Chapter 5
Discussion
DISCUSSION

Destructive harvesting and overexploitation of medicinal plants from their natural habitat has led to devour of the natural wealth at an alarming rate. The regular harvesting of the medicinal plants for their particular use put a thrust on their natural population and has led them to the list of IUCN Red Data List. Thus, it is our responsibilities to conserve this natural wealth and to bring ecological harmony. PTC is rightly acknowledged as one of the leading areas of biotechnology due to its extraordinary potential in multiplying and conserving plant germplasm. The technique is widely used for the propagation of difficult to propagate species and also proved to be economically beneficial for those species that are easy to propagate (Ilczuk and Jacygrad 2016). The present study has been taken to achieve different pathways of regeneration via in vivo and in vitro derived explants for micropropagation of two important endangered plant species namely D. arayalpathra and D. salicifolia respectively. Moreover, viz. physiological and biochemical study were performed during the acclimatization period along with DNA fingerprinting of regenerant, histological analysis of differentiating tissue and SEM analysis. Chemical profiling of methanolic extract of root tuber of in vitro raised and mother plant was also carried out through GC-MS analysis. Elicitation of the 2H4MB through callus and cell suspension culture accompanied with the estimation and quantification of 2H4MB was also conducted and discussed in light of existing literature on the subject.

5.1 Organogenesis

In vitro regeneration system in any plant species can be achieved through shoot organogenesis and it can be achieved either through the direct or indirect mode of shoot buds organization (Shahzad et al. 2017a). Plant cells may retain the ability to differentiate from their current structural and functional state and to initiate new developmental path towards a number of other morphogenic endpoints. At first sight, it is not clear whether there are stem cells in plants. During vegetative propagation, a new plant can arise from a definite cell that terminated its growth or several cells, from one or several tissues. Hence, all the plant cells are totipotent and an entire plant can be formed from a single cell and the process is under control of several signal cascade, synthesis, and transport of phytohormones and other compounds. The top most cells of the root and shoot known as apical meristem and considered as stem
cells of the plant which is found to be similar in many features to the stem cells of animals. According to Hicks (1980), the process of organogenesis can be achieved by two pathways, either it is through the formation of intervening callus stage termed as ‘indirect’ organogenesis, while direct organogenesis is accomplished without an intervening proliferating callus. Generally, direct shoot regeneration is preferred since it lowers the chance of occurrence of somaclonal variation (Liu and Bao 2003). In the present study, the shoot regeneration in D. arayalpathra and D. salicifolia was obtained through direct shoot regeneration only.

5.1 Direct regeneration

Direct regeneration offers a steady and potent procedure to achieve a high frequency in vitro multiplication of plants within a short span of time. It can be obtained via performed meristem (axillary/apical bud proliferation) containing explants or from plant tissues without performed meristem referred to as adventitious regeneration (Sahai and Shahzad 2010, Kone et al. 2013). During our study regeneration was achieved via axillary shoot buds differentiation.

5.1.1 Effect of explants types

The type of explants or plant part as a basic generating material in PTC largely dominates as a pioneering material for a successful regeneration protocol (Vasil 1987). According to Jiang et al. (2012) the explants choice is a fundamental necessity for any plant regeneration protocol. There are several reports available which dealing the effect of explants type and their possible effect on regeneration viz. Macadamia spp. (Gitonga et al. 2010), Citrus jambhiri (Vijaya et al. 2010), Stevia rebaudiana (Sharma and Shahzad 2011) and Tectona grandis (Kozgar and Shahzad 2012). Micropropagation through NS and ST containing pre-existing meristems is a foremost way to get a successful protocol for clonal propagation (Khawar et al. 2005, Dafalla et al. 2016, Ahmad et al. 2018a&b). Other plant parts viz. leaf, petiole, internodal segments, and roots are devoid of organized meristem were also used for mass propagation through adventitious shoot regeneration in various plant species (Sahai and Shahzad 2010, Kumar et al. 2011, Ouyang et al. 2016). In our study, four different types of explants viz. in vivo NS, axenic NS, in vivo ST and axenic ST
explants were mainly involved in direct organogenesis studies in *D. arayalpathra* and *D. salicifolia*.

*In vivo* derived NS of *D. arayalpathra* when inoculated on optimized regeneration media fortified with optimum concentration cytokinin, auxin and growth additives combination resulted in the maximum (11.8 ± 0.1) differentiation of direct shoots with tremendous growth and development in comparison to those obtained through axenic NS (7.10 ± 0.20), *in vivo* derived ST (5.5 ± 0.20) and axenic ST (5.06 ± 0.1) explants on the same PGR composition. Similarly, in the case of *D. salicifolia*, *in vivo* derived NS when inoculated on optimized regeneration medium resulted in the production of as much as 9.97 ± 0.1 axillary shoots in comparison to those obtained through axenic NS (2.56 ± 0.20), *in vivo* derived ST (5.0 ± 0.11) and axenic ST (2.20 ± 0.21) explants on the same PGR combination. These findings are in agreement with many other studies conducted through NS culture in comparison to the ST explants viz. *Aristolochia tagala* (Rajanna and Sharma 2015), *Jatropha curcas* (Rathore et al. 2015), *Pyrus spp.* (Yi et al. 2015), *Salacia chinesis* (Majid et al. 2016), *Pogostemon erectus* (Dogan et al. 2016), *Tylophora indica* (Najar et al. 2018).

### 5.1.1.2 Effect of cytokinins on shoot regeneration

In this study, various PGRs have been applied to evaluate the regeneration efficiency of different explants for the advancement of reliable *in vitro* protocols for the high frequency regeneration of both *D. arayalpathra* and *D. salicifolia*. Among the three cytokinins used in the present study, BA was found to be more effective in terms of a maximum number of shoot formations in comparison to Kn and 2-iP in both the plants species as well as explants types. The superiority of BA over other cytokinins can be understood in the light of their physiological role in the plants. According to the study of George (1993), the addition of the BA to the growth media responsible in the reduction of apical dominance and triggered lateral bud differentiation leading to a high frequency multiplication of shoots. The stimulating effect of BA has also been reported in other medicinal plant species such as *Pistasia lentiscuss* (Kılınc et al. 2015), *Crataegus arotina* (Nas et al. 2012) and *Gymnema sylvatre* (Thiyagarajan and Venkatachalam 2013) etc. BA being as the first generation synthetic cytokinin elicit an efficient proliferation, bud break, multiple shoot formation, it is might be due to its more permeability across the plasma membrane and high cell uptake (Malik et al.)
According to the Rai et al. (2009), BA can trigger the metabolism and synthesis of natural cytokines like zeatin in plant tissue culture.

