reddy et al. (2001) achieved rooting when the microshoots were transfer to the medium containing IBA (8.8 μM) + IAA (1.43 μM). Giridhar et al. (2003) found that IBA (9.8 μM) was efficient for the root induction in the microshoot, while Gururaj et al. (2004) in another study obtained the rooting response when microshoot were transfered to the medium containing 5.38 μM NAA and 400 μM PG. Some reports
have emphasized for the active participation of PG and SA and their compatible interaction to obtain the root formation in in vitro generated microshoot (Giridhar et al. 2003). Ahmad et al. in (2018a) achieved rooting in in vitro regenerated plant of D. arayalpathra using a concentration of 2.5 µM α-naphthalene acetic acid (NAA) augmented in half-strength MS medium with the induction of a mean number of 5.1 ± 0.1 roots per shoot and mean root length of 4.9 ± 0.1 cm. In another study of Ahmad et al. (2018b), root induction in D. salicifolia, emphasized that the use of half-strength MS + 2.5 µM indole-3-butyric acid (IBA) was significantly better which gave rise to 6.10 ± 0.07 roots per microshoot with average root length of 2.30 ± 0.06 cm.

2.4 Different strategies of micropropagation in other plant species

2.4.1 Direct regeneration

Direct induction of shoots found to be genetically identical (Hu and Wang 1983) and it can be obtained through pre-existing meistems (axillary/shoot tip meristems) or from the well differentiated tissue (leaf, stem, cotyledon, petiole, root etc.). Such kind of morphogenesis devoid of any callus formation was first reported by Morel (1960), Morel and Muller (1964) when they cultured the shoot tips for virus free orchids, Cymbidium. Their experiment was considered the most appropriate method for obtaining genetically identical plant in short duration. In plant tissue culture techniques, with the naturally occurring meristems or tissues lacking meristems can give rise to direct organogenesis and the former type of direct organogenesis included axillary bud differentiation, while the latter referred as adventitious regeneration. Several workers have reported response on direct organogenesis in medicinal plants (Khawar et al. 2005, Aasim et al. 2011, Sahai and Shahzad 2013, Sharma et al. 2012 and 2014a, Gaikwad et al. 2015, Barpete et al. 2017, Ahmad et al. 2018a&b, Karatatatas et al. 2018a&b).

2.4.1.1 Axillary and apical meristem culture

The initial work of meristem culture was carried by Ball (1946) through shoot tip culture of Tropaeolium majus. After that Morel and Martin (1952) with their remarkable discovery of making virus free Dahlia plant by culturing their shoot tips in vitro, Morel in 1960 further extended the work for making the virus free plant of
orchids. These exceptional discoveries attract various workers in plant tissue culture technology and it was a beginning of plant tissue culture technology of commercial importance. Now meristem culture becomes an exclusive technique for the production of the pathogen-free plant (Walkey 1978, Bhojwani and Razdan 1983, Biswas et al. 2007). Another breakthrough was achieved by Wickson and Thimann (1958) regarding the role of cytokinin for inhibition of apical dominance and leading to the growth of axillary shoots. Therefore, the use of shoot tip or apical meristem culture was used for mass level clonal propagation and production of virus free plants.

2.4.1.2 Synergistic effect of auxin and cytokinin on shoot multiplication

The first cytokinin was an adenine derivative known as Kinetin (6-furfuryliminopurine) discover by Miller et al. (1955) as a factor responsible for cell division. The presence of cytokinin is witnessed in all the plant part and abundant in root tips, shoot apex and immature seeds (Miller et al. 1955). 6-Benzyladenine (BA) is a synthetic cytokinin which is known for its stability and widely used in plant tissue culture. There are also structurally unrelated phenylurea type cytokinins i.e. diphenyl urea, thidiazuron (synthetic cytokinin). An optimum ratio of auxin and cytokinin regulates the plant tissue either for the formation of shoot or root (Skoog and Miller 1957). It is reported that high concentration of cytokinin to auxin generally promotes shoot formation while a low ratio leads to the formation of root. From several years the choice of explants as shoot tips and axillary buds for the in vitro propagation of many plant species has been successfully utilized i.e. Picrorhiza kurroa (Upadhya et al. 1989), Withania somnifera (Kulkarni et al. 2000), D. hamiltonii (Giridhar et al. 2005a), Glycyrrhiza glabra (Vadodaria et al. 2007), Clitoria tematea (Anand et al. 2011), Nilgirianthus ciliates (Rameshkumar et al. 2017), Origanum acutidens (Kizil and Khawar 2017), D. arayalpathra (Ahmad et al. 2018a) and D. salicifolia (Ahmad et al. 2018b).

Kizil and Khawar (2017) established a micropropagation protocol for Origanum acutidens by using nodal segment. They successfully induced 10.28 shoots/explants with a mean shoot length of 2.46 cm on MS + 0.8 mg/l BA. Rameshkumar et al. (2017) developed a micropropagation protocol for Nilgirianthus ciliates from the nodal segments. They tried various concentrations of cytokinin and a treatment consisting of MS + 3.0 µM BA was found to be optimum with the production of 21.6
± 0.1 shoots/explant and a mean length of 6.4 ± 0.4 cm was reported. Dhir and Shekhawat (2014a) developed micropropagation protocol for threatened plants like *Ceropegia bulbosa* Roxb. var. *bulbosa* and *C. bulbosa* Roxb var. *lushii* by using shoot tips as explants, inoculated on MS medium supplemented with BA and NAA. The combination of both auxin and cytokinin was able to differentiate shoots and a treatment of MS + 8.88 µM BA + 0.54 µM NAA produced an average of 7.8 ± 0.28 shoots per explant. Whereas, a combination of 8.88 µM BA + 0.27 µM NAA gave rise to 8.7 ± 0.15 shoots per explant. In the above study they found that BA was more suitable cytokinin with an optimal concentration of 8.88 µM for the maximum number of shoot regeneration. Sahai and Shahzad (2010) developed a potent approach for the large scale *in vitro* production of *Coleus forskohlii* by using leaf explants. Various combinations of cytokinin and auxin were used and a treatment of 2.0 µM BA along with 0.1 µM NAA was found most effective with production of a 35.0 ± 1.2 shoots/explant with shoot length of 5.4 ± 0.5 cm. Whereas, through nodal explants of *C. forskohlii*, a treatment of 5.0 µM BA was found most effective for the shoot multiplication (Sahai and Shahzad 2013). Thereafter, to improve the shoot multiplication, combination of auxin was used with the optimum BA concentration and a medium comprised of MS + 5.0 µM BA + 1.0 µM NAA was found most optimal with the differentiation and development of a maximum of 18.80 ± 0.1 shoots/explant.

Valizadeh and Valizadeh (2011) developed a micropropagation protocol for *Withania coagulans* using nodal explants. They tried various concentrations of BA alone or in combination with the auxin like IBA. They inoculated the nodal segment on the medium comprises of MS + 2.0 mg l⁻¹ BA + 0.5 mg l⁻¹ IBA, wherein a maximum of 7.2 ± 1.0 shoots/explant with a maximum shoot length of 7.0 ± 1.4 cm was reported. Parveen et al. (2010) established micropropagation system for the *Cassia siamea* by using CN explants, they tried different concentration of cytokinin alone or in combination with auxin and a treatment of MS + 1.0 µM BA + 0.5 µM NAA was found most suitable and gave rise to 12.20 ± 0.73 shoots/explant attaining a maximum shoot length of 6.40 ± 0.07 cm. The supremacy of the BA over other cytokinin is well documented for the other medicinal plants including *Peganum harmala* (Khawar et al. 2005), *Spilanthes acmella* (Deka and Kalita 2005), *Penthorum chinensis* (Cao et al.
2007), Marsdenia brunoniana (Ugraiah et al. 2010), Ricinus communis (Alam et al. 2010) and Origanum acutidens (Kizil and Khawar 2017).

In another study by Parveen and Shahzad (2010), an efficient in vitro regeneration system for C. sophera using CN explant was discussed. They evaluated that the application of TDZ was more advantageous with other cytokinin as an optimal treatment of MS + 2.5 µM TDZ contributed a maximum of 6.7 ± 0.2 shoots per explant with an average shoot length of 2.3 ± 0.1 cm. The regeneration efficiency was significantly improved when they tried the combination of BA with the optimum concentration of TDZ and a treatment of MS + 2.5 µM TDZ + 1.0 µM BA was found to be the best with the formation of 14.9 ± 1.4 shoots per explant with an average shoot length of 5.8 ± 0.2 cm. However, Sharma and Shahzad (2013) reported the treatment of TDZ alone to induced a huge number of shoots (30.0 ± 0.3 shoots/explant) with a mean shoot length of 1.0 ± 0.1 through shoot tip explants when cultured on MS + 0.25 µM TDZ after 4 weeks of incubation period.

Thiyagarajan and Venkatachalam (2013) established a reproducible protocol for the in vitro propagation of Gymnema sylvestre by using nodal explants. They tried different concentration of BA alone or in combination with auxin and a concentration of 1.0 mg l\(^{-1}\) BA + 0.5 mg l\(^{-1}\) Kn was found more effective which gave rise to a maximum number of 14.20 ± 0.1 shoots/explant. Further, they inoculated the in vitro raised axillary shoot bud on MS + 1.0 mg l\(^{-1}\) BA + 0.5 mg l\(^{-1}\) Kn + 1.0 mg l\(^{-1}\) GA and a total of 418.72 shoots/explant were obtained after five subculture passage. Similarly, Rathore et al. (2012) reported an early induction of shoots with enhanced shoot regeneration in Withania coagulans. They used certain growth additives with optimum combination of BA and IBA and best re-growth was obtained on the medium comprised of MS + 8.88 µM BA + 0.57 µM IAA + 100 mg l\(^{-1}\) L\(^{-1}\) ascorbic acid + 25 mg l\(^{-1}\) citric acid on which a maximum 19.1 ± 0.28 shoots per explant with a mean shoot length of 6.15 ± 0.25 cm were obtained.

2.4.1.3 Adventitious shoot formation

The formation of adventitious shoot involves the emergence of shoot buds from any part of the explants other than primary meristem without the intervening callus phase. The technique plays an important role to prevent from somaclonal variation during the culture and proven an effective micropropagation technique for the propagation.
Many studies have been conducted on the adventitious shoot formation through various explants i.e. leaf, internode, hypocotyle, epicotyle, petiole, cotyledon, stem and root. An efficient micropropagation protocol has been developed by Rathore et al. (2016) for direct organogenesis in *W. coagulance* by using *in vitro* derived leaf explants and a treatment of MS + 4.44 µM BA resulted in the formation of a mean of 11.4 ± 0.9 shoot buds/explant.

The use of combination treatment of cytokinin and auxin exhibit significant improvement for the enhanced shoot regeneration in some plant species. Barik et al. (2005) developed a protocol for *Lathyrus sativus* using epicotyls segment and a treatment of MS + 17.76 µM BA + 10.74 µM NAA was found optimum for the production of 8.2 shoots/explant. The combination of BA and NAA was also used by Burdyn et al. (2006) in *Aloysia polystachia* and Seetharam et al. (2007) in *Vernonia cineria* for direct shoot bud induction from leaf explants. Perez-Alonso et al. (2018) developed a micropropagation protocol for immensely important plant *Digitalis purpurea* which is known for its cardiovascular and anticancer activities. They tried MS medium supplemented with the various combination of phytohormone and recorded that the optimal combination of 0.54 µM NAA + 13.2 µM BA exhibited 98.5 % regeneration frequency through the leaf explant with the production of a maximum of 18.9 shoots/explant.

Purohit et al. (2004) achieved direct shoot bud differentiation by using leaf explants of *Achras sapota*. A treatment of 8.88 µM BA + 5.0 µM TDZ supplemented with Schenk and Hildebrandt’s medium (SH medium) was optimal, wherein a maximum regeneration frequency (36.44%) with 25.32 buds/explant was reported. Yang et al. (2014) developed a micropropagation protocol for *Dayaoshania continifolia* using leaf explants. The lower concentration of BA i.e. 1.0-3.0 µM was able to induced adventitious shoot from the leaf explants. However, when the combination of BA and NAA was tried, an improved regeneration was achieved and a compostion of MS + 1.0 µM BA + 1.0 µM NAA was found to be optimal for the maximum production of 24.5 ± 0.1 shoots/explant with a mean shoot length of 8.1 mm.

Similarly, Jani et al. (2015) used a combination treatment of two cytokinin to evaluate the regeneration potential of root explants of *Psoralea corylifolia*. The combination of BA and Kn was tried and resulted in an improved response in terms of maximum
production of the shoot and a treatment consisting of MS $+ 2.22 \mu M$ BA $+ 6.98 \mu M$ Kn responded best with the production of 39.67 shoot buds/explant but only 3.37 mean number of shoot were elongated. However, for improved regeneration, the GA was used and a treatment of MS $+ 2.22 \mu M$ BA $+ 6.98 \mu M$ Kn $+ 2.89 \mu M$ GA contributed an utmost number of $9.3 \pm 0.2$ shoots/explant production with an average shoot length of $4.6 \pm 0.1$ cm.