Among the different concentration of BA used, 5.0 µM BA was found to optimum in all the parameter studied for both D. arayalpathra and D. salicifolia. However, the number of shoots/explant and shoot length varied with the type of explants used. This finding is according to the earlier report on Decalepis hamiltonii (Sharma et al. 2014a). Moreover, the adequacy of BA among the cytokinins on multiple shoot bud differentiation has been investigated by several authors which are in agreement with the present study (Sen et al. 2010, Kilinc et al. 2015, Thakur et al. 2016 and Jun-jie et al. 2017). In our study, higher concentration of BA viz. 7.5 µM was unable to improve the shoot proliferation and related parameters. All the growth parameters studied on a higher concentration of BA exhibited reduced response in overall growth parameters and it might be due to exposure of explants to a higher concentration of BA during induction phase led to the hyper-accumulation of cytokinins (Malik et al. 2005). The obtained results were found to be accordance with the findings in D. arayalpathra (Gangaprasad et al. 2005), A. ehrenbergiana (Javed et al. 2013), D. hamiltonii (Sharma et al. 2014a) and Pistacia lentiscus (Jun-jie et al. 2017). The application of single cytokinin resulted in multiple shoot buds induction from pre-meristem containing explants. However, as the incubation time advanced certain growth abnormalities were observed in both the plant species such as callus formation at the base of the plant, stunted microshoot formation, premature leaf drop, low expansion of lamina, yellowing of leaf and shoot tip necrosis.

### 5.1.1.3 Synergistic effect of cytokinin and auxin on shoot regeneration

For the development of an efficient in vitro protocol coupled with various processes viz, signal transduction, the perception of stimuli from PGRs, redifferentiation of the dedifferentiated tissues, successive cell division, organization, and development of different structures (Almeida et al. 2015, Tambarussi et al. 2017). According to the Dohling et al. (2007), the combination and concentration of PGRs greatly influenced the morphogenesis in PTC. Among the different PGRs, the combination of cytokinins and auxins considered as most appropriate and chiefly responsible for the growth and development of the plant (Aremu et al. 2016). The physiological role of cytokinin and auxin has been well established by several authors and mutual action as a signaling
molecule has also been well established (Sauer et al. 2013, Della et al. 2013). Several workers clearly emphasized that the ratio of auxin to cytokinin varies from species to species (Hossain et al. 2012, Hossain and Dey 2013). In the present study, the optimum concentration of BA (5.0 µM) was supplied with three different auxins viz. NAA, IAA, and IBA at various concentrations (0.1-1.0 µM). The combined treatment of cytokinin and auxin significantly improved the growth parameter in both the plant species. The present finding is in agreement with several reports where the combination of optimized cytokinin and auxin was found to be more effective in terms of enhanced production of shoots compared to that of single cytokinin treatment (Kar et al. 2014, Chavan et al. 2015, Thakur et al. 2016).

A pioneer work of Skoog and Miller (1957) has already established the significance of auxin and cytokinin as key regulators of in vitro morphogenesis. Besides this there are several reports on the synergistic effects of auxin and cytokinin (Aremu et al. 2016). In our study, the combination of BA + NAA was found to be suitable for organogenesis than the other auxins. The experimental results in D. arayalpathra clearly depict that the combination of BA (5.0 µM) and NAA (0.5 µM) was able to induce a maximum of 6.2 ± 0.2 shoots/explant through in vivo derived NS. Similarly, in D. salicifolia, a combination of BA (5.0 µM) and NAA (0.5 µM) induced an average of 4.04 ± 0.05 shoots/explant through in vivo derived NS. While on the similar treatment in vivo ST explants of D. arayalpathra and D. salicifolia produced as many as 4.0 ± 0.1 and 3.0 ± 0.11 shoots/explants respectively. Similar kind of results has also been reported by Chavan et al. (2015), wherein they achieved improved results in Salacia chinesis through different explants on the combination treatments of BA and NAA. In a previous report on D. arayalpathra, Gangaprasad et al. (2005) found that the combination of BA and NAA was more advantageous to produced at least one shoot/node through NS explants in D. arayalpathra. The optimum synergistic effect of BA and NAA was also found in many other plants viz. R. serpentina (Baksha et al. 2007), T. indica (Faisal et al. 2007) and C. siamea (Parveen et al. 2010). The NAA at the higher concentration found to be inhibitory shoot regeneration but formed callus formation at the base end which might hamper the nutrient uptake and thus overall growth was retarded. Such kind of behavior was also observed in Garcinia indica (Malik et al. 2005) and Artemisis judaica (Liu et al. 2003).
The synergistic effect of cytokinin and auxin resulted in multiple buds induction from pre-meristem containing explants in both the plant species, however, the effect was more favorable in *D. arayalpathra* in terms of maximum shoot production and related parameter studied. However, the growth abnormalities that was observed during single cytokine treatment, was also persisted in combination medium as the days advanced.

### 5.1.1.4 Effect of growth additives on shoot regeneration

To develop a successful in vitro protocol for the organogenesis, an interaction of many factors either endogenous or exogenous viz. PGRs, explants type, nutrient composition, and aseptic condition are needed (Bakhtiar et al. 2016). Hence, to eradicate the above mentioned problems observed, another set of experiment was planned for the better growth and development of the plant. Different growth additives viz. Ads, Glu and PG were added to the optimized media. The addition of growth additives significantly improved the growth and development of the plant and also rectified the growth abnormalities that observed during the single and combination treatment of PGRs. The combination of Ads with optimized auxin and cytokinin was more responsive than PG and Glu. Murashige (1974) has demonstrated that the addition of Ads has a stimulatory effect on the shoot bud proliferation.