2.4.2 Indirect organogenesis

Indirect organogenesis termed as the regeneration of the plant via an intermediate or intervening callus phase. The dedifferentiation of the explants takes place first to form an unorganized mass of the cell known as ‘callus’, then re-differentiate to shoot buds and finally developed into shoots and plantlets. The process of indirect organogenesis can be achieved by using various plant parts i.e. leaf, stem, petiole, node, internode, hypocotyle, root, cotyledon etc. (Khurana et al. 2005). The distinct concentration of the PGRs and controlled growth conditions are effective ingredients responsible for the formation of callus and their dedifferentiation followed by re-differentiation (Tripathi and Tripathi 2003). The strategies of the indirect organogenesis have been utilized for the micropropagation of the several medicinal plant i.e. *Jatrina curcas* (Rajore and Batra 2007), *D. hamiltonii* (Giridhar et al. 2004), *Hypericum perforatum* (Wojcik and Podstolski 2007), *Tylophora indica* (Sahai et al. 2010a), *Cassia angustifolia* (Parveen and Shahzad 2011), *Gymnema sylveters* (Kaushalya and Senarath 2013), *C. santapaui* (Chavan et al. 2014), *Hemidesmus indicus* (Pathak and Joshi 2017) and *Ficus religiosa* (Hesami et al. 2018).

Rehman et al. (2014) developed a micropropagation protocol for *Caralluna tuberculata*, they obtained maximum calogenesis on MS $+ 9.04 \mu M$ 2,4-D $+ 4.44 \mu M$ BA by using shoot tip explants. Further, the obtained callus were used for organogenesis and a treatment of MS $+ 13.32 \mu M$ BA $+ 4.52 \mu M$ 2,4-D $+ 2.89 \mu M$ GA3 wherein a mean number of $3.66 \pm 1.53$ shoots/explant with an average shoot length of $4.6 \pm 1.15$ cm was reported.

Parveen and Shahzad (2011) developed a micropropagation technique for the propagation and conservation of the *C. angustifolia* by using callus derived from root explants. Various concentrations of cytokinin alone or in combination with the auxin were tried and a combination of BA and NAA was found most suitable for the
production and proliferation of the shoots. MS medium supplemented with 5.0 µM BA + 0.6 µM NAA was found optimal and produced 35.63 ± 0.75 shoots/culture with an average shoot length of 5.43 ± 0.20 cm. Similarly, Chavan et al. (2014) developed a micropropagation protocol for C. santapauoi which is a threatened medicinal plant. Two types of explants was chosen for the induction of the callus i.e. cotyledonary node and cotyledons. The highest frequency of shoot formation (92.5%) was recorded when callus obtained from cotyledonary nodes explants transferred to fresh media comprised of MS + 2.5 mg l⁻¹ BA + 0.4 mg l⁻¹ IBA, wherein a maximum of 19.7 ± 0.3 shoots/culture was obtained. Thaniarasu et al. (2016) were able to establish a micropropagation technique of indirect organogenesis via using in vitro raised leaf and internodal segment of Plectranthus bourneae. Leaf explants were more responsive as compare to the internodal explants in terms of callus induction followed by shoot formation. The induction of callus was achieved at a concentration of 0.5 mg l⁻¹ BA + 1.0 mg l⁻¹ NAA, further the leaf derived callus was transferred to the medium comprised of MS + 1.0 mg l⁻¹ Kn + 0.7 mg l⁻¹ NAA + 50 mg l⁻¹ CH which gave rise to 29.71 ± 0.1 shoots/culture. Similarly, Pathak and Joshi (2017) reported indirect organogenesis in Hemidesmus indicus using leaf explants. They tried different concentration of cytokinin alone or in combination with auxin and a treatment of 20 µM BA + 1.0 µM IAA was found optimum with 80 % regeneration frequency and an average of 19.97 ± 0.81 shoots per explant were formed. However, Hesami et al. (2018) achieved both direct and indirect organogenesis Ficus religiosa via using seedling derived hypocotyls segments. They obtained maximum callus when they inoculate the explants on the medium consist with 0.5 mg l⁻¹ 2, 4-D + 0.05 mg l⁻¹ BA. For the shoot regeneration experiment, they tried different concentration of TDZ, Kn and BA in combination with IBA and medium comprised of MS + 1.5 mg l⁻¹ BA + 0.15 mg l⁻¹ IBA was found optimal with 96.66 % regeneration frequency and the production of a maximum of 6.26 shoots/explants.

2.5 Rooting in microshoot

The success of any micropropagation protocol lies with the successful rooting and acclimatization of the in vitro raised microshoots. The microshoots of different medicinal plants species have been rooted successfully on full-strength or half-strength medium alone or supplemented with auxins viz. C. forskohlii (Sahai & Shahzad 2010), Tylophora indica (Sahai et al. 2010a & b), Huernia hystric (Amoo et

Thakur et al. (2016) have successfully established rooting system for the *in vitro* raised microshoots of *Pittosporum eriocarpum*. They tried different concentrations of three auxin i.e. IAA, IBA and NAA. Among the three auxins used, IBA was found more effective and treatment of MS + 0.6 µM IBA was able to produce 12.78 ± 2.6 roots per microshoot with a maximum root length of 36.40 cm. In another report of Bhardwaj et al. (2018) on *Rhodiola imbricata*, NAA was found best for the root induction in microshoot on a treatment consisting of MS + 0.5 µM NAA, wherein a maximum of 22.0 roots with maximum root length of 0.6 cm.

Minocha (1987) best explained the efficiency of half-strength MS over full-strength MS for the root induction. Low salt concentration i.e. half-strength MS was found most effective alone or when supplemented with the auxins. Parveen and Shahzad (2010) established rooting system for *C. sophera* and they tried both MS and half-strength MS medium augmented with various concentration of two auxin i.e. IBA and NAA. A treatment of MS + 1.0 µM IBA was found optimal for the best rooting response and a mean of 5.7 ± 0.5 roots/microshoot with an average root length of 5.6 ± 0.5 cm was obtained. Sharma et al. (2014a) have established the rooting system for *in vitro* raised microshoots of *D. hamiltonii*. Different concentration of auxin was tried and a treatment consisting of half-strength MS + 2.5 µM NAA was found optimum on which a mean of 7.80 ± 0.1 roots/microshoot with root length of 6.40 ± 0.3cm were obtained. In another study, Thiyagarajan and Venkatachalam (2013) induced rooting in the excised microshoot of *G. sylvestre* when transferred to the fresh medium supplemented with various concentration of auxin i.e. IAA, IBA and NAA. A treatment consisting of half-strength MS + 2.0 mg l⁻¹ IBA resulted in the production of 12.8 roots per microshoot. Sahai and Shahzad (2013) reported *in vitro* rooting in *C. foskohlii* and a treatment of half-strength MS + 2.5 µM IBA was found to be better and produce a mean number of 4.40 ± 0.4 roots/microshoot along a mean root length of 11.14 ± 0.6 cm. Similarly, in *Ceratonia siliqua*, a treatment of half-strength MS + 10.0 µM IBA was reported best for the induction of a maximum 20.16 ± 0.40
roots/microshoot (Shahzad et al. 2017c). Whereas, Khanam et al. (2018) in *Allamanda cathartica* obtained maximum rooting response on half-strength MS liquid medium supplemented with 0.5 µM IBA with the production of 4.50 ± 0.16 mean roots/microshoot with an average root length of 4.05 ± 0.17 cm. While, Pathak and Joshi (2017) used full-strength, half-strength and one-fourth-strength MS for rooting and reported that one-fourth-strength MS + 20 µM IBA proved optimal with 100% rooting response with the production of 8.83 ± 0.28 roots per microshoot.

### 2.6 Synthetic seeds production

Synthetic seed encapsulation emerges as a new and advance technology for the conservation of germplasm and exchange of plant material (Sharma et al. 2013). Somatic embryos, shoot tip, nodal segment, bulbs etc. can be encapsulated in sodium alginate gel which considered as synthetic seed (Pond and Cameron 2003). Synseed technology has several advantages such as exchange of germplasm of endangered and critically endangered plant, for longer distance transport due to small size of beads and could be stored up to 8 weeks at low temperature (4°C) (Sharma et al. 2013).

During the initial phase of synthetic seed technology many substances were used for the purpose of encapsulation matrix i.e. sodium alginate, sodium pectate, carrageenan and agar. Out of these, Na-alginate was used mostly because of its moderate viscosity, low toxicity, and fast gelation, cost effective and biological compatibility (Redenbaugh 1993). Thereafter new study concluded that the combination of Na-alginate and calcium salt was more effective gelation matrix and it might be due to ease in handling, cost effectiveness, non-damaging ions and effortlessness in germination. Several workers have reported on synthetic seed technology with favorable slight modification ensuring better germination and conversion of capsules i.e. nutrients supply either alone or in combination with a carbon source to the germinating propagules and to prevent microbial contamination addition of antimicrobial agent and vitrofural G-1 (Nieves et al. 2003, Lata et al. 2009, Sundararaj et al. 2010).

Germaná et al. (2011) developed a protocol for the short term storage and conservation of *D. hamitonii* using synthetic seed technology by encapsulating the juvenile nodal segment and they concluded that 4% Na-alginate and 100 mM
CaCl$_2$·2H$_2$O for gelling matrix was optimum for the synthesis of ideal Ca-alginate beads. Further it was reported that the concentration of sodium alginate (Na-alginate) and calcium chloride (CaCl$_2$·2H$_2$O) affects the encapsulation (Sharma et al. 2013). The recent advances in encapsulation technology have served by many different ways viz. as an alternative tool for large scale production or micropropagation along with the properties of re-growth, rooting and thereby conversion into plantlets coupled with high possibility in germplasm exchange between the laboratories (Micheli et al. 2007, Sharma et al. 2013). Therefore, the technique provides a new way of storage and exchange of different elite and endangered germplasm and defeating various problems related with long-distance transport.

The first study of synthetic seed technology was reported by Kitto and Janick (1982) by encapsulating the somatic embryos of carrot. Since this concept revolutionized the scope of plant multiplication, several attempts have been made by different workers to apply in important medicinal plant species i.e. *Bacopa monnieri* (Ramesh et al. 2011), *Decalepis hamiltonii* (Sharma and Shahzad 2012), *R. tetraphylla* (Faisal et al. 2013), *Sterculia urens* (Devi et al. 2014), *T. undulate* (Shaheen and Shahzad 2015), *Digitalis davisiana* (Verma et al. 2016) and *Althaea officinalis* (Naz et al. 2018).

Sharma and Shahzad (2012) developed encapsulation technology for *D. hamiltonii* using nodal segment. They emphasized that the concentration of sodium alginate and calcium chloride was a major parameter for the successful polymerization of spherical beads, and, hence they found that a combination of 4% Na-alginate and 100 mM CaCl$_2$·2H$_2$O was optimal. They recorded a maximum of 77.0 ± 2.0% shoot re-growth in encapsulated nodes when beads were inoculated on to MS + 5.0 µM BA + 0.5 µM IAA + 30.0 Ads and the recovered microshoots were rooted on MS + 2.5 µM NAA. However, Parveen and Shahzad (2014a) encapsulated the nodal segments of *C. angustifolia* by using 3% Na-alginate and 100 mM CaCl$_2$·2H$_2$O for making of identical and uniform beads. About 94% regrowth response of beads was achieved when they were inoculated on MS medium augmented with 2.5 µM BA + 0.4 µM NAA. Both the encapsulated and non encapsulated nodal segments were also stored at 4°C for different time period. For complete plantlets production the
regenerated microshoots were best rooted on half-strength MS + 1.0 µM IBA + 5.0 µM PG.

In another study of Sharma et al. (2009), 4% Na-alginate + 100 mM CaCl₂.2H₂O was found to be optimum for the encapsulation of nodal segment of *Spilanthes mauritiana* and making uniform beads. The *in vitro* conversion of synthetic seed in to complete plantlets achieved on MS medium supplemented with 5.0 µM BA + 0.5 µM IAA, on which a maximum of 83% conversion of beads into complete plantlets was achieved. Similarly, Sharma et al. (2014b) encapsulated the nodal segments of *Salvia splendens* in 4% Na-alginate + 100 mM CaCl₂.2H₂O. A combination of BA and auxin was used for the conversion of synseed into plantlets and a treatment of MS + 5.0 µM BA + 2.5 µM IAA proved to be optimum for the maximum conversion (63.6%). However, 3% sodium alginate was used for the encapsulation of nodal segments of *T. undulata* prepared in MS + 2.5 µM BA + 0.5 µM IAA and 100 mM CaCl₂.2H₂O (Shaheen and Shahzad 2015). They developed an effective protocol in the woody species, otherwise a hard to regenerate plant, for multiplication for better re-growth response. They tried second treatment but a medium comprised of MS + 10.0 µM BA was found optimal media and produced 6.50 ± 0.28 shoots/bead.

While, in another of woody plant species *Vitex trifolia*, Alatar et al. (2016) encapsulated the *in vitro* raised nodal segment in 3% Na-alginate + 100 mmol/ CaCl₂.2H₂O and observed that MS + 2.5 µM Kn + 1.0 µM NAA exhibited optimal response with 90% re-growth efficiency.

Saeed et al. (2018) developed encapsulation technology for the conservation of immensely important antidiabetic liana, *Gymnema sylvestre*. The pretreated nodal segment with 2.5 µM IBA encapsulated in Na-alginate matrix with PGRs and conversion into complete plantlets was achieved on MS + 2.5 µM BA + 2.5 µM GA₃ + 50 µM Ads with maximum recovery of 88.2 ± 0.48%.