Further, the study in *Pterocarpus marsupium* by Husain et al. (2008) suggests that Ads acts as a rich source of nitrogen and its cellular uptake are very high in comparison to the other nitrogen form. The addition of Ads (20 µM) with optimized concentration of BA (5.0 µM) and NAA (0.5 µM) was more thriving and a maximum of 11.8 ± 0.1 shoots/explant was formed from *in vivo* derived NS and 5.5 ± 0.2 shoots/explant from *in vivo* derived ST explants in *D. arayalpathra*. Similar kind of behavior was also observed in *D. salicifolia* wherein, a maximum of 9.97 ± 0.01 shoots/explant were obtained from *in vivo* derived of NS and 5.0 ± 0.1 shoots/explant or *in vivo* derived ST explants. The addition of Ads was not only improved the shoot formation but also increased shoot length was observed. Similar kind of finding has also been observed in *Acacia catechu* (Kaur and Kanta 2000), *Jatropha curcas* (Datta et al. 2007) *Ficus religiosa* (Siwach and Gill 2011) and *D. hamiltonii* (Sharma et al. 2014a). Ads being a rich source of nitrogen might have improved the multiplication rate along with healthy leaf played a vital role in the development and also prevention.
of early leaf fall. It is well established by Murashige (1974) that the addition of Ads acts as a catalyst for the organization of enhanced shoot bud proliferation.

In our study, the addition of Ads also proved to be beneficial in rectifying the associated problems viz. premature leaf fall, leaf necrosis, basal callusing on both the plant. A better lamina expansion with a better growth of leaf was also witnessed on the optimized medium. Similar kinds of results have been reported in plants like *Jatropha curcas* (Datta et al. 2007) and *Syzygium cumini* (Naaz et al. 2014). Naaz et al. (2014) reported that adenine sulphate resulted in the eradication of shoot tip necrosis in *Syzygium cumini* and optimum response was obtained on 10.0 µM BA + 100 mgL⁻¹ Ads which are in agreement with our results.

5.2 Indirect organogenesis

Indirect organogenesis is a pathway that involves an intermediate callus formation. According to Paterson and Everett (1985), a differential response for callus induction is revealed by different explants taken from the same source or plant species because of the fact that the explants were at different biochemical or physiological stage at the time of collection.

In the present study different kinds of callus was obtained, however, differentiation of shoot buds from the callus could not been observed on any treatment even after different subculture passages.

5.3 Effect of different pH levels and sucrose concentrations

Along with the optimized concentration of media, an optimum pH is one of the important factors for the growth and development of plant tissues culture (Andersone and Ivenish 2008). The pH of the media greatly influenced the solidification of a gelling agent (Bhatia and Ashwath 2005). The pH of the medium varies according to the different stages of organogenesis and it can facilitate the nutrient availability to the plant cells (Ostrolucka et al. 2004, Thorpe et al. 2008, Kizil et al. 2017). According to the study of Mimura et al. (2000), if plant cells exposed to low pH levels, the conversion of inorganic phosphate to organic phosphate was taken place at an extracellular region, an overall effect leads to the decreased ATP level (Mimura et al. 2000). Hence, to obtain maximum shoot regeneration, the optimization of the pH
of the media is mandatory. In our study, different pH value with optimized PGRs in MS has been evaluated for both the plant species.

Among all the tested pH values, maximum organogenic response was obtained at pH 5.8 from both in vivo and in vitro derived NS and ST explants in both the plant species. The present finding is according to the earlier reports on several medicinal plants viz. *Pistacia lentiscus* (Kılınc et al. 2015), *Eriocephalus africanus* (Madzikane-Mlungwana et al. 2017). The pH value beyond the optimum level resulted in the reduction of shoot regeneration potential due to hampered nutrient uptake. Generally, higher pH could lead to the formation of hard medium and lower pH could lead to the watery medium. According to the Schubert et al. (1990), plant cells release H⁺ to the extracellular environment affecting the nutrient uptake viz. NH₄⁺ at low pH while at high pH value cell release OH⁻ ions which hampered the absorption of NO₃⁻.

During in vitro culture condition, there is a limited supply of CO₂ in culture vessels and the growing plant unable to fix the sufficient amount CO₂ in the absence of sucrose that is why the supply of sucrose as a carbon source is needed (Gautheret 1955, Grout and Price 1987). In PTC, sucrose was used with MS medium in order to maintain an adequate supply of carbon source in most of the micropropagation protocol (George 1993, Fuentes et al. 2000). However, the concentration and type of energy source also vary according to the stage of culture (Thompson and Thorpe 1987). Sucrose was also found to control the osmotic potential of the cells. In the present study, different concentration of sucrose has been examined viz. 1%, 2%, 3%, 4% and 5%. Among all the treated concentrations, 3% sucrose was found most suitable for healthy growth and development of the shoots via various explant viz. in vivo derived NS, in vitro derived ST explants in both the plant species. Similar kind of results has also been obtained in various medicinal plants viz. *C. ternatea* (Ismail et al. 2012) and *Melastoma malabatricum* (Ghimire et al. 2016). However, sucrose beyond the optimal concentration, found to be the least effective and reduced shoot growth was witnessed. In contrary to our results, 5% and 6% sucrose was found optimal concentration in *Amygdalus communis* (Gurel and Gulsen 1998), 4% in *Eucomis autumnalis* (Taylor and van Staden 2001).
5.4 Effect of different subculture passages on shoot multiplication

The regenerative cultures from the best explants obtained on optimum media were constantly subcultured on to the fresh medium having the same concentration at an interval of 4 weeks. This subculture was carried out for further proliferation and long-term maintenance of in vitro cultures. In our study 6 subculture passages have been undertaken, however, optimum production of the shoot was obtained at 4th subculture passage and thereafter a sharp declined was reported in all the growth parameters in both the plant species. The regenerated shoots up to the 4th subculture passages exhibited healthy growth and development. Similar kinds of results have been also reported in several other medicinal plants such as Santalum album L. (Akhtar and Shahzad 2017) and Gymnema sylvestre (Saeed et al. 2018). The increased in shoot number and their proliferation on a consecutive transfer of mother tissue might be due to the inhibition of the apical dominance during the subculture passages (Shekhawat and Shekhawat 2011).