### 2.7 Acclimatization of plantlets

An advantageous micropropagation technique lies on the efficiency of *in vitro* raised plantlets to face various stresses during the acclimatization. When plants were transferred from *in vitro* to the *ex vitro* condition they face trauma because of severe
environmental and nutritional stress and hence to cope up with these stresses the plant needs proper hardening (Lavanya et al. 2009). There are various factors which restrict the plant survival in natural environment i.e. differential humidity, light, temperature and wind flow etc. and overall effects of these factors leads to pessimistic characteristics to physiology, morphology and anatomy of the plant (Pospíšilová et al. 1992, Chandra et al. 2010). Another study supports the priming of the in vitro raised plantlets with certain chemicals to effectively pre-sensitize cellular metabolism of plants and accelerate the acclimatization efficiency (Hazarika 2003, Nowak and Pruski 2004). Ray and Bhattacharya (2008) for the first time demonstrated the concept of priming of micropropagated plantlets of Eclipta alba with chlorocholine chloride (CCC) a plant growth retardant. The alteration in GA biosynthesis, increased chloroplast and chlorophyll content and enhance secondary metabolism were reported when CCC was applied to the microshoot. These alteration results in increased acclimatization efficiency of the plant in natural environmental. Whereas, Dey et al. (2013) developed micropropagation protocol of Stevia rebaudiana, CCC priming resulted in 92.3% plant survival. Pospíšilová et al. (1998) reported that when tobacco plant transferred to the natural environment, plant faces a transplantation shock and ABA alleviate solely responsible to overcome the shock. The role of ABA to overcome the environmental stresses in enhanced acclimatization efficiency was also established by several workers viz. Aguilar et al. (2000), Hetherington (2001) and Finkelstein and Gibson (2002).

Another effective parameter required for the successful acclimatization is planting substrate which can influence the acclimatization process. The various potting substrate have effected acclimatization of medicinal plant such as soil and sand mixture (2:1) in W. coagulans (Valizadeh and Valizadeh 2009), sand and soil mixture (1:2) in G. sylvestre (Thiyagarajan and Venkatachalam 2013), cocopeat in G. kurroo (Sharma et al. 2014), soil : vermicompost : vermiculite (1:1:1) in P. corylifolia (Jani et al. 2015), sterile soil, sand and leaf litter (2:1:1) in Nardostachys jatamansi (Bose et al. 2016) and soilrite and vermicompost (3:1) in G. sylvestre (Saeed et al. 2018).

2.8 Physiological studies during acclimatization

Micropropagated plantlets when transferred to the ex vitro condition, face high light irradiance and situation demands increased in pigment content to overcome the
effects. The reduction in chlorophyll content and inadequate photosynthetic capacity during the initial days of acclimatization for which Pospíšilová et al. (1999) has found the reason i.e. disordered granna might be responsible for initial fall. The above reason is basic factor responsible for the acclimatization failure of in vitro derived plantlets. Lavanya et al. (2009) concluded that the acclimatization of the in vitro raised plantlets under greenhouse environment is a prerequisite steps before the natural environment. Exposure of high humidity in the environment can prevent water loss to the newly transplanted plantlets (Preece and Sutter 1991). During the early stage of acclimatization when plant faces severe exposure of uncontrolled environmental factors, a biological mechanism cascade started in plant which leads to the production of different protecting compound which is responsible to cure the plant. Preece and Sutter (1991) found that in vitro raised plantlets replaced its leaves with the new leaves.

Several reports are available which support cytokinin and its relation to the synthesis of photosynthetic pigments. Aremu et al. (2012) found that cytokinin effects the chloroplast formation and biosynthesis of photosynthetic pigments. In another study of Bhattacharya et al. (2016), a direct correlation of cytokinin and photosynthetic pigments i.e. chl a, chl b and total carotenoids. They found a significant increase in the photosynthetic pigments of micropropagated Dendrobium nobile. Amoo et al. (2014) demonstrated the correlation of cytokinin and photosynthetic pigment synthesis in Aloe arborescens and Harpagophytum procumbens during acclimatization studies (Amoo et al. 2014).

Sharma et al. (2014a) studied the effects of environmental factors on in vitro raised plantlets of D. hamiltonii during the acclimatization. At the beginning of the acclimatization due to stresses the plant start to shade its leaf and reduction in photosynthetic pigment content and P_N was observed. However, as the days of acclimatization advanced the plant started to grow its new leaf and gradual increase in pigment content as well as P_N was observed. Ahmad et al. (2018a&b) evaluated the photosynthetic parameter and related attributes viz. Net photosynthetic rate (P_N), chlorophyll content and total carotenoid content in D. aryapathra and D. salicifolia respectively. They found initial fall in P_N and photosynthetic pigment and thereafter a linear increase was reported as the days of acclimatization advanced.
2.9 Biochemical studies during acclimatization

The light being as preliminary energy source for the growth of the plant however, excess of light intensities can be harmful for the plant and leads to the photoinhibition of photosynthetic light reaction and the incomplete reduction of oxygen responsible for the successive production of ROS (reactive oxygen species) i.e., hydrogen peroxide superoxide radical and singlet oxygen (Batková et al. 2008). However, plant cells develop strong defense mechanisms including SOD, CAT, APX, and GR (Kayihan et al. 2012, Xu et al. 2012). The SOD converts superoxide to H$_2$O$_2$ and O$_2$. This is the primary defense against the ROS. CAT scavenges H$_2$O$_2$ by converting it into O$_2$ and H$_2$O in peroxisomes. The process of detoxification is performed by different membrane associated and stromal enzymes, such as SOD and APX at the accepter side of photosystem I (Scalet et al. 1995). APX and GR are principle enzyme of ascorbate glutathione cycle. They play an important role by scavenging H$_2$O$_2$ in chloroplast, cytosol, vacuoles, and apoplast (Asada 1999).

Ahmad et al. (2018a) studied the role of antioxidant enzyme viz. SOD, CAT, APX, and GR during the acclimatization of in vitro raised plant of D. arayalpathra. They reported that SOD activity increased after the first week of acclimatization through a decrease was observed up to 28 days of acclimatization. Likewise they found a gradual increase in CAT, APX and GR activity as the days of acclimatization increases.

Ahmad et al. (2018b) reported the defensive role of this antioxidant enzyme in D. salicifolia when the in vitro grown plantlets were acclimatized to the natural environment. The rise and fall in the activity of the enzyme viz. SOD, CAT, APX, and GR witnessed the defense improvement and better acclimatization against the environmental stress. Halliwell and Gutteridge (2007) reported that this antioxidant inhibits or delays the oxidation of biological substrates. Van Huylenbroeck et al. (2000) have found in their study that an antioxidant mechanism triggered in in vitro raised plant. Many reports are available which brings common conclusion about the defensive role of antioxidant enzymes to prepare the plant for better adaption when transferred from in vitro to ex vitro conditions. The consequential changes in antioxidant enzyme activity were reported in Zingiber officinale (Guan et al. 2008), Scrophularia takesimensis (Jeong and Sivanesan 2015) and Albizia lebbeck (Saeed and Shahzad 2015).
2.10 Histological studies of differentiating tissue

Structural analysis during plant morphogenesis is a potent step to be conducted for better understanding of plant morphogenesis (Wetmore and Wardlaw 1951). Since histological studies contributed continuously in the study of \textit{in vitro} plant culture (Yeung 1999). There are several reports available considering the importance of histological studies viz. \textit{C. forskohlii} (Sahai and Shahzad 2013), \textit{C. aungustifolia} (Parveen and Shahzad 2014a) and \textit{Cichorium intybus} (Dakshayini et al. 2016).

Sahai and Shahzad (2013) investigated direct development of shoot bud from the cut end of nodal segment of \textit{C. forskohlii} through histological study and revealed the direct origin and development of shoot buds from basal swollen cut end of nodal explants. Sharma et al. (2014a) performed the histological study of nodular tissue obtained through shoot tip cutting of \textit{D. hamiltonii}. The experiment was conducted to explore the understanding of regeneration and origin of \textit{de novo} shoot formation. During their study they found meristemoids region in calli and further its differentiation into shoot buds. Similarly, Luis and Scherwinski-Pereira (2014) with the help of histological analysis confirmed that the formation of somatic embryos from nodular embryogenic callus of \textit{Acrocomia aculeate}. Parveen and Shahzad (2014a) carried out histological study of \textit{C. aungustifolia} during various stages of embryogenesis and concluded that the embryos differentiated through callogenesis.

2.11 Marker assisted genetic fidelity assay

Larkin and Scowcroft (1981) coined the term somaclonal variation to demonstrate the tissue culture raised plants exhibiting morphological, genetical and epigenetical variation from the mother plants. The occurrence of somaclonal variation during the propagation of an elite species is quite adverse (Rahman and Rejara 2001). Singh et al. (2002) considered somaclonal variation as threat to the genetic integrity of the tissue culture raised plant. To overcome the problem of heterogenous plantlets population, the genetic fidelity test of the tissue culture raised plants is become integral part of micropropagation studies. Use of more than on marker has always been suggested for a better analysis of genetic homogeneity of plants. RAPD and ISSR marker are unsophisticated and profitable in screening of genetic evenness due to its lower cost, highly discriminative, usage of small amount of DNA and non-involvement of radioactive labels compounds for labeling (Kumar et al. 2011). AFLP
and RFLP on the other hands are being sidelined due to its demands of expensive enzymes, radioactive labeling etc.


Singh et al. (2012) study the clonal fidelity of the micropropagated plant of *Eclipta alba* by using RAPD markers. Out of 30 RAPD primers 10 primers were able to give their amplification product, which were monomorphic and validating the genetic similarity of micropropagated plant to that of mother plant. The number of amplified product ranges from 3 to 8 and the size from 250 to 2000 base pair. Ahmed and Anis (2014a) evaluated the genetic fidelity test for the micropropagated plants of *C. alata* by using 20 RAPD primers and 13 ISSR markers. Out of 20 RAPD primers, 18 primers produces reproducible bands and OPL-18 produces a maximum of 6 bands with a ranging 200-800 base pair. A total of 71 bands were obtained by using 13 ISSR markers and UBC 812 produces a maximum of 10 bands. All the bands were clear and monomorphic. Faisal et al. (2014) evaluated the genetic stability test for micropropagated plants of *Mentha arvensis* by using a total of 9 ISSR primers. All primers were able to amplify the DNA segments and produces clear and scorable bands with an average of 14.4 bands per primer. All the produced bands were monomorphic and authenticated the uniformity of the *in vitro* raised plants. Similarly, Kumar et al. (2015) screened the *in vitro* raised plants of *D. hamiltonii* with 12 RAPD primers. All the 12 primers were able to amplify the DNA segment but OPD-20 was found more responsive as it give a maximum of 12 numbers of bands and range of amplification was recorded to be 250-2300 base pair. The banding profiles of all the micropropagated *D. hamiltonii* were monomorphic and similar to that of mother plant. In *P. eriocarpum*, out of 15 RAPD primers only 10 primers showed clear reproducible bands and a total of 44 scorable bands were obtained with an average of 4.4 bands per primer ranging from 250 to 1000 base pare, among the primers, OPL-02 was most prominent which generated 7 bands, while out of 15 ISSR primers screened, 10 primers yielded a total of 34 reproducible bands with an average of 3.4 bands per
primer ranging from 250-1500 base pairs (Thakur et al. 2016). They reported that ISSR-860 gave four monomorphic bands and two polymorphic bands. However, Muthukumar et al. (2016) found ISSR marker analysis much better for the genetic variability in terms of highly reproducible and more inflexible. They used a total of 10 ISSR primers, 4 ISSR primers showed promising banding pattern with a total of 25 reproducible bands. All the bands were monomorphic and no polymorphic bands were obtained. Out of 10 ISSR markers, UBC 810 produces a maximum of 7 bands.

Swain et al. (2016) performed genetic fidelity test for the in vitro raised plants of Hypericum gaitii to confirm the genetic stability. They use 10 ISSR markers and out of those USB-810 was found better to give a maximum of 7 bands. Ahmad et al. (2018b) check the homogeneity of the in vitro raised plant of D. salicifolia with that of mother plant by using RAPD and ISSR marker. No polymorphism was detected during the analysis and uniform banding pattern with no alien bands was observed. They used 10 RAPD primers which gave a total of 42 scorable bands and OPL-3 primer was found to be most effective and produced a total of 8 bands. Similarly, 10 ISSR primers were used and a total of 50 bands were obtained in which UBC-825 contributed 9 prominent bands.

2.12 Scanning Electron Microscopic studies

The use of SEM anatomizing is a significant parameter for the in vitro raised or acclimatized plantlets because the study depicts the stomatal behavior and morphological characteristics of leaves. Hazarika et al. (2002) reported morphological variation in guard cell of citrus leaf when SEM studies were carried out. Sáez et al. (2012) reported differences in shape of stomata by using SEM techniques in the leaves of micropropagated plantlets and nursery grown plantlets of Castanea sativa and reported dense and open stomata in the leaves of in vitro microshoot than those of nursery plantlets. Several workers have also reported the SEM analysis studies to understand the morphological adaptation during hardening and acclimatization to natural environment in different plant species viz. C. crotalifera (Rozali et al. 2014), W. somnifera (Munien et al. 2015), Dendrobium broga (Uddain et al. 2015), Melastoma malabatricum (Ghimire et al. 2016), Anacardium occidentale (Ramos et al. 2016) and Ceratonica siliqua (Shahzad et al. 2017c).
2.13 Phytochemistry

The demands of human being increasing continuously for phytomedicine due to its accuracy and consistency, which creates a thrust towards the plants and their role in phytomedicine bioactivity. A concerted effort would be of great significance to which the availability of phytomedicine in the science of mankind. Gradual destruction in the population of plant species have several reasons including environmental effects on habitat loss, destructive collection, urbanization and industrialization. The overall effects lead to the plant population in danger and most of them are poorly explored and less studied. Hence, such kind of plant species needs greater attention for their industrial utilization and effective conservation and screening of the newer secondary metabolite. The modern techniques for the screening of the metabolites i.e. GC-MS and HPLC analysis had provide us a wide perception to make out the biochemical composition of plant and plant parts.