5.5 Rooting in microshoots

To develop a successful micropropagation protocol, rooting to the microshoot is a mandatory step for the complete plantlets formation. The in vitro rooting process can be divided into different phases such as induction, initiation, and expression (De Klerk et al. 1999). According to Han et al. (2009), root development to the microshoot during in vitro culture is one of the complicated processes in PTC studies. In vitro rooting greatly influenced by media composition, and in the present investigation different strategies have been used for rooting in microshoots. Both full-strength and half-strength MS medium have been used for root induction studies. MS basal medium did not support any root formation in both the plant species. while half-strength MS medium found suitable for root initiation in the microshoot. Root induction in auxin-free medium might be due to the presence of some endogenous auxin in microshoot (Minocha 1987). Similar kind of results for the rooting on half-strength MS basal medium have also been also reported in different plant species such as Caralluma edulis (Patel et al. 2014), Crassocephalum crepidioides (Opabode et al. 2016) and Lasia spinosa (Hore and Tanti 2018). However, auxin viz. NAA, IBA and IAA when supplemented with reduced salt concentration or half strength MS medium was able to improve rooting to the microshoot. Among the different auxin used NAA
was found to be optimum for maximum production of roots/shoot in *D. aryalapathar*, wherein a mean number of 5.1 ± 0.1 roots/shoot were formed after 4 weeks of culture. The roots obtained on NAA (2.5 µM) supplemented media were healthy and branched, while weak and fibrous roots were obtained on IAA and IBA supplemented media. A similar kind of results have been obtained in *Gymnema sylvestre* (Thiyagarajan and Venkatachalam 2013), *Moringa oleifera* (Jun-Jie 2017) and *Rhodiola imbricate* (Bhardwaj et al. 2018). In another study of Sudha et al. (2005) on *D. aryalpathra*, half-strength MS medium augmented with optimum NAA (1.07 µM) was able to induce a maximum of 5.8 ± 0.3 roots/microshoot after 5 weeks of culture. In contrary to the present findings, IBA was found to be most responsive auxin with half-strength MS medium, wherein 6.3 ± 1.24 roots/shoot were produced after 40 days of culture in *D. aryalpathra* (Gangaprasad et al. 2005).

In the case of *D. salicifolia*, half-strength MS medium supplemented with IBA (2.5 µM) was found to be optimum and an average of 6.10 ± 0.07 roots/microshoot were produced after 4 weeks of culture. Similarly, there are several reports available dealing the stimulatory effect of IBA on rhizogenesis in medicinal plants such as, *Cyclea peltata* (Abraham et al. 2010), *Rhinacanthus nasuthus* (Cheruvathur and Thomas 2014), *Glinus lotoides* (Teshome and Feyissa 2015), *Plectranthus bourneae* (Thaniarasu et al. 2016), *Pittosporum eriocarpum* (Thakur et al. 2016), *Lathyrus sativus* (Barpete et al. 2016) *Eriocephalus africanus* (Madzikane-Mlungwana 2017) and *Lasia spinosa* (Hore and Tanti 2018). The present experiment of *in vitro* rooting also depicts the influence of gelling agents in both the plant species. In our study agar was compared with phytogel on root formation and evaluated that the agar gelled medium was more effective in both the plant species. *In vitro* rooting on agar gelled medium resulted in the development of healthy roots and mean number of roots was also higher which might be due to the proper nutrients availability and uptake. Similar results have also been observed in *Pittosporum eriocarpum* (Thakur et al. 2016) and *Moringa oleifera* (Jun-Jie 2017).

### 5.6 Acclimatization of plantlets

To achieved a successful protocol for *in vitro* culture of any plant species there *ex vitro* transfer is one of the important parameters. The quality of micropropagation protocol can be determined with the acclimatization of the *in vitro* raised plantlets.
During *in vitro* growth of the plantlets, they face controlled light irradiance, optimum sugar concentration and PGRs. However, when they transferred to the *ex vitro* condition, they face less air humidity, high light irradiance, high CO₂, low level of sugars, and PGRs. These factors are responsible for the alteration in morphology, anatomy, and physiology of the regenerated plantlets (Pospíšilová et al. 1999). According to the Pospíšilová et al. (1998), during the initial days of acclimatization, shade was quite beneficial to the *in vitro* raised plantlets. Thus, to sustained in a natural environment, a proper duration of hardening is mandatory to cope up with these inadequacies and adapt to field conditions (Pospíšilová et al. 1999). In the present study, after attaining complete plantlets with well-developed shoot and root system in both the species were hardened off accordingly, followed by transfer to the field conditions where they exhibit normal growth without any casualty. A maximum survival rate of 92.3% was witnessed in *D. arayalpathra* while 89.3% in *D. salicifolia*. The obtained results are in agreement with other reports on micropropagation of several medicinal plants viz. *Salvia splendens* (Sharma et al. 2014b), *Caralluma edulis* (Patel et al. 2014) and *Santalum album* (Akhtar and Shahzad 2017).

### 5.7 Synthetic seeds production

The conventional method to grow the plant through the seed is unable to meet the demands due to low viability of seeds. Reports are available for the micropropagation of several medicinal plants using different explants could serve as an alternative means for its vegetative propagation. However, long-distance transportation of germplasm is an unsolved problem which restricts the full utilization of micropropagation system. Synseed technology has been proven to enhance the efforts of micropropagation and conservation of several medicinal plants such as, *Decalpis hamiltonii* (Sharma and Shahzad, 2012), *Tecomella undulata* (Shaheen and Shahzad, 2015), *Vitex trifolia* (Alatar et al. 2016), *Plumbago resea* (Prakash et al. 2018) and *Urginea altissima* (Baskaran et al. 2018). The Recent decade witnessed an upsurge in synseed research and its utilization in several commercially important medicinal plants (Sharma et al. 2013). The synseed technology has been found effective to overcome the problems during long-distance transport and germplasm exchange between the countries (Sharma et al., 2013). The synseed technology involves the two
major steps, preparation and storage of encapsulated propagules in a first step, and retrieval of the complete plantlets in a second step.