2.13.1 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The tuberous root of the plant of the genus *Decalepis* is the most important part and contains 2-hydroxy-4-methoxybenzaldehyde (2H4MB) (97%) is a valuable source for the production of commercially important flavour compound vanillin (Verma et al. 2014). The presence of aroma (2H4MB) in the tuberous root of these species placed them as a potential substitute for *H. indicus* in international market. Several studies have been conducted wherein major and minor metabolites were reported in *Decalepis* species. The tuberous root of the *D. hamiltonii* is rich in inositol, saponin, tannins, phenolics, alkaloids, falvonoids etc. (Murti and Seshadri 1941). George et al. (1998) investigated and confirms the presence of 2H4MB in tuberous root of *D. hamiltonii* as a major metabolites with other minors metabolites of pharmacological value i.e. 2H4MB (37.45%), 2-hydroxybenzaldehyde (31.01%), 4-O-methylresorcyraldehyde (9.12%) and benzyl alcohol (3.16%) as major components. The GC-MS analysis of tuberous root extract of *D. hamiltonii* by Nagarajan et al. (2001) witnessed the presence of benzaldehyde (0.017%), salicylaldehyde (0.018%), methyl salicylate (0.044%), 2-phenylethyl alcohol (0.081%), ethyl salicylate (0.038%), and vanillin (0.45%) in minor quantities which are biologically significant. Nagarajan et al. (2001) reported 2H4MB from the volatile oils (96%), which is found to be an important constituents. Similarly, Nagarajan and Rao (2003)
developed a chromatographic technique in *D. hamiltonii*, they used gas as mobile phase and found varying combination of the aromatic compounds. Other compound i.e. 4-hydroxyisophthalic acid, 14-aminotetradecanoic acid, 4-(1-hydroxy-1 methylethyl)-1-methyl-1,2-cylohexane diol, 2-(hydroxymethyl)-3 ethoxybenzaldehyde, bornerol and ellagic acid have also been reported in *D. hamiltonii* root (Srivastava et al. 2006, Srikanta et al. 2007). Moreover, α-atlantone (2.06% v/w of oil) and β-pinene (2.01%) have been isolated in *D. hamiltonii* by Thangadurai et al. (2002), which are known for their insecticidal and anti-microbial properties.

Ahmad et al. (2017) performed the screening of metabolites for methanolic extract of tuberous root of *D. arayalpathra* and its successive fractions which revealed the presence of the alkaloid, phenolics, tannins, carbohydrate, flavonoids, terpenoids, etc. Further, GC-MS analysis enabled the identification of furaneol, 2H4MB, 4H3MB (4-hydroxy-3-methoxybenzaldehyde), neral acetate, diazoacetone, benzoic acid, neryl acetate ester, hexacontane, mome inositol, inositol, tetrapentacontane, diethyl phthalate, 4-ethoxy-ethyl, hexacontane, methyl commate D, diazoacetone, squalene as a major metabolites with chromelin, anhydride, alcohol, myristate, benzoic acid, lup-20(29)-en-3-yl acetate, 2,6-octadiene-1-ol, phthalic acid, steraloids as a minor metabolites (Ahmad et al. 2017). The high performance liquid chromatography study of methanolic extract of *D. arayalpathra* have also been performed and confirms the presence of 2H4MB as a major constituents (Ahmad et al. 2017& 2018a). In *D. salicifolia* also George et al. (2011) in their initial work witnessed the presence of 2H4MB in the root as a major metabolites.

2.13.2 Analysis of 2-hydroxy-4-methoxybenzaldehyde (2H4MB)

Nagarajan and Rao (2003) estimated the 2H4MB content in the roots of *D. hamiltonii* and *H. indicus*. They developed a gas chromatographic technique and found 2H4MB as a major compound (>90%) in their volatile oils. They used both fresh and dry root of *D. hamiltonii* and *H. indicus* and observed that fresh root of *D. hamiltonii* contained maximum 2H4MB i.e. of 5.4 mg/ml, while in the dry root yielded 5.2 mg/ml of 2H4MB. Moreover, Giridhar et al. (2004) evaluated the flavor compound 2H4MB content in tissue culture raised and acclimatized plant of *D. hamiltonii* and estimation was carried out by thin layer chromatography (TLC).
Hexane and Benzene was used as extracting solvent in 1:1 ratio. The overlapping retention time of both the extracts and standard compounds was the affirmation of the presence of the compound in the sample. They reported that 2H4MB content varies with the age and size of the root.

Ahmad et al. (2017) performed the chemical profiling of *D. arayalpathara* due to its immensely important medicinal value. Both qualitative and quantitative evaluation was carried out for the root tuber of *D. arayalpathra* taken from 3 year old plant. They found methanol as suitable solvent for making of extract and the chromatogram obtained from both the extracts and standard compounds show similarities in their retention time. They reported 11.22 µg/ml of 2H4MB content in the extract. Moreover, Sharma et al. (2014a) evaluated the 2H4MB content in roots of *in vitro* raised plant of *D. hamiltonii*. They have found a maximum of 15.94 µg/ml of 2H4MB after 8 week of acclimatization. While, Ahamd et al. (2018a) analyzed the formation of 2H4MB in the root system of micropropagated plants of *D. arayapathra* with the help of HPLC analysis and found that the formation of 2H4MB gradually increased as the days of acclimatization increased and synthesis was started after two week of acclimatization. Both the qualitative and quantitative analysis of 2H4MB was carried out and presence of the compound confirmed by the chromatogram of standard compound. Maximum content of 2H4MB (9.22 µg cm$^{-3}$) in the root extract was recorded after 8 week of acclimatization. Similarly, Ahmad et al. (2018b) evaluated the 2H4MB content in the roots of *D. salicifolia* and found a maximum of 6.8 µg ml$^{-1}$ 2H4MB content was noticed after 10 week of acclimatization.

### 2.14 Enhancement of 2H4MB content in root tuber through elicitation

Secondary metabolites are large number of specialized compound that do not directly involved in the growth and development of the plants but are necessary for the plant survival. The plant is the reservoir of the secondary metabolites which are occurs in 200,000 structural forms (Hartmann and Thomas 2007). In majority the plant secondary metabolites synthesized by the means of Shikimic acid pathway and Malonic acid pathway for the production of the phenolic compound and nitrogen containing secondary products. Mevalonic acid pathway and Methylerthritol pathway for the production of the terpenes. The tuberous root of the plant contains
2H4MB as a major phenol compound that accumulated in the root of the genus *Decalepis* and chiefly responsible for their aroma (Mishra et al. 2017 George et al. 2011). Despite of its importance, the mechanism involve for the synthesis of the compound is not very much explored. However, preliminary work of Chakraborty et al. (2008) and Kundu et al (2012) guide us towards the synthesis of 2H4MB following the pathway of Phenylpropanoid pathway or Shikimate pathway. In their study, they found the enhance level of PAL as key enzyme for the biosynthesis of 2H4MB. Several attempts have been made to enhance the accumulation of 2H4MB in the excised root through the elicitation in *H. indicus* (Chakraborty et al. 2008, Kundu et al. 2012), *D. hamiltonii* (Kamireddy et al. 2017), *D. arayalpathra* (Sudha et al. 2013) and *Cocos nucifera* (Chakraborty et al. 2009).