In the present study, in vitro derived NS of both *D. arayalpathra* and *D. salicifolia* were used for the alginate beads formation and thereafter subjected for conversion/re-growth studies. The selection of NS for the synseed production was made due to more availability than the ST explants, moreover, both the explants have been found appropriate for the encapsulation studies as they possessing meristematic tissues. However, it is hard to obtained complete plantlets formation from the propagules as they unable to form root.

To produce synseeds, excised nodal segments were mixed with Na$_2$-alginate (an encapsulation matrix) and dropped into CaCl$_2$·2H$_2$O solution (a complexing agent). An ion-exchange process took place by the replacement of Na$^+$ ions with Ca$^{++}$ ions resulted the Ca-alginate beads leads construction which are referred as “synseeds”. The morphology of synseeds with respect to shape, texture, transparency, and rigidity may vary with different concentrations of Na$_2$-alginate and CaCl$_2$·2H$_2$O. Among the different composition of the gelling matrices, 3.0% sodium alginate prepared in liquid MS and 100 mM calcium chloride was found ideal for the beads formation. There are several reports available wherein 3.0% sodium alginate and 100 mM calcium chloride was found most optimum for the ideal synseeds formation (Ozudogru et al. 2011, Javed et al. 2017 and Saeed et al. 2018). However, in contrary to our results, Pinker et al. (2005) reported that 3.0% sodium alginate and 75 mM calcium chloride as an optimum combination for the synseeds formation in *Dendranthema × grandifolia*. Similarly, 5% sodium alginate and 50 mM calcium chloride was found to be a perfect combination to obtained ideal synseed in *cannabis sativa* (Lata et al. 2009).

However, in our study, synseed obtained with 3% sodium alginate and 100 mM calcium chloride showed the highest of 57.7 ± 1.15%, 56.7 ± 1.35% re-growth for *D. arayalpathra* and *D. salicifolia* respectively when placed on PGR-free MS basal medium (control) after 6 weeks of plantation. To further improve the response, PGRs viz. cytokinin and auxin were supplemented to the MS basal medium. In *D. arayalpathra*, combination of 2.5 µM BA, 0.5 µM NAA and 20 µM Ads exhibited maximum re-growth of 71.26 ± 1.12% after 6 weeks of culture. While a combination of 2.5 µM BA, 0.5 µM NAA and 30 µM Ads was found significant to exhibited
maximum germination of 71.30 ± 1.22% after 6 weeks of culture in *D. salicifolia*. Although synseeds failed to induce rooting in the microshoots on germination medium and therefore, another experiment was undertaken to induce rooting in microshoots obtained from synseeds. To induce root in synseed derived microshoot a nutrient combination of half-strength MS + 2.5 µM NAA was found more suitable with the generation of 5.1 ± 0.1 roots/microshoot in *D. aryalpathra*, while as many as 6.1 ± 0.07 roots/microshoot was obtained in *D. salicifolia* on half-strength MS + 2.5 µM IBA after 6 weeks of cultures. Our results are according to the findings of Sharma and Shahzad (2012) in *D. hamiltonii*, where they performed a two-step method to achieve maximum synseed conversion into plantlets, in the first step they achieved maximum re-growth of shoots while in the second step, the microshoots were rooted for complete plantlets production. Similarly, Javed et al. (2017) established two-step processes viz. in first step maximum synseed were gave rise to shoot system and in the second step, the microshoots were transferred to the rooting medium of *Erythrina variegate*. However, Saeed et al. (2018) achieved successful conversion of synseed into plantlets of *G. sylvestre* on the same germination medium.

The technology of synseeds acts as important tool of germplasm exchange between countries and hence, storage of synseeds is an important factor which arbitrates their successful germination after long distance transport. Synseeds required no transfer to fresh medium during cold storage, and thus reduces the cost of maintaining germplasm cultures (West et al. 2006). Hence, proper maintenance of synseeds viability is prerequisites during the transportation that leads to the commercialization of synseeds technology. In the present investigation, three types of encapsulation matrix viz. encapsulation matrix prepared in MS basal medium + 5.0 µM + 0.5 NAA µM + 20 µM Ads, encapsulation matrix prepared in MS basal medium and encapsulation matrix prepared in distilled water and naked nodal segments were stored at low temperature (4°C) to see the effect of storage on regrrowth frequency of synseeds. A maximum of 78.13 ± 1.27%, 77.13 ± 1.22% re-growth frequency was observed during the initial days when encapsulation matrix prepared in optimum PGRs concentration in *D. aryalpathra* and *D. salicifolia* respectively. In both the plant species, as the storage duration increases more than 4 weeks, the germination frequency decreased linearly which might be due to the inhibition of tissue respiration by alginate matrix or loss of moisture due to partial desiccation during storage as
reported earlier (Danso and Ford-Liyod 2003, Faisal et al. 2006, Faisal and Anis 2007). The regrowth frequency of non-encapsulated NS witnessed the maximum regrowth frequency of 90.20 ± 1.15, 89.22 ± 1.13% at 0 weeks for *D. arayalpathra* and *D. salicifolia* respectively, then after a declined gradually as the weeks of storage duration increased and no viability was observed during 8 weeks of storage duration. The encapsulation matrix prepared in distilled water loses its viability after 4 weeks of storage duration. The obtained results for cold stored synseed and their regrowth frequency are in accordance with the previous reports on other plant species (Faisal et al. 2006a, Faisal and Anis 2007 and Sharma and Shahzad, 2012). Most commonly, 4 °C temperature was found most favorable for the synseeds storage and to maintain the viability (Baskaran et al. 2015, Kaminska et al. 2017, Bose et al. 2017 and Saeed et al. 2018). However, in contrary to our results, Sundararaj et al. (2010) found 100% re-growth ability for *Zingiber officinale* stored at 25 °C while no re-growth was observed for synseeds stored at 4 °C.