Elicitation has acted as one of the most significant and widely used plan for the enhancement of plant secondary metabolites (Yue et al. 2016). They are biological factors, which helps the plant in defending by manipulating the process of plant secondary metabolism. Plant synthesizes several chemical compounds for their self defense. This chemical compound can be induced by a range of chemical stimulus known as elicitor which can bind with the receptors on the plasma membrane to trigger the signal cascade to influence the physiological and morphological changes to the plant. The activation of NAPDH cascade, reactive oxygen species (ROS) production, and expression of defense gene, induction of protein and enzyme and accumulation of secondary metabolite are the outcomes of elicitors induced stress in the plant (Ferrari 2010, Zhang et al. 2012). They are capable to interfere in different plant metabolic reaction in targeted organism (Ramirez-Estrada et al. 2016). Two types of elicitors have been used for our study i.e. Chitosin and Yeast extract.

Chitosan (CH), a deacetylated chitin are one of the biotic elicitors that are derived from fungal cell wall and mimic the effects of several pathogenic fungi in stimulating defense related to secondary metabolite production in the plant (Montesano et al. 2003). CH has been widely used for the study of metabolic enzyme such as phenylpropanoid (Chakraborty et al. 2009, Kamireddy et al. 2017), phenylalanine ammonia-lyase (PAL) (Chakraborty et al. 2008), and in the production of secondary metabolites (Cai et al. 2012, Boroduske et al. 2016, Singh 2016, Jaisi et al. 2017). CH is a kind of bioactive polymer having extensive application due to its functional
properties such as antibacterial activity, non-toxicity, ease of modification and biodegradability (Muxika et al. 2017).

YE vitamin B-complex rich elicitors contain chitin, N-acetyl-glucosamine oligomers, β-glucan, ergosterol and glycopeptides (Boller 1995). The compound were found to induce plant defense responses by triggering secondary metabolism (Cai et al. 2012) and they are frequently used as biotic elicitors to improve the secondary metabolite production (Zhao et al. 2005, Kundu et al. 2012, Maqsood and Abdul 2016, Kundu and Mitra 2016, Boroduske et al. 2016, Malayaman et al. 2017).

In vitro enhancement of the compound by the manipulation of the biosynthetic phase of the 2H4MB is now becoming a new era to meet the international market demands of the compound. Giridhar et al. (2005b) reported the improved 2H4MB contents in the root system of D. hamiltonii when treated with TRIA. They tried TRIA soil drenching and showed a better yield of tuber with enhanced flavour compound, they found a significant increase (1.5 times more) as compare to the control when analysed with GC-MS. Agrobacterium mediated transformation of D. arayalpathra has been achieved by using different strain of A. rhizogenes i.e. A4, MTCC 532, TR105 and LBA 5402 (Sudha et al. 2013). The juvenile hypocotyls explants were used for the infection and induction of hairy roots at higher frequency of (53.2 ± 0.3 %) than cotyledons (32.1 ± 0.2 %) when infected with TR105 the most virulent strain. They recorded that the formation of hairy roots was took place by two ways, either directly from the wounds or followed by the formation of gall like structures when co cultivated in half-strength MS basal medium. Irregular gall formation was observed during the culture which was showing the active site for the induction of hairy root. They reported a maximum accumulation of 2H4MB (0.22 % dry weight) recorded at 6th week (Sudha et al. 2013).

Giridhar et al. (2005c) evaluated 2H4MB content in root cultures of D. hamiltonii by using dichloromethane as solvent with ethanol in defined ratio for qualitative and quantitative GC-MS analysis. The maximum content (40 μg·g⁻¹ dry weights) of 2H4MB in root was recorded after 45 days when MS was medium was augmented with α-naphthaleneacetic acid (NAA) (1.0 mgL⁻¹). Normal root culture for D. arayalpathra was also established by Sudha et al. (2001) by using leaf and intermodal explants of in vitro raised shoot culture. They used MS medium
augmented with BA (2.5 mg/L\(^{-1}\)) + 2-iP (0.5 mg/L\(^{-1}\)) + NAA (0.5 mgL\(^{-1}\)) and subcultured at 5 weeks interval. The maximum 2H4MB (0.16%) content was recorded when root explants cultured on the MS liquid medium supplemented with IBA (0.5 mgL\(^{-1}\)) + NAA (0.2 mgL\(^{-1}\)) and also favored maximum branching in roots (5.82 g). Chakraborty et al. (2008) developed a protocol for the enhancement of phenylpropanoid derivatives through chitosan in the cell suspension culture of Cocos nucifera. In their study, they found enhanced accumulation of p-hydroxybenzoic acid as a major phenolic compound. Kundu et al. (2012) found that the Shikimic acid pathway for the biosynthesis of 2H4MB was modulated by the use of elicitors in H. indicus roots. They used different elicitors for their study i.e. chitosan, methyl jasmonate, yeast extract. However, the treatment of yeast extract was more effective for the enhanced accumulation of the 2H4MB with a contact period of 18 h in excised root. During their study they found enhanced level of shikimic dehydrogenase and phenylalanine ammonia-lyase enzyme. Jaisi et al. (2017) enhanced the plumbagin content in the root culture of Plumbago indica root by combined action of chitosan elicitation and in situ adsorption using nonpolar copolymer adsorbent. A treatment of 150 mg L\(^{-1}\) of chitosan to 14 days old culture for 72 h was proved to be most appropriate for the maximum plumbagin content (13.08 mg g\(^{-1}\)).

Kundu and Mitra (2016) describe the YE modulation of three important cell-wall bound phenolic compound i.e. 4-hydroxybenzoic acid, 4-coumaric acid and ferulic acid in H. indicus. They concluded that these phenolic compounds are being modulated via Shikimimate pathway. Maqsood and Abdul (2016) used YE elicitation for the enhancement of two immensely important anticancerous compound, vinblastin and vincristin in Catharanthus roseus. Different concentration of YE has been tried for the elicitation and a concentration of 1.5 mg/l was found optimal with 22.74% vinblastin and 48.49% vincristin content as compare to the control. Kamireddy et al. (2017) hypothesized the biosynthetic pathway of flavour molecule 2H4MB and concluded that the origin of 2H4MB is might be from phenylpropanoid pathway (PPP). The study was carried out using PPP inhibitors i.e. piperonylic acid, MDCA and Propanil in in vitro culture of D. hamiltonii. HPLC analysis revealed that the piperonylic acid unable to inhibit the synthesis of 2H4MB formation, while MCDA and propanil showed the inhibitory effect.