5.8 Physiochemical studies

The duration of acclimatization is very climacteric for successful establishment of regenerated plantlets due to several stresses that is plant going to face during *in vitro* to *ex vitro* transfer. During this period of acclimatization, numerous physiological and biochemical changes take place within the plant to cop up with natural stresses. Hence, evaluation of physiological parameter during the acclimatization period is one of the interesting parameters and several workers have reported these physiological changes during *ex vitro* transfer of *in vitro* raised plantlets (Sahai and Shahzad 2013, Shin et al. 2014, Bhattacharyya et al. 2016). The initial days of acclimatization play a very important role in plant adaptation to the natural environment (Aragon et al. 2005). The very first challenge that micropropagated plantlets face when relocated to the *ex vitro* condition is related to water loss due to a poor stomatal functioning and lack of thick cuticular deposition (Pospíšilová et al. 1999). According to the Walters et al. (2003), the leaves of *in vitro* raised plantlets should get adopted in terms of photosynthetic machinery for higher energy load and to encounter the harmful effects of high irradiance of light. In the present investigation, different photosynthetic parameters and related attributes have been evaluated during the days of acclimatization for both the plant species.
In both *D. arayalpathra* and *D. salicifolia*, chl a, chl b, total chlorophyll, carotenoid content and net photosynthetic rate (\( \text{Ps} \)) increased at their maximum level after 28 d of acclimatization. However, an initial fall was also observed in the first week of acclimatization for which Pospíšilová et al. (1999) has found the reason i.e. disordered granna might be responsible for initial fall. Since the role of carotenoid against photosynthetic damage in the plant is well documented (Van Huylebroeck et al. 2000). A similar trend was also found in the other plant species such as *D. hamiltonii* (Sharma et al. 2014a), *C. forskohlii* (Sahai and Shahzad 2013) and *Plantago algarbiensis* Samp. and *P. almogravensis* Franco (Goncalves et al. 2017). The poorly developed chloroplast containing disorganized granna might be the reason of initial reduction in chlorophyll content (Pospisilova et al. 1999). As the days of acclimatization increased, the formation of new leaves were taken place and chlorophyll content increased gradually that was showing the significance of acclimatization with respect to chlorophyll content. An incremental practice in photosynthetic rate is not surprising as it has been also reported in several medicinal plants during *ex vitro* transplantation (Pospisilova et al. 1998, El-Mahrouk et al. 2016). Similarly, stomatal conductance (Gs) and transpiration rate (Tr) was increased linearly after one week of acclimatization. This kind of rise and fall reflects the strong regulation effects of stomatal and water change under *ex vitro* environment. The results were in accordance with the findings of Wang et al. (2005), Galmes et al. (2007) and Perez-Jimenez et al. (2015).

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 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). The polyunsaturated fatty acid present in the plasma membrane is more prone to damage by ROS and leads damage to the plasma membrane and production of MDA as a consequence of lipid peroxidation. The leakage in electrolytes directly corresponds to the membrane permeability (Gill and Tuteja 2010). During the transfer of *in vitro* raised plantlets of *D. salicifolia* to *ex vitro* acclimatization, the plantlets feel light stress and a maximum increase was noticed in both EL (51.8%) and TBARS [2.06 μmol g\(^{-1}\) (f.w.)] at 7 days of acclimatization. After that, a gradual decrease was found due to improved
acclimatization. Our finding was according to the findings of Faisal and Anis (2009) and Perez-Jimnez et al. (2015) conducted in *Rauvolfia tetraphylla* and *Cynara scolymus* respectively.

### 5.9 Biochemical studies

During the transfer of *in vitro* raised plantlets to *ex vitro* conditions, plantlets have to face various stresses. The availability of low CO$_2$ concentration with low photon flux (PPF) in culture tubes might be the main reason for a decrease in acclimatization rate. A change in the functioning of stomata and cuticle thickness has also been reported as an effect of acclimatization (Van Huylenbroeck et al. 1998). These environmental stresses are responsible for the output of reactive oxygen species (ROS), which is a byproduct of aerobic metabolism. ROS causes several kinds of oxidative blow to the plant such as DNA damage, protein degradation, and enzyme inactivation. However, plant cell develops a strong defense mechanism against the hazardous free radical and oxidative stresses in the form of complex antioxidant defense and enzymatic scavenging system as such as SOD, CAT, APX and GR (glutathione reductase) (Xu et al. 2012, Kayihan et al. 2012). The enzymatic scavenging defense mechanism helps the plant to survive in hazardous condition and regulate their evolution in a cell (Mitrović and Bogdanović 2008).

In the present study, changes in the enzymatic assays of CAT, SOD, APX, and GR were evaluated for both the plant species. After the first week of acclimatization, there was a hike in the activities of CAT, SOD, APX, and GR in both the plant species. Peroxisome with the help of CAT and other enzymes play a prominent role in preventing plants from different kinds of photooxidative damage, CAT scavenges H$_2$O$_2$ by converting it into O$_2$ and H$_2$O in peroxisome. Formation of H$_2$O$_2$ might be the reason for increased SOD activity. SOD converts superoxide to H$_2$O$_2$ and oxygen (O$_2$). This might be the primary defense against the ROS. The process of detoxification is carried out by a series of membrane-associated and stromal enzymes, including SOD and APX at the acceptor side of photosystem I (PS I), this enables the plant to protect themselves against the oxidative stress (Scalet et al. 1995). CAT activities involve the dumping of an electron that is the primary cause of the yield of free radical such as superoxide (O$_2^-$) (Abassi et al. 1998) and hence plays a significant role in normal cellular processes. APX and GR are two important enzymes of
ascorbate glutathione cycle. They play a significant role by scavenging H$_2$O$_2$ in chloroplast, cytosol, vacuoles and apoplastic space (Asada 1999). Moreover, increase in CAT activities and SOD both represents photorespiratory detoxification of H$_2$O$_2$ into O$_2$ and H$_2$O in mitochondrial electron system (Scandalios 1990). CAT scavenges H$_2$O$_2$ by converting it into O$_2$ and H$_2$O and hence reduced the level of H$_2$O$_2$ suggest successful acclimatization. The similar kind of behavior of these enzymes was also found in *Scrophularia takesimensis* (Jeong and Sivaneshan 2015) and *Plantago algarbiensis* and *P. almograensis* (Gonçalves et al. 2017).

Moreover, the elevated level of APX and GR enzymes in both the plant species suggest chloroplast-based detoxification of ROS via Mehler pathway (Foyer and Mullineaux 1998). According to Yan (2009), when SOD, CAT, APX and GR were in coordination with one another, the rate of free radical production and oxidative damage could be minimized and a plant can grow congenitally. The elevation of APX and GR is not astonishing as it is previously reported in *Rauvolfia tetraphylla* and *Tylophora indica* (Faisal and Anis 2009, 2010) during the acclimatization period.

5.10 Histological studies

The structural study is a productive step to investigate the pattern of organization and changes occurred in a plant system. Histological techniques have been widely used to understand the pattern of cellular arrangement during in vitro studies. In this study, histological analysis of regenerating shoots from NS explants of *D. arayalpathra* and *D. salicifolia* was done. Histology of regenerative tissue obtained from optimized media combination through NS explants of both the plant affirms the direct origin of axillary shoot buds through meristemoids formation. The meristemoidal cells were found to have prominent nuclei, and later on the organization differentiated into shoot buds with well distinct apical meristem and leaf primordia. The present findings are according to the previous reports on *C. forskohlii* (Sahai and Shahzad 2013), *C. angustifolia* (Parveen and Shazad 2014a) and *D. hamiltonii* (Sharma et al. 2014a).

5.11 Genetic homogeneity assessment

The present study was carried out to developed *in vitro* regeneration protocol for *D. arayalpathra* and *D. salicifolia*. According to the study of Rahman and Rojara (2001), the occurrence of somaclonal variation is a possible disadvantage during *in vitro*
propagation of elite germplasm and it might be responsible for an acute threat to the genetic integrity of the in vitro raised plants. Therefore to avoid such kind of problem, a quality checkup of genomic DNA has been carried out for the regenerated plants. The present investigation was carried out to check the homogeneity of tissue culture raised plants with that of mother plant by using DNA based molecular markers viz. RAPD and ISSR. These two markers have been used due to their specific characteristics viz. small DNA sample required, highly discriminative, cost-effective and non-involvement of radioactive labels. Both RAPD and ISSR marker are unsophisticated and profitable in screening of genetic uniformness. No polymorphism was detected during the analysis of tissue culture raised plant confirming the genetic uniformity of the plant. The present findings also suggest that micropropagation of \textit{D. arayalpathra} and \textit{D. salicifolia} maintains genetic uniformity even after a prolonged period under \textit{in vitro} conditions. Our results are in accordance to the earlier report on genetic uniformity of \textit{in vitro} derived plantlets of \textit{Swertia lawii} (Kshirsagar et al. 2015), \textit{Helicteres isora} (Mariappan et al. 2016), \textit{Salacia chinensis} (Chavan et al. et al. 2015), \textit{Silybum marianum} (Lv et al. 2017) and \textit{Dioscorea} spp. (Adeniran et al. 2018).

5.12 SEM study

SEM is an active approach to study the surface characteristics of the plant. Several workers have used electron microscopy in the area of industrial and scientific research due to its dynamic application and high resolution (Goodhew 2001, Bhushan and Jung 2006). The leaf surface study of \textit{D. arayalpathra} and \textit{D. salicifolia} explore the stomatal behavior and their presences on both the surface. Study on higher resolution depicts the paracytic type of stomata in both the plant species. The results of SEM analysis revealed that \textit{in vitro} raised plants of \textit{D. arayalpathar} and \textit{D. salicifolia} exhibit no morphological difference with that of the mother plant. Similar kind of results was also depicted by Gnasekaran et al. (2016) where they observed paracytic type of stomata during their study on \textit{in vitro} raised plantlets of \textit{Vanda}.

5.13 GC-MS study

Large members of plant species are being eroded due to various reasons and many of them are poorly understood because of the lack of researches conducted on them.
Hence, this type of orphan plant must be screened for the presence of secondary metabolites and their possible use as a therapeutic agent thus, they could gain attention for their conservation with an objective, and otherwise sincere efforts for their conservation could not be accomplished. Now a day’s several techniques have been developed to screen the metabolic content of the plant. These advancement in science provided us a chance to explore the insight metabolic content of the plants. The combination of gas chromatographic technique and mass spectrometry was proved to be most convenient technique for the screening of metabolic content in the plants and plant parts viz. leaf, root, stem, flower etc. Several workers has been utilized this technique for phytochemical analysysi of the different medicinal plant such as in Ocimum basilicum (Gopal et al. 2014), Decalepis hamiltonii (Prakash et al. 2015), Lavandula angustifolia (Kirimer et al. 2017), Premna tomentosa (Priyadarshini et al. 2017), Thymus vulgaris (Mokhtarzadeh et al. 2018), Carissa spinarum (Rao and Anisha 2018) and Salvia hispanica (Falco et al. 2018).

In the present investigation metabolic profiling has been carried out for in vitro regenerated plants and mother plants of both the species to explore the important chemical compounds found in the plant. The tuberous root is the most important part of the plant and contains several compounds such as 2-hydroxy-4-methoxybenzaldehyde (2H4MB), 3-hydroxy-4- methoxybenzaldehyde (3H4MB), 4-hydroxy-3-methoxybenzaldehyde (4H3MB), chlorogenic acid, benzoic acid, inositol, squalene etc. Similar kind of results has been also found by Verma et al. (2014) on D. arayalpathra where they reported several minor and major meatbolites after GC-MS analysis of root extract. Manickam and Periyasamy (2014) studied the GC-MS analysis of methanolic extract of Decalpeis hamiltonii and 2H4MB witnessed as major compound present in the root tuber of the plant.

5.14 Evaluation of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in the root extract

Considering the pharmacological importance of 2H4MB, a great interest has been developed for separation, identification and quantification of 2H4MB in in vitro raised plants of D. arayalpathra and D. salicifolia through High Performance Liquid Chromatography. The present study was carried out to evaluate the 2H4MB content in relation to the biomass content in the root system of micropropagated plants of D.
arayalpathar and D. salicifolia. The obtained results clearly depict that the formation of 2H4MB is in relation to the biomass content however, formation of 2H4MB took place after 2 weeks of acclimatization. The obtained chromatogram for extract was compared with that of the chromatogram of standard compound and similarity in retention time was the basis of affirmation of the presence of the compound. Moreover, the peak purity was further screened and both compounds showed maximum absorption at 278.5 nm. The quantification was based on the calibration curve. The results support the hypothesis that accumulation of 2H4MB start only after 2 weeks of acclimatization and increased gradually. A 2H4MB synthesis was also reported by Sharma et al. (2014a) in D. hamiltonii. Sudha and Seeni (2001) and Giridhar et al. (2005b) also reported similar results in the normal root culture of D. arayalpathra and D. hamiltonii by using GC-MS and HPLC analysis, respectively.

5.15 Enhancement of 2H4MB content in root tuber of D. salicifolia through elicitation

The tuberous root of D. salicifolia contains 2H4MB as a major phenolic compound that accumulate in the root and is chiefly responsible for the sweet flavor and fragrance (George et al. 2011). Despite of its importance, the enzymatic route of this fragrant methoxybenzaldehyde is not yet clear and has been a challenging task for biologist. In plants there are different pathways exist which provides us regulated mechanism leading to the synthesis of particular benzoate derivatives (Wildermuth 2006). Schmidt et al. (1998) emphasized that the plant hydroxybenzoate derivatives are frequent mediators of plant response to biotic and abiotic stress including elicitors. Previous study with H. indicus excised roots culture suggested a possible origin of 2H4MB via central phenylpropanoid pathway (Chakraborty et al. 2008, Kamireddy et al. 2017). Another study of Kundu et al. (2012) also suggest the role of shikimate pathway for the biosynthesis of 2H4MB. Both the studies suggest that benzoate pathway originate either directly from shikimate or via phenylpropanoid pathway. In their study they found a sharp increase in shikimate dehydrogenase along with phenylalanine ammonia-lyase (PAL) as both the enzymes are involved in biosynthesis of 2H4MB (Chakraborty et al. 2008, Kundu et al. 2012). Several attempts have been made to enhance the accumulation of 2H4MB through elicitation such as in the excised root of H. indicus (Chakraborty et al. 2008, Kundu et al. 2012), D. hamiltonii
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(Kamireddy et al. 2017) and Cocos nucifera (Chakraborty et al. 2009). In this study, we reported a significant effect of CH over YE on the enhancement of 2H4MB. The ability of CH for the enhanced production of 2H4MB and other metabolites could be due to the mimicking of natural fungal infection, that induce a natural defense response (Iriti and Faoro 2009) or it might have enhance the levels of enzymes involved in phenylpropanioid pathway for 2H4MB biosynthesis (Chakraborty et al. 2009, Baque et al. 2012). Typically the effect of elicitors on the production of metabolites in plant tissue or cell culture mainly depends on the dose, contact periods and the age of the culture (Murthy et al. 2014). The physiological or metabolic damage might be occurred if exceeding the optimum conditions and contact period. In our study, the optimal dose was reported to be 200 µM with most favorable contact period of 72 h. Therefore, to achieve the maximum production of 2H4MB along with other metabolites in the suspension culture, optimization of elicitor’s dose and their contact period was of great significance. Most of the tested contact period of CH and YE enhanced the biomass and 2H4MB content. The supremacy of CH for the enhanced production of biomass and 2H4MB content also agreed with the report of Chakraborty et al. (2008) in H. indicus. In contrary to the results, YE has been proven as an ideal elicitor for the production of 2H4MB in H. indicus (Kundu et al. 2012). However, the results proposed by Chakraborty et al. (2008&2009) and Kundu et al. (2012) are based on excised roots culture. Excised root culture might be having a drawback over suspension culture, as excised root in itself act as source and sink for substrate(s) of any biochemical reaction and might not be involve the exposure of the entire cell to the elicitors. A second drawback would be the regular harvesting of the root for elicitation experiment might cause a thrust on to the availability of the explants.

In our experiment, the callus obtained from the root tuber has been used for the elicitation experiment as it is more efficient and might increase the possibility of maximum cell exposure to the elicitors. The use of callus as an explants for the elicitation experiment does not causes the thrust to the availability of the explants as we can maintain the culture for longer duration by using a subculturing passage. There are several reports available for the enhancement of secondary metabolites through suspension culture (Cai et al. 2012, Boroduske et al. 2016). Chong et al. (2005) demonstrated that typical cell exhibit changes in mRNA and protein
production at different stages of their growth, so there is a great chance for the elicitors to induce the production of metabolites. Chitosan being as a mostly deacetylated form of chitin is known to be effective elicitors for synthesizing a range of phenolics compound (Villegas and Brodelius 1990). Chitosan hydrolysis releases oligosaccharides which are responsible to bind with the β-glucan-binding protein on the cell plasmalemma, and, thus triggers the synthesis of secondary metabolite in case of plants (Kneer et al. 1999). Enhanced CH contact period resulted in the significant increase in 2H4MB productions, especially at a contact period of 72 h. However, if contact period is increased further i.e. 96 h, has resulted in decrease in 2H4MB when compared with the contact period of 72 h. Such kind of effect is might be due to the toxicity of CH to the living cell (Amborabe et al. 2008). Hence, the most suitable was 72 h which gave the highest total 2H4MB content (9.7 DW/gl), which correspond to 1.4-fold increase in comparison to the control. However, a significant decrease was reported in biomass as culture age increases i.e. 96 h contact period in both the CH and YE treated cultures. Our finding for the electiveness of CH can be correlated with the finding made by Jaisi and Panichayupakaranant (2017) in Plumbago indica root culture for the enhancement of plumbagin. Such finding is might be due to physiological and metabolic activities of the D. salicifolia callus culture were more active during the mid of exposure.
Chapter 6
Summary and Conclusions